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

Muriel De Bock[a], Dominique de Seny[b], Marie-Alice Meuwis[c], Anne-Catherine Servais[d], Tran Quang Minh[e], Jean Closset[f], Jean-Paul Chapelle[a], Edouard Louis[g], Michel Malaise[b], Marie-Paule Merville[a], Marianne Fillet[a,d,*]

[a] Clinical Chemistry, GIGA Research, University of Liège, CHU, B36, B-4000 Liège 1, Belgium
[b] Rheumatology, GIGA Research, University of Liège, CHU, B36, B-4000 Liège 1, Belgium
[c] Proteomic Platform, GIGA Research, University of Liège, CHU, B36, B-4000 Liège 1, Belgium
[d] Dept. of Analytical Pharmaceutical Chemistry, Institute of Pharmacy, University of Liège, CHU, B36, B-4000 Liège 1, Belgium
[e] Affiland s.a., rue de l'Yser 304, 4430 Ans, Belgium
[f] Biochemistry, Institute of Pathology B23, University of Liège, Belgium
[g] Hepato-Gastroenterology, GIGA Research, University of Liège, CHU, B36, B-4000 Liège 1, Belgium

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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.

* Corresponding author at: University of Liège, GIGA, B34, B-4000 Liège, Belgium. Tel.: +32 43664654; fax: +32 43664347.
*E-mail address:* marianne.fillet@ulg.ac.be (M. Fillet).

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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-