ELSEVIER

Journal of Chromatography A, 893 (2000) 293–305

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Protein mapping by two-dimensional high performance liquid chromatography ☆

K. Wagner[a], K. Racaityte[a], K.K. Unger[a,*], T. Miliotis[b], L.E. Edholm[c], R. Bischoff[c], G. Marko-Varga[c]

[a]*Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg-Universität, Duesbergweg 10–14, D-55099 Mainz, Germany*

[b]*Department of Analytical Chemistry, Lund University, S-22100 Lund, Sweden*

[c]*Department of Molecular Sciences, AstraZeneca R&D Lund, S-22187 Lund, Sweden*

Received 22 February 2000; received in revised form 9 June 2000; accepted 28 June 2000

Abstract

Current developments in drug discovery in the pharmaceutical industry require highly efficient analytical systems for protein mapping providing high resolution, robustness, sensitivity, reproducibility and a high throughput of samples. The potential of two-dimensional (2D) HPLC as a complementary method to 2D-gel electrophoresis is investigated, especially in view of speed and repeatability. The method will be applied for proteins of a molecular mass <20 000 which are not well resolved in 2D-gel electrophoresis. The 2D-HPLC system described in this work consisted of anion- or cation-exchange chromatography in the first dimension and reversed-phase chromatography in the second dimension. We used a comprehensive two-dimensional approach based on different separation speeds. In the first dimension 2.5 μm polymeric beads bonded with diethylaminoethyl and sulfonic acid groups, respectively, were applied as ion exchangers and operated at a flow-rate of 1 ml/min. To achieve very high-speed and high-resolution separations in the second dimension, short columns of 14×4.6 mm I.D. with 1.5 μm *n*-octadecyl bonded, non-porous silica packings were chosen and operated at a flow-rate of 2.5 ml/min. Two reversed-phase columns were used in parallel in the second dimension. The analyte fractions from the ion-exchange column were transferred alternatively to one of the two reversed-phase columns using a 10-port switching valve. The analytes were deposited in an on-column focusing mode on top of one column while the analytes on the second column were eluted. Proteins, which were not completely resolved in the first dimension can, in most cases, be baseline-separated in the second dimension. The total value of peak capacity was calculated to 600. Fully unattended overnight runs for repeatability studies proved the applicability of the system. The values for the relative standard deviation (RSD) of the retention times of proteins were less than 1% ($n=15$), while the RSDs of the peak areas were less than 15% ($n=15$) on average. The limit of detection was 300 ng of protein on average and decreased to 50 ng for ovalbumin. The 2D-HPLC system offered high-resolution protein separations with a total analysis time of less than 20 min, equivalent to the run time of the first dimension.

Keywords: Two-dimensional liquid chromatography; Proteins

☆Presented at the 19th International Symposium on the Separation of Proteins, Peptides and Polynucleotides, Delray Beach, FL, 31 October–3 November 1999.

*Corresponding author. Fax: +49-6131-392-710.

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00736-6

1. Introduction

Proteomics is one of the new approaches in drug discovery which are based on studying the molecular mechanisms of disease and drug effects. The proteome is the entire profile of all the proteins expressed by a cell or a tissue under strictly defined conditions at a given time. In contrast to the genome, the proteome consists of a wide variety of highly complex molecules that are in constant flux in response to various stimuli. Further complicating factors are that amplifications are not possible like in the nucleic acid world and that there are very big differences in amounts [1].

The common separation method used in proteomics is two-dimensional (2D) gel electrophoresis which was established by O'Farrell [2] in 1975, providing very high resolution of proteins and high-sensitivity protein detection based on general post-separation staining methods. In the first dimension proteins are separated according to their isoelectric points (p*I*) in a pH-gradient gel. In the second dimension a highly negatively charged complex is formed after addition of sodium dodecyl sulfate (SDS) and the migration within the electrical field is governed by the molecular mass of the proteins. Disadvantages of the 2D-gel approach are labor-intensive work-up procedures, stringent demands on experimental procedures and reagents to achieve the high reproducibility needed for successful intergel comparisons (the cornerstone of proteomics) and the high effort of on-line coupling to mass spectrometry (MS) for protein identification. Additionally, proteins with a molecular mass of less than approx. 20 000 are generally not well resolved leading to a potential loss of important biological information.

In contrast to 2D-gel electrophoresis, high-performance liquid chromatography (HPLC) is based on different protein–surface interactions. In HPLC of proteins several chromatographic modes are available such as ion-exchange (IEX), hydrophobic interaction (HIC), reversed-phase (RP) and size-exclusion chromatography (SEC). With the exception of SEC all chromatographic separation modes are based on adsorptive interactions. All interactive modes use gradient elution. Separation in SEC results from the partial exclusion of analytes to diffuse into pores of defined size due to their molecular size. SEC can be combined with adsorptive chromatography, the so called restricted access materials (RAMs), where analytes need to access the adsorptive surface inside defined pores in order to be retained whereas the matrix components are excluded [3]. RAMs are ideal for the clean-up of complex biological samples, as they combine the advantages of SEC and RPLC or IEX. The outer surface of the porous silica is modified with non-charged hydrophilic hydroxyl groups, while the internal surface of the pores provides hydrophobic and ion-exchange functionalities, respectively. This results in the matrix eluting in the void volume, while the analyte fraction is reversibly bound to the internal surface. The usefulness of RAM columns in the sample clean-up of peptides and proteins has recently been demonstrated [4,5].

RAMs will be applied in the sample preparation step of biological samples to isolate the low-molecular-mass fraction.

Specially designed RPLC columns for peptide and protein analysis are very powerful in resolving peptides and proteins of lower molecular mass. Nevertheless, the peak capacity of a single RP column is generally not sufficient for the separation of complex mixtures such as cell extracts.

Giddings and co-workers [6,7] have shown theoretically that the peak capacity can be greatly enhanced by coupling HPLC columns, separating according to different retention mechanisms. The total peak capacity of such an orthogonal and comprehensive 2D-HPLC system equals, in the ideal case, the product of the peak capacities of the individual dimensions. To achieve maximum resolution, the separation modes have to be orthogonal and the entire analyte leaving the first dimension has to be transferred to the second dimension (comprehensive system). Any separation accomplished in the first dimension should ideally be maintained upon transfer to the second dimension.

Column coupling in a comprehensive system can be performed in different ways. In order to avoid sample losses and benefit from the speed, on-line coupling is essential. To achieve this without long-time storage of the analytes, the two-dimensional system must either consist of several columns in the second dimension or the separation speeds must be sufficiently different to allow on-line analysis in the second dimension. To keep the instrumental set-up to a minimum, the system using different speeds is the

most elegant way for column coupling, albeit putting high demands on the second dimension stationary phases [8,9].

Various two-dimensional LC approaches have been described in the literature. In a review Regnier and Huang [10] differentiated multidimensional chromatography in either a targeted component mode in which it is the objective to determine specific analytes [11] or a profiling mode where it is the objective to fractionate all components in the mixture.

Jorgenson and co-workers [12–14] realized different set-ups for the profiling mode. They differ in the separation modes and column sizes applied in the first dimension [SEC, cation-exchange chromatography (CIX), anion-exchange chromatography (AIX)] and the use of sample loops or on-column focusing for the transfer to the second dimension. Opiteck and Jorgenson [12] have presented a comprehensive on-line LC–LC–MS set-up for protein analysis comprised of micro-HPLC cation-exchange followed by reversed-phase chromatography using Poros R 2/H perfusion chromatography media and electrospray ionization mass spectrometry (ESI-MS). The effluent leaving the first dimension was alternatively stored in one of two loops switched by an eight-port two-position valve. Separation on the reversed-phase column (10 cm×500 μm I.D.) was performed in 150 s including column reconditioning.

A further set-up by Opiteck and Jorgenson [13] designed for the separation of peptides comprised of SEC followed by RPLC using non-porous 1.5 μm beads in the second dimension. Two parallel RP columns were used in the second dimension to circumvent storage loops.

Our focus was to set-up a very fast, high-resolution 2D-HPLC system based on high-speed reversed-phase separations of peptides and proteins using non-porous 1.5 μm silica beads and conventional low-void-volume chromatographic equipment. Those separations were performed within 1 min including the time for column regeneration and has recently been published by our group [15]. Another focus was to prove the repeatability of such a system.

In order to fulfill Gidding's requirements for multidimensional separations, anion- and cation-exchange chromatography, respectively, were applied in the first dimension followed by fast, high-resolution reversed-phase chromatography in the second dimension. These two HPLC modes are based on separations according to differences in charge distribution and hydrophobicity, respectively, hence they rely on orthogonal interaction mechanisms.

Applied in a 2D system set-up, a cycle time (gradient time plus column regeneration time) of 1 min means that 20 runs in the second dimension can be made during a single 20 min run in the first dimension.

Our 2D system consists of either an anion or cation exchanger in the first dimension and used two equivalent non-porous 14×4.6 mm I.D. RPLC columns in the second dimension which are operated much faster compared to the systems previously described in the literature. While the first RP column was loaded with a fraction of the effluent from the ion exchanger, the analytes on the second column were eluted. The two RP columns were alternatively loaded by enriching the analytes on top of the column under ion-exchange conditions. Column switching was performed using a 10-port two-position valve. Due to high resolution and high-speed columns in the second dimension, the number of chromatograms generated was one chromatogram per min total cycle time. To prove the feasibility of this concept we used UV detection for protein identification. After the 2D-LC separations and a digestion step, protein identification will be performed by MS. Depending on the experimental situation we will use either ESI-MS–MS or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). One approach is to split the flow and to identify the proteins on-line by ESI-MS or ESI-MS–MS. In this case the trifluoroacetic acid (TFA) amount has to be reduced or TFA has to be replaced by formic acid. The second option is to couple the second dimension after flow splitting to a piezoelectric flow-through microdispenser for on-line array fractionation on to targets for MALDI-TOF-MS as discrete spots. The required technology is available in our group [16].

2. Experimental

2.1. Chromatographic support

Ion-exchange columns were based on non-porous

polymeric beads (2.5 μm) commercially available with DEAE and SO_3 functionalities, respectively (TSK-gel NP) packed into 35×4.6 mm I.D. stainless steel columns (TosoHaas, Stuttgart, Germany).

Reversed-phase columns were based on non-porous C_{18} modified silica beads Micra NPS ODS I, particle diameter 1.5 μm in 14×4.6 mm I.D. columns (Micra Scientific, Northbrooke, IL, USA).

2.2. Chemicals

Eluents for ion-exchange separations consisted of (A) 0.01 *M* KH_2PO_4 in water, pH 6.0 and (B) 0.5 *M* KH_2PO_4 in water, pH 6.0.

The flow-rate was 1 ml/min. A typical gradient ran from 10 m*M* KH_2PO_4 to 0.3 *M* KH_2PO_4 (60% B) within 20 min. This was followed by 5 min column washing with 0.5 *M* KH_2PO_4 and 15 min column regeneration using buffer A.

The eluents for RPLC separations were (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. A typical gradient cycle for the reversed-phase columns started with 18% B, which was increased to 70% B within 25 s and further increased to 100% B within 5 s, which was maintained for 5 s before being reduced to the initial conditions (18% B) within a further 5 s followed by 20 s column regeneration. The flow-rate in the second dimension was 2.5 ml/min.

All buffers and TFA were of analytical grade, eluents were HPLC gradient grade and purchased from Merck (Darmstadt, Germany). Pure water was produced using a Milli-Q device from Millipore (Bedford, MA, USA). The standard proteins were all purchased from Sigma (Deisenhofen, Germany).

2.3. Instrumentation

The fully automated 2D-HPLC device consisted of an Integral 100Q workstation (Perseptive Biosystems, Framingham, MA, USA), equipped with an autosampler with sample cooling device and three pneumatically-driven 10-port two-position switching valves (Rheodyne, Rohnert Park, CA, USA). One of those was serving as injector and one was used for column switching. The workstation was equipped with one high-pressure binary gradient pump system, an UV detector with a 1.8 μl flow cell as well as a control unit for data acquisition and system management. The second high-pressure binary gradient system (high-speed) consisted of two HPLC pumps Model 2200 and an HPLC-Central Processor Model 7110 for gradient control. Eluent mixing was performed using a dynamic low-void-volume mixing chamber, all supplied by Bischoff Analysentechnik (Leonberg, Germany).

To minimize void volume, tubing lengths were kept to a minimum and an inner diameter of 0.127 mm was used [polyether ether ketone (PEEK) tubing provided by Upchurch Scientific, Oak Harbor, WA, USA].

2.4. System operation

The samples were stored in 1200-μl vials at 3°C in the temperature-controlled tray of the autosampler. Injections were automatically performed by filling a 10- or 100-μl sample loop attached to the 10-port injection valve (Fig. 1). The first dimension high-pressure gradient pump eluted the ion-exchange column continuously with an increasing gradient of phosphate buffer, resulting in a partial separation of proteins.

Each 60 s the analytes leaving the first dimension were transferred and enriched alternatively on top of one of the two reversed-phase columns (on-column focusing). Column switching was performed using the pneumatically-driven 10-port valve controlled by the method editor software. At the same time a 150 ms electrical contact closure pulse was sent to the central processor device of the second gradient system in order to start the gradient and the column reconditioning procedure. During the time when one of the reversed-phase columns was eluted, all of the analytes corresponding to 1 min (1 ml) ion-exchange effluent were deposited on the other RP column. By the use of this technique, desalting of the proteins was performed automatically. The RP column undergoing elution was attached via the valve to the UV detector which was equipped with a 1.8-μl flow-cell and operated at a wavelength of 215 nm.

In order to avoid errors in triggering the data acquisition units and to simplify the data handling we generated one chromatogram in the second dimension which contained all the 20 RPLC chromatograms. Interpretation of the chromatograms was easy, since the gradient of each RPLC chromatogram

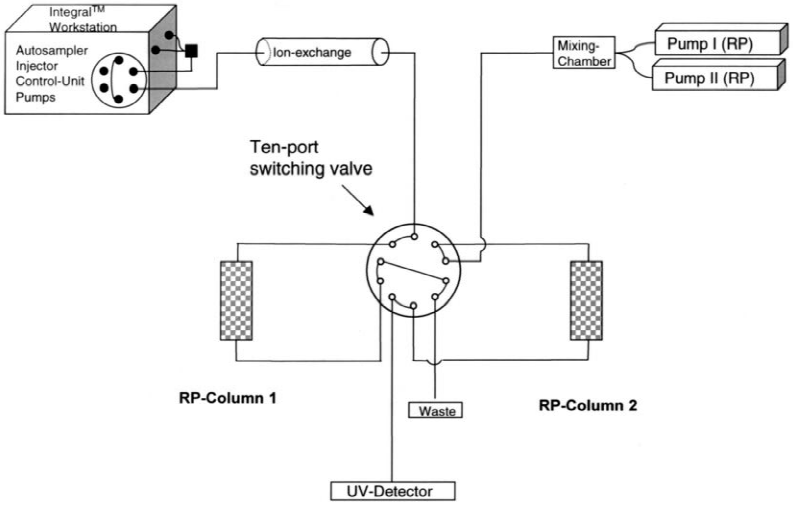


Fig. 1. Scheme of the two-dimensional HPLC system for protein separation.

starts every full minute. In this way relative retention times can be compared.

UV detection after the first dimension was not possible, because the IEX and RP columns were attached through the valve in line. The pressure drop generated to load the RP column would destroy the flow cell which withstands a maximum pressure of 50 bar. UV detection after the first dimension is not indispensable, since the entire analyte is automatically transferred to the second dimension. The chromatograms generated after the first dimension (Fig. 3 and Fig. 4) have been made in the single-column mode.

2.5. Samples

The standard protein mixture for cation-exchange/RP separations contained the following 10 proteins: ribonuclease from bovine pancreas (rib.), insulin from bovine pancreas (ins.), cytochrome *c* from bovine heart (cyt.), lysozyme from chicken egg white (lys.), myoglobin from horse heart (myo.), conalbumin from chicken egg white (con.), ovalbumin (ova.), concanavalin from jack bean (coc.), γ-chymotrypsin from bovine pancreas (γ-chy.) and human albumin (alb.-H).

Separations were performed at pH 6 injecting 10 or 100 μl of the protein mixture at a total protein concentration of 6.8 mg/ml.

The standard protein mixture for anion-exchange/RP separations contained the following 11 proteins: ribonuclease from bovine pancreas (rib.), insulin from bovine pancreas (ins.), cytochrome *c* from bovine heart (cyt.), lysozyme from chicken egg white (lys.), myoglobin from horse heart (myo.), conalbumin from chicken egg white (con.), ovalbumin (ova.), trypsin-inhibitor from soya bean (try.-I), β-lactoglobulin A from bovine milk (β-lac. A), β-lactoglobulin B from bovine milk (β-lac. B), and bovine serum albumin (BSA). This separation was performed at pH 6.0 injecting 10 μl or 100 μl of the 11 proteins at a total protein concentration of 10.2 mg/ml. All protein mixtures were filtered through a 0.45-μm filter unit Milliex-HV (Millipore).

The human fibroblast cell model supernatant was made as follows: human lung fibroblast cultures from ATCC (CCL-153) were used. The cultures were permitted to grow to confluence for 48 h in T 75 flasks and harvested by scraping the cells. The cell layer was washed three times with 3 ml cold phosphate-buffered saline (PBS), thereafter centrifuged at 40 000 *g* (4°C, 20 min). The pellet was resuspended in PBS buffer and sonicated for 40 s. A second centrifugation was performed again at 40 000 *g* (4°C, 30 min), after this, the supernatant was subjected to ultrafiltration using an M_r 30 000 cut-off membrane (YM30; Amicon, Beverly, MA, USA).

3. Results and discussion

Analyzing fibroblast protein expressions by 2D-gel electrophoresis has been carried out extensively within our group (unpublished results). While the molecular mass region down to 20 000 is handled very well by 2D-gel electrophoresis with good repeatability, it is the M_r region below 20 000 that poses a problem due to the high mobility of these proteins which results in poor rigidity and significance of data generated. These are reasons why we developed a 2D-HPLC separation platform dedicated to the M_r<20 000 region. It is to be considered as a strong complement to traditional proteomics. An additional factor of importance for high-resolution HPLC is the favorable diffusion coefficients of small-sized proteins and peptides. Choice and evaluation of HPLC modes:

The chromatographic system developed was based upon separation of proteins in two dimensions. To achieve maximal orthogonality we based the first dimension on electrostatic interactions while the second was based on hydrophobic interactions.

Anion- and cation-exchange chromatography was tested as the first dimension to cover a wide p*I* range at high resolution. Cation-exchange at acidic pH values in the first dimension should be sufficient to use for most biological samples. Different types of commercially available and laboratory-synthesized ion exchangers were evaluated in single-column mode, regarding the peak capacity as a measure of chromatographic resolution. Polymer-based commercially available supports were chosen for the 2D system, since they provide high loadability and high resolution besides high robustness due to their resistance to sodium hydroxide for cleaning purposes.

Phosphate buffer as the eluent showed better chromatographic resolution compared to Tris–HCl containing 0.5 *M* sodium chloride or phosphate buffer containing 0.5 *M* sodium chloride.

Flow-rates and gradient times in the first dimension were adjusted to receive high peak capacities at short analysis times. Analysis times of about 20 min resulted in peak capacities of 20 and more.

Non-porous silica proved to have the required characteristics for the second dimension due to its high mechanical stability and fast mass transfer due to a lack of pore diffusion. This allowed extremely short analysis and reconditioning times in just 1 min providing peak capacities of approximately 30 and higher.

The narrow particle size distribution of ±5% standard deviation adds to the favorable chromatographic properties for fast and high-resolution separations of proteins. In the second dimension, where two different RP columns are used alternatively, it is necessary that both columns must provide a similar separation to get reproducible results. Both RP columns have been compared showing no difference in their respective separation performance (data not shown).

Fig. 2 shows a baseline separation of eight proteins using the 1.5 μm NPS RP columns applied in the second dimension in a single-column run, which clearly confirms the high resolution power of the second separation dimension.

What needs to be considered is the optimal interfacing that is required to build a pressure-driven stable automated 2D separation system. The integrated 2D-HPLC system was operated as a comprehensive analytical system under conditions that allowed achievement of maximum resolution. Fig. 3a shows the anion-exchange chromatogram of 11 standard proteins after the first dimension leading to an incomplete separation, roughly according to their respective isoelectric points. Fig. 3b–e show 16 of the 20 chromatograms generated in the second (RP) dimension. Those proteins which have not been successfully separated based on electrostatic interac-

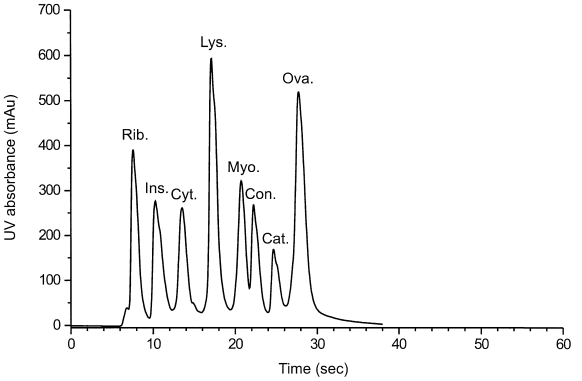


Fig. 2. Baseline separation of eight proteins within 30 s using 1.5 μm NPS reversed-phase columns.

tions can mostly be separated in the reversed-phase mode due to differences in hydrophobicity.

The proteins eluted in the void volume of the anion exchanger are successfully separated on the reversed-phase column and appear mainly in the RP chromatogram of fractions eluting between 1 min to 2 min (Fig. 3b). These non-retained analytes are, however, successfully resolved by the cation-exchange column and vice versa. Fig. 3b shows that the proteins eluted from the ion-exchange column appear in approximately three fractions of the reversed-phase chromatogram but in different amounts which makes it possible to follow the ion-exchange separation. Even that the peak in the ion-exchange chromatogram seems to reach the baseline again, there are still small amounts of protein eluting from the column. This seems to be a specific problem in ion-exchange chromatography limiting the resolution. For the separation of the specific standard protein mixture the ion-exchange separation might be too slow or the sampling rate too high. But in an unknown more complex sample it might be useful to keep the high sampling rate to assess maximal resolution. This is confirmed by a study where the effect of sampling rate on resolution in comprehensive two-dimensional liquid chromatography is investigated [17]. In that paper the authors claim that each peak in the first dimension should be sampled at least three times into the second dimension.

Chromatograms of the same separation at a concentration level, which was 10-times less than in Fig. 3b–e demonstrated that proteins were still detectable despite of baseline fluctuations appearing and slight changes in peak shape (chromatograms not shown).

Similar results are obtained using cation-exchange chromatography in the first dimension and a test mixture of 10 standard proteins (chromatograms not shown).

The fast separation in the second dimension allowed one to analyze all fractions (1.0 ml each) from the ion exchanger on the reversed-phase column without any delay. Sample handling losses during transfer or sample storage were eliminated. Although not all proteins can be resolved in either

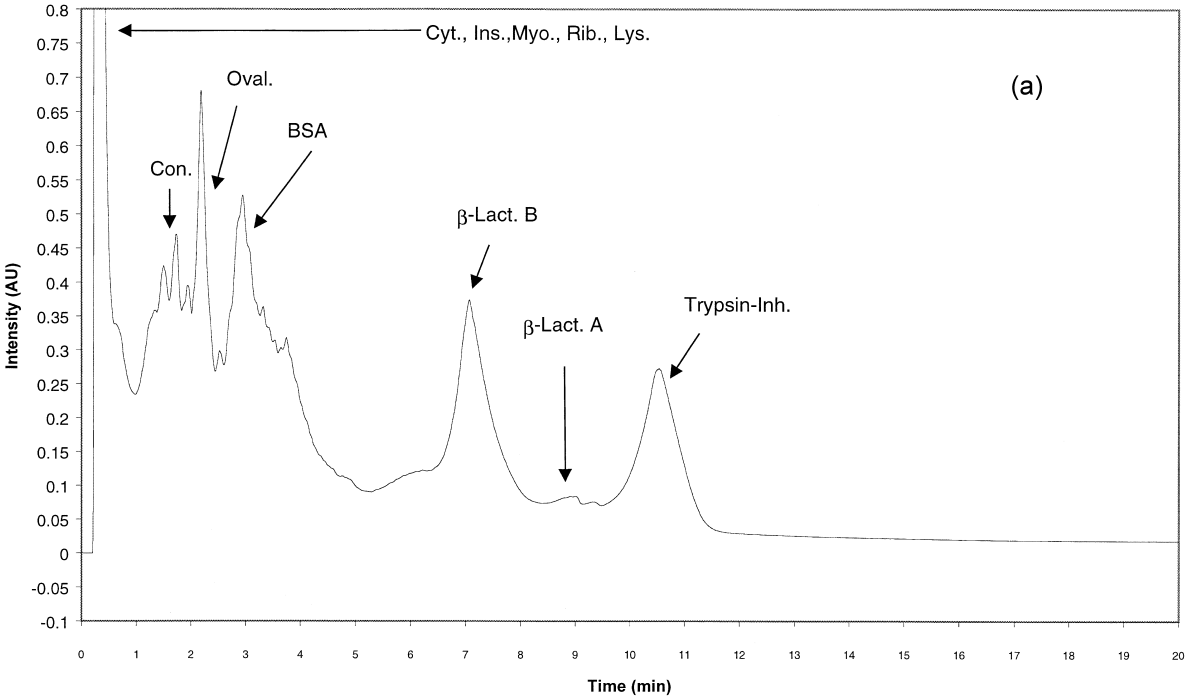
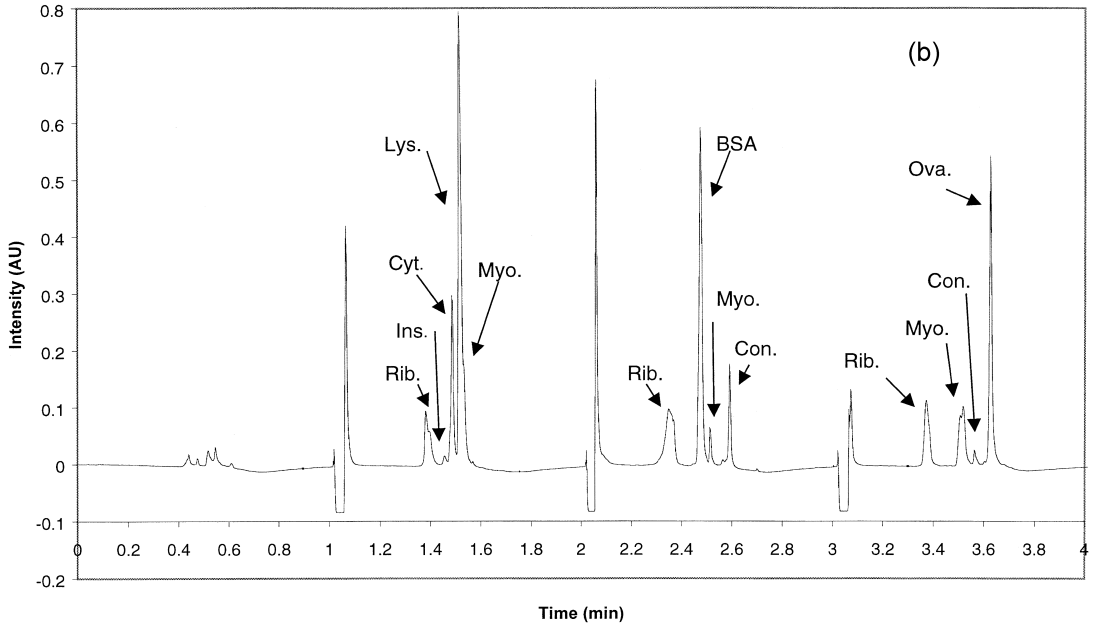


Fig. 3. (a) Incomplete separation of a 11-protein mixture after the first dimension using anion exchange. (b–e) Reversed-phase chromatograms of the 1-min analyte fractions taken from the anion-exchange column.



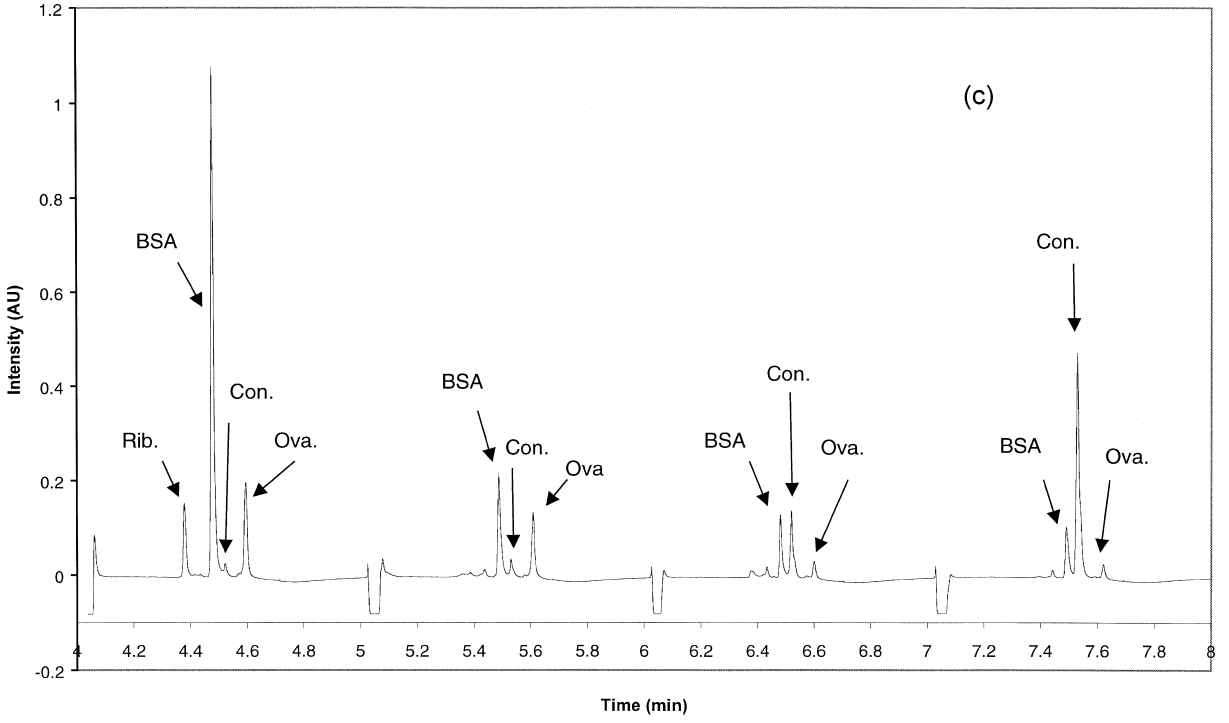
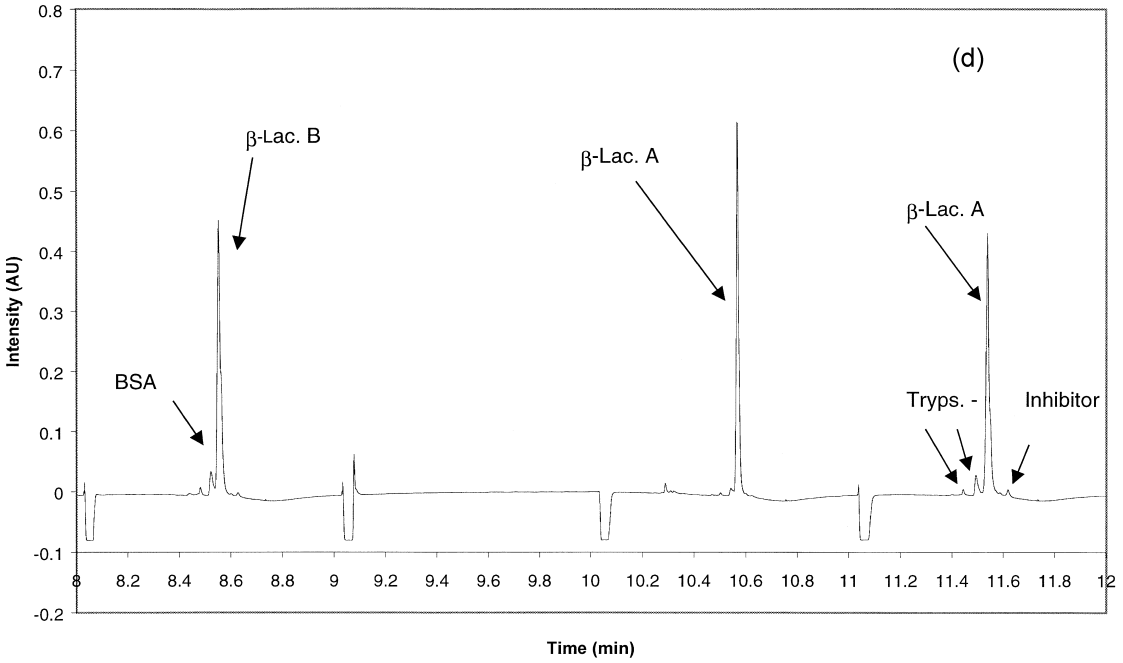


Fig. 3. (*continued*).



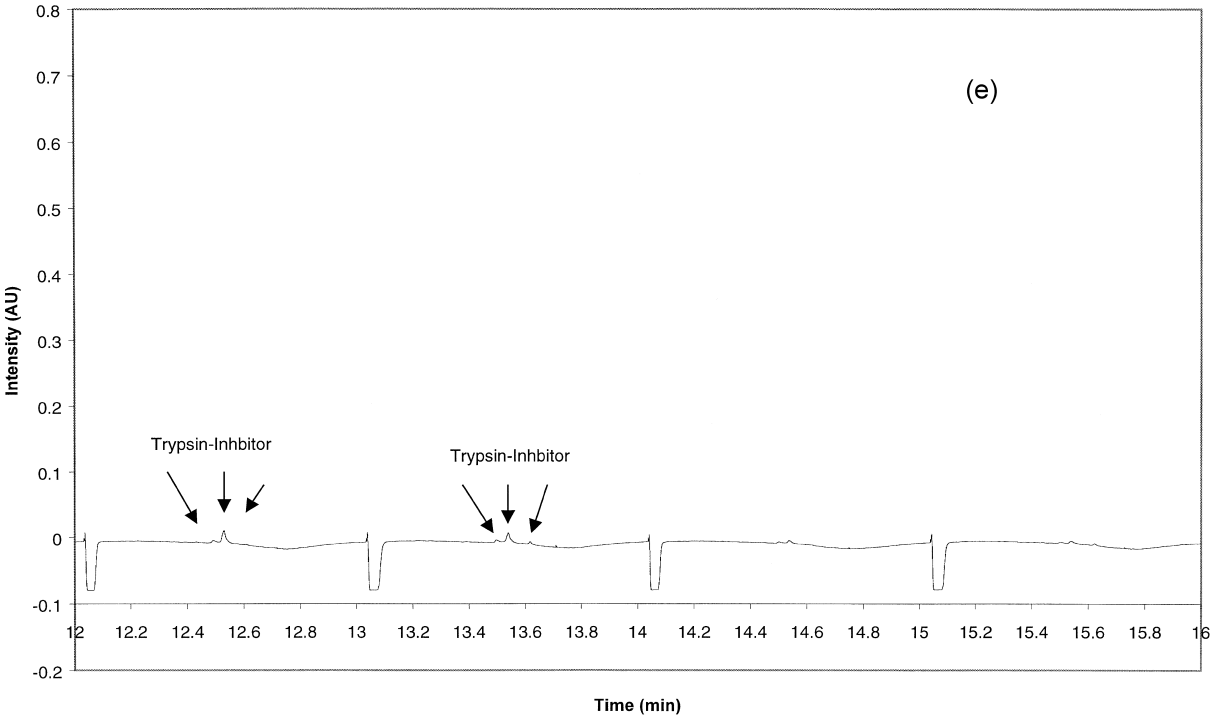


Fig. 3. (*continued*).

Table 1
Repeatability data (n=15) of selected peaks generated with the anion-exchange/reversed-phase HPLC system (102 μg total protein amount)

	Protein										
	Rib., p*I* 9.6	Ins., p*I* 5.7	Cyt., p*I* 10.6	Lys., p*I* 11.0	BSA, p*I* 4.6	Con., p*I* 5.9	Myo., p*I* 7.6	Ova., p*I* 4.7	β-Lac. B, p*I* 5.2	β-Lac. A, p*I* 5.1	Try.-I, p*I* 4.6
Average retention time (min)	1.393	1.469	1.498	1.526	2.486	2.601	3.493	3.614	9.553	10.539	12.546
Standard deviation (min)	0.018	0.011	0.010	0.010	0.014	0.011	0.015	0.018	0.010	0.017	0.014
RSD (%), n=15	1.3	0.8	0.7	0.7	0.6	0.4	0.4	0.5	0.1	0.2	0.1
Average peak area (μAU s)	175 493	14 242	210 618	771 973	452 984	165 335	179 619	443 174	222 346	437 714	8962
Standard deviation (μAU s)	25 619	4777	24 191	58 427	32 021	17 878	19 163	18 780	21 112	12 568	1665
RSD (%), n=15	14.6	33.5	11.5	7.6	7.1	10.8	10.7	4.2	9.5	2.9	18.53
Average peak height (μAU)	131 979	13 527	274 981	749 737	584 525	217 655	160 624	478 140	230 810	542 094	8204
Standard deviation (μAU)	24 693	3214	26 402	43 845	50 213	14 485	28 958	59 698	15 460	54 203	911
RSD (%), n=15	18.7	23.8	9.6	5.8	8.6	6.7	18.0	12.5	6.7	10.0	11.1

the ion-exchange or reversed-phase mode alone, the combined chromatographic resolution of the two dimensions is capable of resolving complex mixtures.

3.1. Repeatability

Repeatability of the anion-exchange/RP system, for example, in terms of retention time, peak height and peak area was based on determinations from all 31 peaks generated from the 11 proteins (some proteins appeared in several fractions from the ion-exchange column). The peaks were dedicated to a protein by their retention time generated in blank runs of each protein in the RP mode and their retention time in the single ion-exchange chromatogram.

Parameters derived from 15 chromatograms (n=15) generated in different runs under the same conditions resulted in a relative standard deviation (RSD) of the retention time of less than 1% on average. The peak areas and peak heights determined by manual integration of each peak showed RSD values ranging from 10% to 20% on average. The reproducibility data for the cation-exchange/reversed-phase chromatograms also derived from 15 different runs were approximately the same (see Tables 1 and 2). The within-day variations were similar to the between-day variations, which proved the operational stability of the 2D-HPLC system.

Table 2
Repeatability data (n=15) of selected peaks generated with the cation-exchange/reversed-phase HPLC system (67.8 μg total protein amount)

	Protein						
	Insulin, p*I* 5.7	Alb.-H, p*I* 5.9	Ova., p*I* 4.7	Myo., p*I* 7.6	Rib., p*I* 9.6	Cyt., p*I* 10.6	Lys., p*I* 11.0
Average retention time (min)	1.433	1.495	1.620	2.537	7.411	8.385	11.467
Standard deviation (min)	0.014	0.011	0.021	0.015	0.023	0.011	0.016
RSD (%), n=15	1.0	0.7	1.303	0.6	0.3	0.1	0.1
Average peak area (μAU s)	14 611	431 919	682 538	133 673	71 748	439 368	106 825
Standard deviation (μAU s)	6345	52 271	41 508	17 151	18 877	76 486	4199
RSD (%), n=15	43.4	12.1	6.1	12.8	26.3	17.4	3.9
Average peak height (μAU)	10 945	379 397	619 659	160 606	89 538	274 920	131 088
Standard deviation (μAU)	1704	39 630	74 406	23 406	45 226	143 205	6148
RSD (%), n=15	15.6	10.4	12.0	14.6	50.5	52.1	4.69

3.2. Reproducibility

The reproducibility of the system was checked by setting it up in two different laboratories in Mainz (Germany) and Lund (Sweden) showing the same results and performance.

3.3. Recovery

The overall recovery of proteins on the cation-exchange/reversed-phase 2D-HPLC system was determined by comparing the total peak areas generated by a mixture of six standard proteins using either the cation-exchange/RP column configuration or no column at all. The test mixture diluted by a factor of 10 was pumped through the tubing, using 50% acetonitrile (0.1% TFA) in water (0.1% TFA) approximating the reversed-phase elution conditions. The total peak area of the separated mixture was calculated as the sum of all single peak areas determined by manual integration. The observed recovery depended on the total amount of proteins loaded and decreased with lower concentrations. At an intermediate amount of injected proteins (66.4 μg total amount) recovery was 70% while decreasing to 22% close to the limit of detection, which was out of the linear range of the system (approximately 6.6 μg total amount of protein).

3.4. Detection limits

The limit of detection (LOD) at a wavelength of 215 nm was dependent on the extinction coefficient of the proteins. The LOD was evaluated by injecting 10 μl of a six-protein mixture. The stock mixture was diluted by a factor of two until the LOD in the 2D-HPLC runs for all proteins was reached. This resulted in a detection limit of approximately 300 ng per protein, for some proteins (e.g., ovalbumin) 50 ng was still detectable. The limit of detection was considered to be at a peak height, which is three-times higher than the average baseline fluctuation. The LODs, the linear ranges for quantification and the linear regression coefficients are shown in Table 3.

3.5. Loadability

The loadability of the 2D-HPLC system is limited by the small surface area of the non-porous 1.5 μm particles in the high-speed reversed-phase columns.

Signs of overloading appear at 1 mg total protein injected.

3.6. Linearity of peak area/peak height and protein amount

The linearity of peak height, peak area and the amount of each protein were checked evaluating six selected peaks from cation-exchange/RP runs at nine different concentration levels using a mixture of six standard proteins. This resulted in a linear correlation for average concentration levels. At higher protein amounts, the curve became more shallow until the maximum loadability of the system at approximately 100 μg per protein was reached. Deviations from the linear correlation also occurred at the concentration levels close to the detection limits where the peak areas were smaller than expected assuming a linear correlation. The regression coefficient (R^2) for the linear part of the curve for each protein is shown in Table 3.

Table 3
Detection limits, and linear regression parameters for a six-protein mixture at different concentrations applied to cation-exchange/reversed-phase chromatography

	Protein					
	BSA	Ova.	Myo.	Rib.	Cyt.	Lys.
Average retention time (min)	1.483	1601	4.526	7.385	8.375	13.478
Limit of detection (LOD) (ng)	178	53	324	325	152	168
Lower limit of linearity (ng)	356	213	648	650	305	336
Upper limit of linearity (μg)	11.4	13.7	10.4	20.8	9.8	10.8
Linear regression coefficient (R^2)	0.9982	0.9909	0.9925	0.9362	0.9928	0.9922

Table 4
Reproducibility data ($n=13$) of cation-exchange/reversed-phase chromatography of fibroblast sample, spiked with six standard proteins

	Protein					
	BSA	Ova.	Myo.	Rib.	Cyt.	Lys.
Average retention time (min)	1.483	1.601	4.526	7.385	8.375	12.439
Standard deviation (min)	0.0073	0.0040	0.0162	0.0090	0.0105	0.0046
RSD (%), $n=13$	0.5	0.3	0.4	0.1	0.1	0.1
Average peak area (μAU s)	355 870	431 554	65 081	120 547	312 462	223 021
Standard deviation (μAU s)	19 474	28 987	5705	1697	31 828	24 358
RSD (%), $n=13$	5.5	6.7	8.8	14.1	10.2	10.9
Average peak height (μAU)	433 433	474 348	113 259	107 968	264 440	310 409
Standard deviation (μAU)	28 331	17 841	25 209	29 860	133 148	39 458
RSD (%), $n=13$	6.5	3.8	22.3	27.7	50.4	12.7

3.7. Applications

In order to evaluate the applicability and stability of the system, a soluble extract from human fibroblasts grown in culture was used. The samples were spiked with a mixture of six standard proteins at a total concentration of 3.3 μg/μl.

A 10-μl volume of this sample was applied to the cation/reversed-phase 2D-HPLC system. No deterioration of resolution between proteins was observed after 13 runs and no column clogging or increase of the pressure drop occurred indicating that column regeneration was sufficiently robust. Those data demonstrated that the 2D-HPLC set-up is still reliable even in presence of a biological matrix. Unattended operation was applied overnight running these cell samples. The RSDs of the retention times were less than 0.5%, $n=13$ in the same range as we found for standard samples. The quantitative data in terms of peak height were found to vary between 5% and 15%. The exact data for selected peaks corresponding to one specific spiked protein are shown in Table 4.

4. Conclusions and perspectives

The experimental data showed the feasibility of utilizing a 2D-HPLC system for the separation of complex protein mixtures based on polymeric ion exchangers in the first dimension and non-porous silica reversed-phase materials in the second dimension. The total peak capacity of this system as a measure for chromatographic performance can be calculated to be as high as 600.

Ion-exchange chromatography with its use of eluents containing salt and buffers in water is favorable regarding protein stability. The short elution times in the RP mode will probably cause less denaturation of the proteins compared to 2D-gel electrophoresis or SDS–polyacrylamide gel electrophoresis. This offers the chance to run bioassays to screen the fractions.

The 2D-HPLC system presented with its on-column focusing makes use of on-line desalting, even though it was proven in a non-comprehensive HPLC system that there is no influence on the separation in the RP mode if the proteins are dissolved and injected in the ion-exchange eluent.

After this first feasibility study it is obvious that the high-speed 2D-HPLC approach using ion-exchange and RP separations was able to perform well.

Improvement of ion-exchange and reversed-phase resolution, regenerating the RP columns in a separate procedure and focusing on smaller proteins and peptides offers the potential for a further increase in resolution.

Miniaturization is an option for increasing the sensitivity and possibly the resolution and will be necessary in cases where there is only a limited amount of sample available.

The 2D-HPLC system with its options for integrated sample preparation using RAMs, miniaturization and on-line coupling to MS could be a valuable

part of a toolbox for protein and peptide mapping, especially for those small proteins which cannot be separated in 2D-gel electrophoresis.

Acknowledgements

This work was supported by AstraZeneca R&D Lund, Lund, Sweden. We would like to thank TosoHaas, Stuttgart, Germany, for supplying us with ion-exchange columns, Bischoff Analysentechnik, Leonberg, Germany for the support with HPLC equipment and Merck, Darmstadt, Germany for supplying us with eluents.

References

[1] F. Lottspeich, Angew. Chem., Int. Ed. Engl. 38 (1999) 2476.
[2] P.H. O'Farell, J. Biol. Chem. 250 (1975) 4007.
[3] K.S. Boos, A. Rudolphi, LC·GC Int. 15 (1997) 602.
[4] K. Racaityte, M. Lutz, K.K. Unger, D. Lubda, K.S. Boos, J. Chromatogr. A (2000) in press.
[5] C.H. Grimm, K.S. Boos, Ch. Apel, K.K. Unger, P. Önnerfjord, L. Heintz, L.E. Edholm, G. Marko-Varga, Chromatographia, submitted for publication.
[6] J.C. Giddings, J. High Resolut. Chromatogr. 10 (1987) 319.
[7] J.C. Giddings, H.J. Cortes, Multidimensional Chromatography, Marcel Dekker, New York, 1990.
[8] M. Hanson, K.K. Unger, LC-GC Int. 9 (1996) 650.
[9] M. Hanson, K.K. Unger, LC-GC Int. 9 (1996) 741.
[10] F. Regnier, G. Huang, J. Chromatogr. A 750 (1996) 3.
[11] M. Frutos, F. Regnier, Anal. Chem. 61 (1989) 17A.
[12] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, Anal. Chem. 69 (1997) 1518.
[13] G.J. Opiteck, J.W. Jorgenson, Anal. Chem. 69 (1997) 2283.
[14] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 161.
[15] T. Issaeva, A. Kourganov, K.K. Unger, J. Chromatogr. A 846 (1999) 13.
[16] T. Miliotis, S. Kjellström, J. Nilsson, T. Laurell, L.E. Edholm, G. Marko-Varga, J. Mass Spectrom. 35 (2000) 369.
[17] R. Murphy, M. Schure, J. Foley, Anal. Chem. 70 (1998) 1585.