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Analysis of human serum by liquid chromatography–mass spectrometry: Improved sample preparation and data analysis

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Abstract

Discovery of biomarkers is a fast developing field in proteomics research. Liquid chromatography coupled on line to mass spectrometry (LC–MS) has become a powerful method for the sensitive detection, quantification and identification of proteins and peptides in biological fluids like serum. However, the presence of highly abundant proteins often masks those of lower abundance and thus generally prevents their detection and identification in proteomics studies. To perform future comparative analyses of samples from a serum bank of cervical cancer patients in a longitudinal and cross-sectional manner, methodology based on the depletion of high-abundance proteins followed by tryptic digestion and LC–MS has been developed. Two sample preparation methods were tested in terms of their efficiency to deplete high-abundance serum proteins and how they affect the repeatability of the LC–MS data sets. The first method comprised depletion of human serum albumin (HSA) on a dye ligand chromatographic and immunoglobulin G (IgG) on an immobilized Protein A support followed by tryptic digestion, fractionation by cation-exchange chromatography, trapping on a C18 column and reversed-phase LC–MS. The second method included depletion of the six most abundant serum proteins based on multiple immunoaffinity chromatography followed by tryptic digestion, trapping on a C18 column and reversed-phase LC–MS. Repeatability of the overall procedures was evaluated in terms of retention time and peak area for a selected number of endogenous peptides showing that the second method, besides being less time consuming, gave more repeatable results (retention time: <0.1% RSD; peak area: <30% RSD). Application of an LC–MS component detection algorithm followed by principal component analysis (PCA) enabled discrimination of serum samples that were spiked with horse heart cytochrome C from non-spiked serum and the detection of a concentration trend, which correlated to the amount of spiked horse heart cytochrome C to a level of 5 pmol cytochrome C in 2 μl original serum.

Keywords: Proteomics; Mass spectrometry; Biomarker; HPLC; Cervical cancer

1. Introduction

Various methods have been applied in recent years for the discovery of biomarkers or biomarker patterns of major human diseases, especially for various types of cancer [1–13]. Amongst these surface enhanced laser desorption ionization mass spectrometry (SELDI-MS), which combines on-chip sample preparation with mass spectrometric analysis, has taken a prominent position [14], although more recent results question the viability of this approach [15,16]. Liquid chromatography coupled on line to mass spectrometry (LC–MS) is one of the most widely used analytical methods with applications going beyond proteomics and biomarker discovery. It has the advantage of combining powerful separation by one- or multi-dimensional chromatography with the exquisite selectivity and sensitivity of modern mass spectrometers. The complexity of a typical LC–MS data set reaches 10^8 data points per sample at a resolution of 0.1 amu in the m/z domain and a chromatographic run time of approximately 2 h (7000 data points). It is thus pivotal to apply data pre-processing algorithms to reduce data complexity and multivariate statistics to reduce dimensionality to be able to compare data sets obtained from longitudinal or cross-sectional patient studies comprising significantly less samples than the number of original variables in the data.

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Sample preparation is an often underestimated problem in comparative biomarker analysis. Notably in serum there are a few highly abundant proteins that will prevent detection of many minor proteins present in the sample. Since it is unlikely that high abundance proteins like albumin or transferrin will be biomarkers for specific diseases, it is necessary for biomarker discovery methodology to detect and quantify proteins present at lower concentrations. One way to reduce serum complexity is chromatographic removal of the most abundant proteins. In human serum, the most abundant proteins are albumin and γ-globulins. Earlier [17] we tested different depletion strategies to reduce the level of abundant proteins based on either specific antibodies, dye ligands (for albumin) [18] or Protein A and G (for γ-globulins) [19,20]. Other approaches based, for example, on ultrafiltration showed lower selectivity for these target proteins but allowed on the other hand to concentrate the sample [21]. Co-depletion of proteins and peptides is a concern when employing such depletion strategies [22].

LC–MS is particularly adapted to the separation and detection of peptides. This has triggered development of the so-called "shotgun" proteomics approach [23]. Contrary to proteomics based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), where the proteins are separated prior to tryptic digestion, trypsin digestion precedes the separation step. The shotgun approach results therefore in extremely complex mixtures of peptides presenting a challenge to any separation methodology. Very high efficiency separation systems have recently been applied to this problem allowing to identify a wide range of proteins in plasma or serum [13]. A new approach developed recently combines the use of immunoaffinity depletion with reversed-phase separation of whole proteins at elevated temperatures to reduce sample complexity prior to identification of proteins in human serum [24]. This approach allowed, for example, to identify hepatocyte growth factor, which is present at a level of 20 ng/ml.

In this study, we combined efficient depletion of high abundance proteins with LC–MS based shotgun proteomics followed by data pre-processing to select information-rich chromatographic traces based on the CODA algorithm [25]. This was followed by multivariate statistical analysis of the selected traces by principal component analysis (PCA) to evaluate the performance of the method to discriminate samples. Initially we tested two approaches for the preparation of human serum for LC–MS analysis. Removal of abundant proteins was performed by dye ligand or antibody-based multiple-affinity chromatography, which eliminates the six most abundant serum proteins. In the case of dye ligand chromatography, a two-dimensional chromatographic system was employed consisting of strong cation exchange followed by reversed-phase HPLC. Proteins remaining after the multiple-affinity removal step were directly digested and analyzed by reversed-phase LC–MS. The performance of each approach was assessed in terms of repeatability of retention times and peak areas for a selected number of endogenous peptides showing that the repeatability of the second method was superior. The discriminatory power of this method was assessed by adding decreasing amounts of horse heart cytochrome C to the original serum proving that a concentration trend was correctly represented in the first principal component after non-supervised data pre-processing and PCA down to a lower level of 50 pmol cytochrome C in 20 μl serum.

2. Experimental

Serum samples were obtained from the Department of Gynecological Oncology (University Medical Center Groningen, The Netherlands) and stored at −80 °C in aliquots. All intermediate fractions were stored at −20 °C. To develop and optimize the analytical procedure, serum from a single cervical cancer patient with a squamous cell carcinoma antigen-1 (SCCA-1) [26] level of 2.2 ng/ml (determined by ELISA) was used.

2.1. Sample preparation of human serum

2.1.1. Method 1

Two hundred and forty microliters of diluted crude serum (60 μl of crude serum mixed with 180 μl of 20 mM NaH_2PO_4 pH 7.0) wer depleted on a 1 ml Bio-Rad Aurum Serum Protein (www.biorad.com) column according to the manufacturer's instructions. Protein concentrations were determined with the Micro BCA™ Protein assay reagent kit (www.piercenet.com) and calculated for an average protein molecular weight of 50 kDa. BSA was used as the calibration standard.

An equivalent of 100 μg total protein of depleted serum were digested with trypsin (1:20, w/w enzyme to substrate) at 37 °C overnight (sequencing grade modified trypsin from Promega, Cat# V5111, USA). One hundred micrograms of digested, depleted serum were fractionated by strong cation exchange HPLC (Poly SEA 2.0 mm × 150 mm, 5 μm, 300 Å column, Michrom BioResources, Auburn, CA, USA) operated at 0.3 ml/min on a Beckman Gold HPLC system (www.beckman.com). The salt gradient ranged from 0 to 1 M KCl with a slope of 10 mM/min. The mobile phase comprised two buffers: A: 5 mM KH_2PO_4/H_3PO_4 pH 3, 25% acetonitrile and B: 5 mM KH_2PO_4/H_3PO_4 pH 3, 25% acetonitrile, 1 M KCl. The following fractions were collected: #1 (0–50 mM) KCl, 17–21 min, #2 (50–100 mM) KCl, 22–26 min, #3 (100–150 mM) KCl, 27–31 min and #4 (150–200 mM) KCl, 32–36 min. Samples were concentrated by vacuum centrifugation (Speed Vac, Univapo 150H, UniEquip, Martinsried, Germany) to approximately 1 ml to remove acetonitrile and fractions #3 and #4 were pooled together because of their low concentration of peptides. The concentrated fractions were passed through a Macro Trap 200 hydrophilic C18 silica cartridge (ODS-AQ; 3 mm × 8 mm; Michrom, USA) for desalting and further concentration by loading at 0.3 ml/min followed by a column wash with 2 ml of 5% acetonitrile, 0.1% formic acid in H_2O. Peptides were eluted with 0.5 ml 70% acetonitrile, 0.1% formic acid at a flow rate of 1 ml/min. Acetonitrile was evaporated by vacuum centrifugation (Speed Vac) and the final volume was adjusted to 150 μl. Pure acetonitrile and pure formic acid were added to reach final concentrations of 5% acetonitrile and 0.1% formic acid, respectively. All LC–MS analyses were performed on an Agilent 1100 capillary HPLC system coupled on-line to an SL ion trap (www.home.agilent.com) equipped with an Atlantis™

dC 18 (1.0 mm × 150 mm, 3 μm) column (www.waters.com). Forty microliters of the pretreated fractions corresponding to ~8 μg or 160 pmol of total protein digest (calculation based on a 50 kDa protein) were injected. Peptides were eluted in a linear gradient from 0 to 70% (0.5%/min) acetonitrile with 0.1% formic acid at a flow-rate of 20 μl/min.

2.1.2. Method 2

Eighty microliters (80% of the total amount of diluted crude serum (20 μl of crude serum mixed with 80 μl of buffer A (Agilent)) were depleted on a Multiple Affinity Removal column (Agilent, 4.6 mm × 50 mm, Part # 5185–5984) according to the manufacturer's instructions.The flow-through fraction (depleted serum collected between 2 and 6 min) of a total volume of approximately 1 ml was collected. Protein concentrations were determined as in Method 1. One hundred microliters (~10% of the total amount, which corresponded to ~7 μg or 140 pmol of total protein considering a molecular weight of 50 kDa) of depleted serum were digested with trypsin (1:20, w/w enzyme to substrate) under the same conditions as described in Method 1. All LC–MS analyses were performed on the identical LC–MS system (Agilent 1100 capillary HPLC; SL ion trap mass spectrometer) except that an in-line trap column was used (Atlantis^{TM} dC 18, 3 μm, 2.1 mm × 20 mm Guard column (www.waters.com)). One hundred microliters depleted and digested serum were injected and washed in the back flush mode for 40 min (0.1% aq. formic acid and 3% acetonitrile at a flow-rate of 50 μl/min) and eluted on-line to the analytical column (Atlantis^{TM} dC 18, 1.0 mm × 150 mm, 3 μm column (www.waters.com)). Gradient conditions were identical to Method 1.

2.2. Polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed in a Mini-Protein III cell (Bio-Rad, www.biorad.com) using 12% gels with 0.1% SDS according to the manufacturer's instructions. Staining was performed with Coomassie Brilliant Blue R concentrate (Sigma, www.sigmaaldrich.com) diluted and used as prescribed by the manufacturer.

2.3. Mass spectrometry

The following conditions were used for mass spectrometry during LC–MS. Nebulizer gas: 16.0 psi N_2, drying gas: 6.0 l/min N_2, skimmer: 40.0 V, cap. exit: 158.5 V, Oct. 1: 12.0 V, Oct. 2: 2.48 V, Oct. RF: 150 Vpp (Voltage, Peak Power Point), Lens 1 : −5.0 V, Lens 2: −60.0 V, Trap drive: 53.3, T°: 325°, Scan resolution: Enhanced, 5500 *m/z* per second scan speed. Target mass: 600. Scan range: 100–1500 *m/z*. Spectra were saved in centroid mode. LC–MS chromatographic data were analyzed with Bruker Data Analysis software, version 2.1 (Build 37).

2.4. Repeatability study

A serum sample from a cervical cancer patient was treated six-times with Method 1 and five-times with Method 2. In Method 2 crude sample of the same patient was also spiked with horse heart cytochrome C (Sigma, www.sigmaaldrich.com) before depletion (210 pmol cytochrome C per 20 μl of original serum) and the procedure repeated four times.

2.5. Standard addition of cytochrome C

Horse heart cytochrome C was added to 20 μl of the original serum over a range of 25 pmol–1.26 nmol, of which 10% were subjected to the final LC–MS analysis to evaluate the discriminatory capacity of Method 2. Cytochrome C was alternatively digested with trypsin and added in the same amounts to depleted and trypsinized serum (2 μl equivalent) prior to LC–MS to evaluate whether the depletion procedure affected the recovery of cytochrome C.

2.6. Data analysis

2.6.1. Data pre-processing

The original Bruker Daltonics LC–MS data files were converted into ASCII-format with the Bruker Data Analysis software. The original *m/z* ratios (0.1 amu resolution) were combined into 1 amu bins by rounding the *m/z* ratios off to the closest integer values. This reduced the amount of data by almost a factor 10 but, more importantly, avoided misclassification of *m/z* traces due to the fact that centroid sampling of the original mass spectra introduced a slight error that could lead to misalignment of *m/z* traces. In order to classify the individual *m/z* traces with respect to their "information content", the component detection algorithm (CODA) developed by Windig et al. was applied [25]. This algorithm compares the raw chromatograms with their smoothed (using a moving average) and standardized versions. The difference between the raw and smoothed chromatogram is small for high quality chromatograms (and the "CODA quality score" with a value ranging between 0 and 1, is high), while the opposite is true for chromatograms containing mainly background noise and/or spikes. By setting a threshold the user can define down to which level of quality *m/z* traces will be considered for the subsequent statistical analysis. In our study mass traces with a quality value higher than 0.98 were retained for further analysis meaning that only about 45 very high quality chromatograms were considered. The total ion current (TIC) was calculated from all mass traces or from the CODA selected high quality ones. The latter represented rather well the main characteristics of the original TIC, while background noise was essentially eliminated. Information of the peaks present in this rather conservative class of mass traces turned out to be sufficient for the detection of interpretable patterns in the data, for example separation of spiked from non-spiked samples.

2.6.2. Multivariate statistical analysis

To perform multivariate statistical data analysis of multiple LC–MS samples, information present in the union set of all selected high-quality mass traces was used. For each high-quality mass trace in the union set of mass traces, the peak with the highest intensity was obtained and entered in a peak list.

This peak list was further analyzed using principal component analysis. PCA is a widely used statistical technique that enables search for and visualization of patterns present in highly multivariate datasets [27]. In this study mainly biplots were used to analyse the available LC–MS peak lists. All statistical data analysis calculations were performed in the MATLAB programming environment (version 3.5.1, release 13).

2.7. Protein identification

1D nanoLC–ESI–MS–MS analysis was performed on an integrated nanoLC system (Agilent) comprising a binary gradient pump with a cooled autosampler, an auxilary pump for loading and washing the trap column, a column switching module configured for trap plus analytical capillary column, and a Q-Star XL API mass spectrometer (Applied Biosystems, MDS Sciex, Framingham, USA) fitted with nano-LC sprayer and operated under Analyst QS 1.1 control. Injected samples were first trapped and desalted isocratically on an LC-Packings PepMap C18 μ-Precolumn Cartridge (5 μm, 300 μm I.D. × 1 mm; Dionex, Sunnyvale, CA, USA) for 5 min with 0.1% formic acid delivered by the auxillary pump at 10 μl/min after which the peptides were eluted from the trap column and separated on an analytical C18 capillary column (5 cm × 75 μm, Atlantis) connected in-line to the mass spectrometer, at 250 nl/min using a 90 min gradient of 5–50% acetonitrile in 0.1% formic acid.

The QStar XL mass spectrometer was operated in information-dependent acquisition (IDA) mode. In MS mode, ions were screened from *m/z* 300 to 1500, and MS–MS spectra were acquired from *m/z* 50 to 2000 (pulsing mode on). In standard acquisition mode, each acquisition cycle was comprised of a 1s MS and a 1s MS–MS scan. In IDA mode the four most intense peaks were selected and MS–MS spectra acquired when their intensities exceeded 30 counts, In product ion mode, MS–MS spectra were acquired for selected precursor ions (*m/z* 619.2, 694.4, 753.5, 682.5 and 909.6) without threshold restriction. Acquired MS–MS spectra were searched against the SwissProt/Trembl database with a mass tolerance of 1.1 Da for the precursor and 0.15 Da for the obtained fragment ions. A hit was considered significant when the score exceeded 2.0, which corresponds to a confidence interval of more than 99%.

3. Results

3.1. Preparation of human serum for LC–MS analysis

Depletion of high-abundance proteins is one way to enhance the capability of proteomic methods to detect subtle changes in the protein profile of human serum. Previously we reported on the efficacy of several depletion columns to remove albumin and γ-globulins [17]. Method 1 (Fig. 1), used here as a first approach, is partially based on a previously published protocol, which was extended and optimized in the current work. Method 1 relies on dye-ligand affinity chromatography to remove albumin and Protein A to remove IgG. The subsequent steps comprise trypsin digestion and strong cation exchange chromatography (SCX) to fractionate the sample prior to LC–MS analysis. This

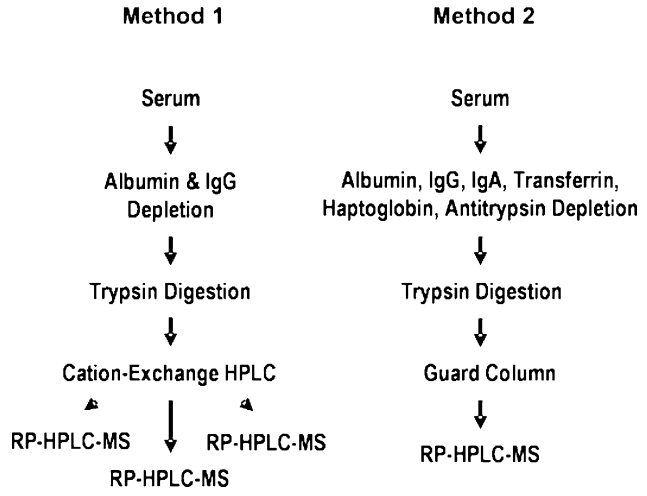

Fig. 1. Schematic description of two methods for sample preparation and analysis of human serum. The main differences between the methods are that Method 1 uses dye ligand affinity chromatography and Protein A for depletion of albumin and IgG (Aurum column, BioRad) while Method 2 employs a multiple affinity removal column (Agilent) based on a mixture of antibodies and Protein A. Method 1 includes a strong cation-exchange prefractionation step, while this step was omitted in Method 2.

method was repeated six-times in order to evaluate the overall repeatability. To this end 10 endogenous peptides from three cation-exchange fractions that covered the entire retention time range of eluting peptides in serum were selected and their respective extracted ion chromatograms integrated. While repeatability in terms of retention times was satisfactory (RSD < 0.8%) peak areas differed over a wide range (RSD between 12 and 160%) (see Table 1). We argued that the low repeatability with respect to peak area of some peptides was due to the cation-exchange prefractionation step, since fraction collection from a highly complex chromatogram of partially resolved peaks can easily lead to arbitrary cutting of component peaks and thus to major quantitative differences. This effect can be depicted in Fig. 2A showing six repetitions of the final LC–MS analysis using Method 1 clearly indicating two groups of three chromatograms. In order to overcome this limitation, we improved the efficiency of the depletion step by employing a multiple-affinity removal column (removes albumin, IgG, IgA, transferrin, haptoglobin, and α1-antitrypsin) and eliminating the cation-exchange chromatography step (Fig. 1, Method 2). Visual inspection of the chromatograms showed already that this approach was superior in terms of repeatability (Fig. 2B). An overview of the efficiency of depletion by both methods was obtained by SDS–PAGE showing that Method 2 (multiple depletion) was more efficient in depleting high-abundant serum proteins (Fig. 3).

This is in agreement with recently published data using 2D gel electrophoresis [28]. While Method 1 removed approximately 70% of total serum protein, Method 2 removed 90–95% according to determination of the total protein content after depletion. This allowed an increased loading capacity of the remaining digested proteins of 10–20-fold on the reversed-phase column.

Table 1
Repeatability of Method 1 (six repetitions) and Method 2 (five repetitions) (see Fig. 2) in terms of retention time and peak area for a selected number of endogenous peptides

m/z	Average RT (min)	%RT dev.	Average area	% Area dev.
Method 1. CEX fraction 1				
772.1	100.72	0.25	4.9×10^6	36.8
472.2	106.20	0.23	3.0×10^7	85.5
552.9	108.95	0.79	9.7×10^5	104.8
713.8	111.62	0.24	5.4×10^7	50.0
682.5	115.25	0.26	7.3×10^6	157.5
Method 1. CEX fraction 2				
619.2	99.75	0.05	2.1×10^7	24.0
694.4	103.95	0.08	4.1×10^7	11.9
753.5	111.62	0.10	9.9×10^7	113.2
909.6	114.37	0.12	2.5×10^6	94.1
Method 1. CEX fraction 3				
525.1	99.34	0.50	2.2×10^7	73.8
Method 2.				
756.7	109.40	0.06	4.5×10^7	13.1
753.5	110.78	0.04	3.3×10^7	13.7
909.6	113.54	0.08	3.8×10^6	27.5
682.5	114.20	0	8.3×10^7	14.1
619.2	99.90	0.05	4.4×10^7	20.1
694.4	104.20	0.04	2.4×10^7	9.1

Neither retention times nor peak areas were normalized. In bold: Peaks detected in both methods (*m/z* 619.2 and 694.4: doubly charged ions of peptides [DLATVYVDVDVLK and VSFLSALEEYTK, respectively] from apolipoprotein A (accession number (P02647, SwissProt/Trembl); *m/z* 682.5: triply charged ion of [LLLQQVSLPELPGEYSMK] and *m/z* 753.5: doubly charged ion of [AAQVTIQSSGTFSSK] from alpha-2-macroglobulin precursor (P01023); *m/z* 909.6: doubly charged ion of [SNLDEDIIAEENIVSR] from human complement C3 precursor (P01024).

3.2. Repeatability

As indicated in Fig. 2, Method 2 resulted in a better repeatability in terms of retention times (Method 1: <0.8% RSD; Method 2: <0.1% RSD). This may be due to the use of an in-line trap column for sample clean-up and focusing. It is noteworthy that retention time differences of five repetitive LC–MS runs ranged from 0 to 6 s in the case of Method 2 without any alignment of the chromatograms. Importantly, Method 2 proved also to be significantly more repeatable with respect to the observed peak areas for identical, selected endogenous peptide peaks (Method 1: 12–160% RSD; Method 2: 10–30% RSD). This was particularly for those peptides that showed large standard deviations with Method 1 (Table 1). This may be attributed to the significantly reduced number of sample preparation steps and in particular elimination of the fraction collection step by strong cation-exchange HPLC.

To estimate the repeatability of the developed methods on a more global scale, CODA was applied to all measured replicates. So instead of limiting ourselves to 6–10 univariate repeatability measures, all CODA-selected mass traces were used to compare the methods. Fig. 4 displays the calculated CODA quality scores for the different mass traces of the replicates prepared according to sample preparation Method 1 (top) or Method 2 (bottom). Since the image plots of the first, second and third SCX fraction gave similar results, only the quality values of the second fraction are visualized. While for Method 2 the quality values of the different replicates are higher and very similar over the whole *m/z* range, Method 1 shows far less repeatable quality values. Replicates 2, 3 and 6 (Method 1) differ significantly from replicates 1, 4 and 5 (see also Fig. 2). This result is confirmed after application of PCA to the peak lists generated from the high quality mass traces (CODA score > 0.98) selected by CODA. In the scores plot (not shown here) the replicates separate into two groups: a group containing replicates 2, 3 and 6 and a group with replicates 1, 4 and 5. Such analytical variability may interfere with detecting patterns of samples when analyzing patient sera or indicate false clusters.

3.3. Data analysis

In view of trying to discover differences in the abundance of peptides amongst samples from cancer patients at various stages of disease, it is pivotal that baseline variations are kept to a minimum due to methodological variability. Thus, Method 2 was chosen for further work. In an effort to evaluate the discriminatory capacity of the analytical and data analysis methodology, 210 pmol of horse heart cytochrome C were added to 20 μl of the original serum sample and analyzed with Method 2 as described above. LC–MS data was acquired for in total four replicate samples spiked with cytochrome C and five non-spiked replicates. Peak lists for all 9 LC–MS data files were obtained after application of the binning algorithm and usage of CODA. Fig. 5 summarizes the results of PCA of the union peak list. The biplot shows besides the scores of the nine samples (4 spiked samples = red diamonds, five normal samples = blue squares) also the loadings of the high quality mass traces (45 mass traces = green triangles) that were used for the statistical analysis. In Fig. 5A the spiked samples are clearly separated from the normal (non-spiked) samples. Especially *m/z* traces 483, 748 and 965 contribute significantly to the discrimination of the spiked from the normal samples (Fig. 5B). All of these *m/z* values are related to tryptic peptides of cytochrome C (the *m/z* values of 483 and 965 correspond to doubly and singly charged ions of the same peptide).

Since depletion of high abundance proteins, notably albumin has been reported to lead to co-depletion of other proteins, peptides and metabolites that are bound to albumin [21,22], we investigated the recovery of the added cytochrome C from 20 μl serum of a cervical cancer patient (same patient serum as used before) after depletion. For comparison, an equal amount of a tryptic digest of cytochrome C was added to the depleted and digested sample just prior to LC–MS analysis. Both analyses were repeated nine-times and the average peak areas for five selected *m/z* traces from peptides related to horse heart cytochrome C were compared. A comparison of peak areas of cytochrome C added to the original serum or added just prior to LC–MS analysis, revealed that 19–27% of spiked cytochrome C were recovered following multiple affinity removal of high abundance proteins (Table 2). Recoveries on the same order were also found when 10–63 pmol of cytochrome C were spiked into 20 μl serum. The repeatability of the peak areas of spiked cytochrome

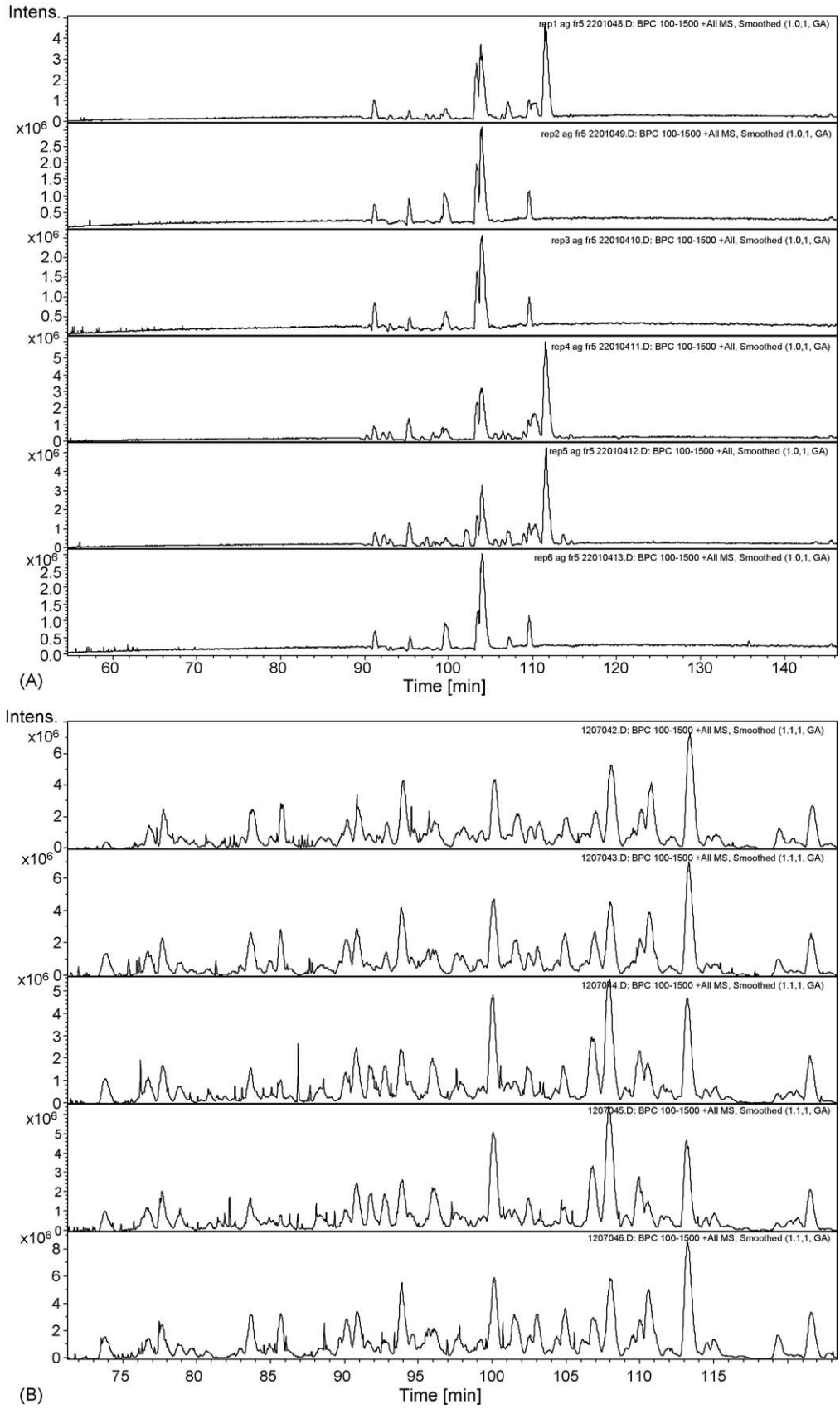

Fig. 2. LC–MS analyses of trypsin-digested serum samples prepared by Method 1 (A) or Method 2 (B). Six repetitions of fraction 2 of the strong cation-exchange HPLC pre-fractionation step (Method 1) are shown in comparison with five repetitions of Method 2.

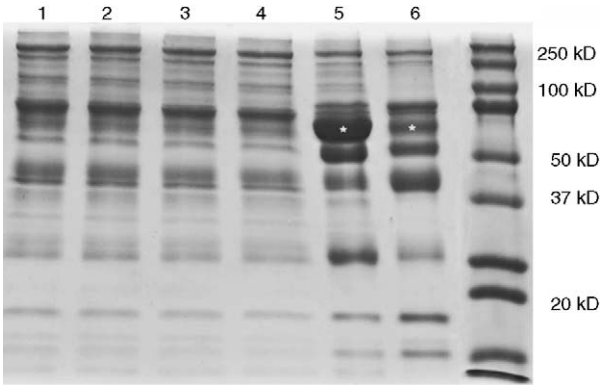

Fig. 3. SDS–PAGE analysis of human serum prior to (lane 5) and after depletion using the multiple affinity removal column (lanes 1–4) or dye ligand/Protein A affinity chromatography (lane 6). Albumin is labelled with an asterisk.

Table 2
Recovery of horse heart cytochrome C (Method 2) added to human serum prior to depletion (210 pmol in 20 μl serum) of high abundance proteins using the multiple affinity removal column (Agilent; depletes albumin, IgG, IgA, transferrin, haptoglobin, and α_1-antitrypsin) based on extracted ion chromatograms (results based on 9 independent experiments with and without addition of cytochrome C)

m/z	Peak area	Recovery %	RSD % ($n = 9$)
302.7	1233449	19.1	6.1
604.4	12538882	27.3	8.6
482.8	27618370	26.0	10.8
748.4	39544944	26.4	7.0
1495.7	504101	20.3	7.5
		Average recovery 23.8	Average RSD 8.0

C was between 12 and 26% RSD (not shown) and thus within the same range as relative standard deviations for other endogenous peptide peaks (see Table 1). Repeatability of the initial depletion step itself was assessed to be better than 5% RSD in terms of

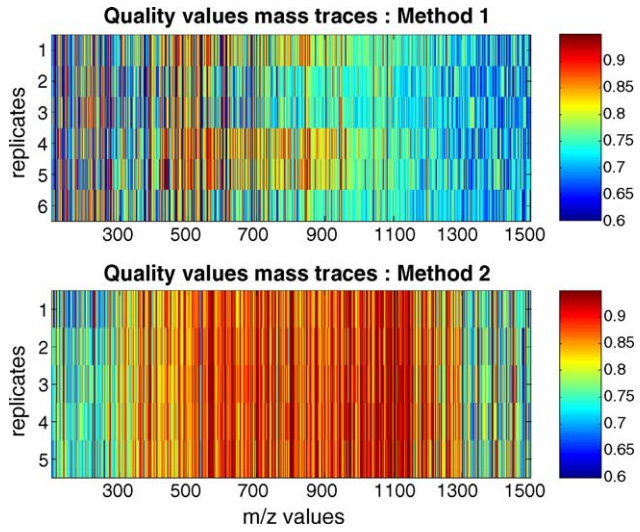

Fig. 4. Image plots showing the colour-coded CODA quality scores of the different mass traces (*m/z* 100–1500). High quality values are red (in web version) and low quality values are blue (in web version). The image plot at the top displays quality values for the 6 replicates (the second fraction is displayed) using Method 1 (see Fig. 2A). The image plot at the bottom shows the quality values of 5 replicates measured using sample preparation Method 2 (see Fig. 2B).

peak area of the flow-through fraction of the multiple affinity removal column.

To investigate whether loss of cytochrome C was due to direct binding to the affinity column or mediated through binding to high abundance proteins, similar amounts of cytochrome C were dissolved in Buffer A and applied to the multiple affinity removal column. Practically all cytochrome C was recovered showing clearly that binding to high abundance proteins, most likely albumin, was responsible for the loss. Therefore, the actual amounts of cytochrome C applied to LC–MS analysis in the comparative spiking studies shown in Fig. 5 were about five times lower

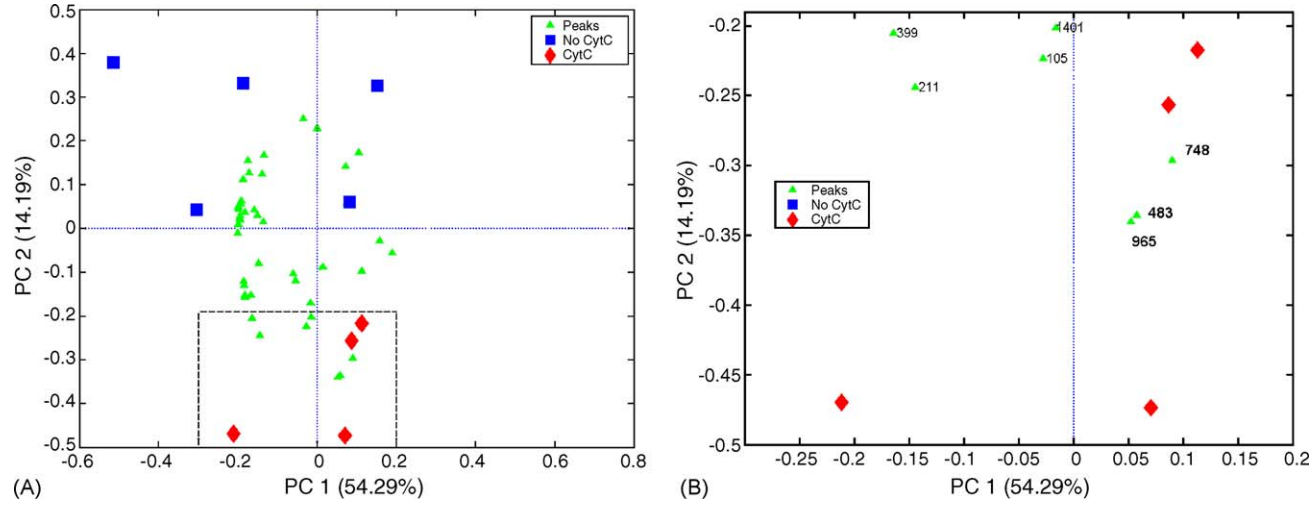

Fig. 5. (A) Principal component analysis (PCA) biplot of trypsin digested, depleted human serum samples with 210 pmol spiked horse heart cytochrome C in 20 μL serum (red diamonds (in web version)) and without cytochrome C (blue squares (in web version)) after selection of high quality mass traces with CODA using a threshold of 0.98. Note that the recovery of cytochrome after depletion is only 20–25% (see Table 2). The used high quality mass traces are indicated by the green triangles (in web version). (B) Enlarged part of the boxed area in (A) showing that peaks at *m/z* 483, 965 and 748 contribute strongly to the discrimination between spiked and non-spiked. All of these peaks are derived from cytochrome C.

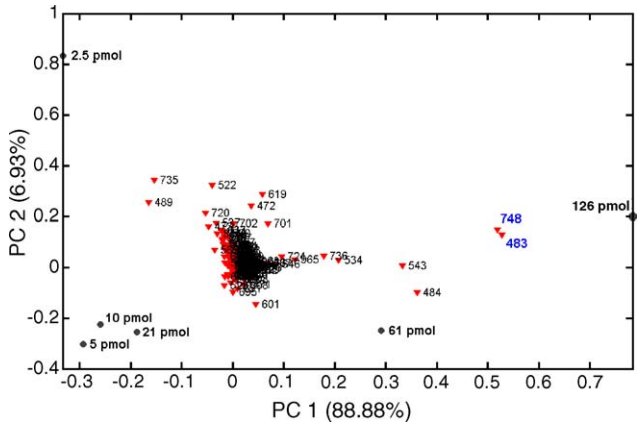

Fig. 6. Principal component analysis (PCA) biplot of human serum samples spiked with increasing amounts of horse heart cytochrome C (25, 50, 100, 210, 610 and 1260 pmol in 20 μL original serum, recalculated for an injected amount corresponding to 10% of the original 20 (l). Peaks (*m/z* values) used for PCA (selected by CODA) are shown as red triangles (in web version). Peaks with *m/z* 483 and 749 contribute strongly to the observed trend in PC1. Both peaks are derived from cytochrome C.

than the amount originally added to the serum sample. These results confirmed the observation that high-abundance proteins may act as "molecular sponges", which bind and transport low molecular weight proteins or peptides an effect that needs to be corrected for when using spiked internal standards.

In an effort to evaluate whether Method 2 in combination with CODA and PCA could detect a trend in concentration of a protein in serum, different amounts of horse heart cytochrome C (25 pmol–1.26 nmol) were added to 20 μl of the original serum sample before multiple depletion and 10% of the tryptic digest was subjected to LC–MS as described above. In Fig. 6 multivariate statistical data analysis (PCA) results are displayed of LC–MS datasets treated with CODA to generate peak lists based on high quality mass traces (CODA score > 0.98). These results show that principal component 1 (PC1), which describes about 89% of the variability in the data, follows the concentration trend generated by the added cytochrome C down to a level of 5 pmol in the equivalent of 2 μL serum (recalculated for an injection of 10% of the original sample). Variability reflected in PC2 (approximately 7%) is not correlated with the concentration of cytochrome C. The serum sample containing 2.5 pmol follows the trend described by PC1 but is separated from the other samples mainly in PC2 most likely due to variability introduced by the analytical procedure. PCA analysis showed again that traces at *m/z* 483 and 748, corresponding to peptides derived from cytochrome C, contributed most significantly to the observed trend (see Fig. 6). Method 2 combined with *m/z* trace selection by CODA and PCA thus correctly identified 2 *m/z* values that correlated with the observed trend in PC1 and the introduced concentrations of cytochrome C added to the serum. In order to evaluate whether there is a good correlation between the detected *m/z* traces and the amount of added cytochrome C, a linear correlation analysis was performed showing that the peak areas of the extracted ion chromatograms at 483 and 748 amu correlated well with the added amounts over the studied concentration range ($R^2 = 0.97$–0.99).

4. Discussion

A procedure for the depletion of high abundance proteins from human serum for subsequent analysis by shotgun proteomics using LC–MS has been described. The overall procedure showed a repeatability of 10–30% for peak area and better than 0.1% for retention times without the use of internal standards or alignment of the chromatograms. Data were pre-processed using CODA at a quality score threshold of 0.98 that selects about 45 of the most "information rich" *m/z* traces. This allowed to reconstruct a TIC highly similar to the original raw data, however, with drastically reduced background noise. PCA of pre-processed LC–MS datasets obtained after spiking different amounts of horse heart cytochrome C into the original serum (range 25 pmol–1.26 nmol in 20 μl serum) allowed to reveal the trend in cytochrome C concentrations in Principal Component 1, which described 89% of the variability in the data. Determination of recoveries for spiked cytochrome C after depletion using a multiple affinity removal column depleting albumin, IgG, IgA, transferrin, haptoglobin, and α_1-antitrypsin showed that only 20–25% of the added protein were recovered and that this loss was due to the presence of high abundance proteins and not to direct binding to the affinity column. The effect of protein co-depletion needs therefore to be taken into account when adding internal standards to serum and likely also to other complex biological samples. Our data indicate that internal standardization using added marker peptides is a viable alternative to stable isotope labeling for quantitative, comparative proteomics but that care needs to be taken to account for limited recoveries. Operating according to a strictly standardized procedure is mandatory. Other groups have also shown that reliable quantitative results in shotgun proteomics of complex peptide mixtures can be obtained based on reproducible peak areas [29–30]. However, repeatability may still be improved using the so-called "Global Internal Standard" strategy based on stable isotope labeled samples [31], an approach that is under investigation.

While our approach shows the feasibility of combining an LC–MS based method including depletion of high-abundance proteins and trypsin digestion with data pre-processing and multivariate statistics to discover trends in concentrations of proteins in complex mixtures like serum, the concentration sensitivity is not sufficient to reach into the range of known tumor markers (ng/ml range). Improvements will therefore have to be made in sample preparation with the goal of being able to treat a larger volume of serum. Our analysis eventually used only 2 μl of the original serum sample, while it is possible to obtain 1ml without difficulty. Due to the high remaining protein content even after depletion (approximately 4–5 mg/ml), it is pivotal to start with a "preparative" separation method and to analyze the prefractionated sample. We are presently investigating prefractionation strategies at the protein level prior to tryptic digestion to reach a better concentration sensitivity. The described method is currently being applied to comparative cross-sectional and longitudinal studies with samples from healthy subjects and cervical cancer patients at different stages of disease to evaluate its suitability to classify different groups of patients.

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