High-Efficiency On-Line Solid-Phase Extraction Coupling to 15−150-μm-i.d. Column Liquid Chromatography for Proteomic Analysis

ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 2003

Impact Factor: 5.64 · DOI: 10.1021/ac0300690 · Source: PubMed

10 AUTHORS, INCLUDING:

Ronald J Moore
Pacific Northwest National Laboratory
125 PUBLICATIONS **5,436** CITATIONS
SEE PROFILE

Christophe Masselon
Atomic Energy and Alternative Energies Com…
71 PUBLICATIONS **3,412** CITATIONS
SEE PROFILE

Kim Hixson
Pacific Northwest National Laboratory
41 PUBLICATIONS **2,027** CITATIONS
SEE PROFILE

Richard D Smith
Pacific Northwest National Laboratory
1,131 PUBLICATIONS **45,995** CITATIONS
SEE PROFILE

Anal. Chem. **2003,** *75,* 3596−3605

High-Efficiency On-Line Solid-Phase Extraction Coupling to 15−150-μm-i.d. Column Liquid Chromatography for Proteomic Analysis

Yufeng Shen, Ronald J. Moore, Rui Zhao, Josip Blonder, Deanna L. Auberry, Christophe Masselon, Ljiljana Paša-Tolić, Kim K. Hixson, Ken J. Auberry, and Richard D. Smith*

Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington 99352

The ability to manipulate and effectively utilize small proteomic samples is important for analyses using liquid chromatography (LC) in combination with mass spectrometry (MS) and becomes more challenging for very low flow rates due to extra column volume effects on separation quality. Here we report on the use of commercial switching valves (150-μm channels) for implementing the on-line coupling of capillary LC columns operated at 10 000 psi with relatively large solid-phase extraction (SPE) columns. With the use of optimized column connections, switching modes, and SPE column dimensions, high-efficiency on-line SPE-capillary and nanoscale LC separations were obtained demonstrating peak capacities of ∼1000 for capillaries having inner diameters between 15 and 150 μm. The on-line coupled SPE columns increased the sample processing capacity by ∼400-fold for sample solution volume and ∼10-fold for sample mass. The proteomic applications of this on-line SPE-capillary LC system were evaluated for analysis of both soluble and membrane protein tryptic digests. Using an ion trap tandem MS it was typically feasible to identify 1100−1500 unique peptides in a 5-h analysis. Peptides extracted from the SPE column and then eluted from the LC column covered a hydrophilicity/hydrophobicity range that included an estimated ∼98% of all tryptic peptides. The SPE-capillary LC implementation also facilitates automation and enables use of both disposable SPE columns and electrospray emitters, providing a robust basis for automated proteomic analyses.

Current proteome analyses often focus on large-scale comprehensive (i.e., global) protein identification and necessarily involve analyses of highly complex mixtures.[1] Recent advances based upon liquid chromatography (LC)/mass spectrometry (MS) approaches for proteome-wide enzymatic digest peptide analysis have circumvented many of the limitations associated with conventional proteome analyses (e.g., due to isoelectric point, molecular mass, and solubility),[2−5] commonly encountered with two-dimensional electrophoresis/MS approaches.[6] Improving the effectiveness and robustness of methods however remains a challenge.

The throughput of analyses is a function of both MS properties and LC separation performance. For a specific mass spectrometer, high-efficiency LC enables more peptides to be resolved. The use of small-inner diameter (i.d.) capillaries greatly benefits the sensitivity of LC/MS when interfaced with electrospray ionization (ESI).[7,8] On the basis of such considerations, we have recently developed a high-efficiency (peak capacities of ∼1000) nanoscale LC/MS, using 15−75-μm-i.d. capillaries (mobile-phase flow rates between 20 and 400 nL/min).[9] Using an optimal ESI emitter orifice size for each capillary column i.d., the sensitivity was found to be linear with the inverse of the mobile-phase flow rate. Due to generally significant sample losses (e.g., in the syringe and connection tubing of autosamplers) when small-injection volume solutions are used to introduce samples, the effective (essentially quantitative) introduction of small samples generally requires loading relatively large sample volumes, which constitutes a challenge for small-i.d. columns. Additionally, to achieve high dynamic range detection for the highly complex samples, the mass loaded to an LC column is generally much larger than that for analyses of simple samples. For example, >400 μg of cellular tryptic peptides was loaded on a 100-μm-i.d. LC column for LC/LC/ion trap MS/MS analysis.[4,5] Loading such large proteomic sample masses to small-i.d. capillary columns may also compromise operational robustness (e.g., due to column clogging).[2]

Although it is commonly recognized that high separation efficiencies are highly beneficial for proteomic analyses, there have been relatively few reports on enhancing LC efficiency for proteomic analyses, with most efforts focusing on the use of multidimension separations. Multidimension fractionations clearly aid complex proteomic sample analyses; however, high efficiency

(1) Wickware, P.; Smaglik, P. *Nature* **2001**, *413*, 869−875.
(2) Shen, Y.; Zhao, R.; Belov, M. E.; Conrads, T. P.; Anderson, G. A.; Tang, K.; Paša-Tolić, L.; Veenstra, T. D.; Lipton, M. S.; Udseth, H.; Smith, R. D. *Anal. Chem.* **2001**, *73*, 1766−1775.
(3) Shen, Y.; Tolić, N.; Zhao, R.; Paša-Tolić, L.; Li, L.; Berger, S. J.; Harkewicz, R.; Anderson, G. A.; Belov, M. E.; Smith, R. D. *Anal. Chem.* **2001**, *73*, 3011−3021.
(4) Washburn, M. P.; Wolters, D.; Yates, J. R. *Nat. Biotechnol.* **2001**, *19*, 242−247.
(5) Wolters, D. A.; Washburn, M. P.; Yates, J. R. *Anal. Chem.* **2001**, *73*, 5683−5690.
(6) Harry, J. L.; Wilkins, M. R.; Herbert, B. R.; Packer, N. H.; Gooley, A. A.; Williams, K. L. *Electrophoresis* **2000**, 21, 1071−1081.
(7) Bruins, A. P. *Mass Spectrom. Rev.* **1991**, *10*, 53−77.
(8) Hopfgartner, G.; Bean, K.; Henion, J. *J. Chromatogr.* **1993**, *647*, 51−61.
(9) Shen, Y.; Zhao, R.; Berger, S. J.; Anderson, G. A.; Rodriguez, N.; Smith, R. D. *Anal. Chem.* **2002**, *74*, 4235−4249.

10.1021/ac0300690 CCC: $25.00
Published on Web 06/07/2003

based upon single-dimension LC separations has the advantage of reducing overall analysis times by eliminating both duplicate detection of peptides in adjacent fractions and the multiple windows at both ends of the individual second-dimension LC separations that are not effectively utilized (i.e., contain no or few peptides). Therefore, we believe that improving single-dimension LC efficiencies to a level appropriate for proteomic analysis is preferable to the use of multidimension LC, which should be limited to the minimal number of (first-dimension) fractions necessary to provide the overall separation efficiency desired. We have recently shown that single-dimension capillary and nanoscale (column i.d., 15−150 μm; flow rate range, 20 nL/min−1.5 μL/min) LC enables separation peak capacities of ∼1000 for peptides to be achieved in 3−4 h when operated at 10 000 psi in an automated format.[2,3,9]

Due to the importance of LC separation efficiencies for proteome analyses, improved sample loading should ideally not impact separation efficiency. Connecting a relatively large i.d. guard column prior to capillary LC columns effectively improves the ruggedness for loading large-mass samples,[2] but provides little practical improvement for the introduction of large sample volumes since the LC capillary column still limits the flow rate used. Placing a split between the guard column and the LC separation column can resolve this problem,[10] but the unswept dead volumes in "corners" (e.g., from the large stationary-phase-packed cross vent and the split tube) are likely to degrade the separation efficiency. Column switching can address this issue, and it has been widely used for on-line coupling solid-phase extraction (SPE) to LC.[11−13] However, SPE-LC has not been widely used for coupling micro (i.e., 1-mm-i.d. columns) and capillary LC because of this degradation of separation efficiency due to dead volume contributions.[14] The off-line connection of a micro-SPE to capillary (250-μm i.d.) LC has been attempted to minimize dead volume,[15] but this mode is not suitable for automation. Adopting conventional-scale SPE-LC column connections (e.g., see: http://www.lcpackings.com/) for SPE-nanoscale LC has provided relatively low separation efficiencies[16] (estimated peak capacities of <60), making this approach less attractive for proteomic analyses.

In this study, we report on the development of combined high-efficiency on-line SPE-capillary and nanoscale LC/MS (column i.d. from 15 to 150 μm) based upon new column connection configurations. The approaches are demonstrated to enhance sample processing capabilities, separation efficiency, and extraction/elution characteristics in the analysis of complex proteomic peptide mixtures.

EXPERIMENTAL SECTION

Capillary Column Packing. Previously described procedures[2,9] were used to manufacture packed capillaries having inner diameters from 15 to 150 μm for this SPE-capillary and nanoscale LC study. Briefly, the 15-μm-i.d. capillaries were packed at 18 000 psi using a 2-propanol-containing organic solution as the slurry solvent and the other i.d. columns were packed at 10 000 psi using acetonitrile (ACN) aqueous solution. After packing, the capillaries were conditioned in an ultrasonic bath for ∼10 min under the packing pressures to stabilize the packing beds.

On-Line SPE-Capillary and Nanoscale LC Experiments. SPE columns of various i.d. and length were coupled to 15−150-μm-i.d. LC columns and evaluated to determine maximal sample processing capability that provided minimal loss of separation efficiency. The SPE columns used 3- and 5-μm C18 with 300-Å pore size Jupiter particles (Phenomenex, Terrence, CA), 3-μm C18 with 120- and 200-Å pore sizes, and 10-μm C18 with 300-Å pore size (Alltech, Deerfield, IL) as packing materials. All capillaries used for LC separations were packed with 3-μm C18 with 300-Å pore size Jupiter particles (Phenomenex). The stainless steel SPE columns (380-μm i.d., 1.59-mm o.d., Valco) were connected directly to the (Valco Instruments, Houston, TX) valve port with stainless steel ferrules. Figure 1 shows the zero dead volume connection configurations that were used to evaluate the influence of column switching on SPE-capillary and nanoscale LC efficiency. Two six-port positive-feedback switching valves (port diameter of 150 μm, Valco Instruments) were used to load samples and couple the two columns.

In Figure 1A, a sample was loaded to the first valve, and then the loaded sample was switched and directed (at 10 000 psi) using mobile phase A to the first column mounted between the two valves. To minimize dead volumes, all column ends were directly connected to the valve ports with PEEK tubing (380-μm i.d., Upchurch Scientific, Oak Harbor, WA) using 2-μm mesh screens (Valco) to support the packed particles (the column connection geometry requires a minimum length of 4 cm for the first column). After extracting peptides on the first column (the extraction time needed was estimated based on the mobile-phase flow rate and sample volume), the second valve was switched to connect the first column to the second capillary, and the sample separated through the two columns during LC gradient. During the LC separation process, both the ports and channel of the second valve (between the columns) contributed to the dead volume.

In Figure 1B, the first column was directly connected, using a geometry-required minimum capillary length of ∼8 cm, to two ports of the second valve at the sample loop. The sample loading procedure was the same as for Figure 1A; however, the extracted sample from the first column was "back-flushed" to the second capillary during the LC gradient. The dead volume region due to the valve was located prior to the second capillary.

The arrangement shown in Figure 1C has the same function as the arrangement shown in Figure 1B, but without the geometry restriction, allowing the use of short (e.g., 4 cm) columns.

Previously described LC instrumentation[2,9] was operated at 10 000 psi. Purified H_2O (Nanopure Infinity ultrapure water system, Barnstead, Newton, WA) containing HPLC-grade 0.2% acetic acid and 0.05% trifluoroacetic acid (TFA, Aldrich, Milwaukee, WI) (v/v) was used as mobile phase A, and an HPLC-grade ACN (Aldrich) aqueous solution (ACN/H_2O/TFA, 90:10:0.1, v/v/v) as mobile phase B.

(10) Licklider, L. J.; Thoreen, C. C.; Peng, J.; Gygi, S. P. *Anal. Chem.* **2002**, *74*, 3076−3083.
(11) Fritz, J. *Analytical Solid-Phase Extraction*; Wiley-VCH: New York, 1999.
(12) Hennion, M.-C. *J. Chromatogr., A* **1999**, *856*, 3−54.
(13) Rossi, D. T.; Zhang, N. *J. Chromatogr., A* **2000**, *885*, 97−113.
(14) Rivasseau, C.; Vanhoenacker, G.; Sandra, P.; Hennion, M.-C. *J. Microcolumn Sep.* **2000**, *12*, 323−332.
(15) Cappiello, A.; Berloni, A.; Famiglini, G.; Mangani, F.; Palma, P. *Anal. Chem.* **2001**, *73*, 298−302.
(16) Hoes, I.; Van Dongen, W.; Lemière, F.; Esmans, E. L.; Van Bockstaele, D.; Berneman, Z. *J. Chromatogr., B* **2000**, *748*, 197−212.

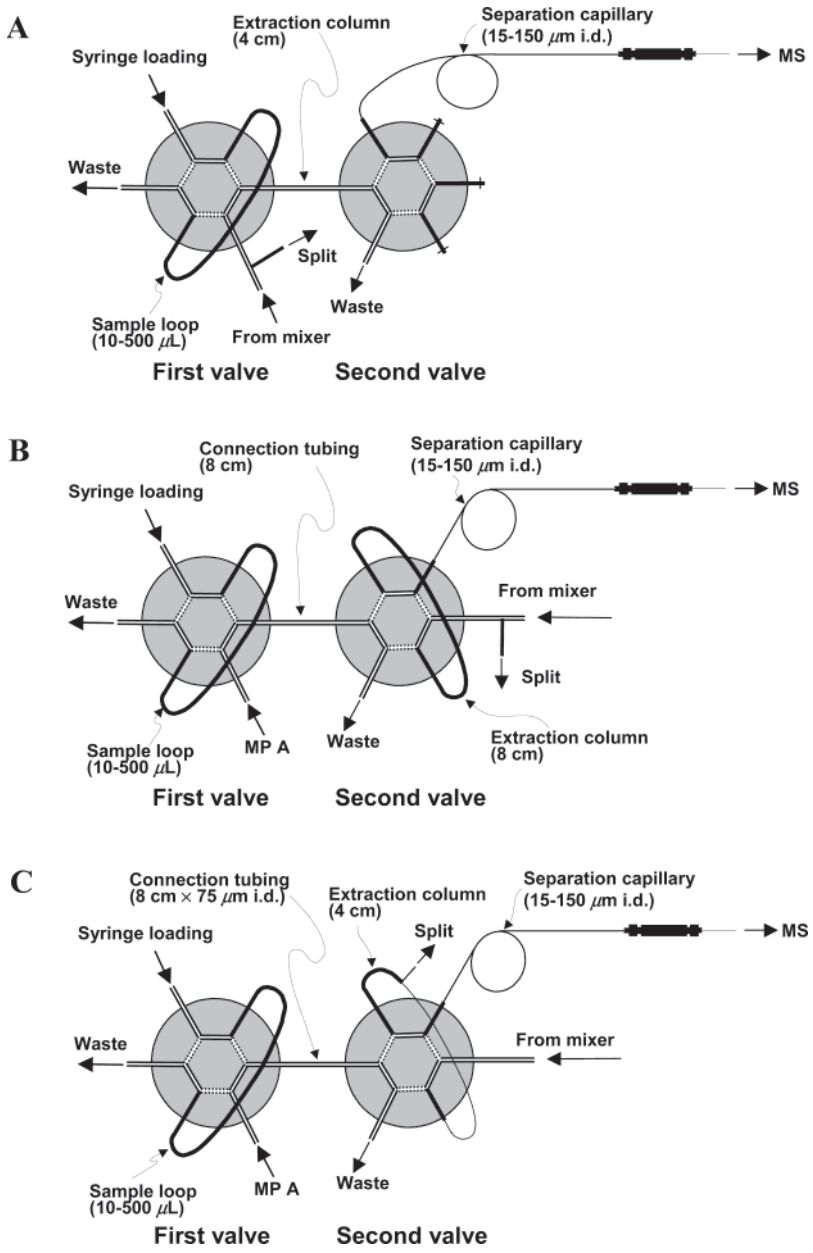

Figure 1. "Zero dead volume" column connection arrangements used for high-efficiency SPE-capillary and nanoscale LC: (A) "front-flash" SPE-LC with minimum SPE column length of ~4 cm; (B) "back-flash" SPE-LC with minimum SPE column length of ~8 cm; and (C) "back-flash" SPE-LC with minimum SPE column length of ~4 cm. MPA, mobile phase A. See text for details.

ESI-MS and MS/MS Studies. The emitter tips for replaceable ESI were fabricated as previously described for microESI (handling the fluid flow rates of approximattely μL/min)[2] and nanoESI (for flow rates of approximattely nL/min).[9] An ion trap mass spectrometer (Finnigan model LCQ XP, ThermoQuest Corp., San Jose, CA) was used with heated capillary temperature and ESI voltage of 120−200 °C and 1.2−2.2 kV, respectively (depending upon the mobile-phase flow rate). For MS experiments, a scan time of ~1.4 s (three microscans with a maximum ion injection time of 300 ms) with an m/z range of 300−2000 was used, while for MS/MS, the three most abundant peaks from each MS spectrum were selected to provide an overall duty cycle of ~9 s. A collision energy setting of 45% was applied for ion fragmentation, and a dynamic exclusion was used to discriminate against previously analyzed ions (data-dependent analysis). A 11.4-T FTICR mass spectrometer developed at our laboratory[17] was used as previously described,[3] except for use of 105 °C ESI inlet capillary temperature and 1.3-kV ESI voltage.

Data Analysis. The chromatographic separation quality was evaluated using the peak capacity concept.[18] The SEQUEST algorithm (ThermoQuest Corp.) was used for peptide assignment and protein identification by searching against the translated predicted proteome databases[19,20] for *Deinococcus radiodurans* and *Shewanella oneidensis* according to the peptide assignment rules of Yates and co-workers.[4] The peptides eluted from the SPE-LC were

(17) Bruce, J. E.; Anderson, G. A.; Wen, J.; Harkewicz, R.; Smith, R. D. *Anal. Chem.* **1999**, *71*, 2595−2599.
(18) Giddings, J. C. *United separation Science*; John Wiley & Sons: New York, 1991; p 105.
(19) http://www.tigr.org.
(20) ftp://ftp.tigr.org/pub/data/ dradiodurans/GDR.pep.

characterized using the Hopp and Woods parameters,[21] and the properties of proteins identified were examined using the ProtParam tool.[22]

Sample Preparations. Yeast lysates, *D. radiodurans* membrane proteins, and $^{14}N/^{15}N$-labeled *D. radiodurans* membrane protein tryptic digests were prepared as described elsewhere.[3,23,24] *S. oneidensis* MR-1 was grown in a Luria broth at 37 °C and harvested at mid-logarithmic phase by centrifugation. The cells were washed twice with phosphate buffer and stored at −80 °C. Cells were thawed and resuspended in NH_4HCO_3 (pH 8.0) by vortexing. Several 200-μL aliquots were dispensed into 600-μL siliconized tubes, and zirconia/silica beads (BioSpec Products, Bartlesville, OK) were added to fill the volume to 500 μL for each tube. Cells were lysed using the homogenize setting for 90 s (minibead beater, BioSpec Products). The supernatant was recovered from the beads by centrifugation (Eppendorf 5815, 13000*g* for 5 min). Cytosolic protein content was determined using a Coomassie Plus assay (Pierce Chemical, Rockford, IL). *S. oneidensis* lysate was denatured at 100 °C for 3 min using 4 M guanidine hydrochloride and reduced using 5 mM DTT. The digestion buffer (NH_4HCO_3, pH 8.0) was added until the concentration of guanidine was less than 1 M, sequencing grade trypsin (1:50, w/w, Promega, Madison, WI) was added, and the sample was incubated overnight at 37 °C. Peptides were isolated on a C18 SPE cartridge (Alltech, State College, PA), washed with water, eluted with acetonitrile, lyophilized, and stored at −80 °C.

RESULTS AND DISCUSSION

SPE On-Line Coupling High-Efficiency Capillary and Nanoscale LC. For the column connection arrangements described, the dead volume between the SPE and LC columns was estimated as ∼40 nL (i.e., the volume of the two valve ports and one channel). We examined the influence of this dead volume on separation efficiency for the three column-switching arrangements using yeast lysate tryptic digests. For the arrangement shown in Figure 1A, a 8 cm × 30 μm i.d. capillary packed with 3-μm particles (300-Å pore size) was used as the first column and a 76 cm × 30 μm i.d. capillary packed with the same type of particles was used in the second longer column. A separation peak capacity of ∼600 was obtained for this 84-cm total length LC column at 10 000 psi, corresponding to ∼40% loss in separation efficiency compared to results obtained with a single, similar-length column.[9] This efficiency degradation is attributed to the existence of dead volume from use of the switching valve. The efficiency is lower than the peak capacity of ∼750 obtained for a shorter 76 cm × 30 μm i.d. capillary (packed with the same type of particles) with directly loading the sample onto the column head using the switching valve. After moving the valve dead volume toward the separation column inlet by rearranging these two columns as shown in Figure 1B, the peak capacity improved to ∼730, although the total column length was shorter (i.e., 76 cm rather than 84 cm). Thus, the influence of dead volume on gradient LC efficiency decreases as the dead volume is displaced toward the column inlet.

Table 1. Influence of SPE Column Properties on SPE-LC Separation Efficiency[a]

3-μm Particles (C18, 300-Å p_d)		
$d_{c,SPE}$ (μm)	F (μL/min)	C_p
30	0.6	∼750
50	1.3	∼750
75	4.0	∼750
100	6.0	∼550
50-μm-i.d. Columns (C18, 300-Å p_d)		
$d_{p,SPE}$ (μm)	F (μL/min)	C_p
3	1.3	∼750
5	3.5	∼750
10	12.0	∼650
50-μm-i.d. Column 3-μm Particles (C18)		
p_d (Å)	F (μL/min)	C_p
120	1.3	∼650
200	1.3	∼750
300	1.3	∼750

[a] Extraction column: the arrangement of Figure 1B was used for these investigations; 8-cm length having various inner diameters ($d_{c,SPE}$) and containing various sizes of particles ($d_{p,SPE}$) and surface pores (p_d); LC column, 76 cm × 30 μm i.d. capillary packed with 3-μm C18 Jupiter particles; the sample extraction and LC separations operated at a pressure of 10 000 psi; an ion trap MS (LCQ) used as detector; F, sample trapping flow rate; C_p, peak capacity (calculated according to ref 18). Other conditions as described in the text.

Optimal SPE effectiveness requires not only a large sample (volume and mass) processing capability but also a minimal pressure drop across the SPE column to facilitate the use of longer LC columns to provide high-efficiency separations at a given operating pressure. Since the SPE column must be connected to the LC column during separation, the influence of the SPE column void volume on gradient delay also has to be considered. According to the pressure drop expression,[25] assuming the SPE and LC columns have identical column resistant factors, the following three expressions apply:

$$F_{SPE} = \frac{\pi \epsilon_{SPE} d_{c,SPE}^2 d_{p,SPE}^2 \Delta p_{SPE}}{4 \phi \eta L_{SPE}} \quad (1)$$

$$\Delta p_{SPE}/\Delta p_{total} = \frac{1}{1 + \frac{L_{LC}}{L_{SPE}} \frac{d_{p,SPE}^2 d_{c,SPE}^2}{d_{p,LC}^2 d_{c,LC}^2}} \quad (2)$$

$$t = \frac{\epsilon_{SPE} \pi d_{c,SPE}^2 L_{SPE}}{4 F_{LC}} \quad (3)$$

where F_{SPE} and F_{LC} are volume flow rates through the SPE and LC columns, respectively; $d_{c,SPE}$ and $d_{c,LC}$ are the SPE column and

(21) Hopp, T. P.; Woods, K. R. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3824−3828.

(22) http://us.expasy.org/tools/protparam.html.

(23) Shen, Y.; Tolić, N.; Masselon, C.; Paša-Tolić, L.; Camp, D. G., II; Hixson, K. K.; Zhao, R.; Anderson, G. A.; Qian, W.; Smith, R. D. *Anal. Chem.*, submitted.

(24) Blonder, J.; Goshe, M. B.; Moore, R. J.; Pasa-Tolic, L.; Masselon, C. D.; Lipton, M. S.; Smith, R. D. *J. Proteome Res.* **2002**, *1*, 351−361.

(25) Knox, J. H. Kinetic Factors Influencing Column Design and Operation. In *Tachniques in Liquid Chromatography*; Simpson, C. F., Ed.; John Wiley & Sons: New York, 1983; p 31.

Table 2. Optimized SPE-Capillary and Nanoscale LC Arrangements at 10 000 psi[a]

LC column (3-μm particle packed)	SPE column (5-μm particle packed)	LC flow rate (μL/min)	SPE flow rate (μL/min)
86 cm × 15 μm i.d.	4 cm × 50 μm i.d.	~0.02	~8
86 cm × 30 μm i.d.	4 cm × 75 μm i.d.	~0.07	~13
86 cm × 50 μm i.d.	4 cm × 150 μm i.d.	~0.14	~75
86 cm × 75 μm i.d.	4 cm × 200 μm i.d.	~0.40	~120
86 cm × 150 μm i.d.	4 cm × 380 μm i.d.	~1.50	~400

[a] The arrangement of Figure 1C was used for these examinations of optimal SPE-LC conditions; LC and SPE flow rates at 10 000 psi.

LC column inner diameters, respectively; $d_{p,SPE}$ and $d_{p,LC}$ are the particle diameters packed in the SPE and LC columns, respectively; L_{SPE} and L_{LC} are the SPE and LC column lengths, respectively; Δp_{SPE} and Δp_{total} are the pressure drop across the SPE and SPE-LC columns, respectively; and ϵ_{SPE} is the SPE column porosity, ϕ is the column resistance factor η is the solution viscosity, and t is the gradient delay time. During the SPE sample extraction, Δp_{SPE} remains constant (e.g., 10 000 psi), and h changes slightly as the sample solution gradually replaces the mobile-phase A that originally existed in the SPE column for conditioning. In eq 3, the volume of the switching valve ports and channel is considered as negligible compared to the SPE column volume.

From expression 1, a short L_{SPE} column having a large $d_{c,SPE}$ and containing large $d_{p,SPE}$ benefits rapid sample extraction. Experimentally, minimization of L_{SPE} is limited by the geometry restriction of column connections, and ~4 cm is the shortest length practical for connecting the SPE column to the switching valve port using the arrangement shown in Figure 1C. Increasing $d_{c,SPE}$ is limited by the consideration of gradient delay as the extracolumn void volume of the SPE column increases with the square of $d_{c,SPE}$ (this situation is different from single LC where, after sample loading to the LC column, the mobile-phase flow can be redirected to avoid passage through the sample loop during the gradient separation). The use of larger $d_{p,SPE}$ is constrained by the influence of the sample SPE focusing on LC efficiency, which was experimentally evaluated. Table 1 lists the separation efficiencies obtained for a 76 cm × 30 μm i.d. capillary LC when coupled with various 8-cm-length SPE columns. The 75-μm-i.d. SPE column and the use of 5-μm C18 packing particles (300-Å pore size) provided the best SPE-LC performance, considering

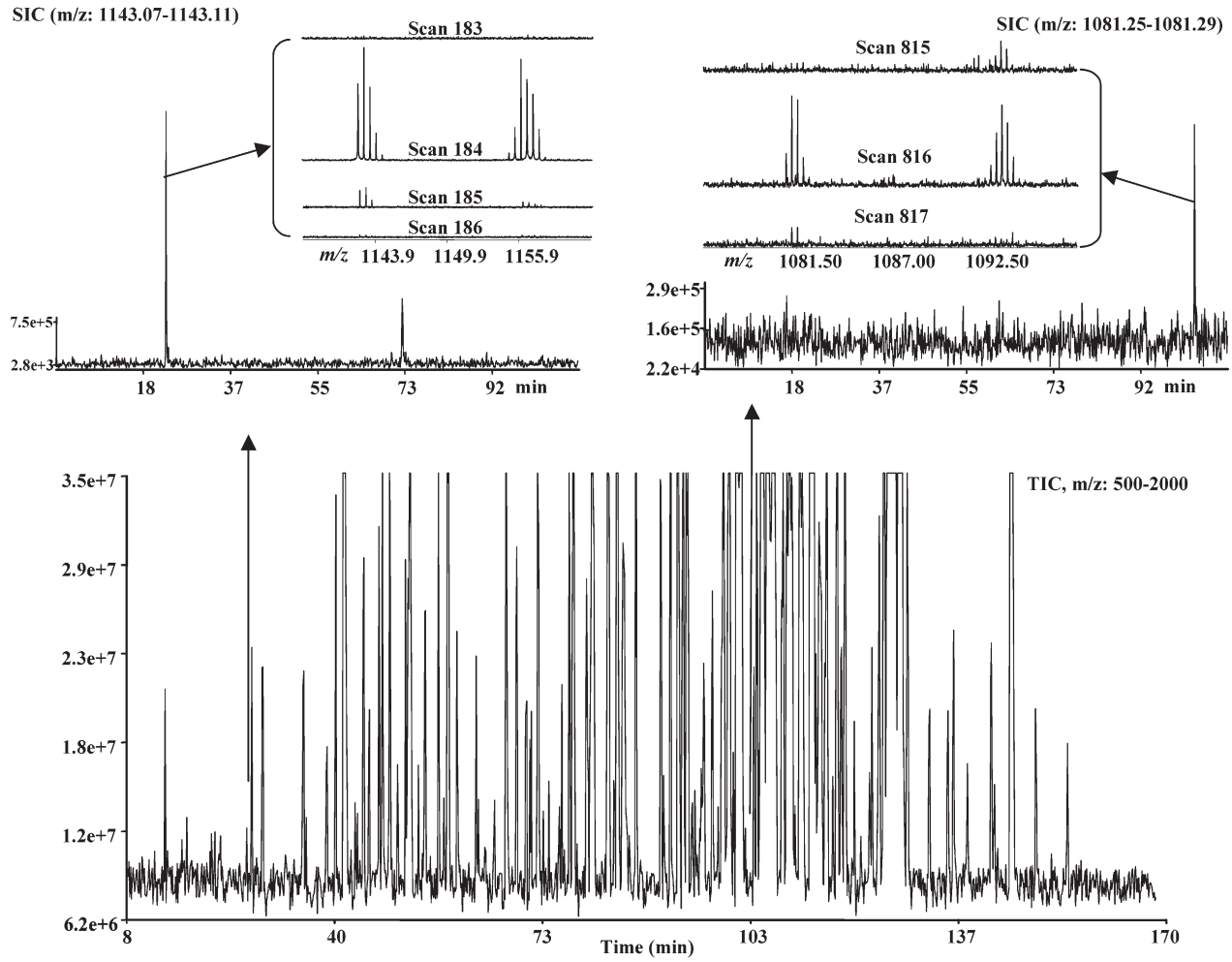

Figure 2. High-efficiency on-line microSPE-nanoLC-nanoESI-FTICR MS of 0.25 ng of $^{14}N/^{15}N$-labeled (1:1) *D. radiodurans* lysate tryptic digest. Conditions: the arrangement of Figure 1C with the optimized SPE-LC listed in Table 2 for 15-μm-i.d LC column (86-cm length) was used; mobile-phase gradient from A (H_2O, 0.2% acetic acid, 0.05% TFA, v/v) to 70% B (ACN/H_2O, 90:10, 0.1% TFA, v/v) in 180 min; the sample was loaded with 10 μL of solution for 2 min. Other conditions are described in the text.

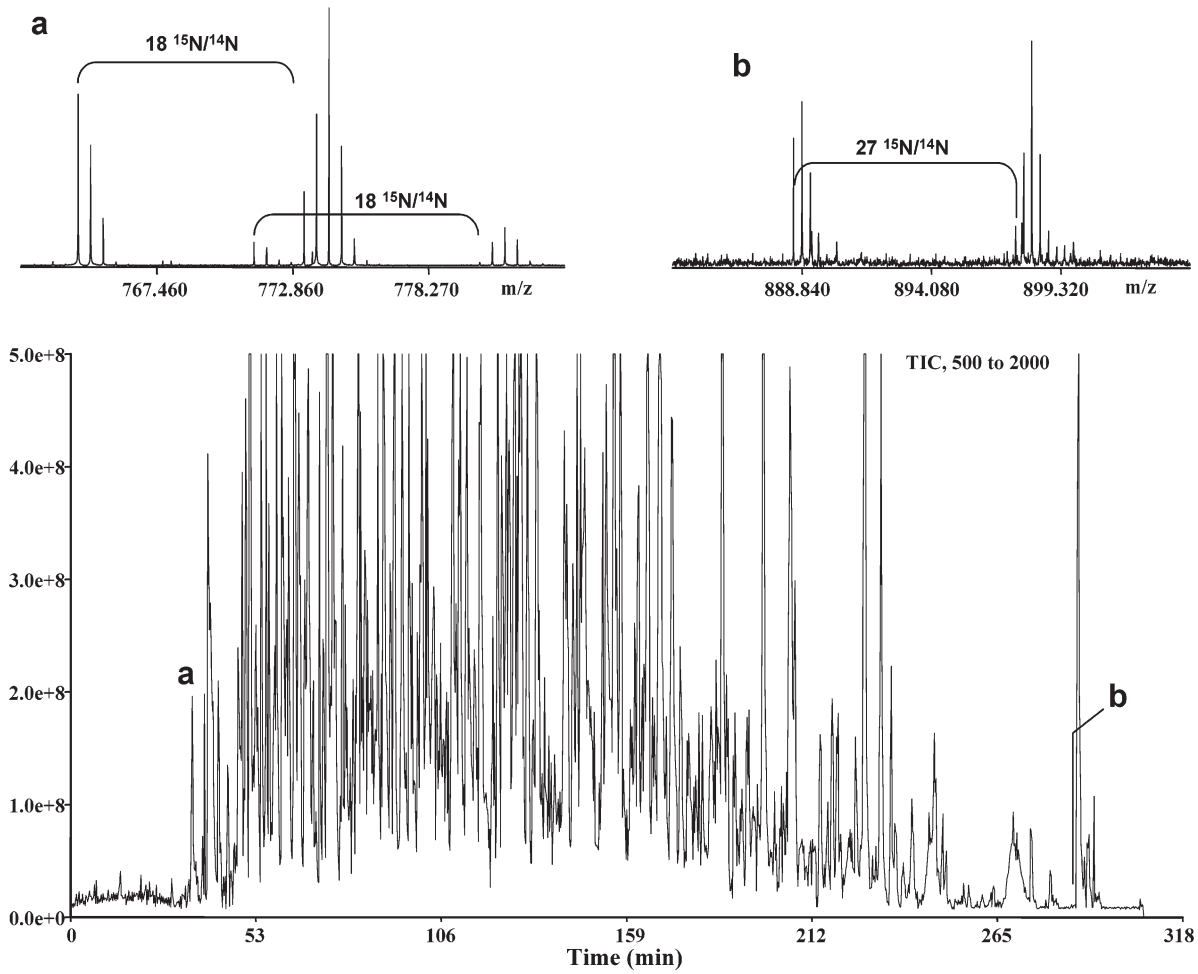

Figure 3. High-efficiency on-line microSPE-nanoLC-nanoESI-FTICR MS operated for enlarged sample size with extended LC gradient. The experiment conditions are the same as for Figure 2 except for loading 2.5 ng of the sample with 10 μL of solution and extending the gradient to 300 min.

the SPE solution-processing rate and the LC separation quality. Decreasing the particle surface pore sizes to increase the SPE sample extraction capability resulted in a slight decrease in separation efficiency.

If $L_{SPE}/L_{LC} \leq 1/20$, $d_{c,SPE}/d_{c,LC} = 2.5$ (e.g., 75-μm i.d. used for the SPE column and 30-μm i.d. for the LC column), and $d_{p,SPE}/d_{p,LC} = 1.6$ (e.g., 5-μm particles for the SPE column and 3-μm particles for the LC column), expression 2 indicates that the Δp_{SPE} is $\leq$0.3% of the Δp_{total}. This suggests that the same LC column length for on-line SPE-LC as used for single LC can be applied to achieve high-efficiency separations. Table 2 lists the column dimensions for various optimized scales of on-line SPE-capillary and nanoscale LC, which covers most capillary and nanoscale LC proteomic applications. These optimized conditions increased the SPE-LC capability by ~400-fold for processing solution volume (~40-fold from the increase in linear velocity and ~10-fold from the increase in cross section) and 10-fold for sample mass (from the increase in cross sectional area) compared to a single-dimension LC column with the same dimensions. The selection of SPE-LC scale depends on the desired sensitivity (using ESI-MS) and sample size availability, as well as the experimental robustness desired. The gradient delay depends on F_{LC} and the void volume between the LC column inlet and the gradient split. The flow rates through optimized SPE-capillary and nanoscale LC were experimentally measured, and the results are also given in Table 2. These measured flow rates allow an estimate using eq 3 of <3 min for the gradient delay due to the SPE column void volume if the SPE column i.d. is ~3-fold larger than the LC column for the arrangement shown in Figure 1C and 4-cm SPE and 86-cm LC columns (assuming both columns have ϵ of ~0.7). This delay time is negligible for the present separation times (typically ~3 h).

Combining these optimized conditions for column switching and dimensions, high-efficiency separations of peptides were achieved even for SPE coupling with the smallest (15-μm-i.d.) LC columns, where the influence of dead volume on efficiency is expected to be most significant. Figure 2 shows separation of 0.25 ng of a *D. radiodurans* lysate tryptic digest sample using on-line microSPE-nanoLC/nanoESI-FTICR MS, where 50-μm-i.d. capillary was used for SPE and 15-μm-i.d. capillary for LC. A 10-μL solution was used for sample introduction to the microSPE column with a loading speed of ~8 μL/min at 10 000 psi, compared to 20 nL/min for loading the solution directly to the 15-μm-i.d. LC column at the same pressure. We estimate that any sample losses due to residue in the syringe, valve ports, and autosampler connection tubes can be reduced to <1% if the loading of a 10-μL sample solution is followed by 30 μL of solution obtained from washing the sample vial and loading devices (to give a total sample loading

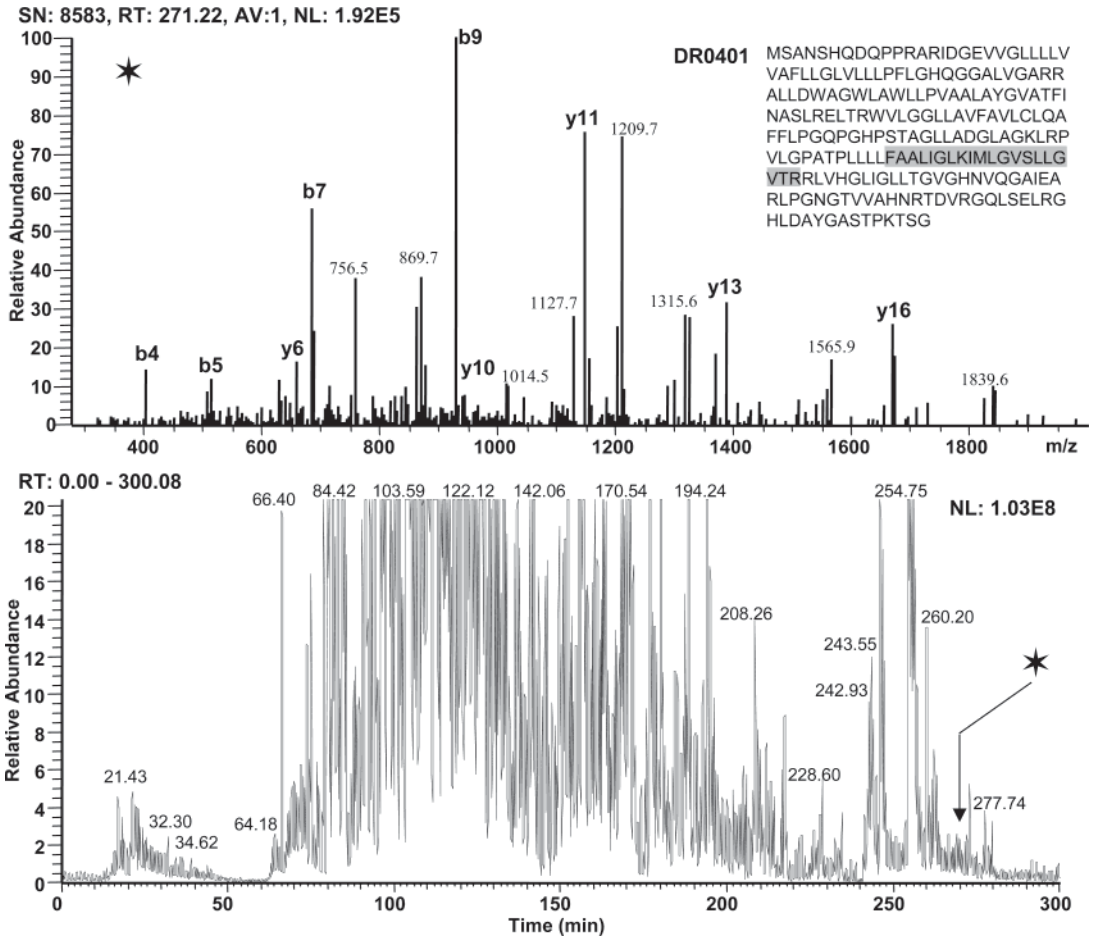

Figure 4. High-efficiency on-line SPE-capillary LC-microESI ion trap MS/MS of *D. radiodurans* membrane protein tryptic digest. Conditions: the arrangement of Figure 1C with the optimized SPE-LC listed in Table 2 for 150-μm-i.d LC column (86-cm length) was used; 150 μg of the *D. radiodurans* membrane protein tryptic digest sample was loaded with 500 μL of sample solution and another 500 μL of solution from washing the sample vial. Others conditions are the same as for Figure 3.

time of ~5 min). During this run, peptides eluted across an effective separation time window of ~160 min with intensity-dependent LC peak widths. Low-intensity (~10^6 signal amplitude) peptides were often observed in only single scans (as marked in Figure 2), moderate-intensity (i.e., 10^7-10^8 signal amplitude) peptides were observed across two to four scans, and high-intensity peaks (i.e., $>10^9$ signal amplitude) were found in larger numbers of scans. An average peak capacity of ~950 is estimated (for moderate-intensity peaks of one to three scans, and 7.25 s/scan in this work) and thus with only ~5% efficiency loss due to the use of on-line coupled SPE.[9] This SPE arrangement is now being used for ultrasensitive proteomics studies (to be reported elsewhere).[23] For tandem MS analyses with an ion trap MS, both extended peptide LC elution and sample mass size aid MS/MS data acquisition, and separation conditions for such purposes were also optimized. Figure 3 shows a separation of a 2.5-ng *D. radiodurans* sample using the on-line microSPE-nanoLC-nanoESI with FTICR MS. Extending the separation to ~5 h yielded a separation efficiency similar to that shown in Figure 2. Below we discuss the applicability of such high-efficiency separations for tandem MS proteomic analyses using 150-μm-i.d. LC columns.

High-Efficiency On-Line SPE-Capillary LC/MicroESI MS/MS proteomics. Loading relatively large sized samples (compared to the LC column dimensions) can improve MS/MS protein identification and proteome coverage and greatly benefits from the use of SPE to improve method ruggedness and ease of automation. For *D. radiodurans* membrane protein tryptic digest samples, we failed to load 150-μg samples directly onto a 150-μm-i.d. capillary column (packed with 5-μm C18 particles) in 10-μL solutions due to column clogging. With the coupled SPE (optimal i.d. of 380 μm, ~6.4 times larger cross sectional area than that of the 150-μm-i.d. LC column; see Table 2), 500-μL sample solution volumes could be successfully loaded within 3 min (being followed by another 500 μL of solution from washing the sample vial) and then efficiently separated after switching the loaded sample to an 86 cm × 150 μm i.d. LC column (packed with 3-μm particles), as shown in Figure 4. Figure 4 also shows an example of MS/MS identification for a late-eluting peak. In this single 5-h LC/MS/MS analysis, 1118 different tryptic peptides from 449 *D. radiodurans* open reading frames (ORFs) (of 3116 total predicted) were assigned using the database search criteria reported by Yates and co-workers.[4] Similarly, for 150 μg of the *S. oneidensis* lysate tryptic digest, 1646 *S. oneidensis* different peptides from 634 ORFs (this organism has 4944 total predicted ORFs) were assigned from a single 5-h run with high-efficiency SPE-capillary LC/MS/MS (Figure 5).

The influence of the LC separation efficiency and sample loading on LC/MS/MS peptide detection was also investigated,

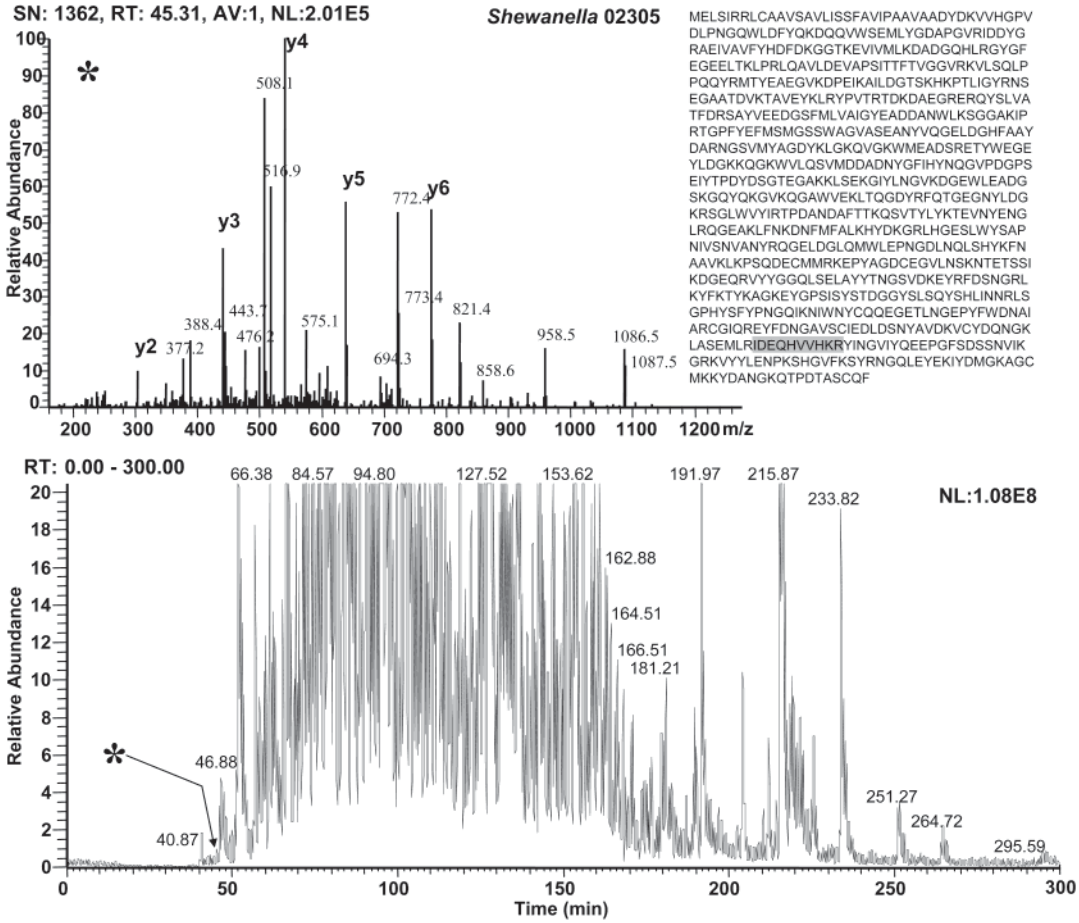

Figure 5. High-efficiency on-line SPE-capillary LC-microESI-ion trap MS/MS of *S. oneidensis* soluble protein tryptic digest. Conditions are the same as for Figure 4 except for loading 150 μg of the *S. oneidensis* soluble protein tryptic digest.

and results obtained for the *S. oneidensis* sample are presented in Table 3. Replacing an 86 cm × 150 μm i.d. capillary containing 3-μm particles with a 60 cm × 150 μm i.d. capillary packed with 5-μm particles (note: both 3- and 5-μm particles are Jupiter stationary phase bonded with C18 and having a surface pore size of 300 Å from Phenominex) degraded the separation efficiency and reduced the numbers of total peptides identified by ~45% for 150-μg samples. These numbers were further reduced by ~40% when the sample size was decreased to 10 μg, an observation attributed largely to an insufficient S/N for low-abundance species due to both sample size reduction and separation efficiency degradation. Slowing peak elution during MS/MS data acquisition can improve data acquisition for coeluted minor components but compromises the detection sensitivity for low-abundance species due to peak broadening. Currently, we are working to further improve capillary LC efficiency by extending the LC operation pressure.

The SPE capability for peptide extraction/desorption was also evaluated by examining the hydrophilicity/hydrophobicity of peptides identified. Tryptic peptides from all *S. oneidensis* and *D. radiodurans* ORFs have hydrophobicity parameters ranging from −1.78 to +2.55, as shown in Figure 6A. The hydrophobicity distribution range of tryptic peptides detected with very high confidence (e.g., with XCorr ≥ 4.0) from both *S. oneidensis* and *D. radiodurans* samples is from −0.93 to +1.82 (the most hydrophilic peptide was observed from the *S. oneidensis* sample, and the most hydrophobic peptide was observed from the *D. radiodurans* membrane protein sample), as shown in Figure 6B. This hydrophobicity range covers ~98% of all tryptic peptides from the two proteomes, suggesting that SPE-LC can allow broad proteome coverage and that limitations will most likely arise due to other factors, such as sensitivity and the effectiveness of peptide identification by MS/MS. The extraction wastes from SPE were also examined using LC/MS/MS, and no peptides were observed.

The large hydrophilicity/hydrophobicity window supplied by the SPE-capillary LC for peptide detection also enabled effective identification of many highly hydrophobic proteins. Table 4 summarizes properties of proteins that were identified from *S. oneidensis* and *D. radiodurans*. For the *D. radiodurans* membrane protein sample, we identified 81 integral inner membrane proteins containing 1−16 mapped transmembrane domains, 30 integral outer membrane proteins, and 29 periplasmic proteins. A total of 46 hydrophobic proteins from the membrane subproteome have positive GRAVY values that ranged from +0.005 to +0.97, suggesting effective coverage for highly hydrophobic integral membrane proteins containing multiple mapped transmembrane domains (TMDs). Highly hydrophobic integral membrane proteins containing multiple TMDs are generally not detectable using 2-D gels.[26] The peptide detection approach is more efficient in

(26) Santoni, V.; Molloy, M.; Rabilloud, T. *Electrophoresis* **2000**, *21*, 1054−1070.

Table 3. LC Efficiency and Sample Loading Influence on MS/MS Peptide Detection*

150 μg of *S. oneidensis* lysate tryptic digest on an 85 cm × 150 μm i.d. capillary packed with 3-μm C18 (300-Å pore size) operated at 10 000 psi
SPE-LC (Figure 1C, SPE dimensions listed in Table 2 for 150-μm-i.d. LC; peak capacity, ~1000)
total peptides and searching scores
10853 peptides, XCorr > 1
5335 peptides, XCorr > 2
1642 peptides, XCorr > 3
822 peptides, XCorr > 4
310 peptides, XCorr > 5
total tryptic peptides
2426 (XCorr > 2)

150 μg of *S. oneidensis* lysate tryptic digest on a 60 cm × 150 μm i.d. capillary packed with 5-μm C18 (300-Å pore size operated at 5000 psi
SPE-LC (Figure 1C, SPE dimensions listed in Table 2 for 150-μm-i.d. LC; peak capacity, ~550)
total peptides and searching scores
4239 peptides, XCorr > 1
2065 peptides, XCorr > 2
728 peptides, XCorr > 3
424 peptides, XCorr > 4
166 peptides, XCorr > 5
total tryptic peptides
1043 (XCorr > 2)

10 μg of *S. oneidensis* lysate tryptic digest on a 60 cm × 150 μm i.d. capillary packed with 5-μm C18 (300-Å pore size) operated at 5000 psi
single LC (peak capacity, ~550)
total peptides and searching scores
3341 peptides, XCorr > 1
1293 peptides, XCorr > 2
464 peptides, XCorr > 3
260 peptides, XCorr > 4
113 peptides, XCorr > 5
total tryptic peptides
645 (XCorr > 2)

[a] The conditions were described in the text. The listed numbers of peptides detected at various XCorr values were those directly output from the SEQUEST program log text.

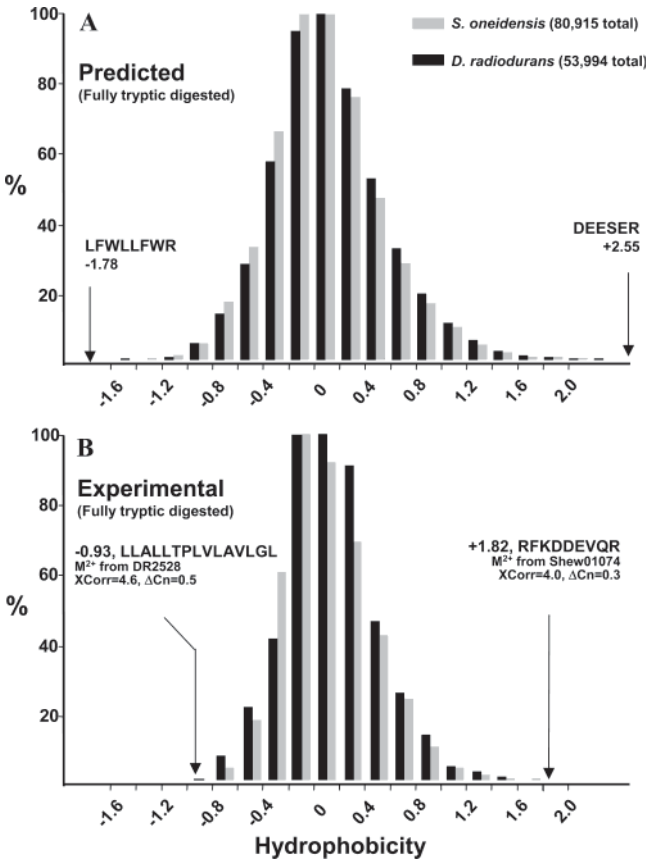

Figure 6. Hydrophobicity distributions for tryptic fully digested peptides from (A) all predicted proteins (open reading frames) and (B) experimental detection using high-efficiency SPE-capillary LC/ion trap MS/MS. The MS/MS data were collected from runs of Figures 4 and 5; the peptides were assigned according to the reported criteria;[4] the hydrophobicity parameter values were calculated according to ref 21.

probing hydrophobic integral membrane proteins;[4,27] however, highly hydrophobic transmembrane spanning peptides have rarely been identified. Using single-dimension high-efficiency SPE-capillary LC/MS/MS combined with the membrane protein enrichment sample preparation, a wide range of highly hydrophobic multiple TMDs containing integral membrane proteins and transporters belonging to the ATP binding cassette (ABC) superfamily were identified (see Table 4). Identification of these proteins enables studies of their import/export functions in the cell membrane.[28,29]

CONCLUSIONS

The ability to more effectively conduct global proteome analyses using practical sample sizes with high-efficiency separations and MS analysis has been investigated. We have shown that effects due to separation void volumes are reduced as their source is displaced toward the column inlet. The optimized connection of the SPE column on-line to the capillary or nanoscale LC (as shown in Figure 1C), the proper matching of the SPE and capillary or nanoscale LC dimensions (as listed in Table 2), and the use of 3-μm C18 packing particles for capillary and nanoscale LC and 5-μm C18 for SPE (both sizes of particles having surface pores of 300 Å) increased the sample mass and solution volume processing capability by ~10- and ~400-fold, respectively. These improvements enabled the loading of ~1-mL sample solutions with capillary LC and >20-μL samples with nanoscale capillary LC, in less than a few minutes. Under optimal conditions, on-line SPE-capillary and nanoscale LC provided separation peak capacities approaching 1000, even with the use of a 15-μm-i.d. capillary LC that provides the greatest sensitivity. These developments allow much smaller samples to be analyzed with minimal losses and also make the analysis of hydrophobic proteins more practical since column clogging problems are greatly reduced. The selection of most appropriate SPE-LC system scales listed in Table 2 is determined by method sensitivity requirements, as well as sample qualities. Currently, we are using 15-μm-i.d. capillary LC for SPE-LC to conduct ultrasensitive proteomic studies, 150-μm-i.d. capillary LC for SPE-LC for more problematic samples available in relatively large quantities (e.g., hundreds of micrograms), and 30- and 50-μm-i.d. capillary LC for typical proteomic analyses. Both separation efficiency and sample quantity significantly influence

(27) Han, D. K.; Eng, J.; Zhou, H.; Aebersold, R. *Nat. Biotechnol.* **2001**, *19*, 949−951.
(28) Higgins, C. F.; Lipton, K. J. *Science* **2001**, *293*, 1782−1784.
(29) Paulsen, I. T.; Sliwinski, M. K.; Saier, M. H. *J. Mol. Biol.* **1998**, *277*, 573−592.

Table 4. Properties of Proteome Proteins Identified Using High-Efficiency Capillary LC-MS/MS[a]

	Total Property Ranges for Detected Proteins	
p*I*	4.25 (ShewORF02927)	12.20 (DR2152)
MW	5605 (DR2152)	285097 (ShewORF02927)
GRAVY[b]	−1.65 (DR2152)	0.97 (DR2589)

	Properties of Identified Membrane Proteins				
	integral inner membrane proteins[c]	integral outer membrane proteins[c]	periplasmic proteins[c]	cytoplasmic proteins[c]	total
no. identified	81	30	29	86	226
hydrophobic proteins[b]	45	7	3	1	46
GRAVY range[b]	−0.751 ± 0.97	−0.681 to + 0.76	−0.81 to +0.31	−1.651 to +0.10	−1.651 to +0.97
mapped TMD range[c]	1−16	na[d]	na[d]	na[d]	1−16
p*I* range	4.66−11.45	4.63−11.13	5.76−12.22	4.59−12.19	4.59−12.19
protein mass (kDa)	10.0−125.9	8.0−123.7	11.4−73.6	5.6−179.0	5.6−179

	Some ABC Transporters Identified		
ORF	note	GRAVY	no. TMD
DR2589	iron ABC transporter, permease protein	0.96	8
DR1037	branched-chain amino acid ABC transporter	0.85	8
DR2155	amino acid ABC transporter	0.65	5
DR0364	peptide ABC transporter	0.61	6
DR0959	peptide ABC transporter	0.61	6
DR1036	branched-chain amino acid ABC transporter	0.61	11
DR0365	peptide ABC transporter	0.58	5
DR1570	peptide ABC transporter	0.52	6
DR0958	peptide ABC transporter	0.47	6

[a] The arrangement of Figure 1C with the optimal SPE-LC conditions for 150-μm-i.d LC column as listed in Table 2; the experimental conditions are the same as in Figures 4 and 5; proteins were identified using the SEQUEST algorithm and the genome translated protein database.[4] [b] Hydropathy values for identified protein GRAVYs were calculated using ProtParam.[23] [c] Subcellular location and TMDs in identified proteins predicted by PSORT (http://psort.nibb.ac.jp/form.html) and hydrophobic proteins were recognized by positive GRAVY values. [d] na, not available.

the number of peptides detected or identified using MS/MS, and the LC efficiencies achieved at 10 000 psi provided good coverage relative to the use of moderate-efficiency LC involving two-dimensional separations. The hydrophilicity/hydrophobicity window provided by the SPE-capillary and nanoscale LC covered up to 98% of all the tryptic peptides.

Although this study focused only on improving sample introduction for capillary and nanoscale LC, the optimized capillary connection and switching approaches described here are also applicable for coupling different capillaries with multidimensional LC, multifunctional LC, or on-line physical/chemical processing (e.g., protein extraction and enzymatic digestion) in automated formats.

ACKNOWLEDGMENT

We thank the U.S. Department of Energy's Office of Biological and Environmental Research and the National Cancer Institute (Grant CA86340) for support of portions of this research. We also thank Stanley Sterns of Valco Instruments for providing switching valves for 10 000 psi operation. Pacific Northwest National Laboratory is operated by the Battelle Memorial Institute for the U.S. Department of Energy through Contract DE-ACO6-76RLO 1830.

Received for review February 19, 2003. Accepted April 25, 2003.

AC0300690