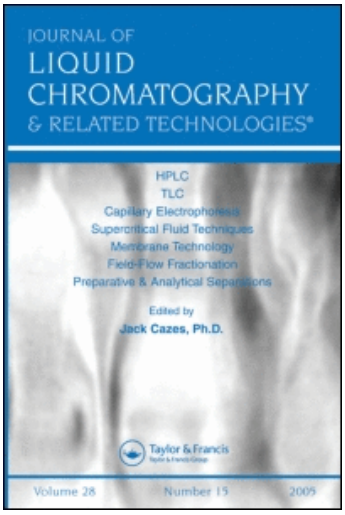

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On-line Method for Identification of Native Proteins using pH-Gradient SAX Chromatography and Reversed Phase Chromatography-Mass Spectrometry of Tryptic Peptides

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Abstract: A method for identification of proteins based on their pI values and peptide mass fingerprint (PMF) using an on-line system has been developed. Proteins were separated by pH-gradient strong anion exchange (SAX) chromatography and trapped on C4 reversed phase (RP) columns. Trapped proteins were on-line transferred to an immobilized trypsin reactor, and the resulting peptides were subsequently on-line transferred to an RP analytical column via a combination of RP and weak cation exchange (WCX) trap columns. The tryptic peptides were identified by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). Three model proteins were used for method development and basic human plasma proteins were subjected to identification. On-column trypsination is compatible with high acetonitrile concentration. The analysis time was 180 min for the first fraction and an additional 80 min for each subsequent fraction selected for protein identification.

Keywords: pH-gradient, Strong anion exchange chromatography, On-line tryptic digestion, Immobilized trypsin, Human plasma

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INTRODUCTION

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used as a standard approach in proteomics. However, major drawbacks are limited dynamic range; low resolution of proteins with extreme size, pI or hydrophobicity; low reproducibility, as well as lack of on-line integration with mass spectrometry (MS) and lack of automation.[1–4] Liquid chromatographic techniques (HPLC), in recent years, have utilized combinations of size exclusion chromatography (SEC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and reversed phase (RP) chromatography to overcome the shortages of 2D-PAGE.[4,5] However, in most cases, proteins were subjected to enzymatic digestion without fractionation before identification by LC-MS.[2,6] Enzymatic digestion is a critical step and has been commonly performed in solution off-line.[7] Limitations of these in solution digestion methods are (i) time consuming reaction, (ii) self digestion of residual protease, which leads to a loss of sensitivity and an increase in complexity of the mass spectra, and (iii) protein losses, especially of proteins present at low concentrations.[8,9] In recent years, immobilized enzymatic reactors, most commonly using the protease trypsin immobilized on various carriers, have been developed and utilized with several advantages over the in solution protocol, such as larger enzyme to substrate ratio, high digestion efficiency in a very short digestion time of several seconds to few minutes,[9] less self digestion and repeatability of use.[8,10–14] Beside these advantages, the on-line application of the reactor in proteome analysis opens the possibility for automation to overcome the drawbacks of the off-line technique as loss of sample, contamination, and difficulty to completely transfer the digest to the next dimension.[15]

The on-line integration of an immobilized trypsin reactor in chromatographic methods has been extensively described.[13,14,16–19] In most cases, proteins were initially on-line digested on an immobilized trypsin reactor and the resulting tryptic digest directly transferred onto the next dimension(s) for further separation and identification. There are a few reports on the utility of a trypsin reactor in an on-line system with two or more separation dimensions,[13,18,20,21] but to our knowledge not in combination with separation of native proteins according to their pIs prior to the trypsination.

In the present work, our goal was to develop a method for identification of proteins based on their pI values and peptide mass fingerprint (PMF) using an on-line system to avoid protein losses and to reduce the analysis time, and using narrow inner diameter columns for increased sensitivity. The system fractionated proteins by pH-gradient SAX chromatography[22,23] with on-line protein transfer via reversed phase trap columns to an immobilized trypsin reactor, and subsequent on-line separation and identification of the tryptic peptides by RP chromatography with electrospray ionization time-of-flight MS (ESI-TOF-MS) detection.

EXPERIMENTAL

Chemicals

1-Methylpiperazine (99+%) was purchased from Across Organics (Geel, Belgium) while 99% imidazole, 98.5% diethanolamine, and 99% piperazine were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Ethanolamine (99%) was obtained from Fluka Chimie GmbH (Buchs, Germany) and Trizma base (tris) minimum 99.9% titration from Sigma (MO, USA). Ammonia solution (analytical grade) was from BDH Laboratory Supplies (Poole, UK). Grade 1 water was produced with a Milli-Q Ultrapure water system (Millipore, Bedford, MA, USA). Formic acid (FA) (50%), 50% acetic acid (AcOH), and 5M ammonium acetate (NH_4OAc) solutions were purchased from Fluka. HPLC grade acetonitrile was from Rathburn Chemicals Ltd. (Walkerburn, Scotland).

Bovine heart cytochrome c, horse myoglobin, bovine erythrocytes carbonic anhydrase II, and citrated and lyophilized human plasma (P9523-5 mL) were obtained from Sigma-Aldrich Inc.

Solutions

A stock amine buffer solution prepared from 1-methylpiperazine, imidazole, diethanolamine, piperazine, ethanolamine, and Trizma base with 2 M concentration of each amine was stored at 4°C for a maximum of 2 weeks. Working solutions and mobile phases were prepared by appropriate dilutions of the stock solution.

Stock protein solutions of 2 mg/mL were prepared in 10 mM amine buffer solution (pH 10.5). A 0.5 mg/mL working protein mixture consisting of the 3 model proteins cytochrome c, myoglobin, and carbonic anhydrase was prepared in the same buffer solution.

For testing protein trapping, the proteins were dissolved in a buffer solution with a pH corresponding to their elution pH from the SAX column, at a concentration of 0.5 mg/mL.

Human plasma was reconstituted in 5 mL water and stored at −18°C. One milliliter human plasma conditioned to room temperature was diluted with 1 mL buffer solution pH 10.5 and filtrated by a 0.45 μm low protein absorption regenerated cellulose syringe filter (Alltech, IL, USA) just before analysis.

Instrumentation

The multidimensional chromatographic system developed is shown in Figure 1 and operated as described in Table 2.

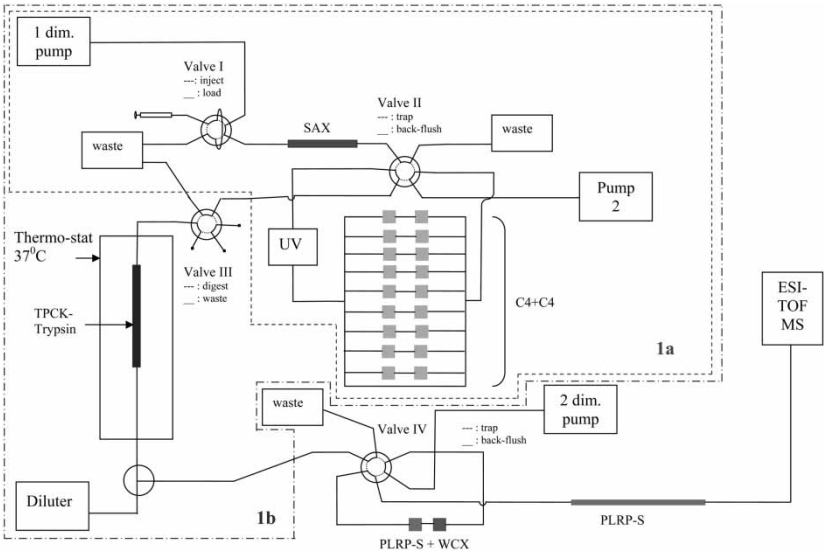

Figure 1. Scheme of the multidimensional chromatographic system.

Protein Separation

A mixture of model proteins (0.5 mg/mL of each) was introduced via a 0.8 μL loop to the first dimension by a Valco 6-port valve (valve I). The same loop was also used to introduce the 1:1 diluted human plasma solution. The separation column was a 25 cm × 1.0 mm i.d. 5 μm PL-SAX column purchased from GT-Septech (Norway). Mobile phases of 10 mM were obtained by dilution of a proportional amount of the stock amine buffer solution with water followed by pH adjustment with 10% formic acid (FA). Gradient chromatography was performed from buffer A (pH 10.5) to buffer B (pH 3.5), delivered by an Agilent binary CapPump 1100 (pump 1) at a flow rate of 40 μL/min (profile A, Table 1). The column was conditioned with buffer A at a flow rate of 40 μL/min for 40 min before each gradient. Separation of proteins was monitored at 280 nm with a Knauer K-2600 UV detector equipped with a 2 μL flow cell, and the data were collected with TotalChrom software version 6.2 (Perkin Elmer).

Protein Trapping Device

The eluent from the SAX column was fractionated using a Valco flow selector with 10 channels each equipped with two 5 × 1.0 mm i.d. 5 μm 300Å

Table 1. Gradient profiles in first and second dimensions, back-flushing and fractionation steps

Profile no.	Solution a	Solution b	Gradient profile
A	A mine buffer (10 mM), pH 10.5	A mine buffer (10 mM), pH 3.5	0–5 min: 0–20%B 5–40 min: 20–40%B 40–42 min: 40–90%B 42–60 min: 90–100%B
A*	A mine buffer (10 mM), pH 10.5	A mine buffer (10 mM), pH 3.5	0–5 min: 0–0%B 5–47 min: 0–100%B 47–60 min: 100–100%B
B	AcN/water/ NH_4OAc 5/95/ 10 mM, pH 7.0	AcN/water/ NH_4OAc 80/20/ 10 mM, pH 7.0	0–2 min: 0–46%B switch valve II (start back-flush) 2–7 min: 46–74%B 7–10 min: 74–74%B 10–20 min: 40–40%B 20 min: 0%B
B*	AcN/water/ NH_4OAc 5/95/ 10 mM, pH 7.0	AcN/water/ NH_4OAc 80/20/ 10 mM, pH 7.0	0–2 min: 0–46%B switch valve II (start back-flush) 2–6.5 min: 46–71%B 6.5–17 min: 0–0%B
C	AcN/water/ NH_4OAc 5/95/ 10 mM, pH 7.0	AcN/water/ NH_4OAc 80/20/ 10 mM, pH 7.0	0–1 min: 100–100%B 1–3.5 min: 60–60%B 3.5–10 min: 0–0%B
D	FA/water 0.1%	FA/AcN/water 0.1/80/20	0–3 min: 2–2%B 3–6 min: 2–15%B 6–60 min: 15–60%B
E	NH_4OAc/water 10 mM	NH_4OAc/AcN 10 mM	0–2 min: 5–40%B switch valve II (start back-flush) 2–7 min: 40–60%B 7–10 min: 60–60%B 10–12 min: 60–80%B 12–15 min: 45–45%B
F	AcN/water/ NH_4OAc 5/95/ 10 mM, pH 7.0	AcN/water/ NH_4OAc 80/20/ 10 mM, pH 7.0	0–2 min: 0–46%B switch valve II @ 3 min 2–6.5 min: 46–71%B stop flow @ 6.5 min in 5 min 11.5–20 min: 0–0%B

Kromasil-C4 Tracy columns (GT-Septech) in series. With the flow selector, up to 9 fractions can be collected per analysis. Gradient profile B (Table 1) delivered by pump 2 (Hotsep, GT-Septech, Norway) was used to elute the proteins from the C4 trap columns of each channel onto the trypsin column at a flow rate of 15 μL/min. Eluent A and B contained 5 and 80% acetonitrile,

respectively, in 10 mM NH_4OAc, pH 7.0, adjusted with 1% NH_4OH. The effluent was monitored at 280 nm by the Knauer K-2600 UV detector.

The recovery was calculated with formula:

$$R = \frac{a/r_r}{b/r_2}$$

where R is the recovery of protein, *a* is the sum area of trapped/backflushed protein peak with trapping columns, r_1 is sum area of reference protein mass peak with trapping columns, b is sum area of non-trapped (direct injection) protein peak without trapping columns, and r_2 is sum area of reference protein peak without trapping columns

The sum area was the combined area of 10 m/z values in the mass range 600–2500.

On-Column Trypsination

A 250 × 0.75 mm i.d. TPCK-Trypsin micro column purchased from OraChrom Inc. (ME, USA) was placed in a Mistral 880 thermo oven (Spark Holland, Emmen, The Netherlands) set at 37°C. A Valco 6-port valve (valve IV) was placed after the trypsin column to direct the eluent to a trap device ("trap" position) or directly to waste ("backflush" position). The trypsin column and the C4 trapping columns after each fraction analysis, were washed and conditioned by the gradient B* at flow rate of 15 μL/min, followed by the gradient C at 45 μL/min with the valve IV set to "backflush" position while the diluter was set to off-flow.

Peptide Trapping Device

Peptides generated on the trypsin column were transferred to and trapped on a pair of trap columns, a 5 × 1.0 mm i.d. 5 μm 4000 Å PLRP-S Tracy column, and a 5 × 1.0 mm i.d. 5 μm 4000 Å WCX Tracy column (both GT-Septech) joined in series. An aqueous solution of 0.1% NH_4OAc adjusted to pH 6.0 with 50% acetic acid was added to the flow by the diluter pump, L-7100 Hitachi isocratic pump set at 90 μL/min.

Following complete trapping, valve IV was switched to "backflush" position, and the peptides were backflushed from the PLRP-S + WCX trap columns onto the analytical column, see Table 2.

Peptide Separation System

Reversed phase separation of the tryptic peptides was performed on a 15 cm × 0.32 mm i.d. 5 μm PLRP-S column purchased from GT-Septech.

Table 2. The time schedule for the complete method

Step	Time (min)	Event	Action	Valve I	Valve II	Valve II	Valve IV
1	0.0	SAX column conditioning	Start 1. dim pump	Load	Back-flush	Waste	Backflush
2	40.0	Sample loading		Load	Trap	Waste	Backflush
3	40.1	Sample injection and 1. dim separation	Start pH gradient, profile 1A (1A*)	Inject	Trap	Waste	Backflush
			Switch selector for protein fracmentation				
4	100.1	Preparing trypsin column for fraction 1	Stop pH gradient	Inject	Trap	Digest	Backflush
			Start pump 2				
			Start diluter				
			Selector at non-column position				
5	100.2	Preparing for peptide trapping	Start gradient on pump 2, profile 1B*	Inject	Trap	Digest	Trap
6	102.2	Trypsination and trapping peptides	Switch selector to fraction 1	Inject	Back-flush	Digest	Trap
7	118.2	2. dim separation	Start gradient on 2. dim pump, profile 1D	Inject	Back-flush	Digest	Backflush
8	119.0	Washing C4 and trypsin columns	Start gradient on pump 2, profile 1B*	Inject	Back-flush	Digest	Backflush
9	135.0	Washing C4 and trypsin columns	Stop diluter	Inject	Back-flush	Digest	Backflush
			Start gradient on pump 2, profile 1C				
10	178.2	End 2 dim separation		Inject	Back-flush	Digest	Trap
11	179.0	Repeat step 5 to step 10 for fraction 2					

Mobile phases comprising 0.1% FA in water (A) and 0.1% FA in AcN/water (80/20, v/v) (B), see Table 1D, were delivered by an Agilent binary CapPump 1100 at a flow rate of 5 μL/min. After each analysis, the column was conditioned with mobile phase A for 15 minutes.

ESI-MS Conditions

The LCT time-of-flight mass spectrometer (Micromass, UK) was used in positive ionization mode. The ESI(+) source parameters were: capillary voltage 3.0 kV, sample cone voltage 35 V, extraction cone voltage 2 V, radio frequency lens voltage 500 V, desolvation temperature 120°C, source temperature 120°C and desolvation flow rate 250 L/h. Signals were recorded in a m/z range of 200–2500 or 400–2500 at 1.0 s scan time. The LCT-MS was controlled by MassLynx V4.0 software.

Data Analysis

The list of peptide mass values resulting from MS analysis was subjected to peptide mass fingerprint for protein identification using MASCOT search engine (www.matrixscience.com) with the NBCI database (version 20071019). Peptide tolerance was set to ±1.2 Da. Peptide masses searched were monoisotopic. Entries with a MASCOT MOWSE score >65 ($p < 0.05$) were identified as significant hits. Each matched peptide was used for one unique identified protein.

RESULTS AND DISCUSSION

The aim of the present study was to develop an on-line method for identification of proteins where the proteins initially are separated by pH gradient chromatography, then transferred on-line to a trypsin column with subsequent on-line transfer of the tryptic peptides to a RP-ESI MS system.

For development of the method the three model proteins shown in Table 3 was used.

Table 3. Properties of model proteins

Protein	Mass (Da)	AAs	pI	No. of tryptic cleavage site (K, R)
Cytochrome c	11704	105	9.28	20
Myoglobin	17083	154	6.88/7.33	21
Carbonic anhydrase	29114	260	6.0	27

Since the method is complex with several steps, each step was investigated separately before the complete method was put together.

Separation of Proteins on SAX Column with pH Gradient Elution

The proteins are separated approximately according to their pIs on a SAX column with pH gradient elution. The conditions and gradient profile chosen were as described in our previous papers[22–24] and are not explained in detail in the present paper. The obtained separation of cytochrome c, myoglobin, and carbonic anhydrase is displayed in Figure 2.

In theory, the eluent containing protein from the SAX column could be led directly onto the trypsination column. However, pH stabilization of the trypsination will be impossible with an eluent containing amine buffers at different pH. In order to obtain the same tryptic digestion condition for each protein (or fraction), a protein trapping step was included.

Fractionation and Trapping of Proteins from the SAX Column onto RP Columns

With the device used in the present configuration (see Figure 1), up to 9 fractions from the SAX column can be collected and trapped. The

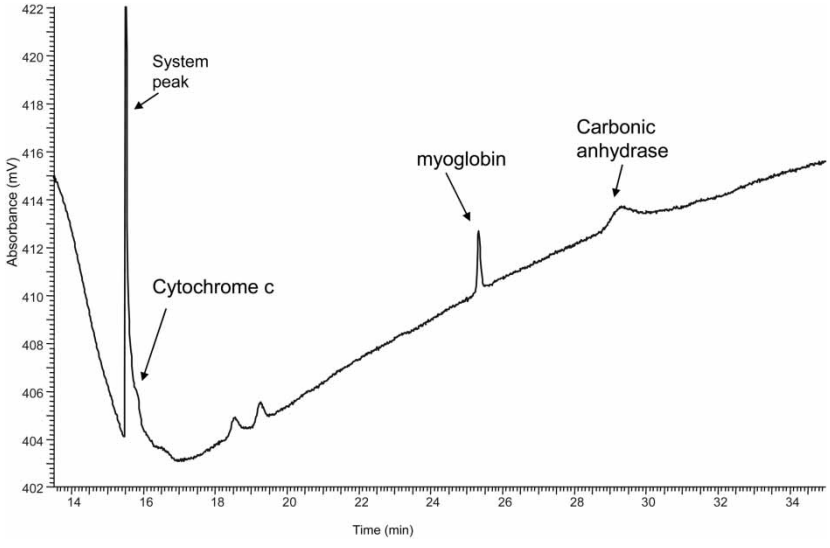

Figure 2. SAX chromatogram of cytochrome c, myoglobin, and carbonic anhydrase. Each protein 0.5 mg/mL, 0.8 μL injection volume, gradient profile 1A.

Table 4. Recovery of cytochrome c and myoglobin on different trapping materials

Material	Cytochrome c (%)	Myoglobin (%)
PLRP-S	51 (n = 1)	53 (n = 1)
C4	64 (RSD = 1, n = 4)	35 (RSD = 3, n =4)
C4 + PLRP-S	88 (n = 3)	68 (n = 3)
C4 + C4	69 (RSD = 1, n = 3)	48 (RSD = 2, n = 4)

fractionation time, based on the peak width of the investigated protein, varied, however, a fractionation time of about 2 min was appropriate to completely collect the individual model proteins. When separating a real sample, where the proteins might not be baseline separated, a 2 min fraction will contain several proteins.

To obtain an effective trapping with high recovery of proteins, the choice of trapping material is important. In preliminary experiments, trapping of the proteins on SAX trap columns was explored by increasing the pH of the SAX eluent by postcolumn addition of a strong basic solution. However, retaining proteins with extreme basic pIs required a high concentration of the basic solution (2% NH_4OH), and this caused protein elution because of high ionic strength (data not shown). Hence, we decided to use reversed phase trapping columns. Both 5 μm 4000 Å PLRP-S and 5 μm 300 Å Kromasil-C4 materials were investigated, using two model proteins, cytochrome c, and myoglobin.

The recovery from the trapping procedure was estimated by comparing the peak area of trapped and backflushed protein with that of direct injection of the same amount of protein, using an injector with a 200 nL loop replacing valve III and coupled directly to ESI-TOF MS (Table 4). The TOF MS was used for detection with an acidified eluent (0.2% FA at 10 μL/min). Proteins were backflushed with gradient profile 1E (Table 1) when trapped on PLRP-S, and a profile 1B (Table 1) when trapped on Kromasil-C4. Since suppression effects could occur and give an error when the protein was introduced to the ESI interface with different mobile phase composition, a reference was necessary to obtain a reliable measurement of the recovery of a protein captured on and eluted from the trapping columns. Despite of providing less recovery than the C4 + PLRP-S combination, the C4 + C4 combination war chosen since this combination did not result in carry-over (data not shown).

To retain the enzymatic activity of immobilized trypsin, it is important to minimize the time the trypsin column is exposed to a high amount of organic solvent. Hence, the elution of protein with AcN from the trap columns should be performed in as a short time as possible. Different gradient profiles were investigated, e.g., 40–80%, 30–50%, and 40–60% AcN. The latter one was most suitable for all three model proteins (see also Figure 3). Protein precipitation was observed with the 40–80% profile and peak broadening with the 30–50% AcN (data not shown). Only the elution fraction containing the

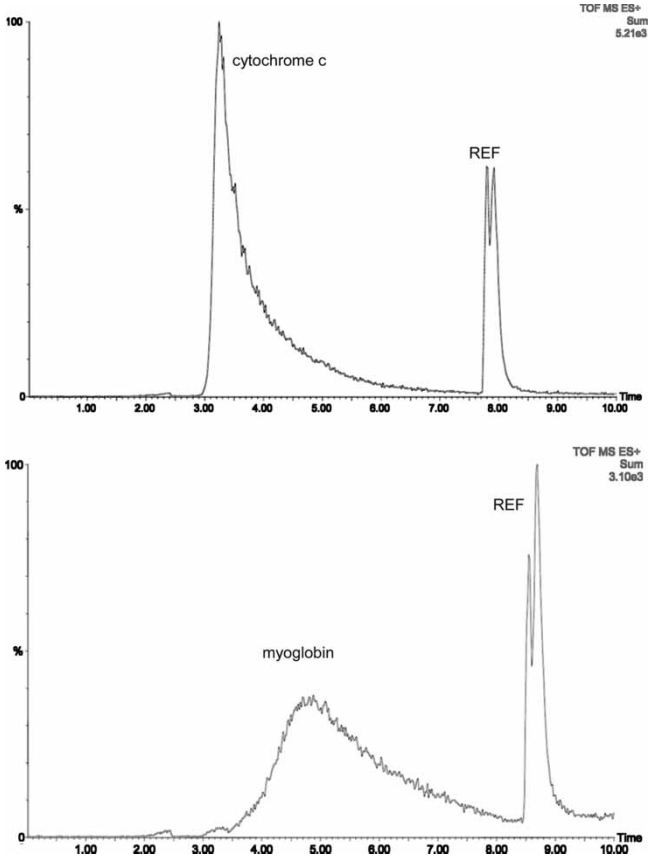

Figure 3. Elution of cytochrome c, myoglobin, and their REFs trapped on C4 + C4 columns.

protein was transferred onto the trypsin column. In addition, to maintain the enzymatic activity of the trypsin column employed in the next step, the increase in AcN concentration was stopped when protein eluted, using gradient profile 1B*, a modification of profile 1B.

On-Line Tryptic Digestion

The immobilized TPCK trypsin column displays high stability toward pH, organic solvents, and high flow rates. In addition, there is no autodigestion due to the absence of contact in the immobilized format between enzyme molecules.

The cleavage obtainable obviously varies with proteins as well as the operating condition such as temperature, flow rate, and pHs.[12,25] The

temperature of the column was 37°C during the digestion process. The column was proclaimed to tolerate a pH range of 5–11 and recommended to operate ideally at pH around 8.2. In addition, the presence of AcN is favorable to obtain efficient digestion because of increased solubilization of proteins.[26] Since the column was to be used in a multicolumn on-line system, the operating conditions needed to be compatible with the steps both before and after. For simplification, optimization experiments were performed with the system outlined in Figure 1b, where a UV detector operated at 280 nm was placed prior to the trypsin column to monitor the elution of protein onto the trypsin column, and a (diluter) pump delivered 0.2% HCOOH to acidify the eluent prior to MS introduction.

To find the best pH, the pH of the mobile phases for eluting C4 + C4 trapped proteins onto the trypsin column was adjusted with 2% ammonia solution to pH 7.0 and 8.5, respectively. There was no significant variation in the number of tryptic peptides of cytochrome c and myoglobin cleaved at different pH at a flow rate of 15 μL/min (data not shown). The pH of 7.0 was therefore chosen, because of the necessity of low pH for efficient peptide trapping in the following step.

Trapping of proteins on the RP-C4 columns with subsequent elution with a mobile phase containing an organic constituent can lead to (partial) denaturation of the protein. This, however, should be beneficial for the trypsination process by improving cleavage site accessibility.[27–30] In our system, the trypsination was carried out for 6.5 min with a concentration of AcN up to 57% produced by the fast gradient profile 1B*. Figure 4 shows the mass spectra of tryptic digest of proteins. The peptide masses were subjected to MASCOT search to obtain protein sequence. Peptides 19, 11, and 12 of cytochrome c, myoglobin, and carbonic anhydrase, respectively, were detected in the m/z range 400–2500. The matched peptides covered 75/105, 109/154, and 112/260 amino acids (AAs) of cytochrome c, myoglobin, and carbonic anhydrase, respectively, with a maximum number of missed cleavages of 2. The high number of matched peptides and the high sequence coverage demonstrated that the TPCK-trypsin column operated effectively in a solvent of high AcN concentration.

Continuous-Flow Versus Stop-Flow Trypsination

As mentioned above, the flow rate of the mobile phases or the time the protein is in contact with the immobilized trypsin is one of the most important factors for digestion efficiency. The trypsination of the three model proteins was investigated at two different continuous flow rates, 15 and 30 μL/min, see Table 5. When 10 or more peptides were used for protein verification, both flow rates could be used, but the flow rate of 15 μL/min gave better cleavage than 30 μL/min for all three investigated proteins (Table 5). The difference in sequence coverage caused by different flow rate was more

noticable for the larger protein carbonic anhydrase (11%) than the smaller cytochrome c (2%).

In order to improve the digestion efficiency of large proteins, the trypsination time was extended by using a stop-flow mode as shown in gradient profile 1F. This gradient profile was a modified version of the profile 1B* adapting to stop-flow operation, in which a heart cut of the protein was transferred to and retained on the trypsin column for the time the flow was stopped. The transfer time was

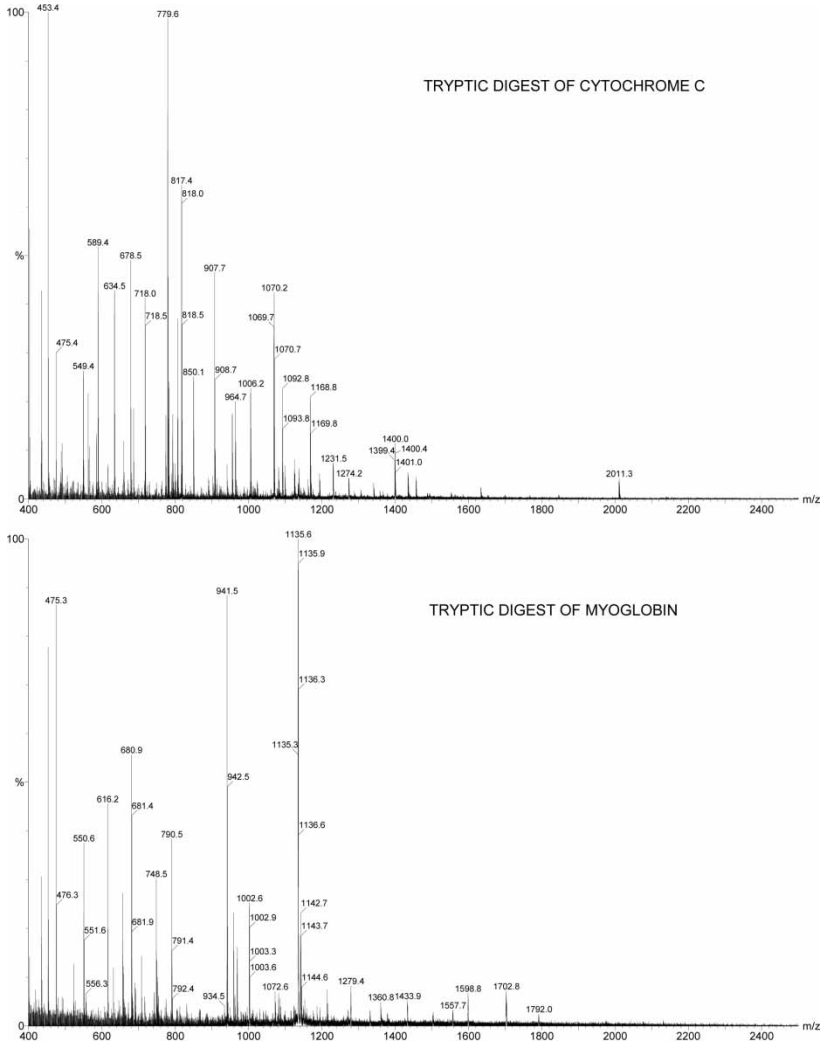

Figure 4. Mass spectra of tryptic digest of cytochrome c, myoglobin, and carbonic anhydrase, using gradient profile 1B* at 15 μL/min.

(*Continued*)

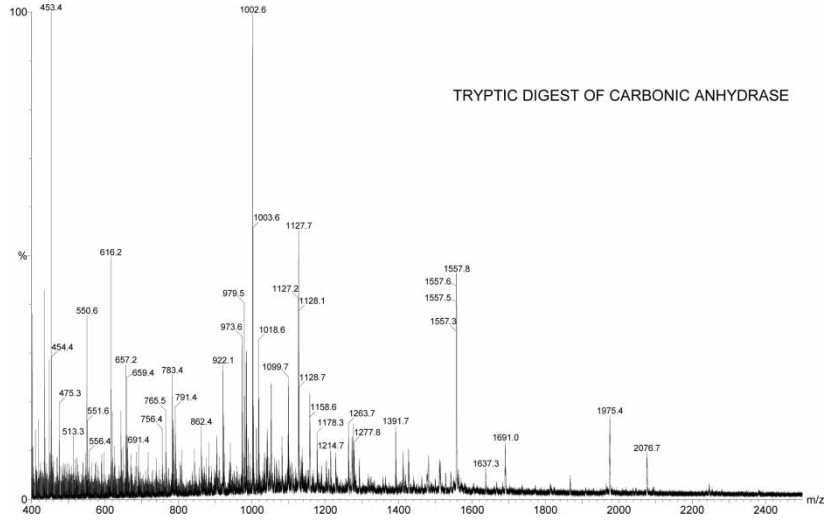

Figure 4. Continued.

chosen based on the flow rate, the dimension of the trypsin column, and the peak width of protein eluting from the dual trap columns, in such a way that almost all of the protein was on the trypsin column at stop conditions. Since most of the protein was eluted from the trap columns with AcN concentration less than

Table 5. Effects of flow rate and flow mode on trypsination of proteins

Flow mode	Flow rate	No. of searched mass values	No. of matched mass values	Score	Sequence coverage (%)
Cytochrome c					
Continuous	15	36	19	108	71
	30	35	17	102	69
Stop	15	26	11	71	65
	30[a]	30	10	52	65
Myoglobin					
Continuous	15	19	11	92	71
	30	14	9	71	57
Stop	15	32	13	89	49
	30[a]	33	11	64	37
Carbonic anhydrase					
Continuous	15	33	12	70	43
	30	28	10	63	32
Stop	15	32	16	89	62
	30[a]	31	14	92	60

[a]Applied after stop-time.

60%, the fast AcN gradient as profile 1B* made the protein elute in a relative narrow band, about 3–4 min in our experiments (Figure 3). To narrow the protein elution window further, a faster gradient elution, 40–60%AcN in 2 min, was also investigated for cytochrome c. Unfortunately, the peptide abundances seriously decreased, most probably because of precipitation prior to the elution of proteins (data not shown). A heart cut time of 3.5 min was, therefore, the most suitable for the proteins investigated, at flow rate of 15 μL/min.

Results of the trypsination of the model proteins performed in stop-flow mode are displayed in Table 5. Due to the risk of protein precipitation and also the potential loss of enzymatic activity of the trypsin column when a high amount of AcN is used for a long time, the stop-flow experiments were carried out for a relatively short stop time of 5 min. Digestion of the largest model protein carbonic anhydrase with 27 tryptic cleavage sites, carried out in stop-flow mode resulted in four more peptides identified, and an increase in sequence coverage from 43% to 62% compared to continuous flow condition. The smaller proteins cytochrome c and myoglobin with tryptic cleavage sites of 21 and 20, respectively, showed a decreased number of peptides and sequence coverage. However, the score and number of matched peptides were high enough to provide protein identification.

Hence, the on-line coupling of SAX chromatography with RP-C4 trap columns with subsequent on-column tryptic digestion was obviously well functioning. Continuous flow mode was the best choice for small proteins while stop-flow mode was an alternative for the larger proteins.

Trapping of Tryptic Peptides

When the protein fraction from the SAX column contains several proteins, as it will for real samples, the resulting tryptic peptide mixture will be complex, and separation of the tryptic peptides is necessary before MS identification. Reversed phase chromatography is commonly used, and also chosen in the present method. However, the peptides cannot be time efficiently trapped and separated on a narrow i.d. column, which is preferred for optimal sensitivity. Hence, a column switching system with trap columns was explored.[31] The PLRP-S and WCX trapping columns employed were evaluated for their efficiency of peptide trapping. In principle, a reversed phase trap column should be sufficient to obtain trapping of the peptides. However, the AcN concentration after dilution is still around 8.5%, and to obtain trapping of the most hydrophilic peptides, a WCX column was added in series to the reversed phase PLRP-S column to get retention because of ionic interactions as well, since trapping occurs at approximately neutral conditions (pH ~6). The ionic interactions are suppressed when the peptides are backflushed with the acidic mobile phase. The RP trap column is placed before the WCX trap column to trap the most hydrophobic peptides with hydrophobic interactions only.

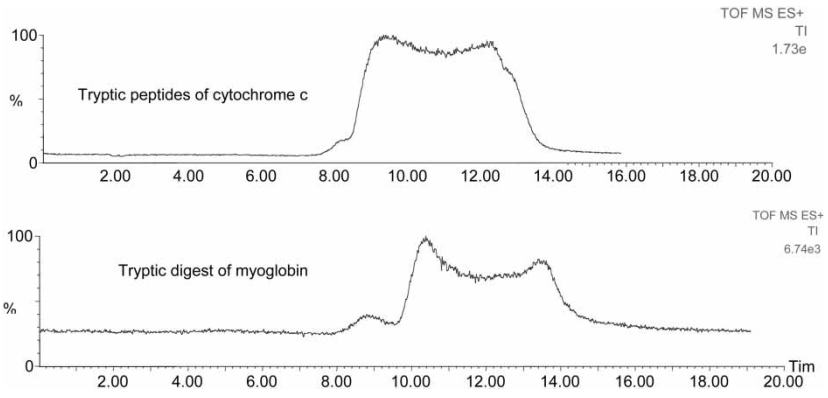

Figure 5. Elution of tryptic peptides from the trypsin column.

Trapping efficiency was investigated using a system as shown in Figure 1, in which the PLRP-S analytical column was replaced with 0.075 mm i.d. fused silica tubing.

Since a high concentration of AcN was used for backflushing of proteins from the C4 trap columns, diluting the eluent after the trypsin column with a 0.1% NH_4OAc aqueous solution (pH 6.0) was necessary to enhance the trapping on the PLRP-S column. Different dilutions (from 1+3 to 1 + 6) using a diluter pump flow of 45 to 90 μL/min were carried out, and the breakthrough of peptides after the trapping columns was monitored (Figure 5). Experiments without trapping columns, called the non-trapped, were also performed and used as a reference to evaluate the trapping efficiency. The percentage breakthrough of peptides was calculated as the percentage of sum area (in mass range 500–2500) relative to that of the non-trapped peptide peaks (Table 6). The breakthrough of cytochrome c and myoglobin peptides decreased when the dilution was increased and was about 7% (RSD = 10%, n = 3) and 5% (RSD = 9%, n = 3), respectively, with the dilution pump set at 90 μL/min. Hence most peptides were well retained on PLRP-S + WCX columns, while only a few peptides were not retained. In the non-trap experiment with the dilution pump set at 45 μL/min, 19 and 11 peptides were detected for cytochrome c and myoglobin, respectively, in the m/z 550–2500 with relative intensity higher than 10%. Between 2 and 4 of them were not detected in the eluent when backflushed from the trapping columns (Table 6) and could be due to breakthrough. The number of lost peptides seemed to be less dependent on the dilution factor. Therefore, the dilution pump was set at 90 μL/min to assure minimal breakthrough.

Elution of all peptides from the trypsin column occurred from 8.0–15.0 min (Figure 5). Therefore, trapping of peptides on the PLRP-S + WCX columns was performed for 16.0 min. Longer trapping time, up to 20 min, was also investigated, however, there was no difference observed (data not

Table 6. Flow rates and percentage of acetonitrile in mobile phase employed for PLRP-S + WCX trapping

Flow rate of trypsin column	Dilution with water pH 6.0	%AcN of MP[a]	Breakthrough (%)[b]		No. of peptide peaks	
			Cytochrome c	Myoglobin	Cytochrome c	Myoglobin
15	45[c]				19	11
15	45	15	16	80	17	9
15	60	12	47	95	17	8
15	75	10	31	16	17	8
15	90	8.5	7	5	17	7

[a]%AcN = (Flow rate for trypsin column/total flow rate) × 60% (max for C4).
[b]Breakthrough = sum area of breakthrough/sum area of non-trapped × 100.
[c]Non-trapped.

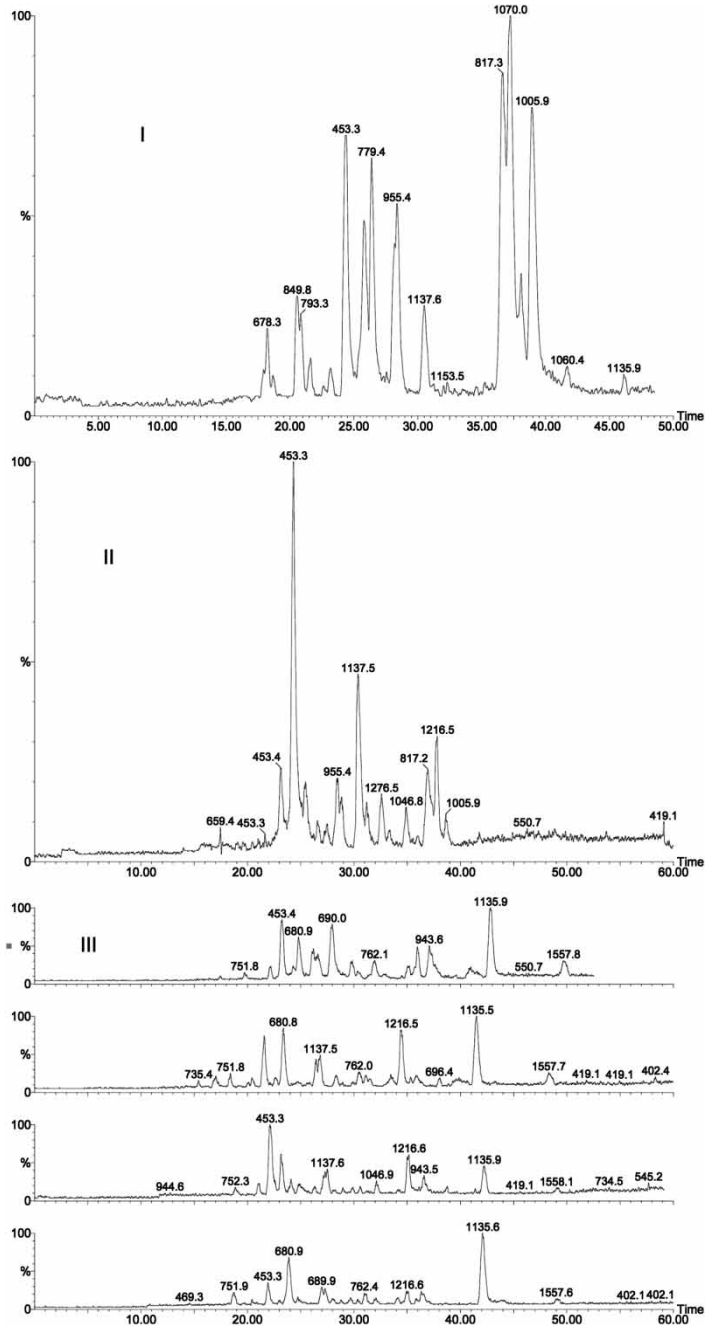

Figure 6. RP chromatograms of tryptic digest of collected fractions. I) Fraction of cytochrome c; II) Fraction of carbonic anhydrase; III) Fraction of myoglobin (4 replicates).

shown). The sum area in mass range 500–2500 of peptides eluted from the trapping columns in 16 min was repeatable with RSD (n = 3) of 2% and 3% for cytochrome c and myoglobin, respectively.

In brief, 1+6 dilution (15 + 90 μL/min) and 16 min trapping time were chosen.

Testing the Complete Method with a Mixture of Three Proteins

A mixture of native cytochrome c, myoglobin, and carbonic anhydrase was subjected to the multidimensional SAX/on-line tryptic digestion/RP-MS method, as outlined in Figure 1, in continuous flow mode. The operational conditions for SAX separation, trypsination, and trapping steps optimized and presented above were utilized. The analytical RP column in the second dimension is of significantly smaller inner diameter than the first dimension SAX column, thus concentrating digested proteins. In order to obtain a complete collection of each protein from the SAX column, the fractionation time was chosen in accordance with its elution pattern and varied from 1 to 3 minutes. For the peptide separation, a gradient elution method D as shown in Table 1 was used at a flow rate of 5 μL/min.

The chromatograms of the peptide fingerprints for each of the model proteins are demonstrated in Figure 6. Data sets of m/z 500–2500 were subjected to MASCOT peptide mass fingerprint search for protein identification and the results are presented in Table 7. All three proteins were correctly identified in their respective fractions with sequence coverage of 88, 48, and 44 for cytochrome c, myoglobin, and carbonic anhydrase, respectively.

With the described system, we demonstrated that the analysis time is quite short compared to that required for an off-line method. The analysis time for the three model proteins were 180 min for the first fraction, plus 80 min for

Table 7. Peptide masses and sequences obtained by SAX/on-line digestion/RP multi-dimensional system (mass tolerance: ± 1.2 Da)

Protein	Sequence coverage (%)	Number of matched peptides	Score
Cytochrome c	88	15[a]	168
Myoglobin	48	6[b]	53
Carbonic anhydrase	44	6[c]	98

List of peptide mass (missed cleavage):

[a]678.3 (0), 634.3 (0), 1633.5 (1), 1168.6 (0), 1584.5 (1), 1698.7 (1), 2010.9 (0), 2138.9 (1), 2798.3 (2), 806.4 (1), 779.4 (0), 617.2 (2), 1434.7 (2), 964.5 (0), 1092.6 (1).

[b]3005.7 (4), 1378.9 (0), 1507.0 (1), 1502.7 (0), 1360.8 (1), 2284.3 (3).

[c]2199.3 (0), 1392.7 (1), 5924.1 (1), 3156.7 (1), 2854.5 (0), 3123.6 (1).

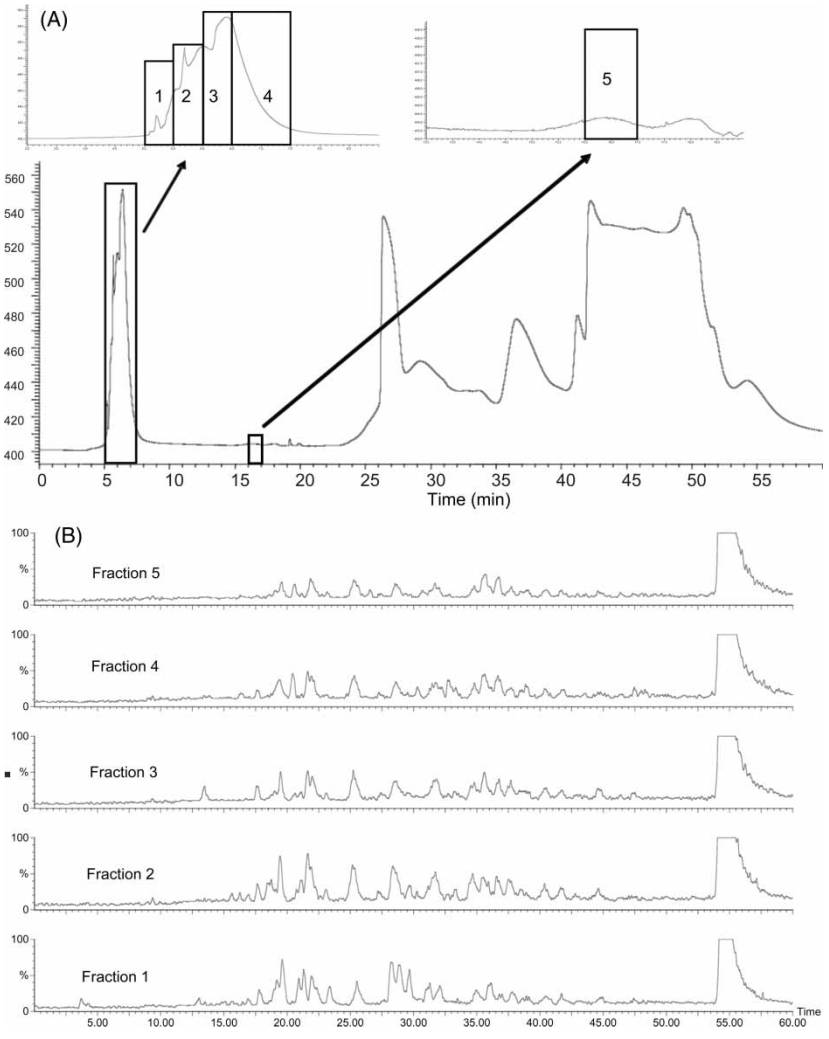

Figure 7. (A) SAX chromatogram of human plasma and expanded view of collected fractions. (B) TIC chromatograms of five SAX fractions.

each subsequent fraction (Table 2). Column cleaning and conditioning can be done simultaneously with trypsination/tryptic peptide analysis. The immobilized trypsin column was used for more than 50 injections and gave highly repeatable results.

Human Plasma Analysis

To show the applicability of the method, a plasma sample was analyzed with the focus on identification of the most basic proteins. Of 1+1 diluted human

Table 8. Proteins identified by MS in pH-fractions of human plasma. Parameters obtained from database search

NCBI no.	Protein name	Mascot score	No. of matched peptides (corresponding sequence coverage rate)	Missed cleavage	pI	Mass (kDa)
Fraction 1: 5.0–5.5 min						
gi|1200089	ORF [homo sapiens]	92	42 (65%)	3	8.60	59.9
gi|113412900	Hypothetical protein [homo sapiens]	72	22 (54%)	3	12.17	22.2
gi|119597471	hCG1980622, isoform CRA_g [homo sapiens]	88	27 (76%)	3	8.61	25.9
Fraction 2: 5.5–6.0 min						
gi|119608862	RNA binding motif protein, X-lined, isoform CRA_b [homo sapiens]	97	31 (89%)	3	11.01	23.5
gi|116812597	Zinc finger protein 586, isoform a [homo sapiens][a]	103	30 (45%)	3	9.14	46.3
gi|119592940	Zinc finger protein 586, isoform CRA_b [homo sapiens][a]	101	31 (50%)	3	9.29	41.6
gi|119606314	hCG2017070 [homo sapiens]	85	18 (48%)	3	9.38	54.7
Fraction 3: 6.0–6.5 min						
gi|113412900	Hypothetical protein [homo sapiens]	80	19 (51%)	2	12.17	22.2

(*continued*)

Table 8. Continued

NCBI no.	Protein name	Mascot score	No. of matched peptides (corresponding sequence coverage rate)	Missed cleavage	pI	Mass (kDa)
Fraction 4: 6.5–7.5 min						
gi|3171045	Immunoglobulin heavy chain variable region [homo sapiens]	67	9 (66%)	1	5.26	15.7
gi|119612068	2, 4-dienoyl CoA reductase 1, mitochondrial, isoform CRA_a [homo sapiens]	68	10 (59%)	1	7.68	21.2
gi|119627129	Deiodinase, iodothyronine, type I [homo sapiens]	66	8 (89%)	1	7.93	10.5
gi|280077093	Retinoblastoma-binding protein 6 isoform 3 [homo sapiens]	91	12 (81%)	1	9.08	13.2
Fraction 5: 16.0–17.0 min						
gi|31377703	Tetratricopeptide repeat domain 13 [homo sapiens]	88	29 (35%)	2	6.58	96.7

[a]Several masses were used in PMF search for both proteins.

plasma, 100 μL was introduced into the multidimensional system with the same analytical conditions as described in the section above, except that gradient profile A* was used for the SAX dimension. Figure 7A shows the UV chromatogram of the human plasma sample and the five fractions selected from the basic elution region from the SAX column. Figure 7B shows the RP separation of the tryptic peptides from these fractions. The proteins identified and their properties are displayed in Table 8. Except for the highly basic hypothetical protein in fraction 3 (also found in fraction 1), the identified human proteins were found to elute from the SAX column approximately according to their pIs. The proteins identified in fraction 1 and 2 with molecular weights higher than 22 kDa showed a slightly higher number of missed cleavage compared to the smaller proteins in fraction 4. Some of the matched peptides with a high charge (3 to 5) demonstrated missed cleavage of the proteins found in fraction 1 and 2 (data not shown).[32]

CONCLUDING REMARKS

We have developed a method for proteome analysis based on an on-line multidimensional chromatographic system. Beside the fact that proteins can be fractionated and identified based on their tryptic peptides and pIs, less contamination and sample loss are the advantages of this on-line method. The use of on-column trypsination was compatible with the high concentration of AcN. The total analysis time is short relative to off-line approaches, and the method can also be automated.

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