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An Automated On-Line Multidimensional HPLC System for Protein and Peptide Mapping with Integrated Sample Preparation

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A comprehensive on-line two-dimensional 2D-HPLC system with integrated sample preparation was developed for the analysis of proteins and peptides with a molecular weight below 20 kDa. The system setup provided fast separations and high resolving power and is considered to be a complementary technique to 2D gel electrophoresis in proteomics. The on-line system reproducibly resolved ∼1000 peaks within the total analysis time of 96 min and avoided sample losses by off-line sample handling. The low-molecular-weight target analytes were separated from the matrix using novel silica-based restricted access materials (RAM) with ion exchange functionalities. The size-selective sample fractionation step was followed by anion or cation exchange chromatography as the first dimension. The separation mechanism in the subsequent second dimension employed hydrophobic interactions using short reversed-phase (RP) columns. A new column-switching technique, including four parallel reversed-phase columns, was employed in the second dimension for on-line fractionation and separation. Gradient elution and UV detection of two columns were performed simultaneously while loading the third and regenerating the fourth column. The total integrated workstation was operated in an unattended mode. Selected peaks were collected and analyzed off-line by MALDI-TOF mass spectrometry. The system was applied to protein mapping of biological samples of human hemofiltrate as well as of cell lysates originating from a human fetal fibroblast cell line, demonstrating it to be a viable alternative to 2D gel electrophoresis for mapping peptides and small proteins.

With the finalization of the Human Genome Project, scientific interest is turning increasingly to the task of converting the DNA sequence information into knowledge that will potentially improve human medicine and health care. One key challenge is understanding proteomics, the postgenomic protein sequencing field. The proteome characterizes the entire set of proteins expressed by an organism, cell or organelle; and it is highly complex and under constant flux in response to various stimuli over time. The low-abundance and high-abundance proteins differ in concentration over 5 orders of magnitude; hence, highly abundant proteins can mask smaller ones.[1]

In proteomics, the focus is on mapping global expression patterns of proteins[2,3] as well as targeted events, such as modulations of regulatory mechanisms in organelles[4] and in specific biochemical pathways.[5] There is an increasing need for new technology developments with high-speed analysis capacities for de novo characterization of proteins and peptides with the potential to provide new insights into human disease mechanisms.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), introduced by O'Farrell,[6] is the most powerful separation system yet developed and is an unsurpassed separation tool for protein mapping. It fulfils Giddings[7] requirements for multidimensional separation, in which isoelectric focusing and gradient gel electrophoresis provide the orthogonal analyte displacements. However, limitations inherently associated with 2D-PAGE are the extensive sample handling, nonlinear response factors for the most commonly used staining techniques, limited loading capacity, low extraction efficiencies of the gel-embedded proteins, and a decreasing resolving power for proteins with a molecular mass of <15 kDa as a result of their high mobility in the gel. Furthermore, it is difficult to isolate proteins with a pI at the acidic and basic extremes of the pH gradient, proteins with a molecular weight higher than 200 kDa, or membrane and other hydrophobic proteins. In addition, the technique is time-consuming, difficult to automate, and there is no simple way for on-line coupling to mass spectrometric detection.

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The improvement in efficiency and selectivity of liquid-phase separations using both pressure- and electrophoretically driven separation techniques coupled on- or off-line to mass spectrometry is beginning to have a major impact on the characterization of the proteome. An intrinsic property of 2D-HPLC separations is the availability of various chromatographic modes with different separation mechanisms, for example, anion/cation exchange, reversed-phase, hydrophobic interaction, and size-exclusion chromatography.

A single HPLC column does not provide enough chromatographic resolution for proteome characterization of complex biological samples; thus, multidimensional liquid chromatography is a prerequisite. The theoretical framework for multidimensional separation techniques has been discussed by Davis and Giddings,[8,9] and different on- and off-line experimental approaches have been described throughout the literature.[10−23]

To achieve fast and reproducible separations, a continuous column-coupling approach is essential. Any off-line system reinjecting the fractions onto a second column is sensitive to loss of sample, vial contamination, sample dilution, and low reproducibility. The on-line system developed avoids any fraction storage and automatically performs sample enrichment and desalting, thus avoiding eluent incompatibilities; however, many off-line multidimensional HPLC approaches have been described throughout the literature as a result of low demands on the equipment.

Wall et al.[12] published a discontinuous two-dimensional liquid-phase separation method in which proteins in cell lysates were separated using isoelectric focusing in the first dimension followed by fast reversed-phase HPLC in the second dimension. The protein screening obtained by the 2D system, as compared with 2D-PAGE, was complementary, and improved results were obtained for lower mass and basic proteins. Vissers et al.[13] presented an automated off-line capillary liquid chromatography approach using either strong anion exchange coupled to reversed-phase columns or two different reversed-phase columns. Bushey and Jorgenson[14] coined the term comprehensive 2D-HPLC, referring to the fact that the entire effluent from the first dimension is transferred into the second dimension without the use of heart-cutting techniques. A new comprehensive on-line column-coupling technique was introduced by Opiteck et al.[15] using two parallel reversed-phase columns, rather than storage loops, for sample transfer into the second dimension. Step-gradient elution in the first dimension for an interrupted sample transfer onto a single secondary reversed-phase column in the second dimension was a further coupling approach putting minor demands on the equipment.[16,17]

Multidimensional separations have been performed on intact proteins present in biofluids[18−20] and cell lysates,[21] as well as digested proteins utilizing peptide mapping.[22]

In contrast to 2D gel electrophoresis, two-dimensional coupled-column high performance liquid chromatography (2D-HPLC) circumvents many of the above-mentioned limitations. 2D-HPLC provides the means for automated separation and detection with short analysis time. Since the analytes stay in the liquid phase all the time, coupling the separation on-line to mass spectrometry is an attractive feature.

The current system setup presented in this work is an improvement on the previously described fast 2D-HPLC system.[23] The aim was to implement novel approaches and system components to significantly improve resolution, speed, reproducibility, and robustness for the analysis of proteins of a molecular weight smaller than 20 kDa. First, two novel sample cleanup precolumn packings were applied with cation and anion functionality based on the restricted access principle.[24] Second, a column-switching technique was developed using four parallel, short, reversed-phase columns that allows one to increase the gradient time up to 7 min, thereby drastically enhancing the peak capacity in the second dimension. Finally, the performance of the 2D-HPLC system was demonstrated on samples from human hemofiltrate and cell lysate from human fetal fibroblast cell lines.

EXPERIMENTAL SECTION

Samples. All samples were filtered through a 0.45-μm filter unit Millex-HV (Millipore, Bedford, MA). Human hemofiltrate was used as a reference sample for characterization of the 2D-HPLC system and the size-selective sample fractionation step. It was obtained as a lyophilized powder from IPF PharmaCeuticals, Hannover, Germany, after a single step of preparative cation exchange chromatography.

CCL-153 human lung fibroblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown to confluence for 48 h in T75 flasks and harvested by scraping the cells. The cell layer was washed three times with 3 mL of cold PBS and centrifuged (40000*g*, 4 °C, 20 min). The pellet was resuspended in 2.5 mL of PBS buffer containing 8 M urea and sonicated for 40 s. A second centrifugation was performed (40000*g*, 4 °C, 30 min). A 2-mL portion of the fibroblast sample was adjusted to pH 3 for subsequent loading onto the cation exchange RAM column.

Sample Preparation by Ultrafiltration. A 3-mL portion of human hemofiltrate at a concentration of 50 μg/μL was subjected to ultrafiltration through a 10 kDa membrane (YM-10, Amicon, Inc., Beverly, MA) using a 3-mL chamber equipped with a magnetic stirrer while applying an air pressure of 3 bar. A 20-μL portion of the filtrate was analyzed by SDS-PAGE.

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Size-Selective Sample Fractionation Using Novel Restricted Access Columns. Two novel restricted access materials (RAM) were applied as column packings (research products of Merck KGaA, Darmstadt, Germany). IEX-DS (ion exchange diol silica) is a spherical LiChrospher 60 (dp: 25 μm)-based silica. The outer surface of it is modified exclusively with electroneutral diol groups, whereas the inner particle surface carries either cationic (diethylaminoethyl) or anionic (sulfonic acid) functional groups. The average pore size of the IEX-DS material is 6 nm, and the column dimensions are 25 × 4 mm i.d.

For comparative experiments, we used an analogous reversed-phase (RP) material, namely ADS (alkyl diol silica), which is available from Merck KGaA, Darmstadt, Germany, as a LiChrospher ADS RP-18 column (25 × 4 mm i.d.)

Chromatographic Conditions. *Operation of RAM Columns for Sample Fractionation*. The starting condition for the reversed-phase RAM column was as follows: A was water (0.1% TFA), and the elution was made with B being acetonitrile (0.1% TFA). The eluents for the cation exchange RAM column and the analytical cation exchanger were A, 10 mM phosphate buffer at pH 3.0 and B, 1 M phosphate buffer at pH 3.0. The corresponding eluents for the anion exchange RAM column and the analytical anion exchanger consisted of A, 10 mM phosphate buffer at pH 7.0 and B, 1 M phosphate buffer at pH 7.0. The column flow rate for loading the RAM column and elution of high-molecular-weight components (matrix) was 0.2 mL/min applying buffer A. The column was washed for 12 min to elute the matrix quantitatively. Elution of the retained fraction from the RAM column was performed at a flow rate of 0.2 mL/min using a step elution applying 100% eluent B.

Assessment of RAM Column Performance by SDS-PAGE. The RAM columns (RP-18, DEAE, and SO_3H) investigated were loaded with 100 μL (50 μg protein/μL) of human hemofiltrate. The sample was dissolved in the initial eluent, and the pH was adjusted accordingly prior to loading onto the RAM column. After injection, the matrix was eluted with the void volume, and the column was washed for 12 min by applying the appropriate initial eluent A conditions. Elution was performed with 100% B until the 215-nm UV signal approached the baseline again. The eluted peaks were collected and subjected to SDS-PAGE analysis. To increase the sample concentration in the lower-molecular-weight fractions eluted from the RAM column, an enrichment step was applied. The eluted RP-18 RAM fraction was enriched by evaporating the acetonitrile and TFA by vacuum centrifugation (SpeedVac Plus SC210A, Savant Instruments Inc., Farmingdale, NY). The residue was redissolved in 100 μL of 10 mM phosphate buffer of pH 3. The enrichment and desalting of the ion exchange RAM fractions were performed using StrataClean resin (Stratagene Cloning Systems, La Jolla, CA). The solid-phase silica-based resin modified with a hydrophobic surface reversibly adsorbed the proteins. A 40-μL portion of StrataClean resin slurry was suspended in each of the retained ion exchange RAM fractions (~2.5 mL) for 30 min.

The suspensions were centrifuged at 12000*g* for 10 min. The supernatant was discarded, and the complete pellet was resuspended in 25 μL of sample buffer for protein desorption.

A 20-μL portion of the ultrafiltrated fraction and 20 μL of the crude human hemofiltrate sample were mixed with 25 μL of sample buffer. A 5-μL porion of 0.5 M DL-dithiothreitol (DTT) was added to all of the samples, which were heated in a water bath (70 °C) for 10 min prior to loading (28 μL) on a 16% tricine gel (Novex, Frankfurt, Germany). The sample buffer was 25% 0.5 M Tris-HCl buffer at pH = 6.8, 20% glycerol, 40% SDS, and 5% bromophenol blue. The running buffer was 2.9% Tris base, 14.4% glycine, and 1% SDS. The samples were analyzed at 125 V for 1 h and 40 min until the dye front reached the lower end of the gel. All electrophoresis equipment was from Novex. The resolved proteins were visualized by silver staining.

2D-HPLC System. *Sample Fractionation Step Using RAM Columns.* The cation or anion exchange RAM column was loaded with 100 μL (50 μg protein/μL) of human hemofiltrate or 2 mL of fibroblast sample at a flow rate of 0.2 mL/min applying 100% buffer A as described previously. The column was washed for 12 min. Elution was performed in a backflush mode in-line with the analytical column at a flow rate of 0.5 mL/min.

First Dimension. The analytical ion exchange column was TSKgel SP-NPR or TSKgel DEAE-NPR providing either DEAE or SO_3H functionality. The columns were based on nonporous 2.5-μm polymeric beads packed in a 35 × 4.6 mm i.d. column (TosoHaas, Stuttgart, Germany). The column flow rate was 0.5 mL/min,and the column was always operated in-line with the corresponding ion exchange restricted access column.

The linear gradient in the first dimension was run from 10 mM phosphate buffer to 1.0 M phosphate buffer in 96 min. Afterward, the first dimension was washed with 1.0 M phosphate buffer in 8 min, followed by 8 min of column regeneration using buffer A prior to the next 2D analysis.

Second Dimension. The four reversed-phase columns were MICRA ODS I consisting of nonporous C-18 modified 1.5-μm silica beads packed in 14 × 4.6 mm i.d. columns (Eichrom Technologies Inc., Darien, IL).

The eluents for RP separations were A, 0.1% trifluoroacetic acid (TFA) in water, and B, 0.1% TFA in acetonitrile. The gradient cycle for the reversed-phase columns started with 4% B, which was increased to 40% B within 6 min and further increased to 100% B within 0.66 min, then was maintained for 0.15 min. Initial column regeneration and tube flushing was performed at 4% B for 1.17 min using a flow rate of 2 mL/min. Additional reversed-phase column regeneration prior to new sample enrichment was performed using water at a flow rate of 0.5 mL/min. All of the buffers and TFA were of analytical grade (Fluka Chemika, Buchs, Switzerland), and eluents were HPLC gradient grade (Merck, Darmstadt, Germany). Pure water was produced using a Milli-Q device (Millipore, Bedford, MA). All separations were performed at room temperature.

Operation of the 2D-HPLC System. The column arrangement was fully automated and controlled by an Integral 100Q Workstation (Applied Biosystems, Framingham, MA). The workstation provided a high-pressure gradient HPLC system consisting of a UV detector, refrigerated autosampler, and three ten-port valves. Two HPLC gradient systems, a pump, and two more ten-port valves were added to build the total system (see Figure 1). The first ten-port valve served as an injector, and the second ten-port valve was used for column switching in the sample fractionation step. In valve position A, the lower-molecular-weight target analytes were enriched on the internal surface of the RAM

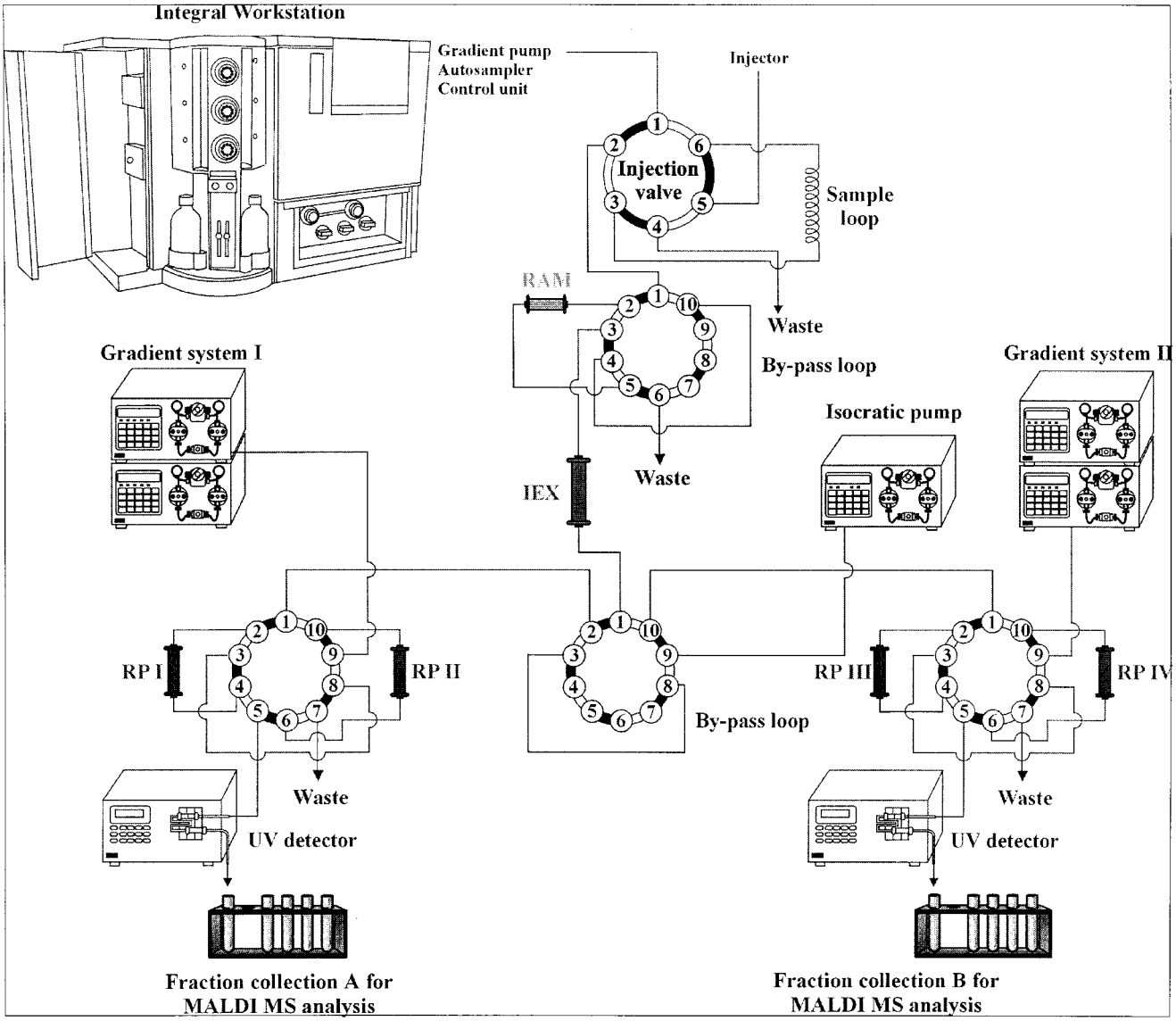

Figure 1. Schematic representation of the on-line comprehensive two-dimensional HPLC system, including an integrated sample preparation step.

packing, but the matrix and larger proteins were eluted directly to waste.

After valve switching, the RAM column was connected in a backflush mode in-line with the ion exchange column. During the 96-min gradient time of the ion exchange column, a total of 24 fractions were further analyzed. Each fractionation occurred over 4 min (corresponding to 2 mL effluent/fraction) and was subsequently transferred to the second dimension. The fractions eluting from the first dimension were enriched on top of one reversed-phase column (on-column focusing) and desalted, but the aqueous elution buffer was directed to waste. At the same time, the second column was regenerated with water while the two other columns were eluted in parallel by the two separate gradient systems. Each column was subjected to cyclic sample enrichment (sample loading), elution, and regeneration. The lower three ten-port valves in Figure 1 were used for column iteration. Table 1 shows the valve positions for the reversed-phase column iteration and the column operating modes over the complete run time.

Three of the ten-port valves are integrated in the Integral 100QWorkstation, pneumatically operated and made of PEEK (Rheodyne, Rhonert Park, CA). Two external electrically driven ten-port valves (Valco Instruments Co. Inc, Houston, TX) were controlled by electrical contact closures from the Integral 100Q workstation. Homemade electronics converted TTL or 12 V pulses from the workstation into contact closures. Two individual high-pressure gradient systems were used for the second dimension. One system consisted of two HPLC pumps, model 2200, and a central processor, model 7110, for gradient control (Bischoff Chromatography, Leonberg, Germany). The second pump set consisted of two LC-10 AD pumps (Shimadzu, Kyoto, Japan). Both reversed-phase gradient systems performed individual gradient control, and the start signal was triggered by an electrical contact closure from the workstation. Gradient mixing was performed using two dynamic low void volume mixing chambers (Bischoff Chromatography). An isocratic pump (LKB 2150, Pharmacia, Uppsala, Sweden) was used for column regeneration. To minimize void volume, tubing lengths were kept to a minimum, and an i.d. of 0.127 mm for the PEEK tubing was used (Upchurch Scientific, Oak Harbor, WA). One UV detector was part of the workstation and was equipped with a 1.2-μL flow cell, and the second detector

Table 1. Column Operational Parameters Indicating Valve Positions and Reversed-Phase Column Iteration Procedure for the 2D HPLC System

time min	valve 1 position	valve 2 position	valve 3 position	column 1 function	column 2 function	column 3 function	column 4 function
4	A	A	A	deposit	elute II	regenerate	elute I
8	B	A	B	elute I	regenerate	deposit	elute II
12	A	B	B	elute II	deposit	elute I	regenerate
16	B	B	A	regenerate	elute I	elute II	deposit
20	A	A	A	deposit	elute II	regenerate	elute I
24	B	A	B	elute I	regenerate	deposit	elute II
28	A	B	B	elute II	deposit	elute I	regenerate
32	B	B	A	regenerate	elute I	elute II	deposit
96	B	B	A	regenerate	elute I	elute II	deposit

was a Lambda 1000 (Bischoff Chromatography) equipped with a 0.8-μL flow cell. Both detectors were operated at a wavelength of 215 nm. Data from the second detector were imported as an analogue auxiliary detector signal into the software. The complete procedure to control the 2D-HPLC system was programmed in the method editor software of the workstation. The samples were stored in 1.2-mL vials at 3 °C in the temperature-controlled autosampler tray. Injections were automatically performed by filling a 100-μL sample loop. Monitoring the UV adsorption after the first dimension during the multidimensional run was not possible. The pressure drop between the columns of the first and the second dimension, which are linked through a valve, would have destroyed the detector cell. The chromatogram after the first dimension was made in a single column mode.

Mass Mapping by MALDI-TOFMS. Effluent fractions (~20 μL) corresponding to selected peaks from the second dimension were manually collected from the two detector outlets and used for MALDI-TOFMS analysis according to the seed layer technique[25] developed in our laboratory. A diluted matrix solution (either α-cyano-4-hydroxycinnamonic acid or sinapinic acid, 2 mg/mL in acetonitrile) was applied (0.5 μL) on the target plate. Then the sample was mixed 1:1 with either saturated matrix solution (15 mg/mL in acetonitrile, 0.2% TFA) and applied (0.5 μL) on the same spot. The MALDI-TOFMS instrument was a Voyager DE-PRO (Applied Biosystems Inc.) with built-in delayed extraction and a linear path of 1.1 m. It was equipped with a 337-nm nitrogen laser. The mass spectra were acquired both in the reflector and the linear mode at an accelerating voltage of 20 kV and a delay time of 150 ns.

RESULTS AND DISCUSSION

Choice and Evaluation of HPLC Modes. Reversed-phase HPLC is a favorable technique for resolving low-molecular-weight proteins and peptides. The chromatographic resolution and sensitivity are inversely proportional to the molecular weight of the protein, but for a gel, resolution and sensitivity are proportional to the molecular weight of the protein.[12] Ion exchange and reversed-phase chromatography were chosen as orthogonal separation modes, and size exclusion chromatography was integrated in the sample fractionation step using the RAM columns. The design of the fully integrated platform for peptide and protein mapping is illustrated in Figure 1. It consists of a sample fractionation coupled on-line with a comprehensive 2D-HPLC system. Polypeptides below 20 kDa can be selectively enriched, whereas higher-molecular-weight matrix components can be flushed directly to waste.

The separation on the RAM column and analytical column with ion exchange functionality is based upon electrostatic interactions, and analytes are resolved according to charge and charge distribution of the protein. Even though ion exchange chromatography does not provide a high-resolution power, it gives the highest possible orthogonality to reversed-phase separation. Cation exchange at acidic pH values (e.g., at pH 3.0) enables one to cover the whole pI range of a biological sample, and anion exchange can be used to expand the lower pI range. Nonporous resins were chosen as packing material for quantitative recoveries without adding an organic modifier, which is a prerequisite for enrichment of fractions on a reversed-phase column. High loading capacities, in the milligram range for both ion exchange columns, are favorable for their use in the first dimension.

n-Octadecyl-bonded nonporous silica particles with an average diameter of 1.5 μm proved to have the required characteristics for the second dimension because of their high mechanical stability, fast mass transfer kinetics due to a lack of pore diffusion, and high recoveries.[26] Using short columns packed with these particles allows short analysis and reconditioning times at peak capacities of ~100−150.

The four MICRA ODS I RP columns used alternatively in the second dimension showed an almost identical resolution pattern with reproducible retention data. The valve positions and the consecutive column usage were identical from run to run. This enabled the direct comparison and correlation of consecutive chromatographic runs despite slight variations in the performance of the four columns. The columns were used for more than 1500 single runs over two years showing high robustness combined with a long lifetime. The use of nonporous MICRA ODS I columns especially designed for high-speed HPLC still required shallow gradients and, thus, long analysis times. The extension of the gradient time to ~8 min led to a large increase in the number of resolved peaks for complex protein and peptide mixtures, as compared to lower gradient times.

The basic idea of an on-line comprehensive 2D-HPLC system is the use of a slow separation in the first dimension followed by a fast separation in the second dimension. To match the requirements for high resolution in the second dimension without

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reducing the sampling rate or slowing down the first dimension, at least two second dimension columns need to be eluted in parallel. The second criterion for economizing analysis time and achieving reproducible results is to allow sufficient time for column regeneration between the gradient elutions. This can be done by regenerating an additional column in parallel to the elutions in the second dimension. The new setup using four parallel columns in the second dimension satisfies the requirements with respect to speed, resolution, reproducibility, and recovery. On-column focusing for fraction transfer to the second dimension was introduced by Opiteck et al.[15] and has considerable advantages. Enrichment of the fractions directly on top of the column does not require any sample storage; hence, there is no vial contamination or sample loss due to other sample handling procedures, such as fraction collection and reinjection. The procedure avoids sample dilutions and automatically desalts the analytes, thus preventing eluent incompatibilities.

Characterization of On-Line Sample Fractionation Using RAMs. Coupling a size-selective fractionation step on-line with a continuous 2D-HPLC system is a new straightforward procedure.

Restricted access materials (RAMs), which were introduced by Pinkerton[27] as early as 1985 and which have been reviewed recently by Boos,[24] combine two chromatographic separation modes in one column, namely, size exclusion and adsorption chromatography. In addition to a defined pore size, the specific feature of RAM is the topochemically bifunctional surface of the particles. In LiChrospher ADS, for example, the outer particle surface is modified with hydrophilic diol groups, whereas the inner pore surface is covered by hydrophobic alkyl chains, for example, C-18. RAM columns allow the direct and repetitive injection, as well as size-selective fractionation, of untreated biofluids, such as plasma, serum, urine, fermentation broth, and supernatant of tissue homogenates.

High-molecular-weight sample components, for example, matrix proteins, cannot penetrate into the pores and, thus, are eluted quantitatively in the void volume of such columns. Only analytes of low molecular mass have access to the binding centers at the inner pore surface and, thus, can be retained and extracted selectively. RAM columns with reversed-phase properties are widely used as precolumns in coupled-column systems for HPLC integrated, extractive, sample cleanup of low-molecular-weight target analytes in raw biofluids.[24] Recently, sample fractionation and extraction of peptides using silica-based ion exchange RAM columns was demonstrated.[28]

The silica-based ion exchange RAM employed in the current study has a narrower pore-size distribution and provided superior discrimination properties, as compared to polymeric based ion exchange RAM columns.[29] Furthermore, silica-based RAMs with cation exchange functionality possess high loading capacities for proteins. The loading capacity is in the milligram range for a 25 × 4 mm-i.d.-sized column and was demonstrated with aprotinin as a standard protein.

Even though the human hemofiltrate had been filtered through a 30 kDa membrane during the dialysis of patients suffering from chronic renal disease, the major component was still human serum albumin with a molecular weight of 67 kDa. Figure 2 shows an image of a slightly overstained SDS-PAGE gel in which different RAM extracts, the crude hemofiltrate sample, and the ultrafiltrated sample were analyzed.

Track 1 was loaded with the human hemofiltrate sample at a concentration of 1 mg/mL (11 μg protein). Even though silver staining is only quantitative over a limited range, human serum albumin is visible as the most intensive band. Compounds below 15 kDa are in low abundance and appear as low-intensity silver-stained bands. Track 2 displays a 50 times higher concentrated hemofiltrate sample after ultrafiltration using a 10 kDa membrane (low mw fraction out of 550 μg protein). Human serum albumin was almost completely eliminated, but proteins and peptides below ~30 kDa were enriched. Tracks 4, 6, and 7 show the separation of the retained fraction on different RAM columns. All of the columns showed an enrichment of lower-molecular-mass components and a reduction in the high-molecular-mass fraction of the sample. Comparable amounts of retained proteins from the SO_3H-RAM column and DEAE-RAM column were loaded onto the gel (low mw fraction out of 5000 μg protein). The low-molecular-weight fractions eluted from the anion and cation exchange RAM columns show less binding of human serum albumin, as compared to the LiChrospher RP-18 ADS column (low mw fraction out of 1000 μg of protein). Nonspecific binding of human serum albumin can be explained by interactions between the relatively hydrophilic albumin and remaining silanol groups on the outer RAM surface carrying the biocompatible diol functionality. It was quantified to be ~8% at an average concentration level, using standard proteins and integration of the UV signal (data not shown); however, inspection of the gel clearly demonstrated the enrichment of components with a molecular weight lower than ~15 kDa, a continuous molecular weight cutoff and some nonspecific binding of higher-molecular-weight components. It should be noted that the protein load onto the gel from the RAM fractions was 100 times higher, as compared to the crude hemofiltrate sample.

Sample Transfer from the RAM to the Analytical Ion Exchanger Column. The RAM column was eluted in a backflush mode in-line with the analytical ion exchange column. In-line column coupling is susceptible to band broadening because of extra volume in the column. Band broadening can be avoided if the binding to the second column is stronger than to the first column and gradient elution is applied. Aprotinin, as standard protein, was loaded onto the SO_3H-RAM column and backflush eluted in-line with the cation exchange column. Comparison to the direct injection onto the cation exchange column proved that band broadening was negligible (data not shown). Alternative approaches for sample transfer using salt pulse elution followed by 1:100 postcolumn dilution proved to be not realizable. Recovery studies on the cation exchange RAM column using aprotinin and the 2D-HPLC buffer system led to almost quantitative recoveries of 95% and higher.

2D-HPLC of Biological Samples. Figure 3 shows the cation exchange separation of a human hemofiltrate sample at pH 3.0. After loading, the cation exchange RAM column was gradient-eluted in-line with the analytical cation exchange column. The chromatogram shows an incomplete separation of the molecular weight fraction below 15 kDa within the 96-min analysis time.

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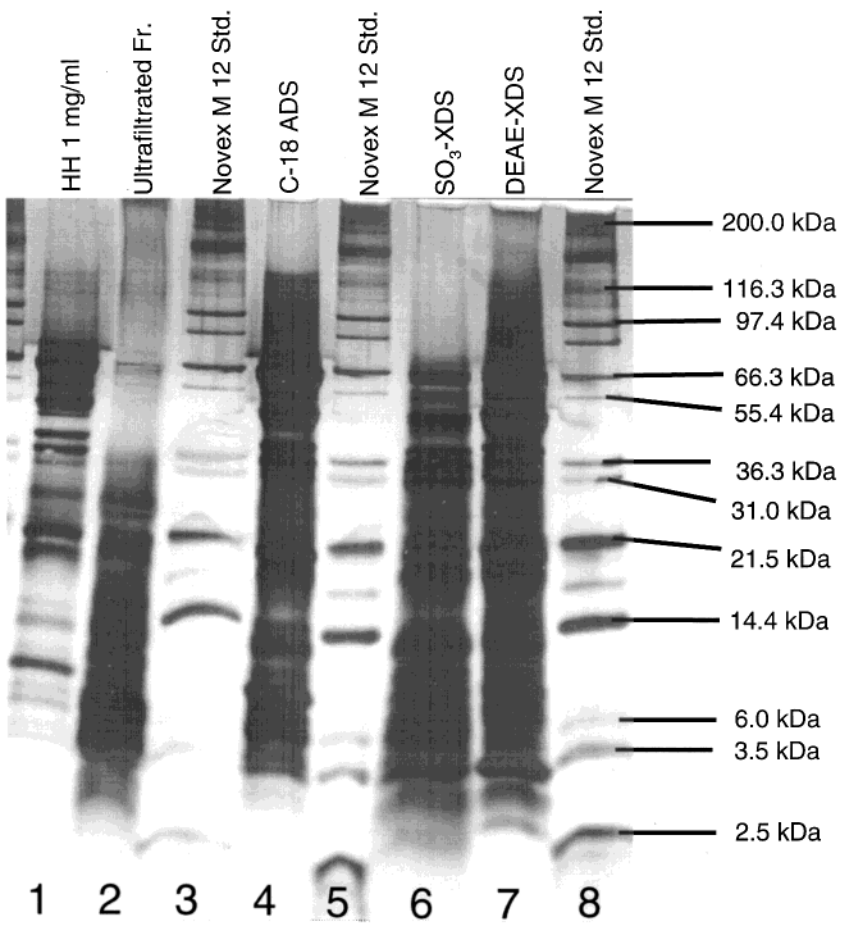


Figure 2. Characterization of RAM performance by SDS-PAGE. Different amounts were loaded onto the gel.

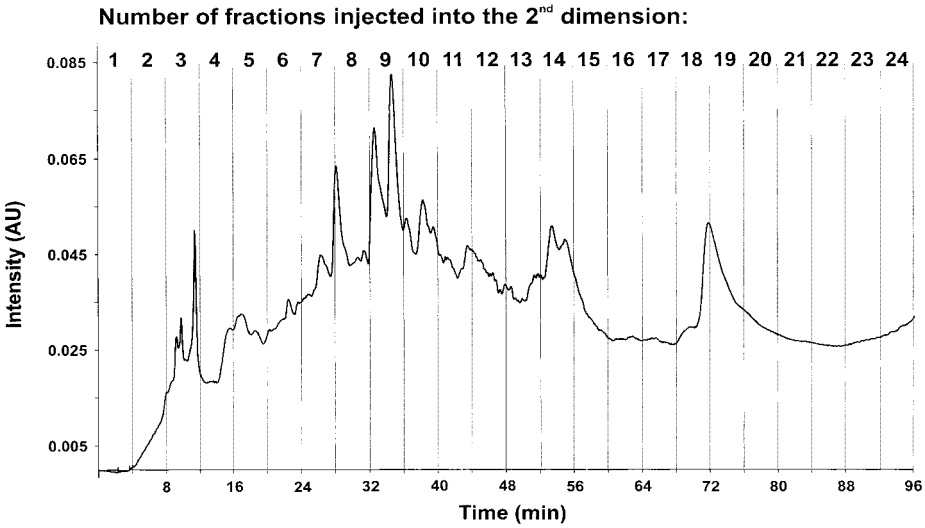
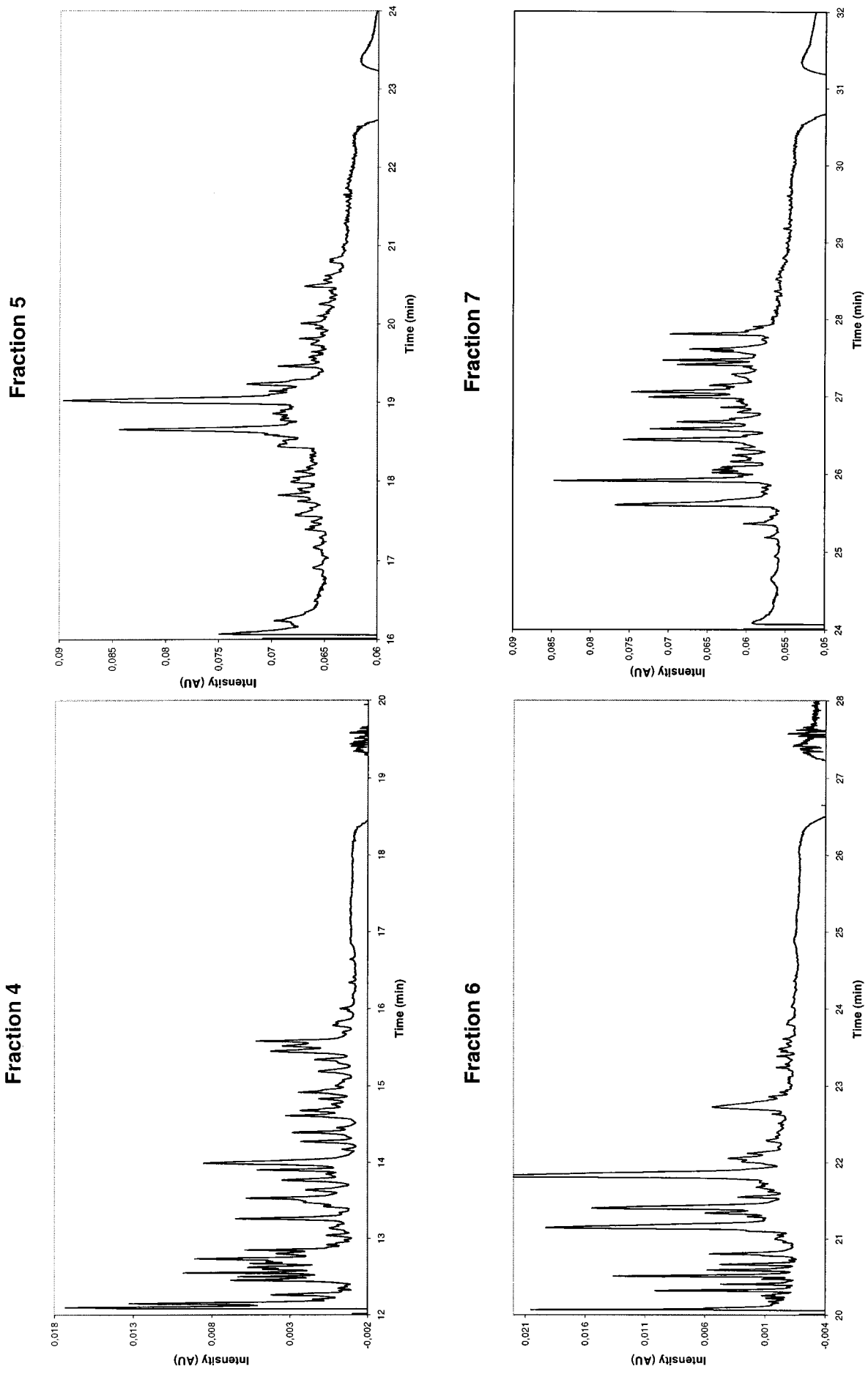


Figure 3. The chromatogram illustrates the separation of human hemofiltrate on the analytical cation exchange column in the first dimension after first being subjected to selective enrichment on a cationic RAM. Fractions (24 in total) were continuously transferred to the second dimension in 4-min intervals for subsequent analysis by reversed-phase chromatography.

Approximately 25 peaks were visible but not baseline-resolved. Each of the 24 fractions of 4-min duration (2 mL of eluent) in Figure 3 was transferred on-line to the second dimension for subsequent reversed-phase chromatography on MICRA ODS I columns. Figure 4 shows 8 typical reversed-phase chromatograms (fractions 4−11) generated during a complete two-dimensional (RAM/cation exchange/RP) run. More than 1000 peaks were resolved within the total analysis time of 96 min.

The fraction number mentioned on the cation exchange chromatogram corresponds to the fraction indication on the reversed-phase chromatograms in Figure 4. Automatic fractionation of equidistant intervals, regardless of the first dimension separation pattern, was applied for sample transfer. The number of first-dimension peaks roughly equals the number of fractions transferred to the RP columns. The peak capacity was inversely proportional to the retention factors, a problem that could be


Fraction 5
Intensity (AU)
Time (min)
Fraction 7
Intensity (AU)
Time (min)
Fraction 4
Intensity (AU)
Time (min)
Fraction 6
Intensity (AU)
Time (min)

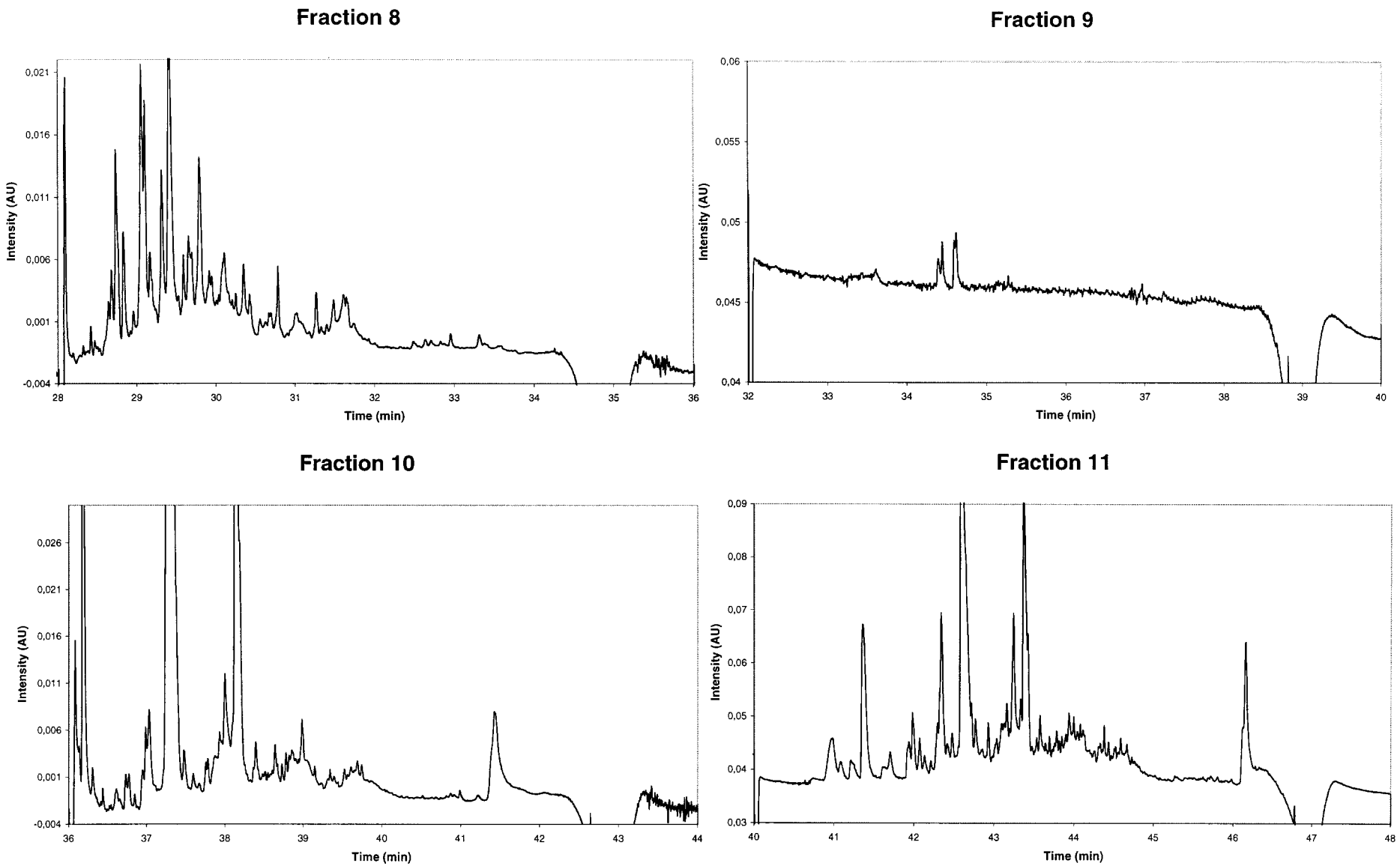


Figure 4. Selected reversed-phase chromatograms corresponding to a complete two-dimensional RAM/cation exchange/reversed-phase run exemplifying the high resolving power within 8 min of analysis time.

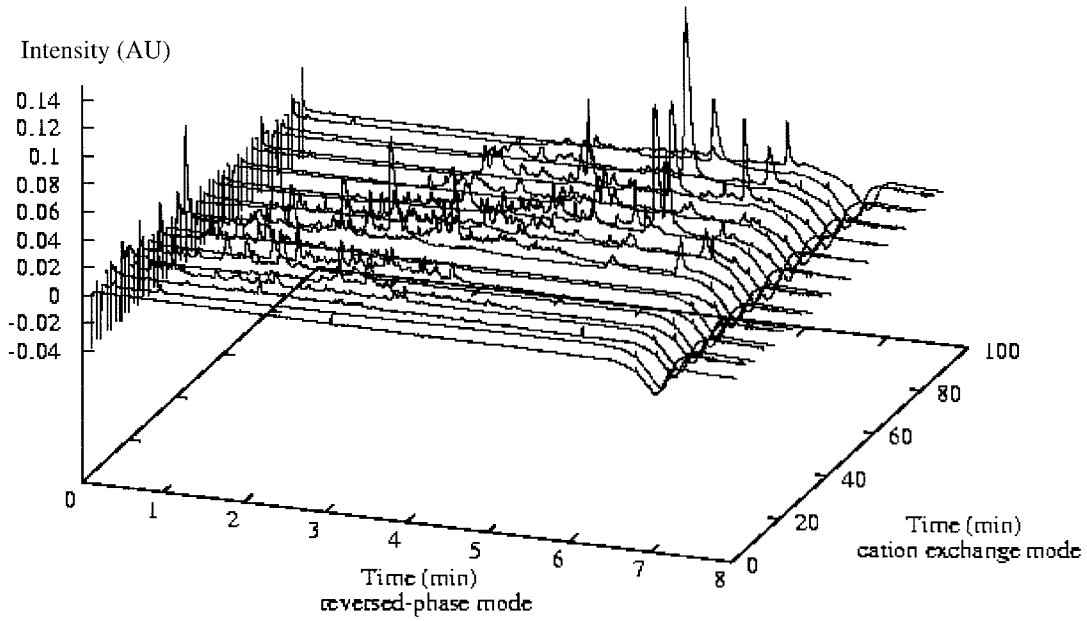


Figure 5. 3D display of the RAM/cation exchange/reversed-phase separation of human hemofiltrate.

circumvented by using a nonlinear gradient for optimization. The size of the fractions that were transferred to the next dimension was not varied because of instrumental limitations. R. Murphy et al.[30] investigated the effect of sampling rate on resolution in comprehensive two-dimensional gel-permeation/RP-chromatography of copolymers. In this paper, the authors claim that each peak in the first dimension should be sampled at least three times into the second dimension to achieve the highest resolution. On the other hand, previous investigations using standard proteins demonstrated that even when 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.

A total number of 24 RP chromatograms were generated in the second dimension. Every second chromatogram was produced with the additional external UV detector, and the start time of each chromatogram must be deducted to compare the absolute retention times in the RP mode.

More than 60 peaks were resolved in some RP chromatograms, clearly demonstrating the resolving power for small proteins and peptides. Comparison of two consecutive RP chromatograms from a single first dimension run showed the orthogonal separation power of the system. The chromatograms are different, with only few peaks appearing in two adjacent chromatograms. The identification of redundant peaks can also be done by a 3D display. The 3D display in Figure 5 indicating the retention times in either separation mode and the UV intensity confirms the orthogonal separation power.

Despite a total of 1000 resolved peaks in the 2D system, it does not necessarily signify that 1000 different components are resolved, as would be expected in a single mode run. This is an intrinsic feature of the column coupling approach.

One peak of a single component can appear in two or more consecutive chromatograms as a result of partial separation in the ion exchanger mode or from splitting a first-dimension peak into two adjacent fractions. The peak capacity in the second dimension can be calculated to ~130 per column. Considering the 24 fractions from the first dimension as the first dimension peak capacity, the total theoretical peak capacity can be calculated to be as high as 3000.

The same findings were achieved by coupling the DEAE RAM column to the cation exchange column at pH 7 followed by the array of RP columns. The anion exchange chromatogram in the first dimension produced fewer and wider peaks, as compared to the cation exchange mode; thus, fewer components were resolved in the RP mode (data not shown).

Figure 6 demonstrates the separation power of the 2D-HPLC system for a biological sample derived from a human fibroblast cell culture. Fraction 13 was chosen as an example out of 24 generated chromatograms. It should be emphasized that no special pretreatment of the sample was necessary prior to sample injection into the multidimensional system, although 8 M urea was used to solubilize the proteins from the primary cells. The separation efficiency was found to be similar to that of the hemofiltrate separation. The total protein amount of the sample was determined by a protein assay to be about 0.5 mg/mL, which required a total sample loading of 2 mL onto the RAM column for sufficient enrichment of the low-molecular-mass analytes. It was demonstrated that high resolution power with minimal sample preparation can be achieved at a concentration level that is accessible by UV detection. This offers the potential to map expression differences (up and down regulations) by comparing the UV traces. Differential displays of cytokine-stimulated fibroblasts are currently being studied utilizing the 2D-HPLC system.

Identification of Selected Components by MALDI-TOF Mass Spectrometry. Small fractions from selected peaks were manually collected from the UV detector outlets and subjected to MALDI-TOFMS analysis. Figure 7 shows a typical chromatographic pattern of 4-min duration of human hemofiltrate with the corresponding MALDI-TOF spectrum correlated to the arrow-marked peak in the chromatogram. For most of the chromato-

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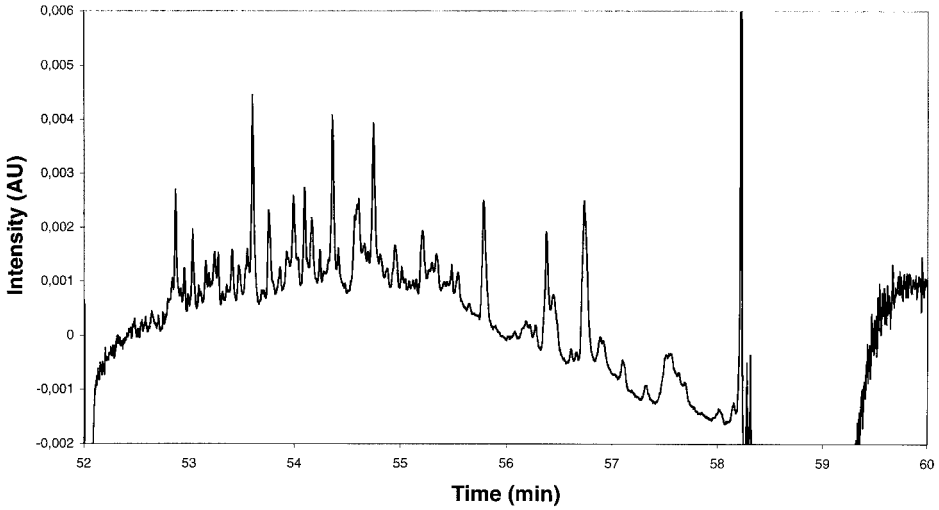

Figure 6. One out of 24 reversed-phase chromatograms originating from the direct injection of the human fetal fibroblast cell line.

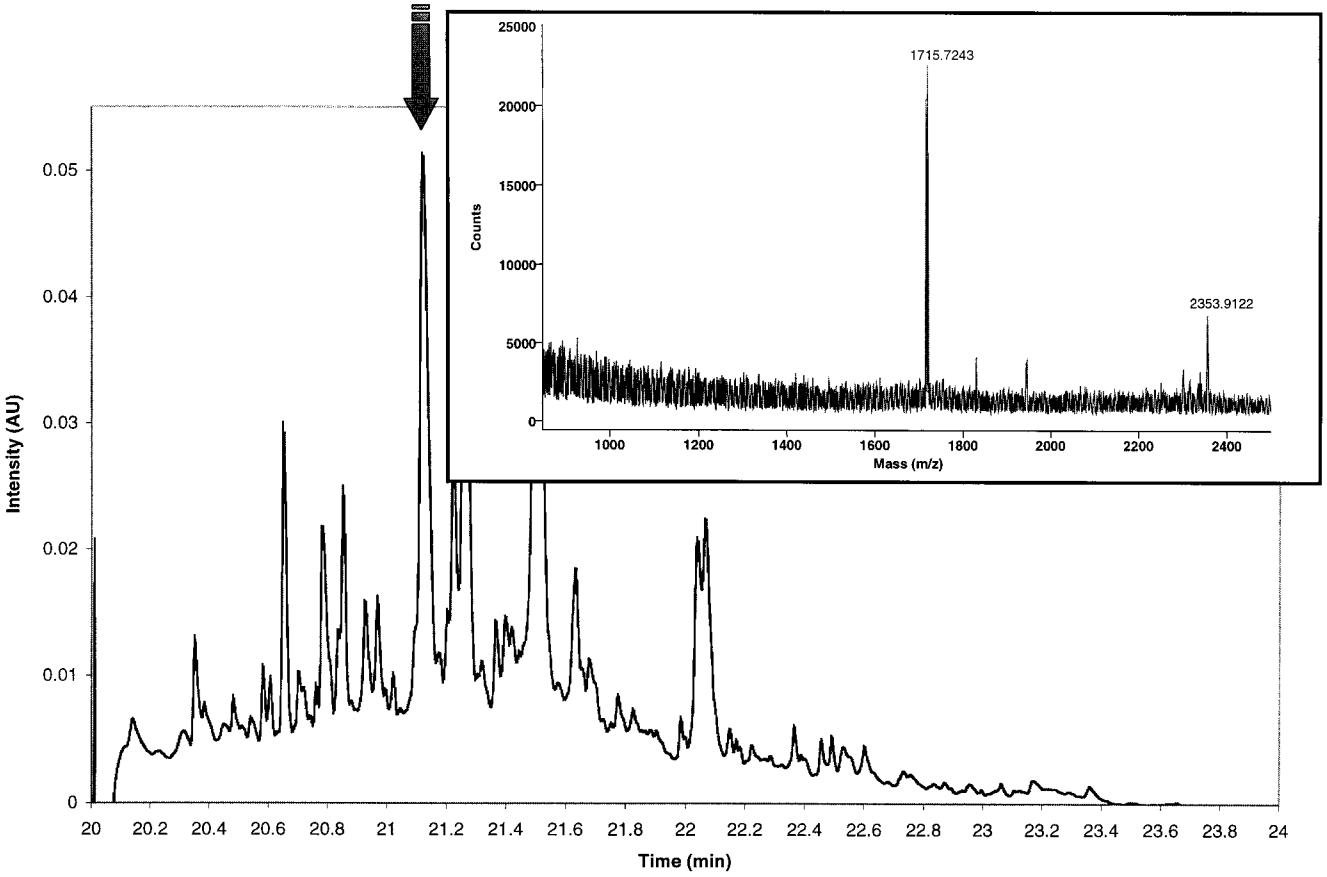

Figure 7. Typical MALDI-TOF spectrum corresponding to the peak (arrow) in the chromatogram, which originates from human hemofiltrate sample. In this case, two masses were observed from the single UV peak.

graphic peaks that have been analyzed by MALDI-TOFMS, only one mass was visible, indicating the high resolving power of the 2D-HPLC system. Some collected peaks contained up to three different components, that is, mass spectrometry provides a third dimension to the system. Although not used in this case, sequencing of the resolved peptides by tandem mass spectrometry (e.g., static nano-ESI) may be an additional option to ascertain identity by database searching. The mass measurements by MALDI-TOFMS revealed that the molecular mass range of the peptides was between 800 and 5000 Da.

Repeatability of the Integrated 2D-HPLC Platform. The repeatability of the cation exchange/reversed-phase system was investigated by assessing the relative standard deviations of the retention time, peak height and peak area. Fifteen randomly selected peaks generated from six consecutive runs of human hemofiltrate were chosen for computation. The repeatability data are shown in Table 2. The RSD of the retention times was less than 0.5% on average. The peak areas and peak heights were determined by employing automatic integration; the RSD values ranged between 5 and 25% with a few exceptions. Hence, the

Table 2. Repeatability Data from Six Consecutive Runs of the Two-Dimensional System

	peak no.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
av retention time, min	21.19	21.80	22.74	28.67	29.42	37.38	38.18	41.44	45.78	55.66	56.72	63.37	64.64	81.17	89.31
RSD retention time, %	0.34	2.41	0.20	0.18	0.16	0.21	0.15	0.13	0.70	0.08	0.03	0.50	0.05	0.05	0.04
RSD peak area, %	14.8	17.6	19.3	13.1	21.1	6.6	7.4	16.9	13.2	22.0	35.6	15.7	12.5	18.2	29.8
RSD peak height, %	16.5	7.4	25.6	12.9	29.3	4.4	2.9	17.8	6.1	10.2	15.4	11.9	13.4	8.6	20.5

obtained RSD values were better than or in the same range as the repeatability of 2D gel electrophoresis. The limit of detection at a wavelength of 215 nm was determined without the sample preparation step using standard proteins. The limit of detection (LOD), as defined by the analyte concentration giving a signal equal to the blank signal plus two standard deviations, was about 50 ng total protein amount for ovalbumine. A typical concentration value is 100 ng/mL. The on-line column-coupling approach, which automatically desalts the samples, easily allows the detection proteins and peptides in the deep ultraviolet region at 215 nm, providing a high sensitivity adsorption in peptide bonds. The limit of detection is in the same order of magnitude as the sensitivity of silver stains.

CONCLUSIONS AND PERSPECTIVES

The resolution power of the 2D-HPLC setup is superior to any other separation method for the specific molecular weight range. This has been clearly demonstrated by the high-resolution separation of small sized proteins and peptides from human hemofiltrate. Hemofiltrate is one of the most complex protein and peptide samples known; thus, similar or even better results are expected for less complex samples, for example, cell lysates. RAM columns are ideally suited for sample preparation of small sized biopolymers, since they enable one to boost the sensitivity for low abundant sample components by loading high amounts of sample and allow one to exclude the higher-molecular-weight fraction.

What has to be taken into account is the low total analysis time of the integrated separation platform of less than 2 h. With the exception of the ICAT approach by Gygi et al.,[22] the separation speed is significantly higher, as compared to other multidimensional HPLC approaches. The techniques using a salt step gradient in the ion exchange mode by Davis et al.[11] takes ∼4 h, but Link et al.[17] use 12 × 60 min plus transfer times for the total analysis. The off-line approaches are usually time-consuming as well, for example, 50 min plus 10 × 45 min for the RPC/CE separation by Udiavar et al. and 14 h plus 20 × 15 min for the IEF/RPC approach by Wall et al.[12]

The reproducibility and robustness have been proven, which is essential for detecting differences in protein patterns. Ion exchange chromatography, with its use of eluents containing aqueous buffers, is favorable regarding protein stability. The short elution times in the RP mode will probably cause less denaturation of the proteins, as compared to 2D gel electrophoresis or SDS-PAGE. This offers the chance to run bioassays to screen the fractions. Miniaturization is an option for a further increase in sensitivity and possibly in resolution and will be necessary in cases in which there is only a limited amount of sample available. Direct coupling to ESI-MS is straightforward, since the analytes stay in a desalted liquid phase all the time.

One further promising option is to on-line couple the second dimension to a piezoelectric flow-through microdispenser for on-line array fractionation onto targets for MALDI-TOFMS. It was demonstrated by Miliotis et al.[31] that the effluent from a micro-HPLC column can be spotted onto a stainless steel target plate that is coated with a thin layer of the matrix crystals. The dispensing unit is fully automated using a robotic equipment.

The new 2D-HPLC method providing high-resolution power with a single injection onto the RAM column can easily be used in high-throughput applications. It has been demonstrated that the high separation power with minimal sample preparation can be achieved at a concentration level that is accessible by UV detection. This offers the potential to map expression differences by comparing the UV traces.

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