ELSEVIER

Journal of Chromatography A, 979 (2002) 241–247

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Miniaturized membrane-based reversed-phase chromatography and enzyme reactor for protein digestion, peptide separation, and protein identification using electrospray ionization mass spectrometry

Yan Li, Jonathan W. Cooper, Cheng S. Lee*

Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, USA

Abstract

A commonly used capillary fitting is employed for housing miniaturized membrane chromatography for performing reversed-phase peptide separations. By placing a hydrophobic and porous polyvinylidene fluoride membrane around the end of a polymer sleeve, the assembly of capillary fitting not only provides the stationary phase, but also establishes the necessary flow paths using capillary connections. The miniaturized membrane chromatography system is coupled with a micro-enzyme reactor containing immobilized trypsins for performing rapid protein digestion, peptide separation, and protein identification using electrospray ionization mass spectrometry. Separation performance of cytochrome *c* digest in miniaturized membrane chromatography is compared with the results obtained from micro-LC and capillary LC. The efficacy and the potentials of miniaturized membrane chromatography in tryptic mapping are reported. The use of miniaturized membrane chromatography allows significant reduction in sample consumption together with enhanced detection sensitivity. By minimizing the void volume in miniaturized membrane chromatography, the elution times of cytochrome *c* peptides are significantly shortened in this study in comparison with our previous results, and are comparable with those in micro-LC and capillary LC using considerably higher mobile phase flow-rates.

Keywords: Instrumentation; Membranes; Proteins; Peptides; Cytochromes

1. Introduction

Membrane chromatography has been introduced for solving two main issues encountered in scaling up chromatographic separations using fine particulate support media, namely technical challenges in overcoming high back pressure and slow solute diffusion kinetics [1]. Subsequently, fast chromatographic separations were achieved without applying extremely high pressure in membrane chromatography [2]. Since then, the applications of membrane chromatography have attracted great attention from pharmaceutical industries [3], particularly for protein separations [4]. Membrane chromatography has also been attempted to perform separations on non-protein target analytes such as DNA plasmids [5], oligonucleotides [6,7], peptides [6,8], amino acids [9,10], and small hydrophobic molecules [6].

In addition to membrane chromatographic separations, various proteins and enzymes have been immobilized on the compact and porous membrane media for performing affinity chromatography and enzyme reactions [11,12]. To miniaturize high-performance membrane chromatography, two layers of

*Corresponding author. Tel.: +1-301-405-1020; fax: +1-301-314-9121.

E-mail address: cl143@umail.umd.edu (C.S. Lee).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01242-6

polyvinylidene fluoride (PVDF) membrane, employed as the stationary phase, were sandwiched between two polydimethylsiloxane (PDMS) substrates containing microchannels [13]. On-line coupling of miniaturized membrane chromatography with a miniaturized trypsin membrane reactor was demonstrated for protein digestion, peptide separation, and protein identification using electrospray ionization mass spectrometry (ESI-MS).

In addition to miniaturized reversed-phase membrane chromatography, two layers of PVDF membrane adsorbed with bovine serum albumin (BSA) were sandwiched between two PDMS substrates for performing chiral separation of racemic tryptophan and thiopental mixtures [14]. Based on the large surface area to volume ratio of porous membrane media, BSA adsorbed onto the PVDF membranes enables the high resolution separation of racemic mixtures with sample consumption of sub-nanogram or less in the integrated plastic microfluidics network. Furthermore, the utilization of membrane pore diameters in the submicron range effectively eliminates the constraints of diffusional mass-transfer resistance commonly encountered during chiral chromatography separations.

The void volume inside the two layers of PVDF membrane exposed to analyte molecules was estimated to be only 80 nl. However, most of the total dead volume in miniaturized membrane chromatography [13,14] was contributed by the capillary connections and microchannels. To significantly reduce the dead volume and the elution time, a commonly used capillary fitting is employed in this study for directly housing miniaturized membrane chromatography. By placing a hydrophobic and porous PVDF membrane around the end of a polymer sleeve, the assembly of capillary fitting, containing a length of fused-silica capillary, provides the necessary flow paths and the stationary phase for performing reversed-phase separations.

The capillary format of miniaturized membrane chromatography allows straightforward integration with UV absorbance detection and ESI-MS for analyte identification. On-line coupling of miniaturized membrane chromatography with a micro(μ)-trypsin membrane reactor through the use of a μ-sample injector is demonstrated for achieving rapid and effective protein digestion, peptide separation, and protein identification in an integrated and miniaturized platform. By comparing with the results obtained from μ-LC and capillary LC, the efficacy and the potentials of miniaturized membrane chromatography in tryptic mapping and protein identification are reported.

2. Experimental

2.1. Materials and chemicals

Bovine pancreatic trypsin and horse heart cytochrome *c* were purchased from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) and ultrapure urea were obtained from Bio-Rad (Hercules, CA, USA) and ICN (Aurora, OH, USA), respectively. Acetic acid, acetonitrile, methanol, and trifluoroacetic acid were acquired from Fisher (Fair Lawn, NJ, USA). All solutions were prepared using water purified by a Nanopure II system (Branstead, Dubuque, IA, USA) and further filtered with a 0.22 μm membrane (Millipore, Bedford, MA, USA).

2.2. Construction of miniaturized membrane chromatography using capillary fitting

A miniaturized membrane chromatography system (see Fig. 1) was constructed using a capillary fitting (μ-tight true ZDV union) obtained from Upchurch Scientific (Oak Harbor, WA, USA). The PVDF membranes with pore diameter of 0.1 μm (Immobilon-P^{SQ}) were acquired from Millipore. A small piece of PVDF membrane was curled widthwise and folded lengthwise over the end of a polymer sleeve containing a length of fused-silica capillary (50 μm I.D.×192 μm O.D., Polymicro Technologies, Phoenix, AZ, USA). It is important to note that the membrane overlapped the face of the sleeve in all directions, and the capillary was positioned flush to the end of the sleeve. The assembly of capillary, sleeve, and membrane was then slid inside the capillary fitting and properly tightened, providing the necessary seal for the membrane sandwiched between the sleeve and the capillary fitting housing.

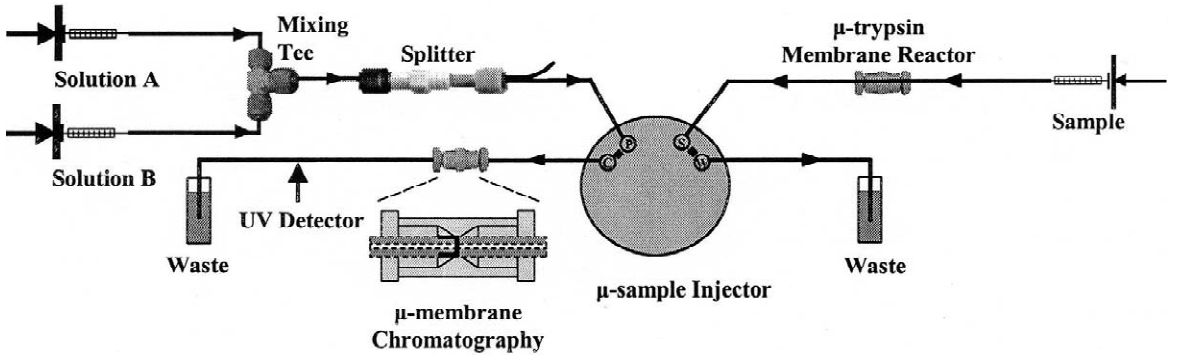

Fig. 1. On-line coupling of μ-trypsin membrane reactor with miniaturized membrane chromatography through the use of a μ-sample injector. The inset presents the enlarged view of capillary fitting containing a piece of PVDF membrane.

2.3. Assembly of μ-trypsin membrane reactor

The components and procedures for assembly of a μ-trypsin membrane reactor inside a capillary fitting were the same as those utilized for the construction of miniaturized membrane chromatography. The immobilization of trypsin onto the PVDF membrane and detailed characterization of rapid and effective proteolytic digestion inside the reactor were reported in our previous work [15]. The ends of the capillary fitting were connected to a Harvard Apparatus 22 syringe pump (Holliston, MA, USA) and the sample port of a two-position/four-port μ-sample injector/switching valve (Valco Instruments, Houston, TX, USA), respectively (see Figs. 1 and 2).

2.4. Coupling of μ-trypsin membrane reactor with miniaturized membrane chromatography

Horse heart cytochrome *c* was completely denatured in a solution containing 8 *M* urea and 10 m*M* Tris overnight at room temperature. Denatured cytochrome *c* was reconstituted in a solution of 10 m*M* Tris and 1 *M* urea using Millipore microcentrifuge ultrafiltration filters (regenerated cellulose membrane, nominal molecular mass cutoff of 5000). A 250-μl gas tight syringe (Hamilton, Reno, NV, USA) was filled with a solution containing denatured and reconstituted cytochrome *c* at a concentration of 10 μg/ml. The protein solution was delivered into a μ-trypsin membrane reactor at a flow-rate of

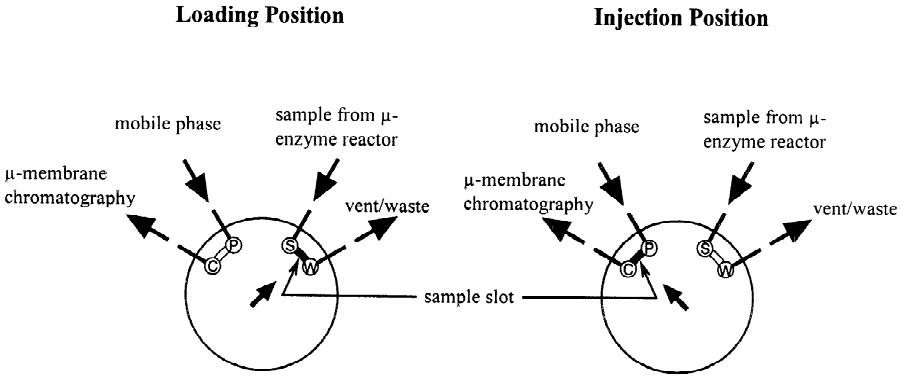

Fig. 2. Schematic of loading and injection positions in a μ-sample injector. The injector contains four ports and can be switched between two positions for sample loading and injection.

0.1 μl/min for performing trypsin digestion. The capillary exit in the reactor was connected to the sample port of a Valco μ-sample injector (see Fig. 1) for peptide sample loading and on-line coupling with miniaturized membrane chromatography.

The miniaturized membrane chromatography system was first flushed with a solution containing 20% (v/v) acetonitrile for 30 min and then equilibrated with deionized water for another 30 min at a flow-rate of 0.1 μl/min. The cytochrome *c* digest from a μ-trypsin membrane reactor was introduced into a 0.1 μl injection loop inside a Valco μ-sample injector at a flow-rate of 0.1 μl/min. By switching from the loading to the injection positions (see Fig. 2), the mobile phase delivered at a flow-rate of 0.1 μl/min performed sample injection and peptide separation through the miniaturized membrane chromatography system.

Solvents A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile) were delivered by two separate Harvard Apparatus PHD 2000 programmable syringe pumps (see Fig. 1) for generating a linear gradient of 5–40% acetonitrile in 20 min. The mobile phase leaving a Upchurch μ-static mixing tee was carried at a flow-rate of 10 μl/min. Prior to the mobile phase port of a Valco μ-sample injector, the flow-rate was reduced to 0.1 μl/min using a Upchurch silica sealtight Y connector.

The other end of the capillary fitting housing miniaturized membrane chromatography was connected to a Linear 100 multi-wavelength detector (Linear Instruments, Reno, NV, USA) or a sheath liquid interface prior to a Perkin-Elmer Sciex (Foster City, CA, USA) API 150 EX single quadrupole mass spectrometer. The sheath liquid interface, which provided the necessary electrical connection for inducing the electrospray process, was constructed as described previously [16,17]. The sheath liquid, composed of methanol–water–acetic acid (50:49:1, v/v) at pH 2.6, was delivered by a Harvard Apparatus 22 syringe pump at a flow-rate of 1 μl/min. An electrospray voltage of 4.0 kV was employed for generating a stable spray of resolved peptides eluted from miniaturized membrane chromatography. Peptides were measured by scanning from m/z 200 to m/z 1800 at a scan rate of 2 s/scan.

3. Results and discussion

High-performance membrane chromatography has been successfully employed for the purification and separation of biological molecules [18–21]. In high-performance membrane chromatography, layers of finely controlled and organized, microporous membrane are used as the stationary phase, and the functional ligands can be attached to the outer and inner surface areas of membrane. The membranes as the porous separation media can be in the forms of a compact and porous disk, a hollow fiber, and a rod. By comparing with high-performance liquid chromatography (HPLC), membrane chromatography exhibits the advantages of low hydrodynamic pressure drop, rapid analysis, and less susceptibility to fouling and clogging. The use of various chromatography modes in membrane chromatography, including affinity, ion-exchange, hydrophobic interaction, and reversed-phase, is dependent on the chemical modification of membranes.

In this study, the miniaturized membrane chromatography was developed by securely placing a small piece of PVDF membrane at the tip of a polymer sleeve inside a commonly used capillary fitting. This PVDF membrane (Immobilon-P^{SQ}, pore diameter of 0.10 μm) is naturally hydrophobic and is designed for Western transfers and protein sequencing procedures. The porous structure of this PVDF membrane provides a large internal surface area (~400 cm^2/cm^2 of front surface) for peptide interaction. Thus, the capillary fitting containing fused-silica capillaries not only provides the stationary phase for performing reversed-phase peptide separations, but also affords all of the necessary flow connections with minimized dead volume.

Furthermore, the miniaturized membrane chromatography device was coupled to a μ-trypsin membrane reactor for achieving on-line protein digestion and peptide separation. As discussed in our previous study [15], the extent of protein digestion in a membrane-based enzyme reactor can be directly controlled by the residence time of protein analytes inside the trypsin-immobilized membrane, the reaction temperature, and the protein concentration. The residence time of protein analytes, in turn, is a function of membrane thickness, polymer sleeve

dimensions (in the capillary fitting), and protein infusion rate. By applying the same experimental conditions as those reported previously [15], complete digestion of cytochrome *c* was anticipated with a residence time of only ~30 s and the resulting cytochrome *c* peptides were directly introduced into miniaturized membrane chromatography using a μ-sample injector (see Fig. 1).

As shown in Fig. 3A, cytochrome *c* peptides were eluted by a linear mobile phase gradient of 5–40% in 20 min and were detected by UV absorbance at 214 nm. Approximately 70 fmol of digested cytochrome *c* sample from the μ-trypsin membrane reactor was injected for the separation. Theoretically, there should be 21 peptides in the cytochrome *c* digest based on the specificity of trypsin and the sequence of cytochrome *c*. For several reasons, less than the theoretical number of peptides is frequently seen and

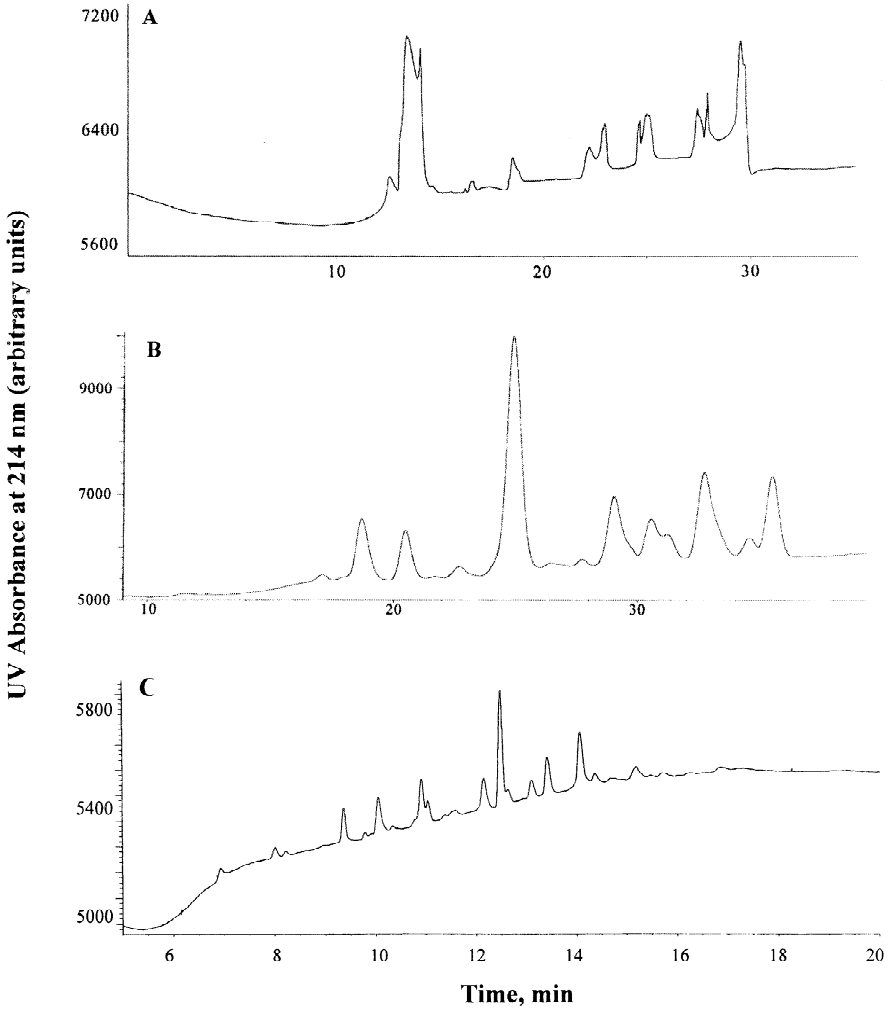

Fig. 3. Comparison of cytochrome *c* peptide separations achieved by (A) miniaturized membrane chromatography: 70 fmol sample loading, a linear gradient of 5–40% acetonitrile in 20 min at a flow-rate of 0.1 μl/min, and UV detection at 214 nm; (B) capillary LC: 25 pmol sample loading, a linear gradient of 5–40% acetonitrile in 20 min at a flow-rate of 2 μl/min, 10 cm×150 μm I.D. reversed-phase C_{18} column, UV detection at 214 nm; (C) μ-LC: 150 pmol sample loading, a linear gradient of 10–40% acetonitrile in 15 min at a flow-rate of 40 μl/min, 15 cm×1 mm I.D. reversed-phase C_{18} column, UV detection at 214 nm.

reported for tryptic digests. One is that very small, hydrophilic peptides elute from a reversed-phase column unretained. Another is that similar peptides may co-elute during the separation.

By comparing with the separation results obtained from capillary LC and μ-LC (see Fig. 3B and C), the number of peaks in the three chromatograms is roughly the same. However, there are differences in the experimental conditions and detection sensitivities that should be noted. One difference was the use of a capillary window with the dimensions of 100 μm I.D.×200 μm O.D. for UV detection in μ-LC and capillary LC. The capillary window employed for miniaturized membrane chromatography had the dimensions of 50 μm I.D.×200 μm O.D. By taking into account the difference in optical path length for absorbance detection, the concentration of eluted cytochrome *c* peptides from miniaturized membrane chromatography (Fig. 3A) was comparable to that measured in μ-LC (Fig. 3C), and was about 30–50% of that obtained from capillary LC (Fig. 3B).

However, approximately 150 pmol of cytochrome *c* peptides from overnight proteolytic digestion using solution trypsin was injected for the separation in μ-LC. The sample consumption for capillary LC was decreased to 25 pmol and was still at least 300 times of that utilized in miniaturized membrane chromatography. The volume flow-rate of organic solvent gradient was 40 μl/min for μ-LC and was reduced to 2 and 0.1 μl/min for capillary LC and miniaturized membrane chromatography, respectively. Furthermore, the separation efficiency measured from miniaturized membrane chromatography was lower than those obtained from μ-LC and capillary LC. The poor separation efficiency of miniaturized membrane chromatography was mainly contributed by potential channeling through the membrane media which was not optimized for performing the separation.

A miniaturized membrane chromatography system was reported and demonstrated in our previous studies [13] by sandwiching two layers of PVDF membrane between two PDMS substrates containing microchannels. By comparing with our previous results [13], an approximately 400-fold decrease of sample consumption was achieved by miniaturized membrane chromatography constructed in this study. Furthermore, the void volume was reduced by at least one order of magnitude. As the result, the elution times of cytochrome *c* peptides were significantly shortened and were comparable with those in μ-LC and capillary LC with considerably higher mobile phase flow-rates (see Fig. 3).

Instead of using UV detection, the capillary format of miniaturized membrane chromatography makes it possible to directly couple with a sheath liquid interface for performing ESI-MS detection of eluted cytochrome *c* peptides. On-line ESI-MS analysis of separated cytochrome *c* peptides from miniaturized membrane chromatography is shown in Fig. 4. By comparing with the results shown in Fig. 3A, the increase in the migration time of cytochrome *c* peptides was attributed to the increase in the dead volume associated with the sheath liquid interface and an additional capillary connection. The sheath liquid interface, which provided the necessary electrical connection for inducing the electrospray process, possibly contributed to additional band broadening as observed in the total ion current (TIC) chromatogram (see Fig. 4). All of the cytochrome *c* peptides in the TIC chromatogram were directly identified on the basis of mass spectra taken from the average scans under the peaks. The mass spectra of peptides taken from the average scans under the peaks with the elution times of 23.3, 25.8, and 27.3 min are shown in Fig. 5. The amino acid sequence of

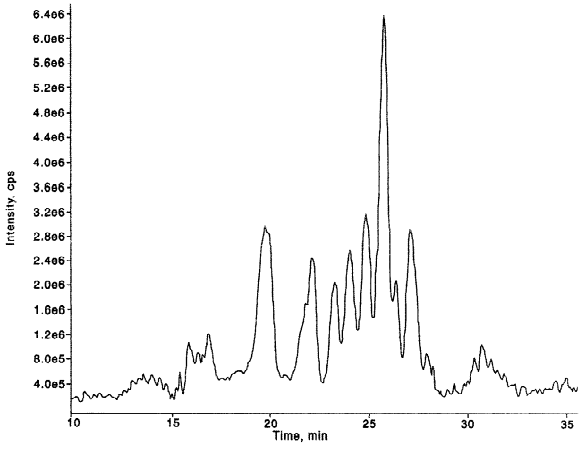

Fig. 4. Total ion current monitoring of cytochrome *c* peptides eluted from miniaturized membrane chromatography using the positive ESI mode.

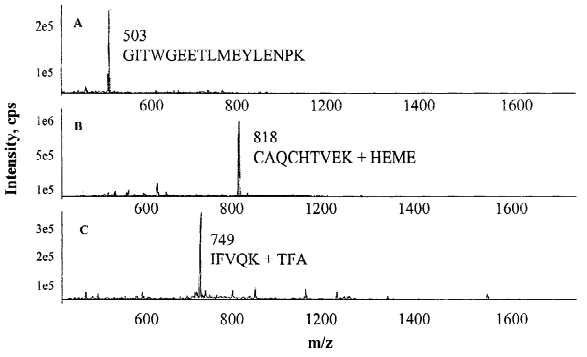

Fig. 5. Positive ESI mass spectra taken from the average scans under the peaks with the elution times of (A) 23.3 min, (B) 25.8 min, and (C) 27.3 min in Fig. 4.

each peptide was obtained by searching the database using the mass as the constraint.

4. Conclusion

On-line coupling of a μ-trypsin membrane reactor with miniaturized membrane chromatography is developed and demonstrated as an integrated microanalytical tool enabling rapid protein digestion, high resolution peptide separation, and sensitive protein identification using ESI-MS. The combination of chromatographic separation with ESI-MS contributes to further enhancement in the dynamic range and the detection sensitivity for the analysis of a protein digest. Such enhancement is particularly attractive for the analysis of complex protein mixtures with a significant difference in their individual concentrations.

Acknowledgements

Support for this work by the National Cancer Institute is gratefully acknowledged.

References

[1] S. Brandt, R.A. Goffe, S.B. Kessler, J.L. O'Connor, S.E. Zale, Biotechnology 6 (1988) 779.
[2] J.A. Gerstner, R. Hamilton, S.M. Cramer, J. Chromatogr. 569 (1992) 173.
[3] D.K. Roper, E.N. Lightfoot, J. Chromatogr. A 702 (1995) 3.
[4] T.B. Tennikova, F. Svec, J. Chromatogr. 646 (1993) 279.
[5] R. Giovannini, R. Freitag, T.B. Tennikova, Anal. Chem. 70 (1998) 3348.
[6] A. Podgornik, M. Barut, J. Jancar, A. Štrancar, T. Tennikova, Anal. Chem. 71 (1999) 2986.
[7] A. Podgornik, M. Barnut, J. Jancar, A. Štrancar, J. Chromatogr. A 848 (1999) 51.
[8] H. Iwata, K. Saito, S. Furusaki, T. Sugo, J. Okamoto, Biotechnol. Prog. 7 (1991) 412.
[9] M. Nakamura, S. Kiyohara, K. Saito, K. Sugita, T. Sugo, Anal. Chem. 71 (1999) 1323.
[10] M. Nakamura, S. Kiyohara, K. Saito, K. Sugita, T. Sugo, J. Chromatogr. A 822 (1998) 53.
[11] D. Josic, H. Schwinn, A. Strancar, A. Podgornik, M. Barut, Y.-P. Lim, M. Vodopivec, J. Chromatogr. A 803 (1998) 61.
[12] S. Xie, F. Svec, J.M.J. Frechet, Biotechnol. Bioeng. 62 (1999) 30.
[13] Y. Jiang, C.S. Lee, J. Chromatogr. A 924 (2001) 315.
[14] P.-C. Wang, J. Gao, C.S. Lee, J. Chromatogr. A 942 (2002) 115.
[15] J.W. Cooper, J. Chen, Y. Li, C.S. Lee, Anal. Chem., submitted for publication.
[16] Q. Tang, A.K. Harrata, C.S. Lee, Anal. Chem. 67 (1995) 3515.
[17] Q. Tang, A.K. Harrata, C.S. Lee, Anal. Chem. 68 (1996) 2482.
[18] D. Lutkemeyer, M. Bretschneider, H. Buntemeyer, J. Lehmann, J. Chromatogr. 639 (1993) 57.
[19] N.I. Dubinina, O.I. Kurenbin, T.B. Tennikova, J. Chromatogr. A 753 (1996) 217.
[20] M.B. Tennikova, N.V. Gazdina, T.B. Tennikova, T. Svec, J. Chromatogr. A 798 (1998) 55.
[21] X. Zeng, E. Ruckenstein, Biotechnol. Prog. 15 (1999) 1003.