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Short communication

Searching biomarker candidates in serum using multidimensional native chromatography
I. Enhanced separation method

Stefan Kreusch[a,1], Margarete Schulze[a], Gerhard A. Cumme[a], Günter Ditze[b], Thomas Moore[c], Heidrun Rhode[a,*]

[a] Institute of Biochemistry, Friedrich Schiller University, Medical Faculty, D-07740 Jena, Germany
[b] Workshop for Research and Development, Friedrich Schiller University, Medical Faculty, Jena, Germany
[c] CyBio AG, Jena, Germany

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ABSTRACT

The microplate-based method developed by our group for non-denaturing multidimensional proteome separation was improved on using improved column arrays and a newly developed robot. Currently size exclusion, anion exchange and lectin affinity chromatography are combined orthogonally. Different samples run simultaneously to enhance reliability of intercomparison. LC–ESI (electro-spray ionization) MS/MS analysis of selected fractions identified 32,288 peptides matching 2669 serum proteins. The present contribution (I) shows the characteristics of the method, whereas "prove of principle" by applying it to search for biomarker candidates with model diseases is reported in an accompanying paper (II).

1. Introduction

The initial surge of publications regarding technological developments in proteomics has been followed recently by more critical views. A growing number of contributions point to the lack of comprehensive information, the lack of reproducibility data, and the inadequate standardization of procedures and tools (e.g., [1–3]). Probably complementary methodologies have to be used to get reliable and comprehensive results, as proposed in [4,5].

To comprehensively characterize a biomarker, information on both concentration and state (complexation, fragmentation, glycosylation, etc.) should be obtained. Whereas information on state of components is lost by many "bottom-up" approaches, classical chromatography may represent an important complementary strategy [4–6] because it preserves all sample constituents. Different separation techniques can be combined orthogonally, miniaturized, and automated. Moreover, liquid sub-fractions are produced throughout. Applying these methods to high-throughput analysis may fulfill the quality criteria essential for multidimensional separation, i.e., resolution, reproducibility, recovery, robustness, simplicity, speed, selectivity, and sensitivity, quoted as "4RS criteria" [7].

Recently a variety of sample separation strategies for serum/plasma have been developed including chromatographical methods (e.g., [8–10]). A native separation technique combining two different chromatographic techniques orthogonally was developed in our group [11]. The technique is compatible with microplate technology and yields liquid fractions with proteins that exhibit defined small ranges of characteristic parameters, such as size or charge. Proteins can be identified by immunological methods, enzyme activity measurements, as well as by mass spectrometric analyses of proteins and peptides and their fragments after tryptic digestion. This method was adapted to human serum for searching indestructible biomarker candidates within blood samples, whereas plasma is generally preferred to serum for in-depth proteome analysis [12]. The separation protocol of [11] was improved with respect to resolution, throughput, reproducibility, robustness, and simplicity. The present paper shows the characteristics of the improved method whereas the accompanying paper describes application of the search principle to model diseases.

2. Experimental

2.1. Materials

N-p-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (EC 3.4.21.4, bovine pancreas) and α-cyano-4-hydroxycinnamic acid were obtained from Sigma–Aldrich Chemie (Sternheim, Germany). Sinapic acid was supplied by Fluka Chemie (Buchs,

* Corresponding author. Tel.: +49 3641 938620; fax: +49 3641 938612.
E-mail address: Heidrun.Rhode@mti.uni-jena.de (H. Rhode).
[1] Present address: Scienova GmbH, D-07743 Jena, Nonnenplan 2, Germany.

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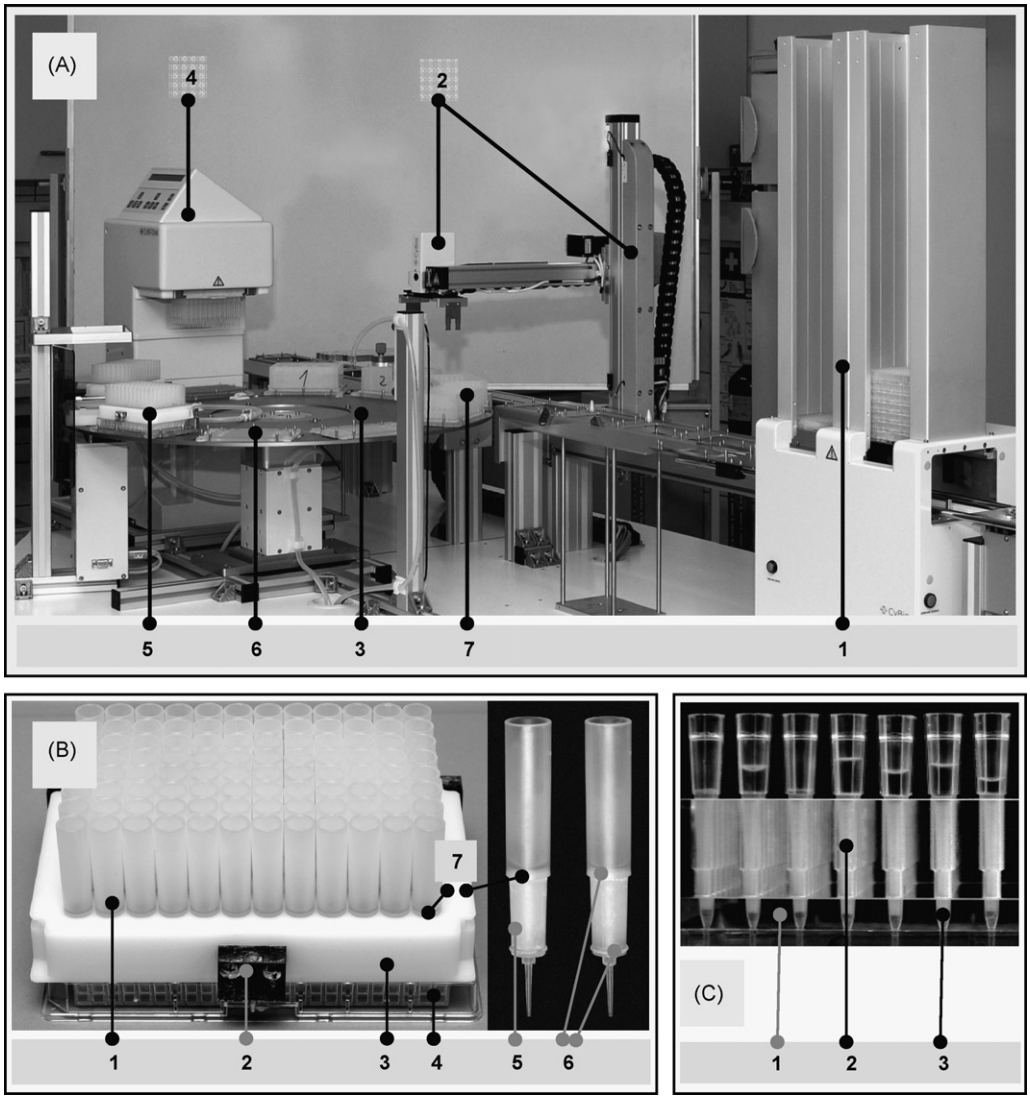

Fig. 1. Parallel AEC and LAC separation. (A) Analysis robot. The robot contains a microplate stacker (1), a robot for carrying microplates (2), a turntable with ten parking places for microplates (3), and a CyBi-Well^{TM} pipetting device (4). (5) One AEC-column array, and (6) represents a free place on the turntable for, e.g., a liquid reservoir (7). (B) Array of AEC columns. Left: 96 AEC columns (1) placed on a partition of a 384-well microplate (4). The adapter tool (3) arranges the columns according to microplate format; whereas adapter tool (2) locates the column block so that the column outlets are centered above the wells. Right: two single columns, each containing 330 μL DEAE-cellulose (5) enclosed between two porous slices (6). (C) Array of LAC columns. The figure shows a part of a frame (1) that contains 8 × 12 holes arranged so that up to 96 Gel-loader tips may be arranged in microplate format. The tips have been modified so as to accommodate 100 μL ConA-Sepharose (2) between two porous slices (3).

Switzerland). Molecular weight standards used for SEC, i.e., thyreoglobulin (porcine), bovine serum albumin, chymotrypsin (bovine), and ribonuclease A (bovine pancreas) were from Serva Electrophoresis (Heidelberg, Germany), and ferritin (horse) and catalase (bovine) were from Amersham Biosciences (Freiburg, Germany). All further reagents used were of analytical grade either from Sigma, Fluka, or Merck KGaA (Darmstadt, Germany). The Peptide Mass Standards Kit Sequazyme, Cs, and Igor peptide were from Applied Biosystems (Darmstadt, Germany).

Serum samples: Normal sera were obtained standardized from healthy volunteers among the laboratory staff (Ethics Commission of the Medical Faculty, 1677-11/05). Twenty millilitre of blood was drawn into 9 mL S-monovettes from Sarstedt (Nümbrecht, Germany) the morning after overnight fasting and permitted to clot for 2 h at ambient temperature. Thereafter serum was separated (1000 × *g*, 10 min, 4 °C) and either used immediately or stored frozen at −84 °C for one to two days until use. Freeze–thaw cycles were avoided throughout.

2.2. First separation dimension (1D): size exclusion chromatography (SEC)

The first separation step and analysis of the resulting fractions are described in [11]. Briefly, a mixture of 0.9 mL serum and 0.1 mL 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl was loaded onto a HiLoad Superdex^{TM} 200 column (16/60, Pharmacia, Uppsala, Sweden) equilibrated with 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl. Ninety six 1-mL fractions were collected in a 96-deepwell microplate using a flow rate of 1 mL/min. Thereafter two 250-μL aliquots from each of the 96 1D-fractions were dialyzed against 10 mM Tris/HCl, pH 7.4, and 25 mM NaCl [11,13].

2.3. Second separation dimension (2D): anion exchange chromatography (AEC)

The separation protocol was improved with respect to [11]. Instead of using a column block, 96 replaceable columns con-

Table 1
AEC elution conditions. 95 μL was loaded onto each column per step. Effluents from each series of AEC steps as indicated in the first column were collected in a 384-well microplate (cf. Section 2.3).

Fraction No.	Solution loaded	Eluent composition, 10 mM Tris/HCl plus NaCl (mM)	n-propanol (%, v/v)	Eluent pH
0, 1, 2	1-D fraction	25		
3-6		25		7.4
7-10		75		
11-14		125	0	
15-18	Elutent	125		
19-22		175		7.2
23-26		225		
27-30		500		
31-34		500	20	

taining 330 μL DEAE-cellulose were framed to microplate format compatible to a liquid handling robot (Fig. 1A and B). They were equilibrated with 10 mM Tris/HCl, pH 7.4, containing 25 mM NaCl. Thereafter these columns were loaded with the 96 corresponding dialyzed 1D-fractions using a CyBi^{TM}-Well device (CyBio AG, Jena, Germany) that had been integrated into a robot. Principally, up to five samples may be automatically separated in parallel with the column arrays. Loading was done in three 95 μL steps. In each of the following elution steps 95 μL of eluent was applied to each column. Proteins were eluted by hydrostatic-driven flow-through. Adhesion effects were eliminated by dipping the column outlet tips into 10 μL of the elution solution which had been preloaded into each microplate well. The eluent NaCl concentration was increased and pH was shifted according to Table 1, instead of solely increasing the ionic strength. The 96 effluent volumes of each loading and elution step were collected in different partitions of 384-well UVstar microplates (Greiner Bio-One, no. 781801, Frickenhausen, Germany). In the end, one microplate with 3 × 96 fractions from loading and eight microplates, each with 4 × 96 eluted fractions, were obtained. Thus, every 1D-fraction was separated into 35 instead of 25 2D-fractions producing 3360 instead of 2400 fractions.

2.4. Third separation dimension (3D): lectin affinity chromatography (LAC)

2D-fractions of still highly complex composition (more than 1 mg total protein per millilitre) were selected by hit picking. Aliquots were placed into a microplate and 3D separated in parallel. Fig. 1C shows mini-columns prepared from Gel-loader tips (Greiner Bio-One, no. 770290). Each column contained 100 μL ConA-Sepharose 4B (Sigma, no. C9017, Sternheim, Germany) and was equilibrated with binding buffer (20 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$). One hundred microlitres of 2D-fractions were loaded directly onto such columns. Fractionated washing three times with 100 μL binding buffer separated non-glycosylated proteins and glycoproteins poor in mannose. Thereafter, glycoproteins rich in mannose were eluted with 3 × 100 μL elution buffer (0.5 M methyl-α-D-mannopyranosid in binding buffer). The columns were regenerated with 100 μL 20 mM Tris/HCl, pH 8.5 containing 0.5 M NaCl. In the end, eight 3D-fractions were obtained from each selected 2D-fraction (one by loading; six by washing and elution; and one by regeneration) and were collected in 384-well UVstar microplates. Fractions were analyzed by UV-absorbance at 280 nm (see below), SDS-PAGE [14], and LC–ESI–MS/MS.

2.5. Quantitation of total protein

Total protein concentration was determined according to [11,15] from UV-absorbance at 280 nm using a Spectramax Plus^{384} microplate reader (Molecular Devices, Sunnyvale, CA, USA), UVstar microplates, and bovine albumin as a standard. One hundred and fifty microlitres of serum samples diluted 1:10 to 1:100 were used; 150-μl aliquots were taken from 1D-fractions, whereas each 2D- and 3D-fraction was used as a whole. Since normal serum was used, interference by bilirubin, nucleotides, and uric acid could be excluded by multi wavelength measurements. Precision of protein concentration of each fraction position was determined from repeated separations using the software package VisualCockpit-Life Science [16].

2.6. LC–ESI–MS/MS

2.6.1. Data acquisition

Aliquots of all fractions were digested as described in [11]. Seven and one-half microlitres of tryptic digest was mixed with 7.5 μL solution A (5%, v/v, acetonitrile and 0.05% formic acid in deionized water). One microlitre of the mixture was subjected to reversed-phase HPLC on an Ultimate system equipped with a FAMOS auto sampler (Dionex, Idstein, Germany). HPLC separation on a PepMap column (15 cm, i.d. 75 μm, C18, 3 μm beads) was performed with a 150 nL/min flow rate and the following gradients: 0–2 min: 5%, 2–57 min: 5–70%, 57–58 min: 70–100%, 58–65 min: 100%, 65–66 min: 100–5%, 66–72 min: 5% (v/v) solution B (80%, v/v, acetonitrile, 0.04%, v/v, formic acid in deionized water) in solution A. The HPLC outlet was coupled through a nano-spray needle (New Objective, Woburn, MA, USA) to the hybrid quadrupole/TOF MS/MS

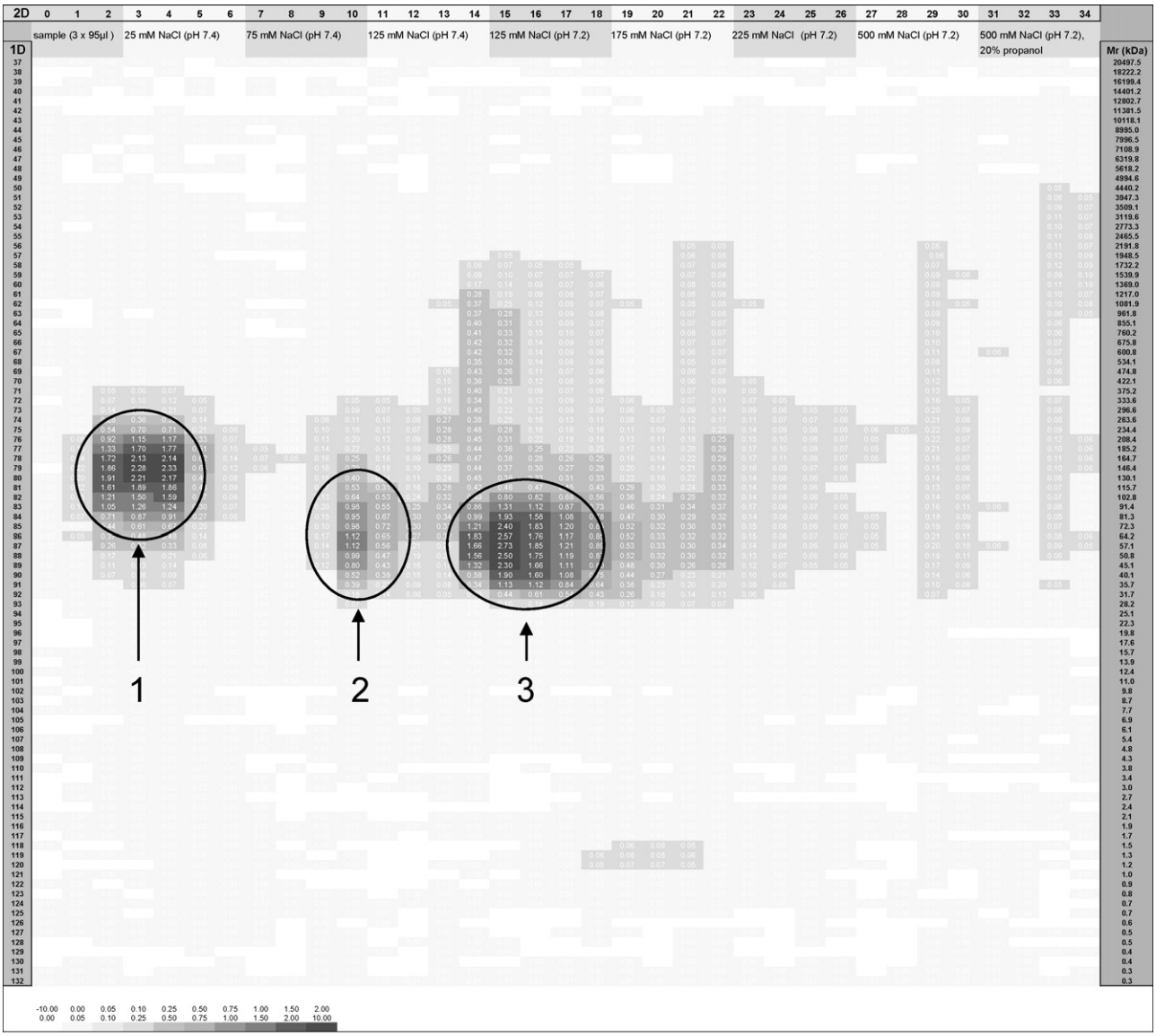

Fig. 2. Distribution of proteins within 2D-fractions. Serum of one healthy volunteer (female, 22 years) was fractionated in two separation dimensions as outlined under methods. Protein concentrations were calculated from UV-absorbance at 280 nm using a BSA-based calibration function and are represented by mg/mL entries as well as by gray tones. The left scale indicates SEC elution volume (1D, mL); the right scale, corresponding molecular weight. AEC elution conditions are indicated below the top scale (cf. also Table 1). Encircled fractions contain mainly immunoglobulin (1), transferrin (2), and albumin (3).

Mass Spectrometer API QStar Pulsar (Applera Deutschland, Darmstadt, Germany). Each data acquisition cycle consisted of one 1.5 s MS-TOF scan of peptides with mass/charge ratios (m/z) 400–1500, followed by three 1.5 s MS/MS quadrupol/TOF scans of peptide fragments with m/z 50–2000. The MS-TOF scan was done in the information-dependent mode and provided m/z of the four most intense peptide peaks with a charge $\geq$2. For each MS/MS scan (product ion mode), one of these peptide peaks was subjected to collision fragmentation after ion acceleration according to the Analyst QS software protocol. MS/TOF was calibrated with Cs (132.905 Da) and Igor peptide (829.5393 Da). At m/z 829.5393, mass accuracy was about 60 ppm, and resolution as given by the ratio, peak mass/full width at half height, was $\geq$9.000.

2.6.2. Database search

MS/MS data of tryptic digests of chromatographic fractions were analyzed against 7500 human amino-acid chain entries drawn from the SwissProt database (01/26/2006). The software pack ProID version 1.e and stringency criteria for peptide identification as provided by Applied BioSystems were used [11] and supplementary material (http://www.db-thueringen.de/servlets/DocumentServlet?id=4054, for starting, hit the “Details” button).

3. Results/discussion

3.1. Second dimension, enhanced separation using AEC

3.1.1. Protein distribution

By 2D fractionating normal serum with SEC and AEC, proteins were distributed in nine 384-well microplates within nine hours. Protein concentrations within fractions as determined by absorbance at 280 nm are shown in Fig. 2. The 2D chromatogram exhibits regions containing mainly immunoglobulin, transferrin, and albumin as found in [11], however, with enhanced AEC resolution, e.g., a large proportion of proteins with higher charges are now separated from the fractions in which albumin pre-

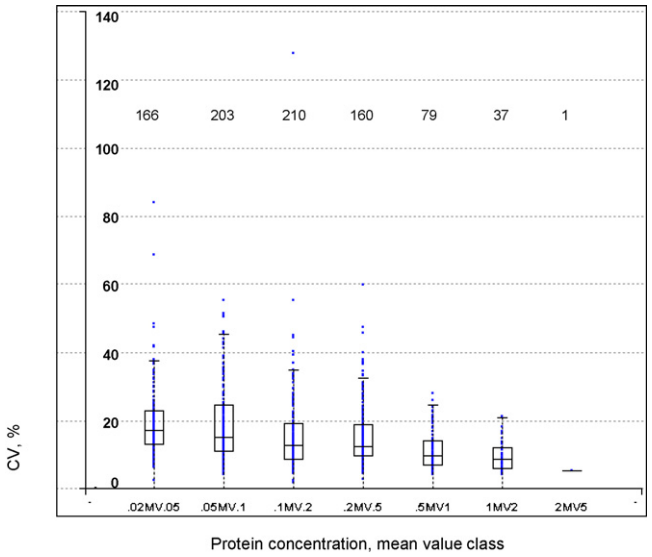

Fig. 3. Overall precision after AEC separation: distribution of CV for selected ranges of mean concentration. Serum of one healthy volunteer (female, 64 years) was fractionated in two separation dimensions as outlined under methods. Fractionation was repeated six times within three days. Mean and CV of protein concentration values were calculated for each position. Abscissa, ranges of mean concentration, mg/mL. Ordinate: Data points indicate single CV values, boxes enclose median value (horizontal bar), 25%, and 75% percentile. Antennas indicate 25% percentile minus 1.5 × IQR and 75% percentile plus 1.5 × IQR; IQR = inter-quartile range = 75% percentile minus 25% percentile. Numbers of 2D chromatogram positions belonging to each concentration interval are given as inset. CV evaluation was confined to sets of six homologous 2D fractions yielding ≥0.02 mg protein per millilitre for each separation performed.

dominates. The same holds for proteins with higher molecular weights.

3.1.2. Precision and recovery

Recovery of total protein was 103.2 ± 16.1% with respect to the initial material as calculated from protein content of all fractions (26 separations using different samples).

Reproducibility of separation, i.e., precision of protein concentration at each 2D position, was determined with six separation runs using one healthy serum sample (f/22y). Fig. 3 shows box plots of CV (coefficient of variation) values for various intervals of protein concentration. The median of CV decreases from 17% for concentrations between 0.02 and 0.05 mg/mL to 5.2% between 2 and 5 mg/mL, indicating remarkable improvement in reproducibility in comparison to [11]. The reproducibility achieved thus permits more reliable detection of differences between homologous fractions belonging to samples of different origin and, therewith, of fractions that may be expected to contain biomarker candidates.

3.1.3. Proteins identified using tryptic digestion and ESI–LC–MS/MS

Out of all 2D-fractions from two normal serum samples from different donors, fractions with ≥0.1 mg of protein per millilitre were selected. These fractions contained 91.6% and 83.1% of total protein of samples 1 and 2, respectively. Due to the bell-shaped distribution of constituents in successive chromatogram fractions, the selected fractions are located in closely neighboring positions of the 2D chromatogram. Thus, analyzing only every second from the selected fractions was considered sufficient for a protein search. The 265 and 206 fractions analyzed contained 42.1% and 40.4% of total protein from sample 1 and 2, respectively.

Analysis which was performed by LC–ESI–MS/MS of tryptic digests (sample 2 in duplicate) compared peptide fragment spectra to 7500 human amino-acid chain entries from the SwissProt database. Matches of 32,288 database entries with fragment spectra, confidence and score data (cf. supplementary material, Table S1 were found using software and stringency data provided by Applied BioSystems. In this way peptides of 2669 different human proteins were stringently identified, 520 proteins by two or more tryptic peptides (cf. supplementary material, Table S2). Examples of RP-LC–MS are given in Fig. S1 (supplementary material).

3.2. Third dimension, LAC

3.2.1. Protein distribution

The still highly complex fractions from chromatogram regions with prevailing immunoglobulin, transferrin, and albumin (Fig. 2) were LAC separated, and the 3D-fractions obtained were analyzed by UV-absorbance measurement, SDS-PAGE, and LC–ESI–MS/MS. As expected albumin is found mainly in fractions containing non-glycosylated mannose-poor proteins, so it is separated from other constituents. On the other hand, transferrin is found both in mannose-poor and mannose-rich fractions and immunoglobulins are separated into at least two glycoforms (data not shown, some examples are given in supplementary material, Fig. S2).

3.2.2. Reproducibility and recovery

2D-fractions from characteristic regions (Fig. 2) were pooled to get sufficient initial material. Hundred microlitre aliquots of pooled fractions were LAC separated in parallel. Mean %recovery ± SD found with 20 separations was 105.2 ± 6.05% and 96.4 ± 1.33% for pools containing mainly albumin and glycosylated transferrin, respectively.

LAC may be applied optionally to still highly complex fractions. Although used here for protein fractionation, parallelized LAC may also be applied to glycoproteomics and glycomics.

3.3. General remarks

The results obtained with the improved method show that orthogonal combination of chromatographic procedures and subsequent MS analysis does not require the sample to be depleted of high-abundance proteins in order to identify a similar number of proteins as found with recent techniques [12,17,18], even if only a subset of the fractions produced is analyzed. Protease inhibitors were not used throughout the analyses to avoid possible affecting of fractionation, subsequent tryptic digestion, and MS analysis (cf., e.g., [9]). Moreover, sufficient recovery was found for a panel of proteins in 2D-fractions by immuno-reactivity and enzyme activity [11,19,20], indicating that heavy proteolysis did not take place. Furthermore, for clinical application, solely biomarker candidates that resist endogen proteolysis will be useful.

Although absorbance at 280 nm is not specific for proteins, it turned out to be useful for selecting fractions with protein level in the sensitivity range of our current MS equipment. Measurement at 215 and 205 nm can be used to improve resolution at low concentrations [11,15]. Furthermore, with multi-wavelength analysis, fractions may be selected that contain constituents such as nucleotides, metabolites, and protein modifications (e.g., advanced glycation endproducts [21]), thus also including non-protein species in the search for biomarker candidates. As all sample constituents are retained in their natural state, the fractions may be analyzed by various techniques, thus permitting the separation method to be used also for other “omics” technologies. A further

advantage of the method is that it does not produce adducts with acrylamide or dyes.

On the one hand, both the native separation and the detection by UV absorbance have some advantages as outlined above. On the other hand, although it drastically reduces the complexity of final fractions, multidimensional chromatography increases dilution with every separation dimension added. This leads to reduced sensitivity, which affects measurements of fractional constituent concentration and identification of constituents. Thus, adding high-throughput concentration steps that increase concentration of fractional constituents will enhance the efficiency of the method. Related development is in progress.

4. Conclusion

Improvement on the first version of the top-down procedure [11] led to simultaneous 3D separation of up to five serum samples into 96 classes of molecular weight, 35 classes of molecule charge, and 8 classes of glycosylation. The sample and its fractions stay in a liquid state throughout the separation, enabling automation based on the use of liquid handling devices. The extensive partitioning does not require the samples to be depleted of high-abundance proteins, thus separation does not discard proteins that may carry important biomarkers (e.g., [22–24]).

Moreover, not only proteins but also other sample constituents are preserved, among them potential biomarkers in the form of peptides, lipids, sugars, nucleic acids, metabolites, etc. Information on the original states of constituents (complex formation, modification, fragmentation, activity) is available as shown in [11,19]. Summarizing, our method may be used complementarily with 2-DE, SELDI-procedures, peptidomics investigations, and various bottom-up proteomic schedules to achieve more comprehensive characterization of biological samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.09.040.

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