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ORIGINAL ARTICLE

High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins

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Abstract In clinical and pharmaceutical proteomics, serum and plasma are frequently used for detection of early diagnostic biomarkers for therapeutic targets. Although obtaining these body fluid samples is non-invasive and easy, they contain some abundant proteins that mask other protein components present at low concentrations. The challenge in identifying serum biomarkers is to remove the abundant proteins, uncovering and enriching at the same time the low-abundance ones. The depletion strategies, however, could lead to the concomitant removal of some non-targeted proteins that may be of potential interest. In this study, we compared three different methods aimed to deplete high-abundance proteins from human serum, focusing on the identification of non-specifically bound proteins which might be eventually removed. A Cibacron blue-dye-based method for albumin removal, an albumin and IgG immunodepletion method and an immunoaffinity column (Multiple Affinity Removal System) that simultaneously removes a total of six high-abundance proteins, were investigated. The bound proteins were eluted, separated by two-dimensional gel electrophoresis and identified by Nano LC-CHIP-MS system. Flow-through fractions and bound fractions were also analysed with the ProteinChip technology SELDI-TOF-MS. Our results showed that the methods tested removed not only the targeted proteins with high efficiency, but also some non-targeted proteins. We found that the Multiple Affinity Removal Column improved the intensity of low-abundance proteins, displayed new protein spots and increased resolution. Notably, the column showed the lowest removal of untargeted proteins, proved to be the most promising depletion approach and a reliable method for serum preparation prior to proteomic studies.

Keywords High-abundance proteins · Human serum · Nano LC-CHIP-MS · Non-targeted proteins · SELDI-TOF-MS · Two-dimensional gel electrophoresis

Introduction

Highly sensitive biomarkers are very important in detecting the early onset, progression and prognosis of human diseases. Biomarker discovery and their validation represent the essential parts of the proteomic studies (Penque 2009). The major goal of plasma and serum clinical proteomics is to obtain accurate information for diagnostic and therapeutic purposes (Palmblad et al. 2009). This requires the establishment of comprehensive baseline data for the serum proteome, including as many of the low-abundance proteins as possible, since protein biomarkers are usually present at low concentrations (Zhou et al. 2005).

Serum is the richest and the most complete informative proteome from a medical point of view, since almost all cells communicate with blood and many of them release part of their contents into the bloodstream upon damage or death (Anderson et al. 2004). Although simple in principle,

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the ability to analyse the serum proteome is extremely difficult in practice (Veenstra et al. 2005). Serum contains 60–80 mg of protein/ml, in addition to various molecules, including salts, lipids, electrolytes and small metabolites, such as peptides and aminoacids (Chan et al. 2004). Of these proteins, albumin comprises ~50–55% of the serum protein content (Anderson and Anderson 2002). Along with albumin, only other few proteins (immunoglobulins, transferrin, haptoglobin and lipoproteins) constitute 90% of the total protein content of serum. Almost all the remaining 10% is made up of 12 proteins. Indeed, only 1% of the entire protein content of serum consists of proteins that are considered to be of low-abundance and of great interest in the search of potential biomarkers (Tirumalai et al. 2003).

This complexity and huge dynamic range in protein analyte concentration and protein properties, including mass, isoelectric point, extent of hydrophobicity and post-translational modifications, make the serum the most difficult sample to investigate for proteomics studies (Hortin et al. 2008). The sample complexity can be reduced by depletion of the high-abundance proteins; this represents an essential first step in the analysis of the serum proteome.

In the past, the fractions containing the most abundant proteins were assumed to be diagnostically unimportant and usually not analysed. In the recent proteomic analysis, many different approaches have been developed to remove abundant proteins from human serum. The classical depletion strategy involves the use of a hydrophobic dye Cibacron blue, a chlorotriazine dye which has a high affinity for albumin. This strategy of removing albumin is still used in serum proteomic analysis because of its relatively low cost (Ahmed et al. 2003; Shaw and Riedere 2003). Some authors have used monoclonal antibodies against albumin to develop an immunoaffinity resin that is effective in the removal of both full-length albumin and many of the albumin fragments present in serum (Steel et al. 2003). More recently, an affinity chromatography method to remove albumin has been developed, consisting of anti-albumin antibody (anti-HSA), which helps to isolate albumin-bound proteins and to investigate the so-called "albuminome" (Gundry et al. 2007).

Immunoglobulins G (IgG) represent the second most abundant proteins in the plasma or serum; therefore some methods have been developed to remove not only albumin, but also this class of proteins (Govorukhina et al. 2003; Greenough et al. 2004; Fountoulakis et al. 2004; Fu et al. 2005); fractionation approaches employing liquid chromatography-mass spectrometry have been used as well (Martosella et al. 2005; Govorukhina et al. 2006). Currently, depletion kits for the simultaneous removal of albumin and IgG are commercially available. Since the removal of these two proteins has been shown to improve the detection of some low-abundance proteins, analytical efficiency was expected to be improved even further by increasing the number of depleted proteins. The recent trend in proteomic analysis goes towards the use of immunoaffinity media, which are made up of matrices with covalently attached different antibodies against the most abundant proteins (Yocum et al. 2005; Liu et al. 2006; Desrosiers et al. 2007). Depletion of 6 and 12 proteins is expected to remove about 85 and 90%, respectively, of the total protein content (Echan et al. 2005). Some comparative studies concerning the various depletion approaches have been recently performed (Chromy et al. 2004; Björhall et al. 2005; Zolotarjova et al. 2005).

Unfortunately, the probability of removing some low-abundance proteins along with the abundant species is a real problem associated with any protein separation method (Adkins et al. 2002). Albumin is the most abundant protein present in serum, but at the same time, it is a transport protein, binding various compounds including hormones, lipids and aminoacids. For this reason, removal of albumin from serum may also result in the specific loss of some low-abundance peptides or small proteins of interest, such as cytokines (Granger et al. 2005). Another drawback to keep in mind, when using depletion systems, is the possibility to simultaneously remove some untargeted proteins, due to the depletion method used.

In this article, we evaluated and compared three different strategies which are able to remove the high-abundance proteins from human serum: the Aurum™ Affi-Gel Blue kit (Bio-Rad), the ProteoPrep® immunoaffinity albumin and IgG depletion kit (Sigma), and the Multiple Affinity Removal System (MARS column, Agilent Technologies).

The Bio-Rad kit consists of spin columns filled with Affi-Gel Blue affinity support, a beaded, crosslinked agarose gel with covalently attached Cibacron Blue dye, which allows the removal of albumin from serum or plasma. The Sigma kit is designed to specifically remove albumin and IgG from human serum. The prepacked spin columns contain the ProteoPrep immunoaffinity medium, a mixture of two beaded mediums comprising recombinantly expressed, small single-chain antibody ligands. Finally, the MARS column is an affinity column packed with immobilized affinity-purified polyclonal antibodies for simultaneous and specific removal of six targeted proteins: albumin, transferrin, IgG, IgA, haptoglobin and antitrypsin.

The main focus of this study was to estimate the specificity of these depletion methods, by the analysis of bound fractions and the identification of non-specifically bound proteins removed along with the specific ones. Two different proteomic techniques were employed in this work: two-dimensional gel electrophoresis (2-DE) coupled to Nano Liquid Chromatography-CHIP-Mass Spectrometry (Nano LC-CHIP-MS) system, and the ProteinChip

technology Surface Enhanced Laser Desorption Inization-Time of Flight-MS (SELDI-TOF-MS).

Materials and methods

Materials

Immobilized pH-gradient (IPG) strips, acrylamide/bis solution 29:1, dithiothreitol (DTT), iodoacetamide, ampholytes pH 3–10, agarose, Tris/Glycine/SDS buffer 10× and H-50 ProteinChip array were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Silver-nitrate and other reagents for gel staining, urea, thiourea, CHAPS, Tris, TEMED, ammonium persulfate, ammonium bicarbonate, glycerol, potassium hexacyano-ferrate(III), sodium thiosulphate and Protease Inhibitor Cocktail were bought from Sigma–Aldrich (Milan, Italy). Acetonitrile (ACN), ethanol, acetic acid glacial ACS grade were obtained from Carlo Erba (Milan, Italy). Formaldehyde, trifluoroacetic, formic and sinapinic acids were obtained from Fluka (Buchs, Switzerland). Trypsin mass spectrometry grade was from Promega (Madison, WI, USA). Protein concentration was determined utilizing the classical Bradford method (Bradford 1976), using the Protein Assay Dye Reagent purchased from Bio-Rad.

Serum samples

Venous blood was collected from healthy donors into vacutainer serum separation tubes, and allowed to clot at room temperature for 1 h. Serum was separated by centrifugation at 2,000×*g* for 10 min at 4°C. After the addition of a protease inhibitor cocktail (Sigma–Aldrich) to prevent protein enzymatic breakdown or modifications, samples were divided into aliquots and kept frozen at −80°C until use.

Depletion of high-abundance serum proteins

Three different methods of depletion were used in this study, following the manufacturer's protocols.

Using the Bio-Rad kit, 125 µl of serum was diluted threefold in 20 mM Tris–HCl, pH 8.3, and then allowed to flow by gravity through the columns. After centrifugation, columns were washed with 400 µl of the same buffer, and flow-through and wash were pooled, constituting the low-abundance protein fraction. Bound proteins were eluted using the ReadyPrep Sequential Extraction Reagent 3 (Bio-Rad).

The Sigma affinity columns were first equilibrated with the Equilibration Buffer, then 100 µl of two-times diluted serum were applied to the columns and incubated at room temperature for 10 min. After centrifugation, flow-through fractions were collected. Finally, high-abundance proteins were eluted from the spin columns by adding the Protein Extract Reagent Type 4 (Sigma).

Using the MARS column (4.6 mm ID × 100 mm), 200 µl of serum were diluted four times in the manufacturer's Buffer A and filtered through a 0.22 µm spin filters, for removal of particulates. The liquid chromatography separations were conducted on an automated HPLC Beckman System Gold (Beckman Coulter, Fullerton, CA, USA). Briefly, 200 µl of diluted sample was injected onto the MARS column in 100% Buffer A, at a flow rate of 0.5 ml/min for 10 min. After collection of the flow-through fraction, the column was washed and the bound proteins were eluted with 100% Buffer B (a low pH urea buffer) at a flow rate of 1.0 ml/min for 7 min. Afterwards, the column was regenerated by equilibration in 100% Buffer A for 11 min, for a total run cycle of 28 min. The flow-through fractions (containing unbound proteins) and the bound fractions from three sequential injections were collected, pooled and buffer-exchanged into 20 mM Tris–HCl, pH 7.4, using spin concentrators with 5 kDa MW cutoff (Agilent Technologies). The samples were subjected to three rounds of buffer addition, with centrifugation at 7,500×*g* for 20 min at 10°C every time. For each method tested, the low-abundance fractions and the high-abundance proteins eluted were either analysed immediately or aliquoted and stored at −80°C until use.

Two-dimensional gel electrophoresis and proteins visualization

Samples were separated in the first dimension by isoelectric focusing (IEF) in a PROTEAN IEF® cell (Bio-Rad). Samples (100 µg of total proteins), were diluted to 300 µl with rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 25 mM DTT, 0.2% ampholytes) and then loaded onto 17 cm IPG strips, non-linear, pH range 3–10 (Ready Strip™, Bio-Rad). The IPG strips were actively rehydrated at 50 V for 12 h at 20°C. The rehydration step was followed by IEF at 250 V for 15 min, ramping up to 10,000 V for 3 h, and finally focusing to reach 75,000 V-hours.

All the strips were reduced and alkylated for 15 min, with 1% DTT, and then with 2.5% iodoacetamide in equilibration buffer (6 M urea, 50 mM Tris–HCl pH 8.8, 30% glycerol, 2% SDS and a trace of bromophenol blue). Equilibrated IPG strips were embedded into 0.5% agarose on top of the 8–16% polyacrylamide gradient gels (20 × 20 cm size), and run in a PROTEAN® II xi Cell Vertical System (Bio-Rad) connected to a refrigerated bath circulator set at 10°C (Cryostatic bath, Instruments S.r.l., Milan, Italy). Lower and upper running buffers contained 25 mM Tris–HCl, 192 mM glycine and 0.1% v/v SDS,

pH 8.3. Electrophoresis was initially performed at 80 mA/gel for 30 min and then increased at maximum of 500 V and stopped when the bromophenol blue dye front reached the bottom of the gel. After 2-DE, the protein spots in the gels were visualized following a silver nitrate staining protocol, as previously described (Bellei et al. 2008). Then, the gel images were acquired using a GS-800 Calibrated Densitometer (Bio-Rad) and spots of interest were immediately cut and stored at −20°C until analysis. The 2-DE separations were performed in triplicate.

"In-gel" protein spot digestion

Protein spots excised manually from the gels were subjected to a tryptic digestion "in-gel". First, each sample was de-stained using a destain solution (1:1 v/v, 30 mM potassium hexacyano-ferrate(III)/100 mM sodium thiosulphate) and then washed with ultrapure deionized water and ACN before the reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide. After drying, samples were rehydrated in 20 µl of 25 mM ammonium bicarbonate, containing 120 ng of sequencing grade trypsin (Promega, Madison, WI, USA), and then incubated overnight at 37°C. After digestion, the peptides were extracted using 1% trifluoroacetic acid in 50% ACN, and finally concentrated in a vacuum dryer (Savant Speed-Vac concentrator).

Protein identification by Nano LC-CHIP-MS analysis

Dried samples were resuspended in 5% formic acid to reach a final concentration of 300 femtomoles/µl, and analysed by a Nano LC-CHIP-MS system, consisting of the Agilent 6520 Accurate-Mass Quadrupole-Time-of-Flight Liquid Chromatography-Mass Spectrometry (Q-TOF LC-MS), coupled with a 1200 Nano HPLC-Chip microfluidic device (Agilent Technologies Inc., Santa Clara, CA, USA). Four microlitres of samples was loaded from the autosampler to the Chip enrichment column (Zorbax C18, 4 mm × 5 µm i.d., Agilent Technologies, Palo Alto, CA, USA) by a capillary pump, with a loading flow of 4 µl/min using a mobile phase composed of 0.1% formic acid/2% methanol/97.9% water. Nitrogen was used as the nebulizing gas. A separation column (Zorbax C18, 43 mm × 75 µm i.d., Agilent Technologies, Palo Alto, CA, USA) was used for peptide separation, setting the analytical flow rate at 0.4 µl/min. Elution was obtained with increasing concentration of 0.1% formic acid/2% water/97.9% methanol (buffer B): 3% for the first 30 s, 40% for 17 min, 60% for additional 3 min, and finally to 80% for other 3 min. One min after, the percentage of buffer B was decreased to 3% for 4 min and maintained constant for 10 min, allowing column re-equilibration. Total run time was nearly 37 min.

The MASCOT search engine (http://mascot.cigs.unimo.it/mascot) was used for peptide sequence searching (MS/MS ion search). Swiss-Prot and MSDB protein databases were selected, with the application of some restrictions: species Homo sapiens (Human), two missed trypsin cleavages, peptide tolerance ±20 ppm and a MS/MS tolerance error of ±0.1 Da. Moreover, the databases were searched using the assumption that peptides were carbamidomethylated at cysteine residues (as the fixed modification). The highest score hits among MASCOT search results were selected. Protein identification was repeated at least once, using spots cut from replicated gels.

SELDI-TOF protein profiles

Surface Enhanced Laser Desorption Inization-Time of Flight mass spectrometry Series 4000 (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to analyse serum samples. To improve reproducibility, all steps were automated using the robotic system Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). Furthermore, a pooled serum sample was used as the quality control (QC), to verify the suitable performance of the instrument. Samples (30 µg of total proteins) were mixed with ProteinChip H50 binding buffer (Bio-Rad) to achieve a final volume of 100 µl and then were added to the pre-equilibrated H50 reverse-phase ProteinChip array (Bio-Rad). After 45 min of incubation at room temperature with gentle horizontal shaking, the unbound proteins were removed by washing three times with the same binding buffer. Afterwards, the chip surfaces were quickly rinsed with ultrapure deionized water and air-dried for 15 min. Finally, 1 µl of saturated sinapinic acid solution (matrix), prepared in 50% ACN and 0.5% trifluoroacetic acid, was applied twice to each spot and allowed to air-dry. Each sample was loaded in duplicate, randomly, in order to minimize any systematic error. The mass accuracy was calibrated externally using the "Protein Chip All-in-One Protein Standard II" (Bio-Rad).

SELDI-TOF statistical analysis

Spectra analysis was carried out using the ProteinChip Data Manager software version 3.0 (Bio-Rad Laboratories, Inc. Hercules, CA, USA). We analysed the low and high mass/charge ratio (m/z) spectra separately to optimize their reading. The protein chip arrays were assayed with a laser intensity of 2,500 nJ for low m/z measurements, and 6,000 nJ for high m/z readings. A total of 901 laser shots were collected and averaged for each spot. The spectra were baseline subtracted, normalized for total ion current (TIC) in the range of interest (5,000–30,000 Da for low m/z and 30,000–150,000 Da for high m/z, respectively) and finally

mass aligned. Supervised clustering was performed using the following settings: five times signal-to-noise (S/N) ratio and 20% min peak threshold on the first pass for peak identification, and two times S/N ratio on the second pass, for cluster completion. Statistical analysis was carried out using the unpaired Student's t test and P value of <0.05 was considered statistically significant.

Results

Serum analysis by two-dimensional gel electrophoresis and protein identification

In this study, we compared three different affinity techniques to remove the most abundant serum proteins and to enrich proteins of lower-abundance. Figure 1 shows the representative images of the flow-through fractions collected from the Bio-Rad kit (b) and from the Sigma kit (d), in comparison with a crude serum sample (a). Proteins removed by the two different kits are indicated with square (albumin) and ellipses (IgG). As shown in (b) and (d), the removal of the high-abundance proteins was incomplete.

The specificity of the affinity depletion methods was evaluated by 2-DE analysis of bound fractions and by the subsequent identification of bound proteins with LC-CHIP-MS. The results showed that the Affi-Gel dye-based affinity support (Bio-Rad), which should include only albumin, actually contained the largest number of non-specifically bound proteins (39 spots, corresponding to 24 unique proteins), as shown in Fig. 1c. All the identified non-specifically bound proteins are listed in Table 1, where we also reported their experimental MW and pI values and data from MS analysis, i.e. the Swiss-Prot primary protein accession number and entry name, the highest score, the number of peptides matched and the sequence coverage, which is the percentage of amino acids sequenced for the detected protein. The protein numbers in the table correspond to the spot numbers assigned in Fig. 1c. Some small protein spots evident in the albumin area were identified as albumin fragments (as shown in Online Resource 1). The 2-DE gel of the bound fraction from the Sigma immunoaffinity albumin and IgG removal kit (Fig. 1e) showed the additional removal of five non-targeted spots (corresponding to four unique proteins), namely complement C3 fragment, complement C4-A, apolipoprotein A-I and alpha-1-antitrypsin, which are reported in Table 2. We also identified some minor spots, corresponding to the specific proteins, namely albumin and IgG chains (as reported in Online Resource 1).

In Fig. 2a is shown a typical immunoaffinity chromatogram, with the peaks relative to fractions collected from the MARS column. In Fig. 2b is shown a crude serum sample, with the six most abundant proteins removed by the column enclosed in ellipses (albumin, transferrin, IgG, IgA, alpha-1-antitrypsin, haptoglobin). As is evident in Fig. 2c, which represent the gel image of the flow-through fractions, the six targeted proteins were removed with high efficiency, since they were absent in the picture. All the major protein spots identified in bound fractions from the MARS antibody column were the six specific proteins (Fig. 2d). Most of the moderate or very faint silver-stained spots were identified as the proteolityc fragments or minor forms of these six proteins (as shown in Online Resource 1). Only three non-specifically bound proteins were detected: complement C3 fragment, apolipoprotein A-I and transthyretin. However, these proteins were not completely removed, since they were also evident in the flow-through fraction. These proteins are shown in Fig. 2d and are listed in Table 3. Moreover, flow-through fractions collected from the MARS column (corresponding to low-abundance proteins), were further analysed by 2-DE using narrow range IPG strips (pH between 4 and 7) in the first dimension, which improved spots resolution compared with a broad pH 3–10 range (additional data are given in Online Resource 2).

SELDI-TOF-MS analysis

We further evaluated the depletion efficiency and the binding specificity of the selected depletion methods by SELDI-TOF-MS analysis. Initially, we considered the two most abundant serum proteins, albumin and IgG. The albumin peak (m/z 66.577) obtained from the bound fraction of each depletion method is shown in Fig. 3b–d, in comparison with albumin peak present in the undepleted serum sample (Fig. 3a). The relative intensity of the albumin peak was higher with the MARS column (b) and the Sigma kit (c), with a mean intensity of 80.71 and 53.60, respectively, compared with the Cibacron Blue-dye kit (d), with a mean intensity of 15.07. The same investigation was conducted for the IgG chains. The IgG peak (m/z 133.860) is shown in Fig. 3 for each depletion methods tested (f, g, h), in comparison with IgG peak visualized in the untreated serum sample (e). Similarly, the antibody column (f) and the Sigma albumin/IgG depletion kit (g) removed the IgG chains with high efficiency (99 and 66%, respectively). The Cibacron Blue-dye kit, which is designed to specifically remove only albumin from human serum, showed a very low IgG peak, with a relative intensity of 0.35 (H).

The SELDI-TOF-MS analysis of the bound fractions in the low m/z range (5,000–30,000 Da) confirmed the results previously displayed by the 2-DE gels. Actually, the spectra obtained from the bound fraction analysis using the Cibacron Blue-dye kit (Fig. 4b) clearly reveals the presence of some non-targeted low-molecular weight proteins, as well

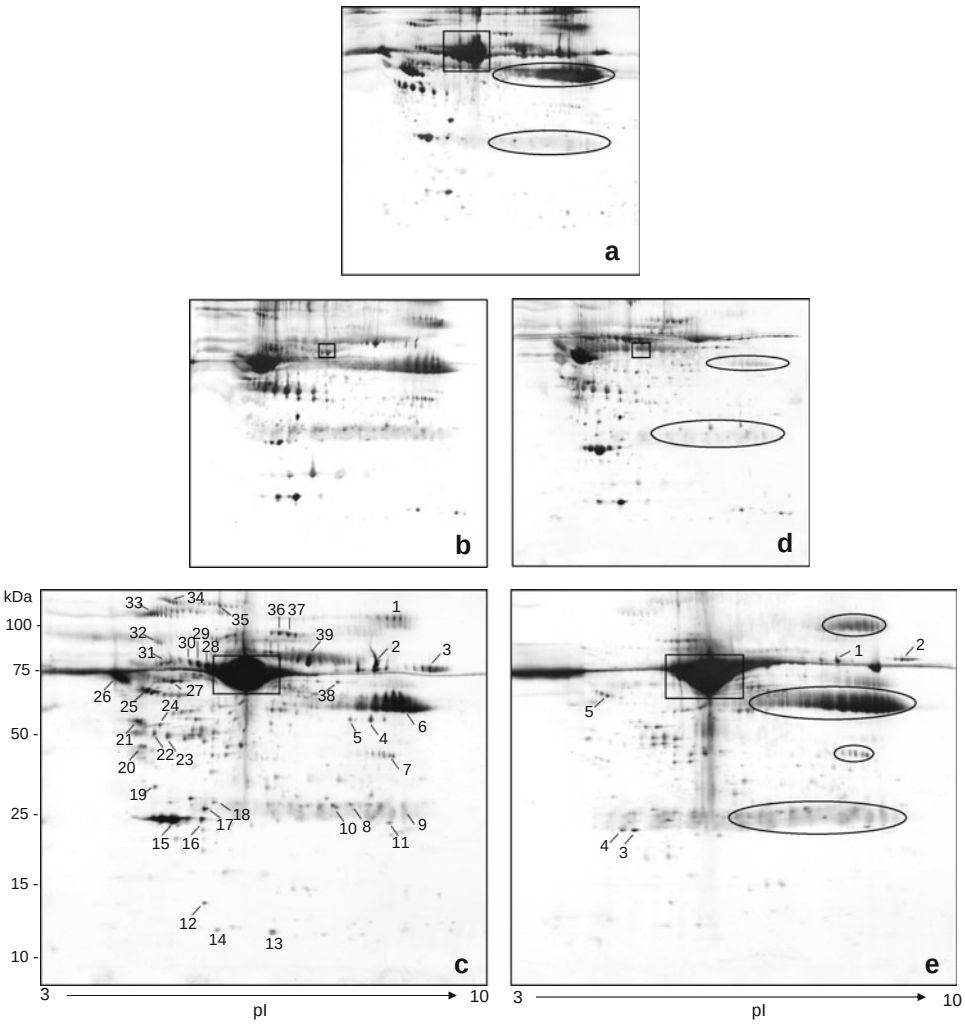

Fig. 1 Comparison of a crude serum protein profile (**a**) with those obtained from the unbound (**b**) and bound fractions (**c**) collected after the use of the Bio-Rad Affi-Gel kit, and unbound (**d**) and bound fractions (**e**) obtained with the Sigma immunoaffinity kit. High-abundance proteins removed by the kits are indicated with *squares* (albumin) and *ellipses* (IgG). In (**b**) and (**d**), the square and elliptic regions show incomplete removal of the abundant proteins. In (**c**) and (**e**) are displayed the non-targeted proteins removed by the two kits, along with the specific ones. 2-DE analysis was performed as described under "Materials and methods". Proteins were "in-gel" trypsin digested and identified using a Nano LC-CHIP-MS system. Protein spot numbers in (**c**) and (**e**) reflect the numbers reported in Table 1 and in Table 2, respectively

evident in comparison with crude sample shown in Fig. 4a. In contrast, these peaks were absent in the spectra obtained from the Sigma kit and the MARS column (data not shown).

Furthermore, we analysed by SELDI-TOF the flow-through fractions collected from the MARS column, both in the low and high m/z range. The visual inspection of the spectra accurately displayed the column efficiency, showing the detection of higher and new protein peaks, in both m/z range. These results were in accordance with those obtained by 2-DE and MS analysis (additional data are given in Online Resource 2).

Finally, we estimated the number of peaks detected in the unbound fractions after removal of high-abundance proteins, for each method used. The fold-increase of SELDI-TOF peaks number, in comparison with undepleted serum sample, was also calculated (Table 4). After removal of a single protein (albumin) with the Bio-Rad kit, protein peaks in the unbound fraction were 39.7 ± 2.1; after immunocapture of albumin and IgG (Sigma kit), and the top six high-abundance proteins (MARS column), protein peaks were, respectively, 44.3 ± 1.5 and 59.0 ± 2.6, in comparison with the initial amount of protein peaks (20.0 ± 3.5). Statistical analysis highlights a significant increase in recovery for each depletion method. Specifically, the major increase in the number of protein peaks detected was observed upon the removal of the top six high-abundance proteins with the column; here, a threefold change was obtained, in comparison with crude serum.

Discussion

Our goal was to verify if the serum depletion strategies examined in this study could cause the removal of different protein components, in addition to the targeted high-abundance proteins.

Recent studies have been conducted in order to resolve the problem concerning the high dynamic concentration range in the analysis of the serum/plasma proteome (Hortin and Sviridov 2009; Roche et al. 2009). At present,

Table 1 Non-specifically bound proteins collected from the Affi-Gel blue-dye albumin depletion kit (Bio-Rad)

Spot no.	Accession no.	Protein full name	Entry name	MW	pI	Score/Queries	% cov.
1	P01857	Ig gamma-1 chain C region	IGHG1	36.6	8.46	85/25	19
2	P01024	Complement C3 fragment	CO3	188.6	6.02	1,249/239	17
3	P0C0L4	Complement C4-A	CO4A	194.2	6.66	333/47	6
4	P01024	Complement C3 fragment	CO3	188.6	6.02	397/27	6
5	P01024	Complement C3 fragment	CO3	188.6	6.02	310/58	7
6	P01859	Ig gamma-2 chain C region	IGHG2	36.5	7.66	56/2	20
7	P01857	Ig gamma-1 chain C region	IGHG1	36.6	8.46	98/19	16
8	P01842	Ig lambda chain C regions	LAC	11.4	6.91	75/17	38
9	P01842	Ig lambda chain C regions	LAC	11.4	6.91	149/38	56
10	P01842	Ig lambda chain C regions	LAC	11.4	6.91	69/14	30
11	P01834	Ig kappa chain C region	IGKC	11.6	5.58	173/32	56
12	P02766	Transthyretin	TTHY	15.9	5.52	54/14	17
13	P02735	Serum amyloid A protein	SAA	13.6	5.89	348/70	63
14	P02735	Serum amyloid A protein	SAA	13.6	5.89	103/9	40
15	P02647	Apolipoprotein A-I	APOA1	30.7	5.56	1,058/297	72
16	P02647	Apolipoprotein A-I	APOA1	30.7	5.56	317/111	48
17	P02743	Serum amyloid P-component	SAMP	25.5	6.12	345/66	35
18	P0C0L4	Complement C4-A	CO4A	194.2	6.66	167/25	3
19	P02760	Alpha-1 microglobulin	AMBP	39.9	5.95	98/16	7
20	P02760	Alpha-1 microglobulin	AMBP	39.9	5.95	86/11	6
21	P27169	Serum paraoxonase/arylesterase 1	PON1	39.7	5.08	170/37	22
22	P00738	Haptoglobin	HPT	45.8	6.13	296/96	20
23	P00738	Haptoglobin	HPT	45.8	6.13	131/50	9
24	P06727	Apolipoprotein A-IV	APOA4	45.4	5.28	1,096/181	69
25	P01009	Alpha-1 antitrypsin	A1AT	46.9	5.37	421/38	26
26	P01011	Alpha-1-antichymotrypsin	AACT	47.8	5.33	368/88	21
27	P01008	Antithrombin-III	ANT3	53.0	6.32	370/109	25
28	P02790	Hemopexin	HEMO	52.4	6.55	341/73	24
29	P02790	Hemopexin	HEMO	52.4	6.55	68/15	7
30	P02790	Hemopexin	HEMO	52.4	6.55	192/38	13
31	P04217	Alpha-1B-glycoprotein	A1BG	54.8	5.65	247/45	17
32	P43652	Afamin	AFAM	70.9	5.58	200/32	16
33	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	103.5	6.51	393/110	13
34	P00450	Ceruloplasmin	CERU	123	5.41	191/23	7
35	P01023	Alpha-2-macroglobulin	A2MG	164.6	6.00	51/11	12
36	P00751	Complement factor B	CFAB	86.8	6.66	829/218	33
37	P00751	Complement factor B	CFAB	86.8	6.66	626/169	39
38	P00751	Complement factor B	CFAB	86.8	6.66	266/59	19
39	P02787	Serotransferrin	TRFE	79.3	6.70	2,731/875	61

Proteins were separated on 2-DE gels and identified by Nano LC-CHIP-MS, as described under "Materials and methods". Proteins are indicated with their primary accession numbers and entry names of Swiss-Prot database. The highest score, the number of matching peptides (queries) and the percentage of amino acids sequenced for each detected protein are reported. The spot numbers correspond to the numbers shown in Fig. 1c

depletion strategies are applied in many biomarkers discovery platforms, such as in the study of ovarian cancer (Lin et al. 2009), prostate cancer (Dekker et al. 2007), or for the characterization of the human uterine secretome (Hannan et al. 2008). Moreover, serum depletion is an important step to estimate the concentrations of targeted biomarkers, such as the circulating forms of some low-abundance peptides (Hawkridge et al. 2008).

Currently, several depletion kits and columns are available; studies have been performed to focus either on their usefulness, or to evaluate their efficiency. Stempfer et al. for example, quantified the effectiveness of human

Table 2 Non-specifically bound proteins eluted from the immunoaffinity albumin/IgG depletion kit (Sigma)

Spot no.	Accession no.	Protein full name	Entry name	MW	pI	Score/Queries	% cov.
1	P01024	Complement C3 fragment	CO3	188.6	6.02	183/13	14
2	P0C0L4	Complement C4-A	CO4A	194.2	6.66	362/68	17
3	P02647	Apolipoprotein A-I	APOA1	30.7	5.56	228/62	35
4	P02647	Apolipoprotein A-I	APOA1	30.7	5.56	32/7	33
5	P01009	Alpha-1-antitrypsin	A1AT	46.9	5.37	179/54	13

The proteins were separated on 2-DE gels and identified by Nano LC-CHIP-MS, as described in "Materials and methods". The spot numbers correspond to the numbers reported in Fig. 1e

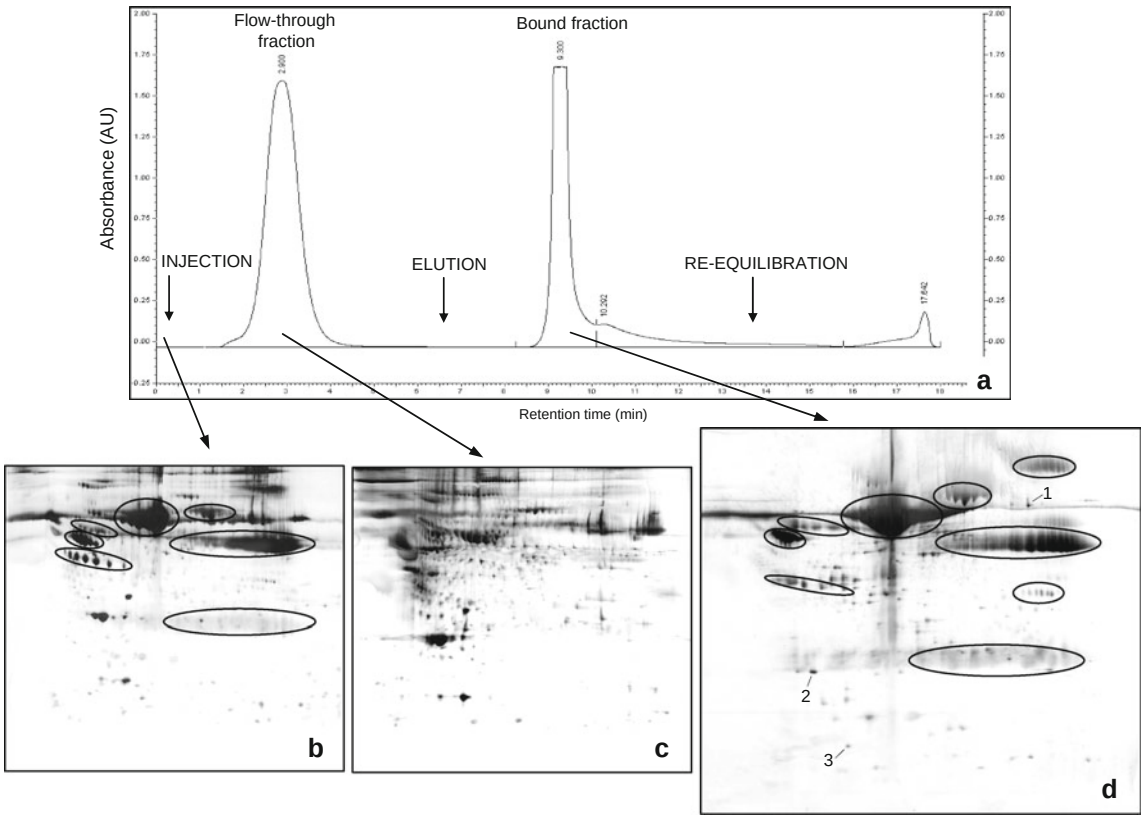

Fig. 2 Representation of the chromatogram (**a**) for the immunoaffinity removal of high-abundance serum proteins with the MARS column. Diluted serum was injected onto a 4.6 mm ID × 100 mm antibody column and flow-through fractions were collected (peak 1.5–4.8 min). The column was washed with Buffer A and the bound fractions were eluted with Buffer B (peak 8.5–10 min). An equal amount (100 µg) of crude serum (**b**), flow-through (**c**) and bound fractions (**d**), collected during HPLC fractionation, were analyzed by 2-DE as described in "Materials and methods". In (**b**), spots enclosed in *ellipses* (albumin, transferrin, IgG, IgA, alpha-1-antitrypsin, haptoglobin) represent the six targeted abundant proteins removed by the column, and (**c**) shows their complete removal. In (**d**) the *spot numbers* indicate the non-specifically bound proteins removed by the column; each *spot number* corresponds to the numbers listed in Table 3

Table 3 Non-specifically bound proteins removed by the MARS column

Spot no.	Accession no.	Protein full name	Entry name	MW	pI	Score/Queries	% cov.
1	P01024	Complement C3 fragment	CO3	188.6	6.02	913/169	16
2	P02647	Apolipoprotein A-I	APOA1	30.7	5.56	413/132	47
3	P02766	Transthyretin	TTHY	15.9	5.52	78/21	16

The proteins were identified by Nano LC-CHIP-MS, as described in "Materials and methods". The spot numbers correspond to the numbers visualized in Fig. 2d

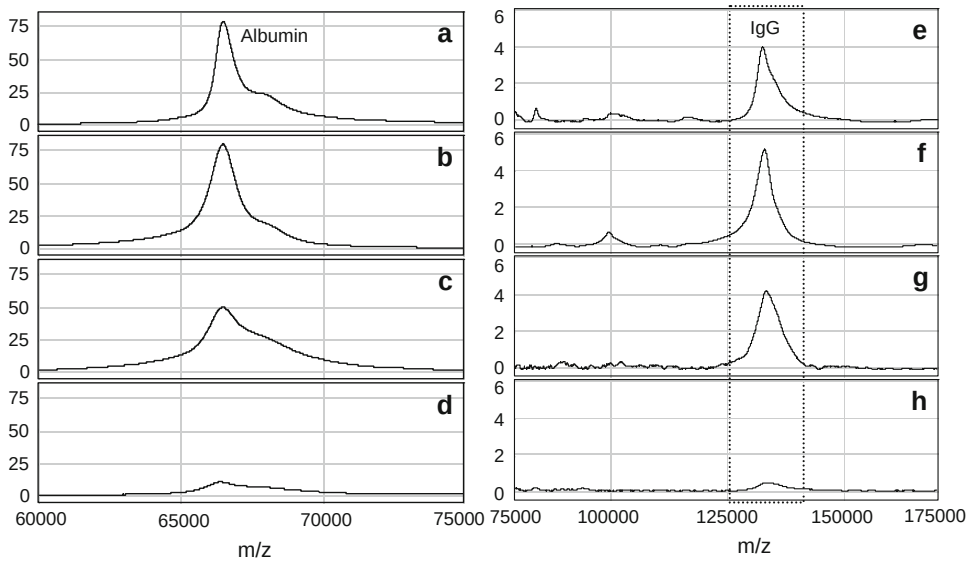

Fig. 3 Albumin and IgG peaks obtained by SELDI-TOF-MS analysis, before and after depletion of the high-abundance proteins. The same quantity of proteins (30 μg) was loaded on the surface of H50 reverse-phase ProteinChip array. *Left panels*: albumin peaks detected in undepleted serum (**a**) and in bound fractions collected after the use of the MARS column (**b**), the Sigma immunoaffinity kit (**c**), and the Bio-Rad kit (**d**). The MARS column and the Sigma kit showed higher albumin depletion efficiency than with the Affi-Gel dye kit. *Right panels*: IgG peaks obtained from the crude serum (**e**) and from bound fractions eluted from the MARS column (**f**), the Sigma kit (**g**) and the Bio-Rad kit (**h**). The column and the Sigma kit confirmed a satisfactory depletion; the Bio-Rad kit, which should remove only albumin, showed a low-intensity IgG peak

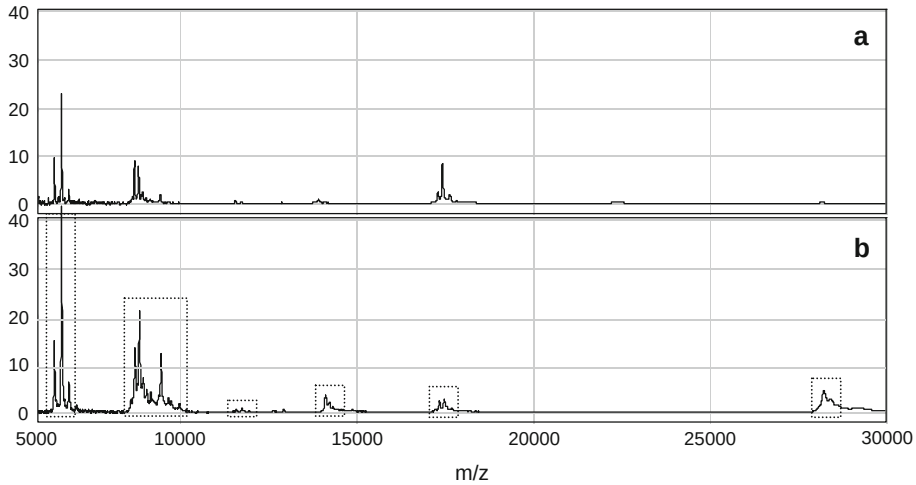

Fig. 4 Mass spectral view (low m/z range) of bound proteins recovered from the Bio-Rad Affi-Gel blue-dye depletion kit (**b**), compared with untreated serum sample (**a**). The spectra showed some peaks, enclosed in *rectangles*, corresponding to the proteins unspecifically bound to the Cibacron blue dye, and removed by the use of the kit

Table 4 Quantitative analysis of SELDI-TOF peaks detected in the unbound fractions after removal of high-abundance proteins with the depletion methods investigated

Depletion methods	No. of proteins depleted	No. of peaks in unbound fractions	Fold increase (depleted/untreated)
Aurum™ Affi-Gel blue-dye albumin depletion kit (Bio-Rad)	1	39.7 ± 2.1*	+2.0
ProteoPrep® Immunoaffinity albumin and IgG depletion kit (Sigma)	2	44.3 ± 1.5*	+2.2
Multiple Affinity Removal Column (Agilent Technologies)	6	59.0 ± 2.6**	+3.0

The fold increase values were obtained by the comparison with the number of peaks present in the undepleted serum samples (20.0 ± 3.5). Statistical analysis was performed considering the criteria for peaks identification described in Materials and methods. Peaks' numbers are expressed as means ± standard deviation. Statistical significance was tested using unpaired Student's *t*-test; * $P < 0.01$; ** $P < 0.001$ versus undepleted serum

high-abundance serum and plasma proteins depletion, in order to improve protein identification of disease marker candidates. These authors also assessed the risk of concomitant removal of relevant marker proteins. By resolving the fractions that were eluted from the matrix upon depletion, they found unspecific binding of relevant proteins in plasma sample obtained from acute myocardial infarction patients (Stempfer et al. 2008). The same problem was approached in earlier studies by using immunoaffinity chromatography to remove the most abundant serum proteins, followed by sequential anion-exchange and size-exclusion chromatography (Pieper et al. 2003).

Since the risk of removing important proteins may well occur when depletion strategies are adopted, we focused our study on the binding specificity of three different methods, identifying the non-specifically bound proteins removed by each of them.

We have demonstrated that the depletion methods tested removed albumin with great efficiency with the exception of the Affi-Gel Blue kit. In detail, this kit removed the higher amount of untargeted proteins (24 different proteins), as detected on 2-DE gels of bound fraction and as confirmed by SELDI-TOF analysis. It is known for some time that the Cibacron Blue resin has a variable affinity for different serum proteins, with albumin and lipoproteins scoring at the top (Gianazza and Arnaud 1982a). Since that time, it was observed that fractionation of plasma proteins on immobilized Affi-gel Blue was modulated under different experimental conditions, such as pH, ionic strength and temperature (Gianazza and Arnaud 1982b). As reported in the kit's protocol, when using spin columns filled with Affi-gel Blue support, the binding of serum proteins other than albumin could not be excluded, even if the product has been developed to minimize this non-specific adsorption. Having this well in mind, in the present study we chose to closely follow the manufactures's protocol, being our purpose that of evaluating the specificity and the efficiency of the kit on the basis of the instructions.

Regarding the Sigma kit, we found that it offered a depletion method with a satisfactory binding specificity, since it proved to remove only four non-targeted proteins: complement C3 fragment, complement C4-A, apolipoprotein A-I and alpha-1-antitrypsin. Moreover, this kit showed an efficient protein recovery in the flow-through fractions.

However, based on our evaluation of binding specificity and depletion efficiency, the MARS column has proved to be the most promising depletion approach. Only three non-specifically bound proteins, complement C3 fragment, apolipoprotein A-I, and transthyretin, were detected in the bound fraction after 2-DE gels analysis, making this method the most selective one among those tested in our study. Two proteins, complement C3 fragment and apolipoprotein A-I, were found also in the bound fractions from the Sigma kit. Antibody cross-reactivity may explain this finding, as in these tests, antibodies could cross-react with similar epitopes on other proteins. In addition, the process for purifying the polyclonal antibodies and the type of resin may be similar. Another possible explanation may reside in the presence of specific protein–protein interactions in both the procedures, and/or generic chemicophysical interactions, generated during the antibody-protein binding. Furthermore, using the MARS column we observed a substantial increase in protein spots resolution in the "albumin area" and improvement in the detection of low-abundance proteins on the 2-DE gels, with the identification of proteins that were not previously detected in the crude serum gels.

Other comparative studies regarding high-abundance proteins depletion have been performed. Björhall et al. for example, compared five different columns, including the Agilent MARS column (Björhall et al. 2005). The bound protein fraction eluted was separated on a monodimensional SDS–PAGE gel, and the obtained bands were identified by MS. Differently from our outcomes, they reported the finding exclusively of the six targeted proteins.

Studies aimed to deplete major serum proteins, in order to favour the subsequent SELDI-TOF-MS analysis, were also performed. Roche et al. used an immunocapture method, based on chicken antibodies against various abundant proteins, to realize a pre-fractionation of serum prior to SELDI-TOF profiling. Analysis of the unbound serum fractions revealed new peaks, most likely due to the low-abundance proteins. Analysing the bound fractions, the majority of the peaks were still detectable, as well as additional ones related to proteins co-purified with the 12 proteins retained by the column (Roche et al. 2006). Recently, the same authors compared the effects of depleting one, six, twelve or twenty blood proteins. They observed that increasing the number of depleted proteins from twelve to twenty had a limited beneficial impact. Actually, this might cause an increase in drawbacks by removing associated peptides and proteins (Roche et al. 2009). Seam et al. assessed the effect of albumin depletion on SELDI-TOF-MS reproducibility. They demonstrated that removing albumin with the IgY immunoaffinity spin columns, SELDI-TOF analysis revealed sixfold more protein peaks than those detected in the unfractionated serum, at both high and low m/z (Seam et al. 2007). In our study, the analysis by SELDI-TOF of unbound protein fractions gave similar results; additional protein peaks were found in both m/z ranges, after the use of each method tested. In particular, the higher fold-increase was observed after immunodepletion with MARS column. Both, protein peaks' number and the presence of some peaks with higher intensity, were evident when compared with untreated sample protein profiles.

Our results confirmed the necessity of depleting major serum proteins for the detection of low-abundance components. SELDI-TOF and 2-DE analysis, along with the identification of bound proteins by Nano LC-CHIP-MS system, clearly indicated that the depletion by MARS column resulted in a highly efficient removal of specific proteins, with subsequent rising in loading capacity of the low-abundance proteins, increasing the probability of their identification. The column enabled the removal of the top six most abundant proteins in a single step, corresponding to 85% of total protein serum content, allowing higher depletion than with the other two kits tested. Moreover, the MARS column offered further interesting advantages. In the first instance, it is compatible with automation, decreasing sample handling and labour. Furthermore, its cost per sample is low, taking also in consideration that the column can be regenerated and re-used for about 200 injections, unlike the disposable, single-use, products.

In conclusion, our study showed that the MARS column proved to be a powerful tool for rapid, reliable and specific depletion of high-abundance serum proteins, even if it has been shown to partially remove also three untargeted proteins. Nevertheless, in the study of serum proteome, depletion strategies are essential to reduce the complexity of the samples, allowing an easier investigation of low-abundance proteins, to favour the discovery of potential useful biomarkers. Whit this study we want to emphasize that, for some specific applications, the removed bound fraction should also be analysed, to ensure that no important proteins are inadvertently omitted.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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