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Protein- *versus* peptide fractionation in the first dimension of two-dimensional high-performance liquid chromatography-matrix-assisted laser desorption/ionization tandem mass spectrometry for qualitative proteome analysis of tissue samples

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ABSTRACT

The availability of robust and highly efficient separation methods represents a major requirement for proteome analysis. This study investigated the characteristics of two different gel-free proteomic approaches to the fractionation of proteolytic peptides and intact proteins, respectively, in a first separation dimension. Separation and mass spectrometric detection by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) were performed at the peptide level in both methods. Bottom-up analysis (BU) was carried out employing well established peptide fractionation in the first separation dimension by strong cation-exchange chromatography (SCX), followed by ion-pair reversed-phase chromatography (IP-RPC) in the second dimension. In the semi-top-down approach (STD), which involved intact protein fractionation in the first dimension, the separation mode in both dimensions was IP-RPC utilizing monolithic columns. Application of the two approaches to the proteome analysis of proteins extracted from a tumor tissue revealed that the BU method identified more proteins (1245 in BU *versus* 920 in STD) while STD analysis offered higher sequence coverage (14.8% in BU *versus* 17.5% in STD on average). The identification of more basic and larger proteins was slightly favored in the BU approach, most probably due to higher losses of these proteins during intact protein handling and separation in the STD method. A significant degree of complementarity was revealed by an approximately 33% overlap between one BU and STD replicate, while 33% each of the protein identifications were unique to both methods. In the STD method, peptides obtained upon digestion of the proteins contained in fractions of the first separation dimension covered a broad elution window in the second-dimension separation, which demonstrates a high degree of "pseudo-orthogonality" of protein and peptide separation by IP-RPC in both separation dimensions.

1. Introduction

The investigation of complex proteome samples as obtained from human tissues, comprising ten thousands of protein species present in an extremely broad dynamic range, constitutes a highly demanding challenge [1]. The availability of efficient analytical methods is a major prerequisite for the study of such samples in order to attain a deeper understanding of the dynamics and functionality of the human proteome, as well as its role in disease. Two principal technologies have evolved for the analysis of complex protein mixtures, namely bottom-up and top-down proteomics [2,3]. In bottom-up (BU) approaches, multidimensional chromatographic separation and analysis of the proteins are typically performed at the peptide level, subsequent to digestion of the entire extracted proteome [4]. The most common separation platforms applied in this strategy employ combinations of liquid chromatographic modes, mostly strong cation-exchange – with ion-pair reversed-phase chromatography (SCX × IP-RPC) [5–7] or reversed-phase – with ion-pair reversed-phase chromatography (RPC × IP-RPC) [8,9]. In contrast, intact proteins are

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separated in top-down proteomics mainly by two-dimensional gel electrophoresis involving isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis [10].

Because of the fact that the chromatographic separation of complex mixtures of intact proteins is accompanied by a number of technical problems, such as limited solubility of proteins, inadequate separation efficiency and low peak capacity due to slow mass transfer, the use of chromatography based top-down approaches is still widely underrepresented. Nevertheless, several successful top-down approaches based on multidimensional chromatography have been elaborated [11–13]. Size exclusion chromatography [14], ion-exchange chromatography [15,16], and chromatofocusing [17] are the most widely used techniques for the first-dimension separation of intact proteins. More recently, also RPC has been implemented as first separation dimension into multidimensional protein fractionation schemes [16]. More specific enrichment of proteins relying on the presence of certain amino acids, post-translational modifications, or specific epitopes has been realized with immobilized metal affinity chromatography [18–20], metal-oxide affinity chromatography [21], lectin-affinity chromatography [22,23], or antibody-based affinity chromatography [24]. Finally, multidimensional intact protein separation schemes were combined with BU approaches for the identification of middle- to low-abundant proteins in tissue samples [25].

Because of their favorable mass transfer properties, nonporous silica- or polymer based stationary phases have been extensively implemented in separations of intact proteins [26–28]. Compared to microparticulate separation columns, monolithic separation columns [29,30] have been shown to offer improved mass transfer and higher permeability (compare Figs. 3 and 6 in Ref. [31] with Fig. 2 in Ref. [32]), which facilitated the rapid and highly efficient separation of intact proteins [32,33]. We recently incorporated monolithic poly(styrene-divinylbenzene) columns into a semi-top-down separation scheme for tumor-related proteomes, encompassing separation of intact proteins using chromatofocusing in the first dimension, followed by IP-RPC fractionation and digestion of the proteins directly on a target plate for subsequent matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) [34]. In a similar approach, we separated proteins by IP-RPC in the first dimension and analyzed the digested proteins in the fractions by IP-RPC off-line combined with MALDI-MS/MS for protein identification [35]. With respect to conventional BU approaches, this so-called semi-top-down (STD) approach revealed higher protein sequence coverage and distribution of identified peptides related to a particular protein over fewer fractions, however, at the cost of a lower total number of identified proteins. Combining the datasets both of two replicates of bottom-up and three replicates of semi-top-down proteome analysis, we were able to provide an extensive dataset of 2660 proteins identified in *glioblastoma multiforme* tissue.

In the course of this study it became evident that the two approaches delivered quite complementary results. Nevertheless, a direct comparison of both methods is challenging since proteins are separated in the first dimension in the STD approach, while peptides are fractionated in the BU scheme. The characteristics of peptide separation in the first dimension by SCX or RPC have been described in detail earlier [7,9,36]. It has been shown that peptides eluted in SCX according to their charge in defined elution windows, while a more homogenous distribution of eluting peptides was observed in RPC at high pH [7,9,36]. In due consequence, we aim in this study at a more differentiated characterization of the implications of first-dimension separation at the protein level on the results of proteome analysis. Moreover, we evaluate the similarities and differences between the two methods, which will allow a more rational selection of the appropriate analytical method for a given biological problem. For this purpose, we perform (i) a systematic optimization of operational parameters for the separation of intact protein mixtures by the use of a mixture of ten proteins and (ii) the subsequent analysis of a highly complex proteome sample obtained from a cell extract of *glioblastoma multiforme* tissue either by SCX × IP-RPC on the peptide level or by IP-RPC-separation of intact proteins followed by IP-RPC-MALDI-MS/MS analysis. Parameters including peak capacity, reproducibility, properties of the identified proteins, and orthogonality of the separation dimensions are investigated and compared for both approaches.

2. Experimental

2.1. Chemicals and materials

Sodium dihydrogen phosphate (NaH_2PO_4, min 99%), was purchased from Merck (Darmstadt, Germany). Trifluoroethanol (TFE, ≥99.5%), tributylphosphine (TBP, 97%), acetonitrile (E Chromasolv), α-cyano-4-hydroxycinnamic acid (CHCA), ≥98%) and human [Glu^1]-fibrinopeptide B ([Glu^1]-fib) as well as the single proteins for the protein mixture including cytochrome C (CYC, type V-A, from bovine heart), carbonic anhydrase (CAH2, bovine), serum albumin (BSA, bovine, ≥97%), β-lactoglobulin A (LACA, bovine milk), catalase (CATA, from bovine liver), lysozyme (LYSC, chicken egg, 95%), myoglobin (MYG, equine, 95–100%), ribonuclease A (RNAS1, from bovine pancreas, 85%), transferrin (TRF, bovine, 98%), α-lactalbumin (LALBA, type I, from bovine milk, 85%) were obtained from Sigma–Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA, ≥99.5%), heptafluorobutyric acid (HFBA, ≥99%), ammonium formate (NH_4OAc, ≥97%) and ammonium bicarbonate (NH_4HCO_3, ≥99.5%) were purchased from Fluka (Buchs, Switzerland) and trypsin (sequencing grade modified) from Promega (Madison, WI, USA).

2.2. Preparation of human brain tissue protein extracts

The *glioblastoma multiforme* tissue was obtained with patients' informed consent from the Department of Neurosurgery, Saarland University Hospital. The tumor biopsy sample used in this study was derived from a 54-year-old male patient. Histopathological analysis revealed WHO grade IV *glioblastoma multiforme*. Following a published protein extraction method [37], 100 μL phosphate buffer (5 mM, pH 7.0) were added to 10–15 mg of *glioblastoma multiforme* tissue. After 3 min shaking (Vortex Genie 2, Bender & Hobein AG, Zurich, Switzerland), the samples were incubated for 1 h at room temperature with gentle shaking and then sonicated for 5 min in ice-water. Addition of 100 μL trifluoroethanol (TFE), incubation for 2 h at 60 °C, and sonication for 2 min in ice-water followed. After 5 min of centrifugation (4 °C, 8500 rpm) the protein concentration of the sample supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). The sample was incubated for 30 min at 60 °C after adding 50 mM tributylphosphine (TBP) to a final concentration of 4.5 mM to denature the proteins and reduce the disulfide bonds. The proteins were injected immediately after incubation into the first separation dimension for the semi-top-down approach or digested for the bottom-up approach, respectively.

2.3. IP-RP-HPLC prefractionation of intact proteins in the first dimension of the semi-top-down approach

Approximately 860 μg of total protein (according to the Bradford assay) in 500 μL were injected into an analytical HPLC system (Model 1050, HP, Waldbronn, Germany). The proteins were separated employing two 50 mm × 4.6 mm i.d. monolithic reversed-phase columns (ProSwift RP-1S, Dionex Corporation, Sunnyvale, CA, USA) connected in series using a flow rate of

0.80 mL/min and operating at 55 °C. The external six-port injection valve (Model 7125, Rheodyne, Rohnert Park, CA, USA) was equipped with a 1.0 mL sample loop. Proteins were eluted with a 60-min gradient of 15–60% acetonitrile in 0.050% TFA, followed by a washing step of 60–100% acetonitrile in 0.050% aqueous TFA in 3 min and holding for 10 min at 100% acetonitrile containing 0.05% TFA. UV chromatograms were recorded at 280 nm and 2-min fractions were collected and stored at −30 °C.

2.4. Tryptic digestion of the extracted proteins for the semi-top-down approach

For the STD analysis, every fraction obtained from the first dimension was evaporated to dryness in a vacuum concentrator (Model Concentrator 5301, Eppendorf AG, Hamburg, Germany) and 100 μL of 50 mM ammonium bicarbonate were added. The total protein concentration obtained from the Bradford assay and the relative peak areas in the UV chromatogram of the first separation dimension were used to adjust the enzyme-to-protein ratio to approximately 1:20 (40 μg trypsin to 860 μg proteins). The enzyme was activated at 37 °C and 550 rpm for 30 min in a thermo mixer (Model Comfort, Eppendorf) and then added to the fractions of the first dimension. The reaction was quenched after incubation over night by addition of 1.0% (v/v) TFA. The fractions then were frozen at −30 °C. Prior to injection into the nano-flow HPLC-MALDI-TOF/TOF system, 100 μL of 0.10% aqueous HFBA solution were added to each fraction.

2.5. Tryptic digestion of the extracted proteins for the bottom-up approach

The reduced protein solution was diluted 1:4 with ammonium bicarbonate (50 mM, pH 7.9) to reduce the TFE concentration. The enzyme-to-protein ratio was adjusted to approximately 1:50 with a solution of 1.0 μg/μL trypsin in 50 mM acetic acid. After activation, 8.5 μL trypsin solution were added to the protein extract and incubated for 15 h in the thermo mixer at 37 °C and 550 rpm. The digest was quenched by addition of TFA to 1.0% (v/v) final concentration. The solvent was evaporated to dryness in the vacuum concentrator and stored at −30 °C. Prior to injection into the first separation dimension, peptides were re-dissolved in 200 μL ammonium formate (10 mM, pH 3.0 which is the starting eluent in SCX-HPLC).

2.6. SCX-HPLC prefractionation of peptides for the bottom-up approach

About 426 μg/200 μL of peptides (quantified with Bradford assay) were injected into an analytical HPLC system (Model 1050, HP). The peptides were separated on an SCX column (polysulfoethyl A, 200 mm × 2.1 mm i.d., 5 μm, 200 Å, PolyLC, Columbia, MD, USA) using a 10 mm × 2.1 mm i.d. guard column and a flow rate of 0.20 mL/min operating at 25 °C. The external six-port injection valve (Model 7125, Rheodyne) was equipped with a 200-μL sample loop (Rheodyne). Eluent A consisted of 10 mM ammonium formate, pH 3.0, and eluent B of 500 mM ammonium formate, pH 6.8. Both mobile phases contained 25% (v/v) acetonitrile. After injection, the system was held for 10 min at 10 mM ammonium formate, pH 3.0. Then a gradient of 10–250 mM ammonium formate was applied within 40 min, followed by ramping to 500 mM ammonium formate (pH 6.8) within 10 min. For the last 10 min the system was held at 500 mM ammonium formate (pH 6.8). The UV chromatogram was recorded at 214 nm. Two-minute fractions were collected, evaporated to 10 μL and frozen at −30 °C. Prior to injection into the second dimension, 100 μL of 0.1% aqueous HFBA solution were added to the fractions.

2.7. Nano-flow IP-RP-HPLC-MALDI-MS of peptides

The instrumental setup consisted of a nano-flow HPLC unit (Model Ultimate, LC Packings, Amsterdam, The Netherlands), an automatic injection unit (Model Famos, LC Packings) and a loading pump (Model K-1001, Knauer, Berlin, Germany) with a 10-port switching valve (Model C2-1000D (stator) and 06A-8029C (rotor), VICI, Schenkon, Switzerland) as described earlier [22]. The detector was equipped with a 3 nL Z-shaped flow cell (Model Ultimate, LC Packings). The UV chromatogram was recorded at 214 nm. The separation column (60 mm × 0.10 mm i.d.) as well as the preconcentration column (10 mm × 0.20 mm i.d.) contained a monolithic poly(styrene-divinylbenzene)-based stationary phase material synthesized according to the published protocol (available from LC Packings, Dionex Corporation, Sunnyvale, USA) [34].

Ten microliters of the peptide sample were injected into the second-dimension nano-flow HPLC system and pre-concentrated and desalted in the monolithic precolumn for 3 min with a 10 μL/min flow of 0.10% aqueous HFBA. After switching of the valve, the peptides were eluted onto the separation column and separated with a 50-min gradient of 0–30% acetonitrile in 0.050% aqueous TFA at a flow rate of 0.70 μL/min, followed by ramping to 100% acetonitrile in 10 min. The eluate was spotted with a spotting frequency of 5.0 s per spot onto a stainless steel target (Opti-TOF 123 mm × 81 mm, Applied Biosystems, Framingham, MA, USA) with a spotting unit (Model Probot, Dionex, Germering, Germany). A matrix and internal calibrant solution containing 3.0 mg mL^{-1} α-cyano-4-hydroxy-cinnamic acid and 15 fmol/μL [Glu^1]-fib in 70% acetonitrile and 0.10% aqueous TFA was added through a T-piece with a flow rate of 3.1 μL/min.

A matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (ABI 4800 TOF/TOF Analyzer, Applied Biosystems, Foster City, CA, USA) was utilized for protein identification. The spectra were acquired in positive ion reflector mode in a mass range of m/z 800–3000. In MS mode, 25 laser shots were accumulated to one sub-spectrum and 40 sub-spectra were summed to the final spectrum (1000 shots) with a frequency of 200 Hz and a laser wavelength of 355 nm. Fragmentation of the six highest-abundant peptide ions per spot was conducted in MS/MS mode exploiting metastable decay with re-acceleration [3]. The fragment ion data acquisition stopped when 3000 laser shots or a signal-to-noise ratio (S/N) of 35 for at least 10 peaks was achieved. The mass spectrometer was calibrated using a six-peptide calibration mix (Applied Biosystems), on eight external calibration spots for each MALDI plate (Plate Model Calibration) resulting in a mass accuracy better than 50 ppm (=default calibration). For optimized mass accuracy (5 ppm), internal calibration on the m/z of [Glu^1]-fib was performed during data acquisition. When this additional calibration failed due to signal suppression especially in spectra with high sample peptide signal intensity, default calibration was automatically applied (50 ppm) by the software. Calibration of the instrument in MS/MS mode was performed using four fragments of [Glu^1]-fib. The mass-to-charge ratio of [Glu^1]-fib was set on the precursor selection exclusion list for sample peptide ions to circumvent its fragmentation and inclusion into data analysis.

2.8. Protein identification

The MS/MS spectra were smoothed with the Savitsky-Golay algorithm, which averaged three neighboring raw data points into one employing least squares regression with a polynomial order of four. For generating peak lists and exporting data to create Mascot generic files (.mgf) the following settings were used in the MS/MS peak filter of the 4000 Series Explorer Software (Applied Biosystems, version 3.5.3): mass range: m/z 60 to pre-

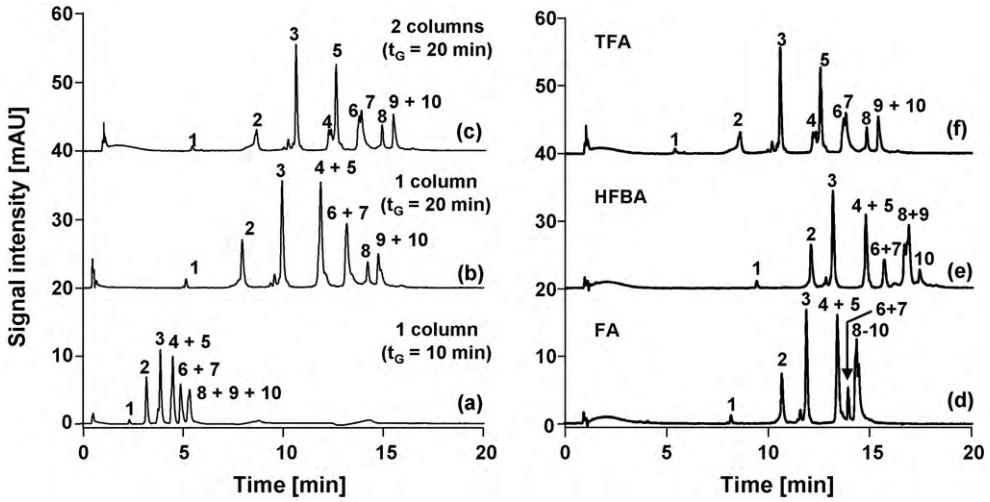

Fig. 1. Separation of ten intact proteins. Columns, monolithic PS-DVB, (a, b) 50 mm, (c–f) 2 × 50 mm length, 4 mm i.d.; gradient, 15–50% acetonitrile in (a–c, f) 0.050% aqueous TFA, (d) 0.050% FA, and (e) 0.050% HFBA in (a) 10 min and (b–d) 20 min; flow rate, 0.80 mL min^{-1}; temperature, 55 °C; detection, UV, 280 nm; sample, 5.0 μL containing 12.6–80.9 pmol each of 1, ribonuclease A; 2, cytochrome C; 3, lysozyme; 4, transferrin; 5, α-lactalbumin; 6, bovine serum albumin; 7, β-lactoglobulin B; 9, catalase; 10, carbonic anhydrase.

cursor mass minus 35 Da; peak density: maximum 20 peaks per 200 Da; minimum S/N: 10; minimum area: 200 and maximum 65 peaks/precursor. To identify proteins, the Mascot generic files (.mgf-files) were sent to the Mascot software (version 2.2.03, Matrix Science, London, UK) which uses the MOWSE (molecular weight search) algorithm [32]. The applied settings were: database: Swiss Prot (version 54.7, January 15th, 2008); taxonomy: Homo sapiens (18,117 sequences); enzyme: trypsin; variable modification: methionine oxidation; peptide tolerance: 50 ppm; MS/MS tolerance: 0.2 Da; maximal missed cleavages: 1 and ion-score cut off for peptides: 0.05 ($P \geq 95\%$). The probability of an identification as a random event for proteins was set to 0.01 ($P \geq 99\%$). The false positive discovery rate (FDR) was determined by Mascot for the protein identifications using an automatically generated randomized decoy database. Moreover, for all identified proteins Mascot directly delivered the theoretical isoelectric points and intact molecular masses calculated on the basis of the primary amino acid sequence.

3. Results and discussion

3.1. Optimization of column dimensions and elution conditions for intact protein separation

A mixture of ten proteins covering a reasonably broad spectrum of protein properties, which are commonly utilized in the evaluation of protein separations [27,38,39], was separated to optimize the chromatographic separation of intact proteins by IP-RPC in monolithic, poly(styrene-divinylbenzene) (PS-DVB)-based columns in the first dimension. As only 50 mm long monolithic columns of 4.6 mm i.d. were commercially available, the performances of a single column and of two columns connected in series were compared. In order to elute a broad range of proteins in terms of hydrophobicity and size, a gradient of 15–50% acetonitrile in 0.050% aqueous TFA was applied. Six peaks were observed in the UV chromatograms for ten components separated with a single column and a gradient time of 10 min (Fig. 1a). Upon increasing the gradient time by a factor of two (Fig. 1b), seven peaks were observed including three unresolved peak pairs. When both gradient time and column length were doubled according to the gradient volume concept (Fig. 1c), all components except the pair catalase/carbonic anhydrase were at least partially resolved, which qualifies this configuration as best suited for intact protein separation. The corresponding peak widths at half height given in Table 1 clearly corroborate the general trend of better performance with the longer column, although in some cases increased peak widths with the longer column indicate the beginning separation of protein variants or impurities, which are frequently observed for intact proteins. It can be seen that the peak widths even for larger proteins being present in isoforms such as transferrin and catalase were significantly below 0.3 min. Consequently, we can expect that fractionation in 2-min fractionation intervals should lead to elution of a single protein (species) in not more than two consecutive fractions.

The peak capacities, measured for three gradient times and two column lengths are shown in Table 2. Because we could expect that the whole elution window will be covered during the elution of a highly complex sample such as a protein extract form a tumor tissue, we calculated the peak capacities simply by division of the gradient times through the average peak widths at base of

Table 1
Peak width at half height w_h for the ten-protein mixture.

Protein	Peak number	One column		Two columns
		w_h [min], t_G = 10 min	w_h [min], t_G = 20 min	w_h [min], t_G = 20 min
RNAS1	1	0.11	0.09	0.08
CYC	2	0.15	0.12	0.18
LYSC	3	0.14	0.13	0.09
TRF	4	0.17	0.14	0.23
LALBA	5			0.10
BSA + MYG	6 + 7	0.17	0.17	0.28
LACB	8	0.25	0.11	0.09
CAT + CAH2	9 + 10		0.12	0.13

Table 2
Peak widths at base w_b and peak capacities PC for two column lengths and different gradient times t_G.

One column (50 mm)			Two columns (100 mm)		
t_G [min][a]	$w_{b\ average}$ [min]	PC	t_G [min]	$w_{b\ average}$ [min]	PC
7.5	0.19	40	15	0.23	66
15	0.24	62	30	0.31	80
30	0.37	82	60	0.49	124

[a] Gradient, 15–50% acetonitrile in 0.050% TFA, 0.80 mL min^{-1}.

the ten standard proteins. As predicted by theory [40], an increase if the gradient time by a factor of two resulted in a rise of the peak capacity by about the square root of two. For example, increasing the gradient time from 15 to 60 min led to an approximately twofold increase of peak capacity (66–124). Due to their smaller diffusion coefficients, multi-point adsorption, multi-mode interaction, and the possibility of conformational and posttranslational isoforms [41], the peak capacities determined for the separation of intact proteins were generally lower as compared to separations at the peptide level. For instance, peak capacities of 249 and 113, respectively, were achieved in the separation of peptides applying a 50-min gradient in a 150 mm C_{18} RP column or in a 50 mm SCX column with a gradient time of 80 min [15].

The influence of mobile phase additives on the selectivity and efficiency of the protein separations was investigated with formic acid (FA), which serves only as an acidic additive, as well as with trifluoroacetic acid (TFA), and heptafluorobutyric acid (HFBA), which, besides being strong acids, also function as ion-pairing reagents (Fig. 1d–f). The longest retention times were observed with the most hydrophobic ion pair reagent, HFBA. The average peak widths at half height were smallest with FA (0.12 min) and slightly increased with HFBA and TFA (0.14 and 0.15 min, respectively). The best selectivity and the broadest elution window were, however, achieved using TFA as ion-pair reagent (Fig. 1f). The critical peak pair bovine serum albumin and myoglobin (peaks 6 + 7 in Fig. 1) was at least partly separated only with TFA as additive. As TFA provided the best selectivity it was applied in the further experiments.

The repeatability of the established IP-RP-HPLC method for protein separation was extracted from three replicates with the two columns connected in series and a 20-min gradient of acetonitrile in 0.050% TFA. The relative standard deviations of retention times for the components of the mixture of ten proteins were within 0.1 and 0.7% with the exception of ribonuclease A, showing a 3.7% relative standard deviation. Since we have observed similar fluctuation for ribonuclease A in analogous investigations, we assume that this is caused by structural variability and/or the fact that this proteins elutes very early in the gradient at 24.8% acetonitrile. Finally, a recent study has shown that intact protein recoveries from PS-DVB-monoliths range between 50 and 100%, depending on the proteins, and are substantially higher as compared to silica-based stationary phases. Moreover, no carryover of intact proteins was observable between runs [42].

3.2. Complementarity of protein identifications in the semi-top-down and bottom-up approaches to proteome analysis

For the characterization of the performance of different proteome analysis strategies it is necessary to involve highly complex protein mixtures such as the proteome extracted from a *glioblastoma multiforme* cancer tissue. We utilize the protein identification results [35] of two technical replicates each of the fractions collected in the first dimension involving intact protein and tryptic peptide fractionation, respectively, to compare the characteristics of bottom-up and semi-top-down proteome analysis.

Table 3
Results obtained from the bottom-up and semi-top-down approach.

Parameter	BU	STD
Annotated spectra [%]	28.3	30.5
Identified peptides	7122	4919
Identified proteins	2056	1507
"One-hit wonders"	811	587
Identified proteins without "one-hit wonders"	1245	920
Peptides in non-redundant proteins[a]	6311	4332
Without homologous peptides[a]	5717	3880
False positives [%]	1.3	1.8
Average mass accuracy (MS mode) [ppm]	18	17
Average peptide score	66.5	70.4
Average peptides per protein	3.4	3.3

[a] For peptides corresponding to proteins identified with more than one peptide.

Sample preparation involved extraction of the proteins from the biological tissue at pH 7 using trifluoroethanol as membrane disintegrating agent [37]. The disulfide bonds of the intact proteins were reduced prior to first-dimension separation using tributylphosphine to unfold protein structure. Following the classical BU strategy, the whole protein extract was tryptically digested, separated into 35 fractions, which were subsequently analyzed in two replicates of IP-RPC-off-line-MALDI-MS/MS. In the alternative semi-STD route, the whole extract of intact proteins was subjected to IP-RPC fractionation, and the resulting 28 fractions were again investigated by two IP-RPC-off-line-MALDI-MS/MS analyses under identical conditions as in the BU approach. Following peptide separation and tandem mass spectrometry, the peptide fragment spectra were submitted for database searching using Mascot with the parameters given in Section 2.

The results of peptide and protein identifications when using the dataset each of the merged (BU1 + BU2) and the merged (STD1 + STD2) analyses are collected in Table 3. A complete list of all identified proteins can be found in the supplementary material published online. After elimination of proteins represented by only one peptide ("one-hit wonders"), 1632 proteins were identified in total upon merging BU1 + BU2 and STD1 + STD2 (Fig. 2a), of which 533 or 32.7% were identified in both approaches, and 712 (43.6%) and 387 (23.7%), respectively, were found in the BU and STD approaches only (Fig. 2a). This suggests a certain degree of complementarity of both methods. Nevertheless, the increase in the number of proteins identified in the four analyses with respect to the two replicates of BU or STD is not necessarily related only to the complementarity of the two methods but could also be due to the higher number of replicate analyses in the combined data evaluation. The total analysis times for duplicate analysis of all fractions were 917 h in BU and 734 h in STD analysis. Normalization of the number of protein identifications to the time invested for measurement yields 1.36 protein identifications per hour for BU and 1.25 protein identifications per hour for STD analysis. These very similar performances indicate that measurement time for mass spectrometric investigation is an important determinant in proteome analysis.

As expected, the average mass accuracy and average peptide score were similar both for BU and STD because the same second-dimension instrumental setup and settings for the MASCOT search engine were employed. The average MOWSE Score for successful protein identification with $P > 99\%$ was computed as 32. The average peptide scores were 66.5 for BU and 70.4 for STD (Table 3). Of all identified peptides, 20.0 and 24.9%, respectively, were identified with a score above 90, and 22.5 and 21.5%, respectively, with a score below 40. A randomized decoy database was applied for estimation of the false positive identification rate. As given in Table 3 false positive rates of less than 2% were observed for both analytical

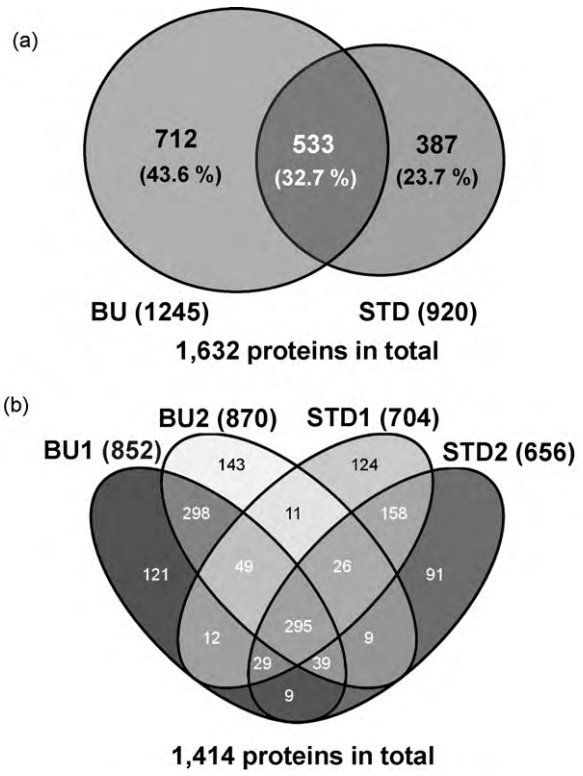

Fig. 2. Venn diagram showing the overlapping and individually identified proteins of both approaches for the (BU1 + BU2) and (STD1 + STD2) datasets (a), and Venn diagram of the four individual datasets (b); STD = semi-top-down, BU = bottom-up.

approaches. This is equivalent to the setting of a 99% probability for correct protein identification.

In order to study the complementarity of the BU and STD approaches in more detail, we prepared a Venn diagram that contains the numbers of both the overlapping and unique protein identifications of all four individually measured replicates (Fig. 2b). In the four non-merged datasets, 852 (BU 1), 870 (BU2), 704 (STD1) and 656 (STD2) proteins were identified. Only 295 (20.8%) of a total of 1414 proteins were detected in all four proteomic analyses. The total number of identified proteins after individual evaluation of all replicates is lower as compared to the merged dataset (1632 *versus* 1414 proteins), because the number of eliminated one-hit wonders is higher in the individual database searches, whereas a second peptide identified in another replicate can lead to the acceptance of a protein identification in the merged dataset. Comparing the first and second bottom-up analyses, BU1 and BU2, we found that 508 (59%) of the proteins were detected in both runs, while 196 (23%) and 148 (17%) were only found in one of the two runs. The picture is very similar in the two STD data evaluations, in which 681 (65%) of the proteins were common to STD1 and STD2, while 171 (16%) and 189 (18%) of the identified proteins were unique. If we now compare two complementary analyses, *e.g.* BU1 and STD1, we can see that only 385 proteins (33%) are in common while 467 (40%) and 319 (27%) were unique in the BU1 and STD1 analyses. All other combinations of BU and STD analyses revealed equivalent numbers, which clearly corroborate the complementarity of the two methods with respect to protein identifications.

3.3. Differences in solubility of the analytes in bottom-up and semi-top-down strategies

The very broad range of protein solubilities in whole proteomes, ranging from highly soluble in water for small and hydrophilic proteins to practically insoluble in water for large and/or hydrophobic proteins represents a real challenge in proteome analysis [43]. In order to improve the solubility especially of hydrophobic proteins, detergents are frequently added to the protein extraction solvents [44]. The recovery of proteins is further hampered, especially in multidimensional analyses requiring sample transfer between different dimensions, by their tendency to irreversibly adsorb to the surfaces of pipette tips, sample vials, and components of the liquid chromatography equipment [45].

The problem of poor solubility and adsorption of proteins is significantly alleviated by digestion of the proteome to peptides, which are generally more soluble in aqueous solvents and less prone to adsorption, already at the beginning of the BU method. We believe that the lower total number of proteins identified in the STD methods is partly due to protein losses because of poor solubility and/or irreversible protein adsorption during the first-dimension separation and the following protein digestion in the individual fractions. For both the BU and STD methods, the proteins were reduced already before the first-dimension separation in order to obviate the necessity of performing these sample preparation reactions in all individual fractions of the STD method. Alkylation was performed only in the BU analysis. While the resulting denaturation of the proteins additionally helps to improve the selectivity of the IP-RP chromatographic separation in the first dimension, it may also result in a further decrease in the solubilities of some proteins, ensuing their precipitation and loss for further analysis. Problems related to protein solubility may also result from difficulties in re-solubilizing proteins after complete evaporation of the solvent after separation in the first dimension of the STD method.

We utilized trifluoroethanol as solubilizing agent, which has been shown to facilitate the solubilization of a substantial fraction of the hydrophobic membrane proteins in mouse brain tissue [37]. Indeed, we were also able to find a significant number of 236 membrane proteins in the proteome extracted from human brain tumor tissue [35], of which 191 were detected in the BU analysis and 104 in the STD approach, suggesting that BU analysis is more efficient in membrane protein identification, Nevertheless, when we look at the portion of membrane proteins in relation to the total number of proteins found, we find that the yield of 15% membrane proteins in the BU approach is not so significantly different from the 11% membrane proteins detected in the STD analysis, which implies that hydrophobicity of the proteins or peptides led to almost equivalent losses in both approaches.

3.4. Retention behavior of proteins and peptides in the first separation dimension

In order to elaborate the characteristics of the BU and STD platforms, the retention behavior of proteins and peptides in the first-dimension separation was investigated in more detail. The following discussion is based on the two datasets obtained from two merged technical replicates for each approach. In strong cation-exchange chromatography employed in the first dimension of BU analysis, peptides mainly elute as function of their net charge [7,9]. For peptides of the same net charge, retention is additionally influenced both by the charge distribution within the peptide and, depending on the nature of the stationary phase, also solvophobic or hydrophilic interactions of uncharged amino acids with hydrophobic or hydrophilic areas that are usually present at the surface of ion-exchange stationary phases.

In the BU method applied in this study, at salt gradient of ammonium formate was applied for elution of the peptides from a strong cation-exchange column bearing sulfonic acid groups. The starting eluent contained 10 mmol L^{-1} ammonium formate, pH 3.0, whereas the gradient forming eluent contained 500 mmol L^{-1} ammonium formate titrated to pH 6.8. Although this configuration did not entail the formation of a continuous pH gradient, the

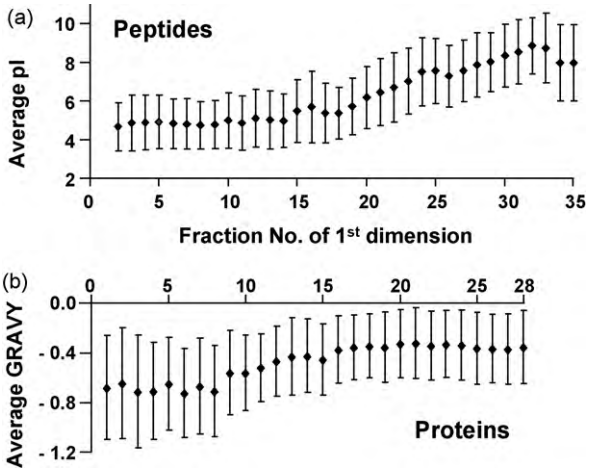

Fig. 3. Retention behavior of peptides and proteins in the first-dimension separation of (a) BU and (b) STD analysis. (a) p*I* as a function of peptide retention. (b) GRAVY index as a function of protein retention.

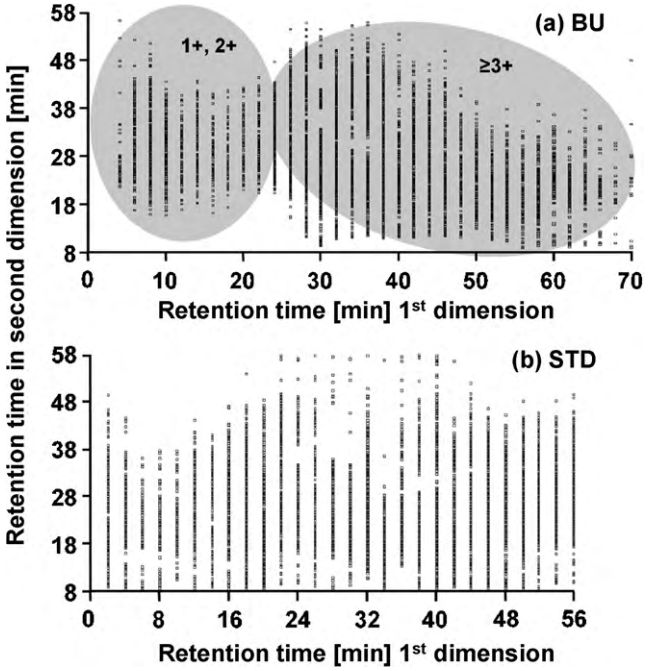

Fig. 4. Orthogonality of separation dimensions in both two-dimensional separation schemes tested.

higher pH of the gradient former helped to reduce the net charge of the peptides in the late phase of elution and hence facilitated the elution of highly charged and strongly adsorbed peptides. Both mobile phases contained 25% acetonitrile to suppress solvophobic interactions.

Fig. 3a illustrates the range of the calculated theoretical p*I* values for the peptides (computed with MW/p*I* tool on www.expasy.org) eluting in the different fractions of the first separation dimension in the BU analysis. The values were arithmetically averaged for each fraction and only non-redundant peptides were included for one fraction. It can be seen that the average p*I* values increased with proceeding elution during SCX chromatography. However, the range of observed p*I* values in each fraction was considerably broad leading to the conclusion that, besides p*I*, retention is determined by additional parameters such as total net charge and hydrophobicity.

Since retention of proteins on a reversed-phase stationary phase in the first dimension of the STD method involves interaction with a hydrophobic surface, we investigated the hydrophobic character of the separated proteins by calculating the Grand Average of Hydropathy (GRAVY) index (Protein GRAVY tool on www.bioinformatics.org [46]). Fig. 3b indicates an only moderate increase in the average GRAVY index for the proteins eluting in the 28 fractions of the first-dimension STD separation. However, within a single fraction, the distribution of the individual GRAVY indexes was considerably broad. Hence, it becomes clear that the retention of proteins is mediated both by solvophobic interactions between the hydrophobic stationary phase and the hydrophobic moieties of the proteins and by electrostatic interaction with the adsorbed ion-pair reagent trifluoroacetate [47].

3.5. Orthogonality of separation dimensions and total peak capacity

To achieve maximum separation selectivity in multidimensional separations, the individual separation dimensions must be orthogonal, *i.e.* separate the analytes according to different and independent molecular properties [48]. The combination of SCX and IP-RPC as applied in our BU approach, has been already shown to offer a high, although not complete degree of orthogonality in two-dimensional peptide separations [7,8]. Fig. 4a plots the retention times of peptides measured in IP-RPC *versus* the retention times observed in SCX. It can be seen that the peptide identifications are quite well distributed over the two-dimensional separation space. Nevertheless, because of elution of the peptides in the first dimension in clusters according to charge, namely singly and doubly charged peptides between 0 and 15 min and triply or higher charged peptides between 25 and 70 min, some of the separation space is not fully utilized. More homogenous peptide elution during BU analysis has been achieved by employing a reversed-phase separation at high pH in the first-dimension separation [49].

Because of the separation of two different species in the STD approach, peptides and proteins, respectively, the term orthogonality of separation is not applicable in its strict sense. Nevertheless, it is possible to plot the retention times of all peptides identified in the second dimension as a function of the elution time of the corresponding proteins in the first dimension, as shown in Fig. 4b. We can see here that the distribution of peptide identification is more homogenous and that the available separation space is utilized to a higher portion, such that this type of two-dimensional separation offers a high degree of "pseudo-orthogonality". Interestingly but not unexpectedly, rather hydrophobic proteins eluting in later fractions of the first-dimension separation show a homogenous distribution of peptides over the whole separation window of the second separation dimension.

The peak capacity of a separation, giving the maximum number of compounds (peaks) that can be separated in an ideal separation within a given time window, represents an appropriate means for characterization of the separation power of a chromatographic system [50]. For fully orthogonal separation dimensions, the total theoretical peak capacity is computed by multiplying the peak capacities of the individual dimensions [51]. Because of time

Table 4
Theoretical peak capacities for both two-dimensional chromatographic separation systems. BU = bottom-up, STD = semi-top-down, t_G = gradient time.

	t_G	Average w_b	PC
1st dimension SCX peptides = BU (a)			34
1st dimension IP-RP proteins = STD (b)			28
2nd dimension IP-RP peptides	50	0.26	190
2D-HPLC (a)			6460
2D-HPLC (b)			5320

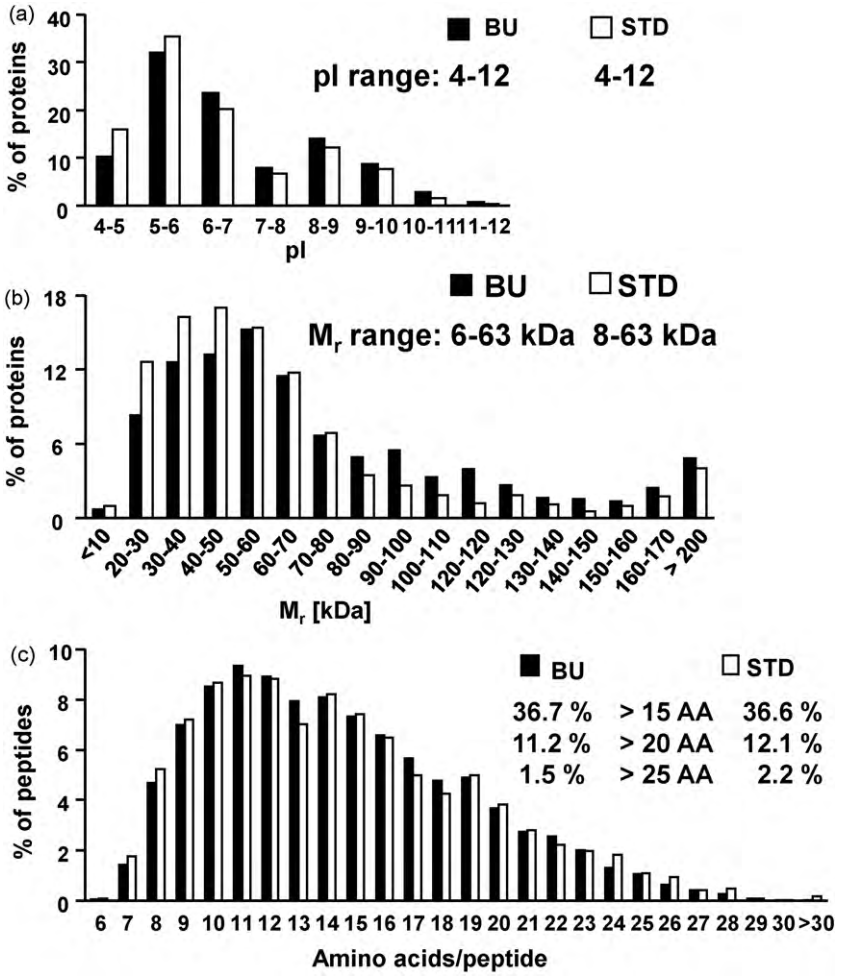

Fig. 5. Comparison of the semi-top-down and the bottom-up approach with respect to (a) the theoretical p*I* for the identified proteins, (b) the computed molecular weight of the intact proteins, and (c) the size of the identified peptides.

restrictions and the necessity of relatively long gradient runs in the second dimension, the two-dimensional separations employed in proteome analysis are usually not comprehensive in the sense that each peak obtained in the first dimension must at least be sampled ten times in the second dimension in order not to sacrifice the separation achieved in the first dimension [48]. In practice, since the peak widths of the eluting peaks are usually significantly smaller than the fractionation window, the number of fractions collected in the first dimension represents a good estimate of the maximum peak capacity. The peak capacity in the second dimension, on the other hand, can be obtained from the average peak width of the eluting peptides and the gradient time [51]. As the second-dimension setup is the same in both approaches, the peak capacity of the whole chromatographic system in our study is limited by the number of fractions collected in the first dimension.

The peak capacities of both tested approaches are summarized in Table 4. The peak capacity in the second dimension was calculated using the average peak width at 13.4% peak height ($w_b = 4\sigma$ [52]) for four standard peptides separated during the same 50-min gradient as employed for the second dimension of the proteome analysis (0–30% acetonitrile in 0.05% aqueous TFA). The total peak capacities for both methods of 6460 and 5320, respectively, are well within the range of previously described peak capacities for 2D-HPLC systems [52]. Collecting more fractions in the first dimension and/or increasing the gradient time in the second dimension represent appropriate means for improving the total peak capacity, however at the cost of an increase in the total analysis time.

3.6. Distribution of pI, molecular mass, and sequence coverage among identified proteins

An important factor in the selection of an analytical strategy is the question, whether it favors or discriminates a certain class of proteins, *e.g.* acidic, neutral or basic proteins or proteins of a distinct molecular mass range. Fig. 5a and b depicts the distribution of the theoretically computed isoelectric points and the computed molecular masses of identified proteins. In both approaches, a broad p*I* and molecular mass range was covered. In the STD approach, proteins of lower p*I* and lower molecular mass were slightly favored. Nevertheless, the difference is rather small and does not suggest that one of the two methods discriminates a certain class of proteins. The lower percentage of large and basic proteins found in the STD method was most probably caused by the higher loss of these proteins during sample preparation and intact protein separation.

Another crucial feature of protein characterization at a global scale is the sequence coverage, which not only determines the confidence of protein identifications but also to which extent sequence variations and posttranslational modifications can be detected. The number of peptides identified per protein, in this context including one-hit wonders, in the BU and STD analyses is shown in Fig. 6a. Both approaches show practically the same percental distribution of proteins identified with one to five or more peptides. The sequence coverage of the individual proteins for both approaches was also examined, as illustrated in Fig. 6b, taking into account only proteins identified with more than one peptide. In the BU

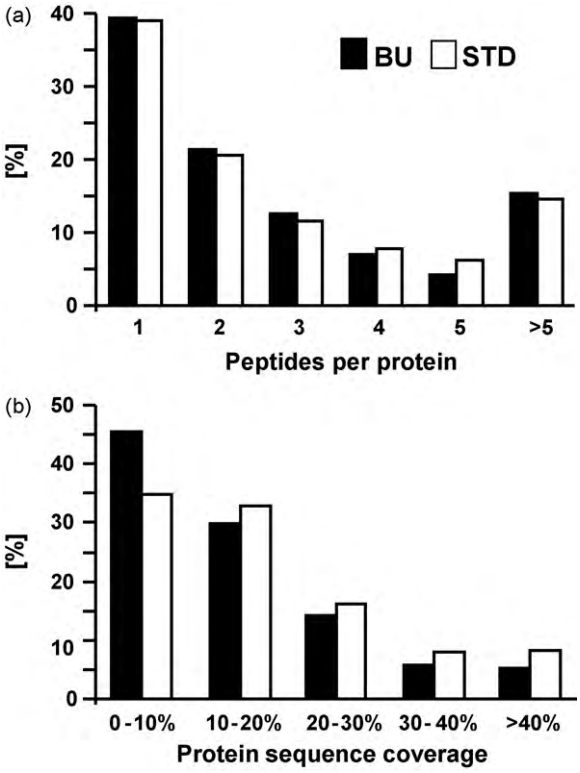

Fig. 6. Distribution of the number of peptides identified per protein (a) and distribution of sequence coverage (b) for all 2056 proteins identified in the BU analysis and the 1507 proteins identified in the STD method.

approach, 10.6% more proteins were found with a sequence coverage of less than 10%. In contrast, using the STD method, 3.1% more proteins were identified with more than 40% sequence coverage. This implies that more relatively long peptides were detected during STD analysis, as demonstrated in Fig. 5c. The average protein sequence coverage determined over all identified proteins of 17.5% in the STD methods was higher than that of 14.8% in the BU method. This implies that STD is more suitable to detect and characterize proteins on a global scale with higher sequence coverage. Moreover, as we have already outlined earlier [35] this approach is also more suitable for the targeted analysis of selected proteins in complex proteomic samples.

4. Conclusions

We have characterized a STD method for gel-free proteome analysis and compared it to the classical BU approach. In the STD approach the retention time is obtained as characteristic information of a protein while no such protein-related information can be retrieved from the BU analysis. More proteins were identified in BU analysis, however with lower sequence coverage. To increase the number of protein identifications for the STD approach, the peak capacity in the first-dimension peak capacity can be increased by extending the gradient time or decreasing the fraction collection time interval. The BU method shows a slight tendency to identify more basic and larger proteins. The STD approach offers the advantage of a high degree of "pseudo-orthogonality" and more straightforward handling of ion-pair reversed-phase separation as compared to ion-exchange separation. Moreover, it is a useful tool for the identification of known target proteins, as the peptides related to the same protein are contained only in one or a few fractions of the first-dimension separation.

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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.chroma.2010.07.044.

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