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Comparison of three methods for fractionation and enrichment of low molecular weight proteins for SELDI-TOF-MS differential analysis

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ABSTRACT

In most diseases, the clinical need for serum/plasma markers has never been so crucial, not only for diagnosis, but also for the selection of the most efficient therapies, as well as exclusion of ineffective or toxic treatment. Due to the high sample complexity, prefractionation is essential for exploring the deep proteome and finding specific markers.

In this study, three different sample preparation methods (i.e., highly abundant protein precipitation, restricted access materials (RAM) combined with IMAC chromatography and peptide ligand affinity beads) were investigated in order to select the best fractionation step for further differential proteomic experiments focusing on the LMW proteome (MW inferior to 40,000 Da). Indeed, the aim was not to cover the entire plasma/serum proteome, but to enrich potentially interesting tissue leakage proteins. These three methods were evaluated on their reproducibility, on the SELDI-TOF-MS peptide/protein peaks generated after fractionation and on the information supplied.

The studied methods appeared to give complementary information and presented good reproducibility (below 20%). Peptide ligand affinity beads were found to provide efficient depletion of HMW proteins and peak enrichment in protein/peptide profiles.

1. Introduction

New biomarkers are expected to improve diagnosis, to guide molecularly targeted therapy and to monitor activity and therapeutic response across a wide spectrum of diseases. From a clinical point of view, it is easy to understand why blood biomarker discovery is very attractive. Its sampling is minimally invasive and can be performed repeatedly. To analyze circulating proteins and peptides, cellular components of blood can be removed, either in the presence of anticoagulants or after blood coagulation, yielding to plasma and serum, respectively.

Proteomic profiling of biological fluids for disease biomarker discovery has already improved drastically and is still in constant evolution. Indeed, potentially interesting biomarkers have emerged in literature for several diseases, including cancers and chronic inflammatory diseases [1–4]. Nevertheless, only a few of these have been validated. Much criticism has been made on the poor specificity of some of the discovered biomarkers [5,6]. Actually, most of them are abundant proteins or truncated forms, such as acute phase reactant proteins or proteins linked to clotting or platelet activation during blood sample preparation. However, even if one single marker shows poor specificity, the combination of several candidates could provide a powerful diagnostic tool, as demonstrated by the recently FDA approved OVA1 test combining 5 markers for ovarian cancer diagnostic. However, sample pre-

Abbreviations: CM10, weak cationic exchanger arrays; HAP, highly abundant proteins; HMW, high molecular weight; IMAC-RAM, restricted access materials (RAM) combined with IMAC chromatography; LMW, low molecular weight; PF4, platelet factor 4; PRM-30, Proteomics-30® resin for molecular mass <30 kDa; RSD, relative standard deviation; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI-TOF-MS, surface-enhanced laser desorption/ionisation-time-of-flight-mass spectrometry.

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fractionation appears essential for exploring the deep proteome and highlighting early disease stage biomarkers rather than host response biomarkers.

Analysis of plasma or serum is challenging because of its huge protein abundance dynamic range. It is well known that blood protein concentration covers 10 orders of magnitude, ranging from albumin (35–50 mg/ml in serum) to IL6 (0–5 pg/ml in serum) [7]. The 20 most abundant proteins, including albumin, immunoglobulin, fibrinogen, alpha 1-antitrypsin, alpha 2-macroglobulin, transferrin and lipoproteins, represent approximately 97% of the total protein mass [8–10]. The remaining 3% belong to a complex mixture of middle and low abundance proteins, including proteins of the complement family, hormones or proteins originating from normal tissue secretion or leakage upon cell death or damages. As the dynamic range of the protein amount that can be detected in a single mass spectrum is typically around 2–3 orders of magnitude, it is thus not possible to cover the entire range present in blood samples within one experiment [11]. To overcome this, several fractionation procedures have been developed and are now available to narrow the sample protein concentration dynamic range [12–14]. The most commonly used methods based on physicochemical peptide/protein properties are centrifugal ultrafiltration, precipitation by organic solvents, electrophoresis and chromatography (on-column or on-magnetic beads) [15–18]. However, these fractionation methods have not yet been evaluated in terms of high throughput capacity and reproducibility in proteomics [19]. Additionally, some proteins can be distributed over several fractions challenging the comparison of their abundance between samples.

Another widely used approach for HAP removal in serum and plasma is their depletion using specific antibodies [20]. But it is worth mentioning that some of the HAP act as carriers for minor abundance proteins, explaining the codepletion of almost 3000 species as observed by several groups, both fractions being thus interesting to investigate [21,22]. Moreover, this kind of affinity depletion shows also a degree of unspecific binding with non-targeted proteins due to cross reactivity of the antibodies used [23,24].

SELDI-TOF-MS is an instrument used for disease biomarker discovery over a large and fully automated scale. It provides biomarker patterns for a high number of individuals aiming at overcoming the limitation of single markers (i.e., lack of sensitivity and specificity) and may lead to consistent statistical data for a large population [25]. Using SELDI-TOF-MS, many key LMW proteins/peptides with molecular masses below 40 kDa were highlighted [26–28]. Some of these could be used to determine the onset of a given disease [29]. Indeed, LMW proteins/peptides in the serum/plasma include members of several physiologically important classes, such as cytokines, chemokines, and peptide hormones, along with proteolytic fragments of larger proteins, including those generated by disease-specific exopeptidases [30]. SELDI-TOF-MS combines the pre-selection of proteins and peptides on a specific chromatographic surface with a linear time-of-flight-mass spectrometer. Different types of surface are available (hydrophobic, ion exchanger, etc.) and determine the proteins that will be analyzed. Nevertheless, this pre-selection step is limited by the small number of activated groups available on this small surface promoting fixation of the most abundant and sometimes less informative proteins. Therefore, the reduction of sample complexity is essential to ensure the detection of proteins that are present at low concentrations.

In this study, we evaluated three different sample preparation methods (i.e., HAP precipitation, restricted access materials (RAM) combined with IMAC chromatography and equalization) to select the best fractionation step for further differential proteomic experiments focusing on the LMW proteome (MW inferior to 40 kDa). The aim was not to cover the entire plasma/serum proteome, but to enrich potentially interesting small MW tissue leakage proteins. The evaluation was based on the number and/or redundant information and on the reproducibility of the tested methods. Those three methods were chosen for their relatively high throughput capacity compared to HPLC, IEF or differential centrifugation. Precipitation is of course very rapid. Proteomics-30® and ProteoMiner® are now being developed in mini-spin columns and 96-well plates, respectively. Indeed, we intended to deal with clinical material presenting a large biological heterogeneity that requires the comparative analysis of a large number of samples.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile, trifluoroacetic acid, CHAPS, sodium chloride, Trizma base, Trizma hydrochloride, Na_2HPO_4, imidazole, thiourea were supplied by Sigma–Aldrich (St. Louis, MO, USA), whereas urea was from Amersham and acetic acid from Vel. Sodium acetate, ammonia solution 25% and ammonium chloride were from Merck. All reagents were of analytical grade. RC-DC protein assay kit, weak cationic exchanger arrays (CM10) and sinapinic acid (SPA) were provided by Bio-Rad (Hercules CA, USA).

2.2. Human samples

EDTA plasma and serum were provided from healthy donors. Serum, after 30 min of clotting, and plasma were centrifuged at $800 \times g$ for 10 min at room temperature prior to being aliquoted and stored at −80 °C. Before each sample treatment, thawed serum and plasma were centrifuged at $16{,}100 \times g$ for 15 min to remove most of the lipids and insoluble materials.

2.3. Peptide ligand affinity beads

Peptide ligand affinity beads, also called ProteoMiner®, were provided by Bio-Rad. Each column contains 500 μl of beads (20% beads, 20% ethanol, 60% water). One milliliter of crude serum or plasma was directly loaded on column without previous dilution. Loading such an important sample volume should ensure the concentration of low and medium abundance proteins [31].

Plasma and serum samples were analyzed in six independent experiments. Briefly, beads were washed successively by the addition of 1 ml of deionised water and 1 ml of wash buffer (150 mM NaCl, 10 mM NaH_2PO_4 pH 7.4). Then, 1 ml of sample was loaded on columns and incubated with beads for a period of 2 h at RT. Columns were centrifuged twice for periods of 2 min and 1 min and all column flowthroughs were collected for further analysis (called FT). Columns were then washed 3 times for 5 min. Proteins and peptides retained on beads were eluted by 300 μl of a solution made of 8 M urea, 2% CHAPS in 5% acetic acid buffer and then directly stored at −80 °C.

2.4. Precipitation

First, serum and plasma were denatured with 1.5 vol. of a solution made of 7 M urea, 2 M thiourea, 2% CHAPS in a 50 mM Tris pH 9 buffer for 30 min at room temperature. Then, 1.25 vol. of an acetonitrile/0.1% trifluoroacetic acid solution was progressively added to the sample and incubated for 30 min at RT. Next, samples were centrifuged at $16{,}100 \times g$ for 20 min and supernatants were collected and adjusted with HPLC water to obtain a final dilution of 1/6.

2.5. IMAC-RAM

These resin column materials, also called Proteomics-30®, were provided by Affiland (Belgium) in a context of scientific collab-

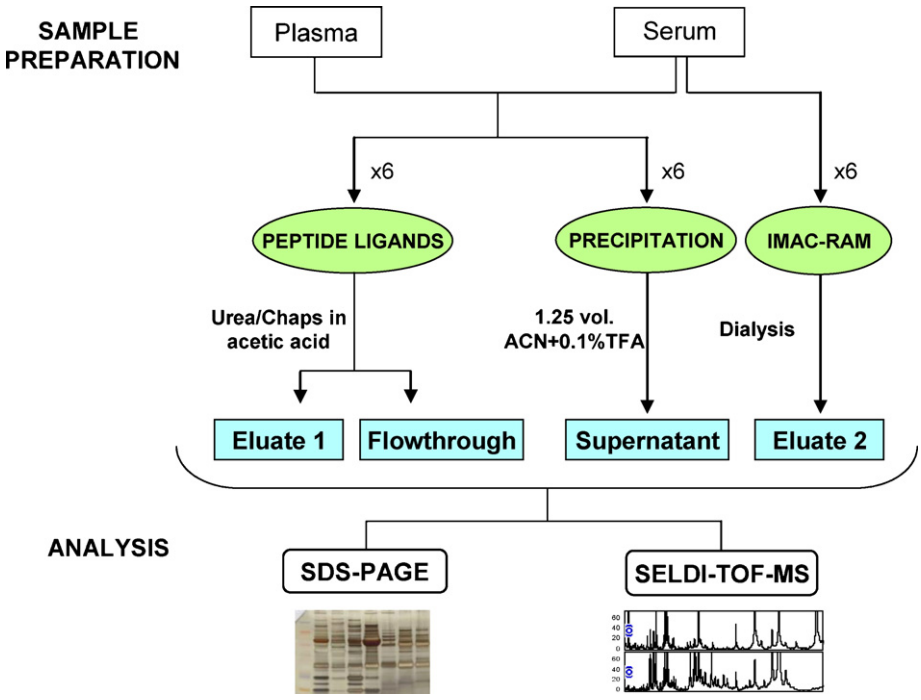

Fig. 1. Experimental layout.

oration. Each kit is composed of Proteomics-30® resin columns, washing buffer and elution buffer. 100 μl of crude serum was loaded into the resin after equilibration with 4 ml of washing buffer. After incubation, 900 μl of washing was added to obtain a final volume of 1 ml. The column was then washed twice with 1 ml and once with 7 ml of the equilibration buffer. Elution was performed adding 3× 1 ml of elution buffer. 500 μl of the second elution fraction was finally dialysed against acetate buffer pH 4 or Tris buffer pH 9 before for further steps.

2.6. One dimension (1D)-gel electrophoresis

The concentration of each sample, including crude samples used as the reference, was measured using the RC-DC protein assay kit (Bio-Rad). SDS-PAGE analysis was carried out loading 5 μg of proteins on NuPAGE 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The gel was further stained using a SilverQuest silver staining kit (Invitrogen). All samples were processed according to the manufacturer's instructions.

2.7. ProteinChip array preparation and analysis

Samples were analyzed on anionic (CM10) ProteinChip arrays (Bio-Rad, Belgium), as previously described [28].

The supernatant obtained after the precipitation process and ProteoMiner® samples (eluate and flowthrough) were respectively diluted 6- and 10-fold in binding buffer: 100 mM acetate, pH 4 or Tris buffer, pH 9. ProteoMiner® eluates were equilibrated by the addition of ammonium buffer pH 10.5.

CM10 arrays were equilibrated 3 times with pH 4 or pH 9 binding buffer. 10 μl of sample were applied on ProteinChip arrays and incubated for 1 h, at RT, in a humidity chamber. The spots were washed with binding buffer (10 μl) followed by a quick rinse with Milli-Q water (10 μl). After 20 min of air drying, 1 μl of saturated sinapinic acid (SPA) solution (prepared following the manufacturer's recommendations) was applied to each spot. CM10 arrays were then analyzed in a PCS4000 SELDI-TOF-MS reader (Bio-Rad). Spectra were calibrated using external calibration against peptides and proteins from an All-in-one Peptide kit (1.5–8 kDa mass range) and an All-in-one Protein kit (8–40 kDa mass range). Laser intensity was optimized for ion detection in these two mass ranges averaging 1560 shots per spot and avoiding signal saturation. Autodetection of peaks was performed for m/z ranging from 1500 to 40000. A minimal signal-to-noise ratio threshold of 3 and a valley depth between 0.68 and 1.9 were the two criteria used for peak cluster formation. Baseline subtraction and normalization on total ion current were performed for all spectra using Protein Chip data manager software (Bio-Rad).

3. Results and discussion

The presence of HAP in serum and plasma such as albumin and IgG is detrimental to the detection of low abundant biomarkers. To address the complexity of these samples, it is essential to remove HAP and to concentrate proteins of low abundance before proteome analysis. Due to the high number of samples required for clinical proteomics (ideally between 100 and 1000 samples), the high throughput capacity of the whole procedure is also an important aspect.

The present study consists in the comparison of three depletion methods of abundant proteins in serum and plasma samples, namely protein precipitation, IMAC-RAM (or Proteomics-30®) and peptide ligand affinity beads for equalization (or ProteoMiner®). Crude serum and plasma were used as reference samples. The experiment layout is presented in Fig. 1. After sample pretreatment, protein content was quantified and analyzed by SDS-PAGE and surface-enhanced laser desorption/ionisation-time-of-flight-mass spectrometry (SELDI-TOF-MS).

SELDI-TOF-MS profiling based on weak cationic exchanger arrays (CM10) was used to evaluate the gain of information (profile enrichment), the reproducibility ($n = 6$) and complementarities between profiles. To broaden the field of investigation, samples were analyzed on CM10 at two pH binding conditions (pH 4 and pH 9). Peak detection was performed within two mass ranges (1.8–8 kDa and 8–40 kDa) with properly mass calibration using two different and adequate calibration curves.

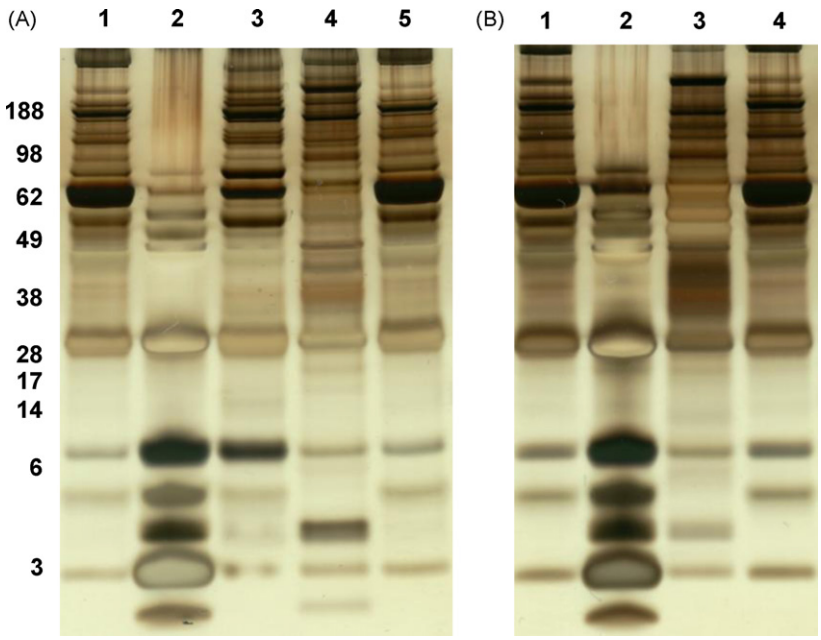

Fig. 2. Representative silver stained SDS-PAGE for each prefractionation method. Protein profiles observed in 2000–200,000 *m*/*z* range. (A) Serum: (1) crude serum, (2) organic precipitation, (3) Proteomics-30®, (4) ProteoMiner® eluate, (5) ProteoMiner® flowthrough. (B) Plasma: (1) crude plasma, (2) organic precipitation, (3) ProteoMiner® eluate, (4) ProteoMiner® flowthrough.

3.1. Sample pretreatments and 1D-gel

3.1.1. Precipitation

Major protein depletion from plasma and serum was investigated using precipitation with various organic solvents (acetonitrile, isopropanol and methanol) at different percentages. After centrifugation and pellet removal, determination of the remaining protein content was performed. Plasma and serum supernatants were then analyzed by 1D-gel and SELDI-TOF-MS. As described by other authors [21,32], acetonitrile added to 0.1% TFA was found to give the best results in terms of number of protein peaks detected below 40 kDa and resolution. Denaturation of sample before organic solvent addition also improves the protein profile (data not shown).

As can be seen in Fig. 2A (column 2) and B (column 2), the majority of the high molecular weight proteins (HMW) (>40 kDa) are depleted after ACN/TFA protein precipitation, compared to crude serum (Fig. 2A, lane 1) and plasma (Fig. 2B, lane 1). Most of the high abundant proteins in blood are larger than 40 kDa. The addition of 1.25 vol. of acetonitrile (ACN) containing 0.1% TFA leads them to precipitate. Subsequent centrifugation removes 97–98% of the proteins, as determined after total protein content determination.

Protein precipitation is not a specific method like immunodepletion [33]. Indeed, non-targeted proteins, including potential valuable biomarkers, may be lost during precipitation. Some proteins may also remain partly soluble and are present in both fractions, compromising differential analysis. Another disadvantage could also be the important dilution of the sample by the addition of solvent. However, the solvent-precipitation method is rapid, simple and cheap. Moreover, the presence of an organic solvent dissociates protein complexes which may facilitate the detection of potential biomarkers associated to HAP.

3.1.2. IMAC-RAM

Proteomics-30® resin combines two principles, IMAC-Cu chromatography and size fractionation with a cut-off of 30 kDa. It is an Affiland patented metal pentadentate chelator (PDC) resin (EP0972566 B1) which recognizes principally all proteins and/or peptides with MW < 30 kDa. PDC coupled to a resin is able to form complexes with all polyvalent metal ions and to give an octahedral Metal ion-chelator complex with five coordination sites occupied by the chelator. It provides a high stability of the Metal ion-chelator complex. It also results in one free site for interaction and selective binding of accessible cysteine/histidine residue and chiefly histidine containing biomolecules. PDC-Cu chromatography is used to bind mostly peptides/proteins with MW below 30 kDa and get rid of HMW proteins, salts and lipids.

Because of the presence of EDTA in the plasma, this sample pretreatment method was only investigated on serum samples (Fig. 2A, lane 3).

The major difference between crude serum and Proteomics-30® pretreated samples visible on 1D-gel is a strong decrease in albumin content, also observed by SELDI-TOF-MS profiling (cf. Section 3.2). However HMW protein depletion is less efficient compared to the two other fractionation approaches. The total protein content was decreased by 76% after Proteomics-30® procedure.

3.1.3. Peptide ligand affinity beads

This new fractionation approach, recently developed by Righetti and Boschetti, implies the use of a combinatorial library of hexapeptides grafted on micro-beads on which, in theory, only one copy of a unique ligand binds. This approach, named ProteoMiner®, simultaneously dilutes HAP and concentrates low and medium abundant proteins [34,35]. The main interest of this equalization method is the dynamic range reduction between high and low abundant proteins and peptides. However, it was shown that, despite the decrease in dynamic range, this technology used for differential studies was only applicable for proteins or peptides which do not reach saturation, i.e. the range of low and medium abundance proteins [36]. ProteoMiner® was also found to reduce the risk of codepletion that may occur with immunoaffinity methods and presents a much higher loading capacity. Simo et al. showed that interactions between protein and amino acid baits are mainly due to hydrophobic interactions, especially with aromatic moieties in priority, followed by hydrogen bonding, and finally ionic interactions [37].

As shown in Fig. 2A (lane 4) and B (lane 3), equalization of the protein concentration range promotes the detection of new protein bands, as compared to crude serum (column 1). Interestingly, 1D-gel profiles from eluates (Fig. 2A, lane 4 and B, lane 3) and flowthroughs (Fig. 2A, lane 5 and B, lane 4) obtained after ProteoMiner® sample pretreatment gave complementary information.

Concerning the total protein content, a decrease of 96 and 98% was measured for plasma and serum, respectively.

3.2. Abundant protein depletion

To have an idea of the fractionation method efficiency for abundant protein depletion, *m*/*z* values of peaks detected in our spectra were correlated to those described and identified in the literature using the same binding conditions on chip arrays [38].

Moreover, we consider that if a prefractionation method is efficient, proteins abundantly present and detected in crude sample should be significantly depleted in fractionated samples.

For example, ProteoMiner® fractionation was found to present a good efficiency for abundant protein depletion after comparison between ProteoMiner® eluate, ProteoMiner® flowthrough and crude sample profiles (cf. Fig. 3). As can be seen in Fig. 3A, several peaks in the 2500–7000 *m*/*z* range nearly disappeared in serum ProteoMiner® eluate profile compared to crude serum profile. These should correspond to fibrinogen α-chain fragments (2932 Da, 3191 Da, 3240 Da, 3262 Da, 5902 Da) and inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) (3157 Da) [39,40]. Apo-C1 variants (6431 Da, 6629 Da) were found to be decreased in ProteoMiner® eluate and precipitation profiles; while it is completely depleted in Proteomics-30® profiles [41]. As expected, these proteins were largely present in the flowthrough profile (cf. Fig. 3A). Moreover, using ProteoMiner®, Fig. 3B and C shows the efficient depletion of transthyretin (13,765/13,886 Da), hemoglobin alpha and beta chains (15,121 Da and 15,863 Da), B2-microglobulin (11,728 Da) and albumin (~66,000 Da) proteins [42–44]. These proteins are among the most abundant ones in serum/plasma [38].

Similar observations could be made with precipitation and Proteomics-30® prefractionation methods (cf. Fig. 3A–C). After Proteomics-30® treatment, Apo-C1, lysozyme (14,685 Da), Apo A1 (28,084 Da) and albumin were depleted [45,46]. Precipitation seemed to be less efficient for depletion of abundant proteins below 40 kDa. However, B2-microglobulin, lysozyme and albumin were depleted while signals of other abundant proteins were increased (transthyretin, hemoglobin chains).

As protein binding onto chromatographic surface depends on its affinity, its concentration, but also on chip surface binding capacity, one can imagine that competition between different proteins for binding sites is rather complex. One can also assume that when high abundant proteins are depleted, low or medium abundant proteins should bind to the available free activated groups of the protein chip surface. This was supported by new peaks appearance in SELDI profiles after sample prefractionation (cf. Fig. 3A–C).

3.3. SELDI-TOF-MS protein profiles obtained after fractionation

SELDI-TOF-MS profiles were studied more particularly within the 1.8–40 kDa mass range. It is noteworthy that these profiles were extended to 80 kDa in order to detect the presence/absence of albumin (*m*/*z*: 66,000). Using CM10 array at pH 4, 104 peaks were detected in the crude serum sample (cf. Table 1). Fewer peaks (73) were observed with crude plasma, probably due to the presence of a high amount of fibrinogen or coagulation related proteins, which might saturate protein arrays [47].

As can be seen in Table 1, precipitation of serum and plasma with ACN/TFA showed nearly the same number of peaks (91 and 73, respectively) at pH 4 compared to the crude sample and only few proteins were observed above 30 kDa (Figs. 2, 4B and Supplementary data 1B).

Using Proteomics-30® material, 110 peaks were detected for serum. Despite the less efficient albumin depletion compared with precipitation and peptide ligand affinity beads (cf. Fig. 4B), the SELDI-TOF-MS profile obtained at pH 4 was significantly enriched compared to crude serum (cf. Fig. 4A).

Eluate and flowthrough obtained after ProteoMiner® treated serum samples were also studied by SELDI-TOF-MS (cf. Fig. 4). ProteoMiner® eluate showed a gain of peaks compared to crude serum (115 vs 104). In addition, the treatment of plasma using ProteoMiner® was rather efficient since the number of peaks almost doubled: 122 versus 73 peaks at pH 4. It is worth noting that rather efficient albumin depletion was also observed (Supplementary data 1B).

In this pH 4 condition, serum treated by Proteomics-30® and ProteoMiner® profiles showed a similar number of peaks (~110) while ACN/TFA treatment gave less information (~90 peaks) despite efficient albumin and IgG removal (cf. Fig. 4B). For plasma samples, the ProteoMiner® approach showed profile enrichment compared to precipitation (cf. Table 1).

Experiments were also carried out at pH 9 as albumin does not bind on cationic exchange surface at this pH (albumin p*I*: 4.7). In theory, proteins with a p*I* > 9 should bind to the chip surface. As can be seen in Table 1, the spectra of serum and plasma pretreated by ProteoMiner® presented almost the same number and more peaks than the crude samples, respectively (92 peaks for serum and 136 for plasma eluates compared to 98 and 86 peaks for crude serum/plasma sample profiles).

Precipitation of serum and plasma at pH 9 revealed a very poor profile (only 27 and 48 peaks, respectively). Proteomics-30® serum eluate profile gave 78 peaks. Finally, ProteoMiner® eluate profiles revealed more peaks (136 peaks) than the two other approaches.

From these experiments, it can be concluded that SELDI-TOF-MS profiles obtained after ProteoMiner® pretreatment showed enrichment on cationic chip arrays at both pH conditions, especially for plasma. Information gain was mostly observed in the 2000–10,000 *m*/*z* range.

Table 1
Number of detected peaks and RSD (%; $n = 6$) for each prefractionation method. Studied mass range: 1.8–40 kDa.

	Serum				Plasma			
	pH 4		pH 9		pH 4		pH 9	
	Peaks	RSD (%)	Peaks	RSD (%)	Peaks	RSD (%)	Peaks	RSD (%)
Crude	104	12	98	10	73	12	86	14
Precipitation	91	8	27	18	73	10	48	15
Proteomics-30® eluate	110	12	78	17	ND	ND	ND	ND
ProteoMiner® eluate	115	10	92	13	122	13	136	16
ProteoMiner® FT	90	14	82	14	91	14	69	15

ND: not determined.

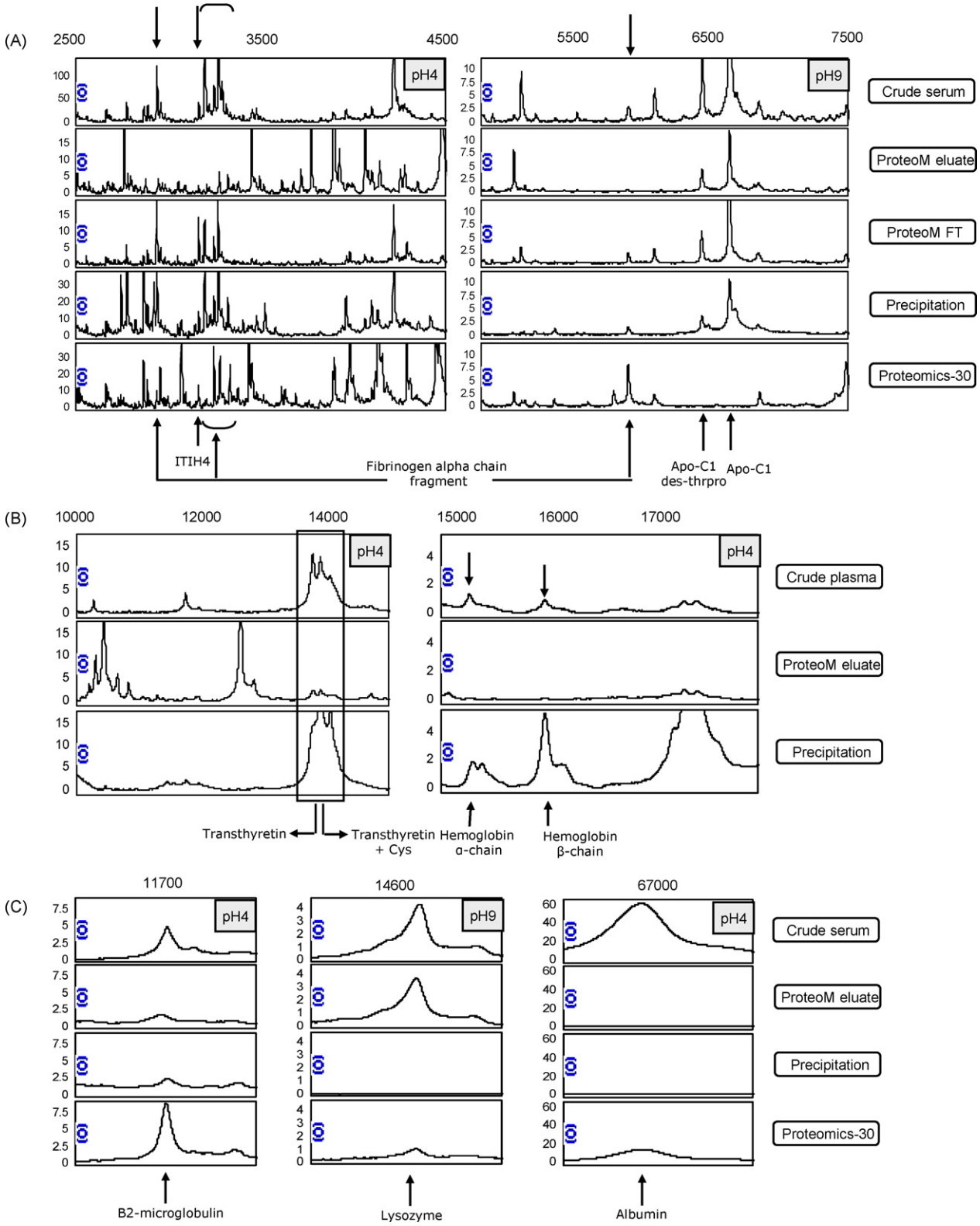

Fig. 3. Spectra examples of HAP depletion. (A) 2500–7000 *m*/*z* range. (B) 10,000–18,000 *m*/*z* range. (C) B2-microglobulin, lysozyme and albumin depletion.

3.4. Sample pretreatment reproducibility

The reproducibility is a prerequisite for accurate differential proteome analysis of clinical samples process as well as for biomarker quantification.

Reproducibility was evaluated on six independent experiments for all sample preparations (cf. Table 1). Relative standard deviations (RSDs) were calculated on the intensity of all SELDI-TOF-MS peaks detected within the 1.8–40 kDa range, after replicates clustering. The reproducibility of the experiments performed with

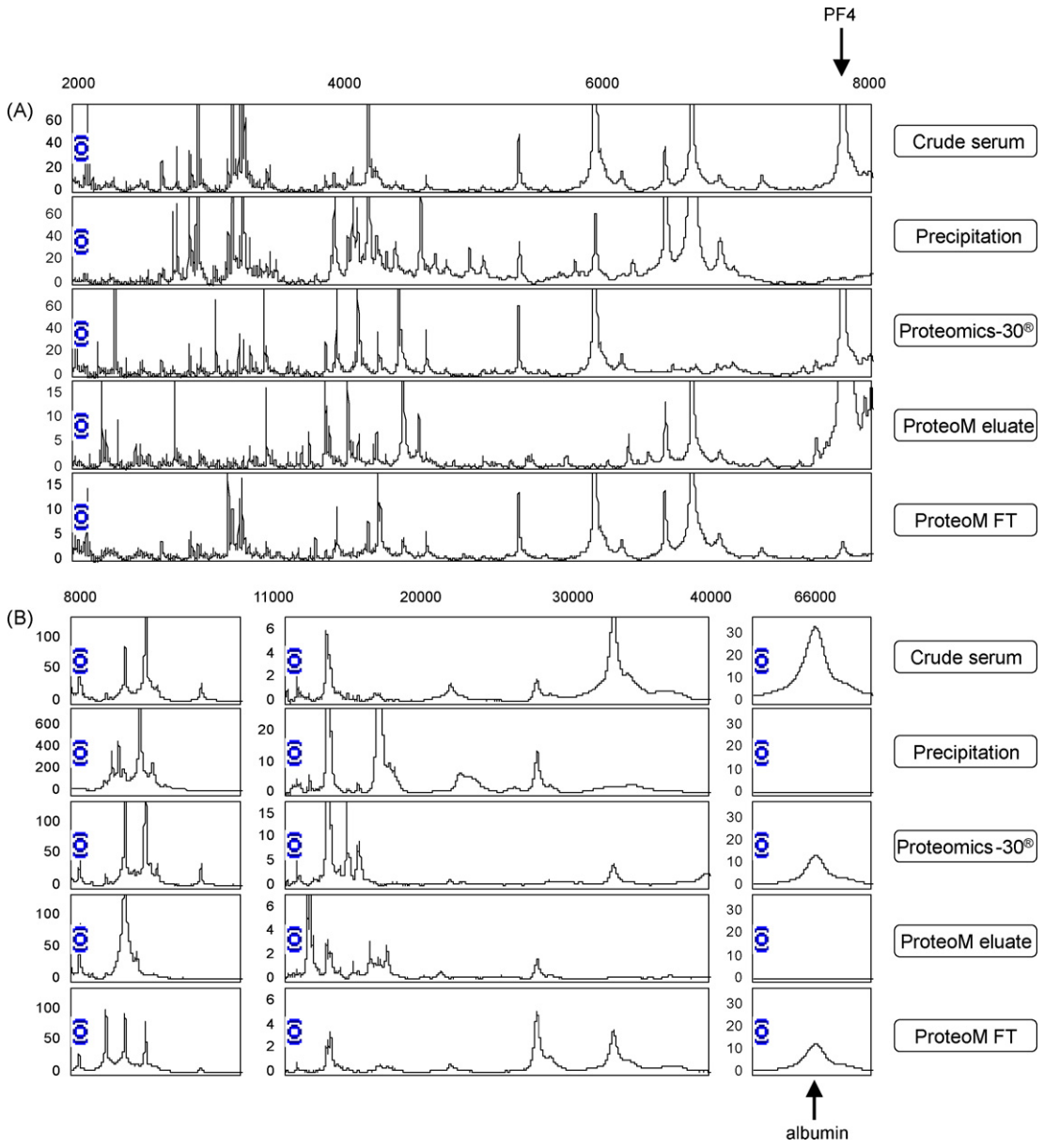

Fig. 4. Comparison of serum prefractionation methods by SELDI-TOF-MS using CM10 at pH 4. (A) Protein profiles obtained in 2000–8000 *m*/*z* range. (B) Protein profiles obtained in 8000–70,000 *m*/*z* range.

crude sample was also evaluated for comparison with treated samples. The amount of peaks detected for a specific prefractionation protocol was found to be the same. However, as mentioned in Table 1, the final number of peaks depended on the prefractionation method considered (i.e., 104 peaks detected in crude serum compared to 115 peaks detected in ProteoMiner® serum eluate) and on the sample type (serum vs plasma).

All the conditions tested on CM10 showed satisfactory RSD values (below 20%, this is the maximal tolerance of the FDA for bioanalysis (cf. FDA guidelines)). RSD values obtained for crude serum and plasma, used as reference samples, were both 12% at pH 4 and, at pH 9, 10% and 14%, respectively. The lowest variability was observed with acetonitrile precipitation at pH 4 (8% and 10%). For Proteomics-30® method, RSD values were also satisfactory (12% and 17% at pH 4 and pH 9, respectively).

As shown in Table 1, ProteoMiner® eluate and flowthrough gave similar RSD values than the reference sample at pH 4, while at pH 9, they were slightly higher.

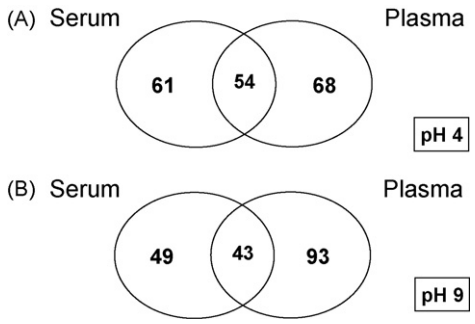

Fig. 5. Venn diagrams showing information overlaps obtained by SELDI-TOF-MS between ProteoMiner® eluate of serum and plasma analyzed at pH 4 (A) and pH 9 (B).

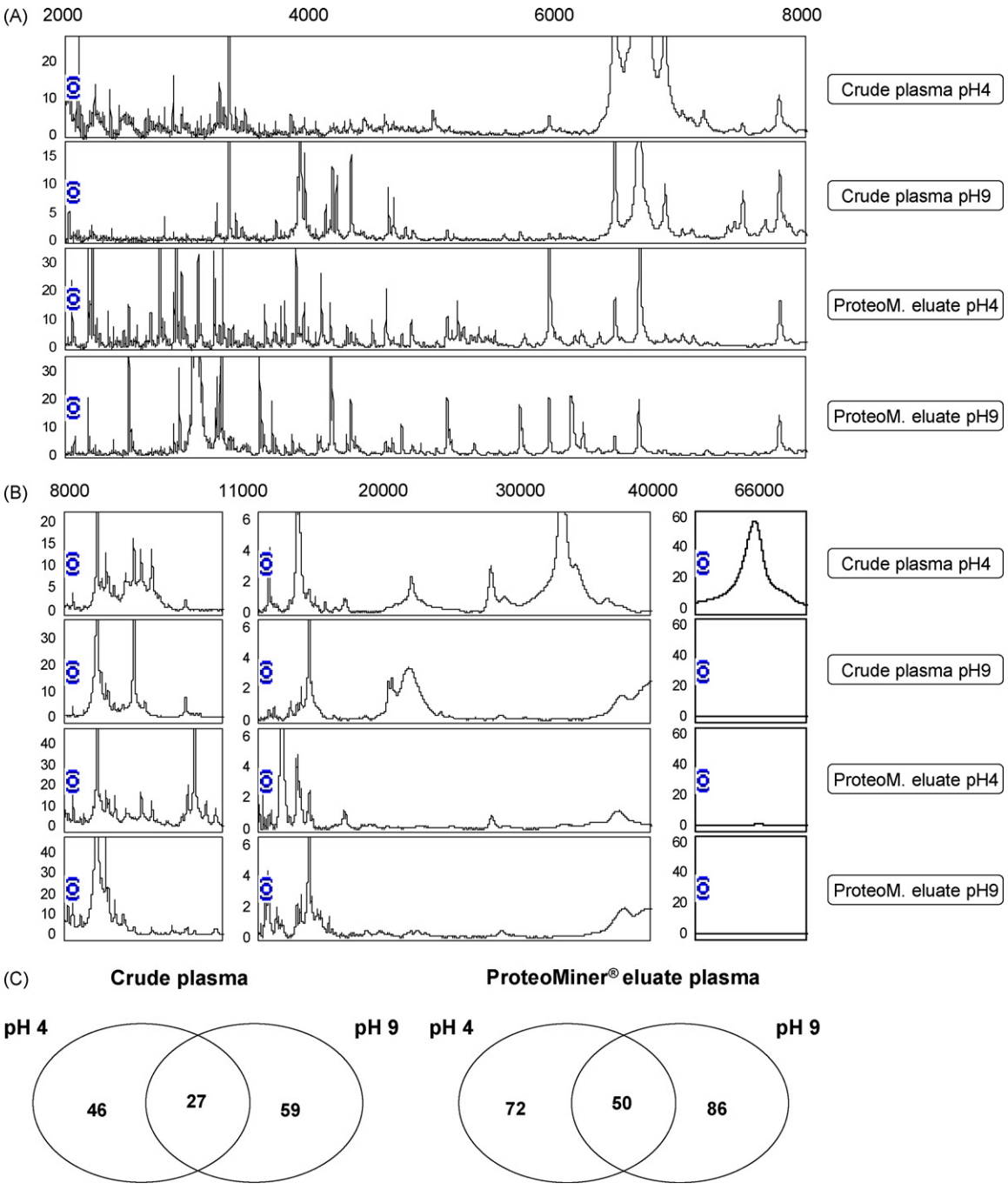

Fig. 6. Representative SELDI-TOF-MS spectra and Venn diagrams from plasma prefractionation with ProteoMiner® at pH 4 and pH 9. (A) Protein profiles obtained in 2000–8000 *m*/*z* range. (B) Protein profiles obtained in 8000–70,000 *m*/*z* range. (C) Venn diagrams.

In semi-quantitative and quantitative proteomic studies, it is important to keep in mind the risk of unselective loss and the functional changes of prefractionation material adsorption ability [48,49]. To our point of view, it is therefore critical, for proteomic analysis, to implement single-use devices which avoid carryover between samples. Indeed, incomplete elution with multiple-used devices can lead to a decrease of binding capacity and to some carryover on subsequent samples, compromising reproducibility and then efforts to find proteins and peptides in relation with disease process. Furthermore, during sample handling, protein degradation might occur. Duration of sample pretreatment processing is therefore an important point to take into account. This step is critical in preserving proteins/peptides integrity.

3.5. Sample pretreatment recovery

To evaluate the overlap between the three methods, Venn diagrams, based on the comparison of *m*/*z* values of the detected peaks across the different samples, were constructed (Figs. 5, 6C, 7 and Supplementary data 4C and 5).

Fig. 5 provides Venn diagrams showing information overlaps obtained by SELDI-TOF-MS between serum and plasma ProteoMiner® eluates bound at pH 4 (A) and pH 9 (B). As can be seen

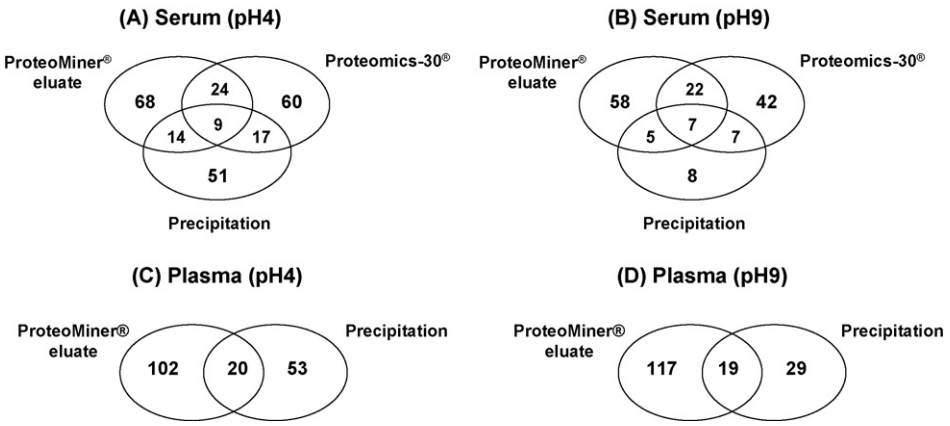

Fig. 7. Prefractionation method information overlaps on SELDI spectra. (A) Serum (bound at pH 4). (B) Serum (bound at pH 9). (C) Plasma (bound at pH 4). (D) Plasma (bound at pH 9).

in this figure, at both pH conditions, plasma generated more information than serum. The comparison of plasma profiles obtained with ProteoMiner® eluates at pH 4 and pH 9 showed 50 common peaks (Fig. 6C), pH 9 condition being more informative (86 non-common peaks vs 72 at pH 4). Profiles obtained at pH 4 and pH 9 bring complementary information (cf. Fig. 6C). In a biomarker discovery trial, it is interesting to combine information from different conditions.

Fig. 7 shows the prefractionation method information overlaps for serum and plasma at both pH conditions. These Venn diagrams clearly indicate that the three fractionation methods are complementary as the information overlap is poor. Indeed, only 9 and 7 peaks were in common when comparing SELDI spectra obtained after serum prefractionation at pH 4 and pH 9, respectively. For plasma samples, almost 30–40% of the peaks detected after precipitation were also present in ProteoMiner® eluate profiles, while these common peaks represented only 15% of total peaks obtained from ProteoMiner® eluate profiles.

In Supplementary data 5, Venn diagrams comparing SELDI profiles obtained at pH 4 with crude samples and ProteoMiner® serum eluate, shows 65 new peaks that emerged after ProteoMiner® compared to the 32 new ones in crude serum. Proteomics-30® showed the same gain of information compared to crude sample (65 peaks), while precipitation is less informative (46 vs 59 peaks in crude serum). The same comparison performed with plasma also showed a gain in information compared to crude plasma (75 vs 16 peaks). As expected, there was an important overlap between flowthrough and crude samples (~30 peaks for serum and ~25 peaks for plasma, cf. Supplementary data 5).

Finally, the comparison of crude serum, Proteomics-30® chromatography and ProteoMiner® eluates showed a more important information gain at both pH conditions after ProteoMiner® treatment (Supplementary data 5). However, we observed low information redundancy between the three approaches at both pH conditions (Fig. 7). Then, we can consider that these three prefractionation methods are complementary.

4. Conclusions

In this paper, three methods of serum and plasma preparation were evaluated according to their capacity of high molecular weight protein depletion and gain of new potential biomarkers. The methods are based on three different approaches: proteins precipitation, metal affinity coupled to restricted access material and equalization by peptide ligand affinity. All three appeared to give complementary information and presented good reproducibility (<20%). The organic solvent-precipitation did not supply a real gain in new peptide/protein peaks when studied by SELDI-TOF-MS but the depletion of the abundant proteins with a MW > 40kDa was very efficient. On the contrary, despite of the less efficient depletion of HMW proteins, IMAC-RAM treatment led to additional peaks with low MW. Finally, peptide ligand affinity beads were found to provide efficient depletion of HMW proteins and peak enrichment in protein/peptides profiles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.04.029.

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