MEDICINAL CHEMISTRY MEETS PROTEOMICS: Fractionation of the Human Plasma Proteome

András Kovács (1)

1) Horváth Laboratory of Bioseparation Sciences, Research Centre for Molecular Medicine, Medical and Health Science Center, University of Debrecen, H-4032 Debrecen, Nagyerdei krt. 98., Hungary

András Guttman* (1,2)

1) Horváth Laboratory of Bioseparation Sciences, Research Centre for Molecular Medicine, Medical and Health Science Center, University of Debrecen, H-4032 Debrecen, Nagyerdei krt. 98., Hungary

2) Barnett Institute, Northeastern University, Boston, MA 02115, USA

Correspondence:

András Guttman*

Horváth Laboratory of Bioseparation Sciences, Research Centre for Molecular Medicine, Medical and Health Science Center, University of Debrecen, H-4032 Debrecen, Nagyerdei krt. 98., Hungary

Tel: +36 (52) 411 717/ex 64182; Fax: +36 (52) 255-539; Email: a.guttman@neu.edu

Running title: Fractionation of the Human Plasma Proteome

Keywords: affinity-based methods, biomedical science, biomedicine manufacturing, chromatography, depletion, fractionation, multi-step separation, plasma, precipitation, proteomics

Abstract

Human plasma and its fractions/derivatives are frequently used materials in biomedicine as it contains thousands and thousands of proteins representing the majority of human proteome. Several important methods were developed in the past for the fractionation of this important biological fluid and their use for medicinal purposes. One of the greatest challenges is the very large dynamic range of plasma proteins ranging up to 10-12 orders of magnitude. Early attempts were mainly based on methods such as salting out or cold ethanol precipitation, as well as chromatography utilizing affinity, size exclusion, ion exchange and hydrophobic interaction techniques. More recently, fractionation applications started with the depletion of the high abundant plasma components, such as serum albumin and immunoglobulins, before isolating lower abundant proteins of interest. Plasma volumes were utilized from the milliliter scale for diagnostic applications to hundreds of liters for industrial scale plasma fractionation (e.g., medicinal product manufacturing). In this paper we review this important part of medicinal chemistry, highlighting the traditional methods along with some of their variations as well as the most significant recent achievements of the field.

Table of contents

1 Introduction

About 20% of the extracellular fluid in the human body is comprised by plasma, which is one of the most studied and processed biological substance in the pharmaceutical industry and biomedical sciences. Its exceptional importance is due to the high complexity consisting of thousands and thousands of proteins originating from the proteomes of practically all body tissues [1]. Millions of liters are fractionated over the world annually for isolation and purification of therapeutic products under carefully designed, strictly hygienic conditions in accredited plasma fractionation plants, mainly based on conventional precipitation and chromatographic techniques [2]. The most abundant plasma constituents are human serum albumin (HSA) and the

immunoglobulins, representing ~80% of all plasma proteins. HSA is accounted for about the half of the therapeutic plasma protein products sold worldwide during the last decades and used for treatment of e.g. exsanguination, hypoproteinemia or hypoalbuminemia just to mention a few important ones [3]. In addition, the biomedical research also consumes significant amounts of plasma as a source to isolate different important plasma components or for biomedical and diagnostic research. For the latter one, it is of great importance to remove the highest abundant proteins, especially HSA and immunoglobulins since these components are mostly considered to be non-informative and obscure the ones of diagnostic biomarker potential present in much lower abundance. Accordingly precise, sometimes exhaustive and expensive preparation/pre-fractionation techniques are needed to deplete the most abundant plasma constituents prior to deep proteomic analyses, e.g. with two-dimensional electrophoresis or mass spectrometry. The role of traditional precipitation and chromatographic methods of size exclusion, ion exchange or hydrophobic interaction - frequently arranged in combination for enhanced effectiveness - to fulfill this aim is still unquestionable. However, there is a recent spread of affinity-based methods, representing a significant portion of sample preparation techniques. These affinity-based techniques are able to simultaneously remove the most abundant plasma components quickly and easily, with high recovery. In this review, selected examples of the most important and widespread methods are described together with recent developments and techniques to fractionate the human plasma proteome.

2 Precipitation based methods

2.1 Salting out with inorganic salts

About a hundred years ago, the famous German chemist, Franz Hofmeister and his coworkers elaborated the salting out method making a contribution of historical importance on the effects of inorganic salts on protein precipitation. They used salts with mono- and bivalent cations for the treatment of serum and coined the main separated fractions as ‘globulins’ (precipitated at lower salt concentrations) and ‘albumins’ (precipitated at higher salt concentrations). The original papers were written in archaic German but their English translation can be found in [4]. While precipitation methods have not changed so much since their introduction, it is of great importance to

choose the most suitable buffer (concentration, pH, ionic strength) to achieve the best possible reconstitution of the protein precipitate without leaving clumps or aggregates in the solution. Although the establishment of the mechanism was developed on serum and the first experiments were also performed using serum, the same principles were also adapted to plasma precipitation.

2.2 Precipitation with organic solvents

The Cohn method [5] is considered as affecting the practice of large-scale plasma fractionation to the greatest extent. The main goal of this cold ethanol fractionation was the production of pure albumin for therapeutic purposes but the immunoglobulins were separated too. This technique was easy, effective and aseptic, thus still being used, mainly in the U.S. [6]. The original process was further developed by Kistler and Nitschman [7] by including multi-step processes. Coagulation factors were removed in the form of cryoprecipitate, while plasma was being thawed first followed by fibrinogen precipitation with 8% cold ethanol. During the second cold ethanol precipitation step (please note that the Cohn method used 25% ethanol at pH 6.9, while the Kistler-Nitschman method used 19% ethanol at pH 5.85) the immunoglobulins were removed and the remaining solution contained the albumin product. HSA was purified from α- and β-globulins by the addition of more ethanol (to 40%) in one (Kistler-Nitschman) or two steps (Cohn). Final albumin precipitation took place near its isoelectric point at pH 4.8. In spite of the large ethanol consumption, this latter method is more cost-effective thus became more popular in Europe [8].

The combination of ethanol and heat treatment of plasma was another labor- and cost-effective technique resulting in high quality albumin [9]. Another precipitation option employed ether, an apolar solvent used for e.g. lipid extraction in the laboratory practice, but therewith it was described to be suitable for the fractionation of human plasma even for clinical purposes. Using ether precipitation the main plasma proteins, albumin and γ-globulin were separated together with fibrinogen and prothrombin. Ether concentrations, pH, ionic strength and temperature were well defined and the treatment was performed under strict aseptic conditions [10].

2.3 Rivanol precipitation

Hořejší and Smetana [11] found that Rivanol (2-ethoxy-6,9-diaminacridine lactate), a positively charged acridine derivative, precipitated blood plasma proteins. First the agent was applied at the concentration of 0.3% and all plasma proteins were precipitated except the γ-globulin fraction. The supernatant was collected by centrifugation and activated charcoal was used to remove the Rivanol. The resulting 97% pure γ-globulin was found to be suitable for clinical use. They have also performed a quantitative study on serum precipitation and found that with the increase of the concentration of the substance the amounts of the precipitated albumin, α- and β-globulin and also the γ-globulin were increased. Since the introduction of this method, several important plasma proteins were produced by the rivanol precipitation technique and antibodies were raised against some of them [1].

2.4 Polyethylene glycol precipitation

Polyethylene glycol (PEG) is a water-soluble synthetic polymer, usually used at industrial scales to isolate important plasma components [12]. It is considered to be non-toxic and chemically relatively inert [13]. Schneider and co-workers combined heat fractionation with polyethylene glycol precipitation yielding at least 90% of the original albumin content of the plasma [14].

3 Chromatographic methods

3.1 Large-scale processing for biomedicine manufacturing

Besides the widespread laboratory level applications, chromatographic methods were developed and utilized for the purification of different plasma proteins for therapeutic purposes, since these techniques allow the isolation and purification of high purity proteins both in the laboratory and industrial scales. Since most plasma proteins bear charges, ion exchange chromatography is a good fractionation tool, frequently coupled with other chromatographic methods. Ion exchange can also be used for the elimination of proteins of no interest. Affinity ligands are usually used to partition proteins of interest as well as to remove impurities from the final product. Size exclusion chromatography or gel filtration is another complementary step to a multi-stage fractionation process to eliminate contaminants [15]. In this section we discuss selected

examples on the application of chromatographic methods for the production scale purification of plasma proteins for therapeutic use.

High purity (>95%) albumin was produced by means of QAE-Sephadex A-50 ion exchange resin from the supernatant after the precipitation of immunoglobulins with PEG [16]. Another abundant plasma component, immunoglobulin G was separated from human plasma cryosupernatant by the combination of several methods such as ion exchange chromatography (DEAE-Sepharose), affinity chromatography (arginine Sepharose 4B) and size exclusion chromatography (Sephacryl S-300 HR) as the final purification tool obtaining a yield of 3.5 g IgG from one liter plasma [17].

In a novel approach, anion exchange was the anterior step followed by metal chelate affinity chromatography for the manufacture of α-1 antitrypsin (AAT), which protein in purified form can possibly cure AAT deficiency when administered intravenously [18].

Coagulation factors that are used in the treatments of different hemophilias are also usually purified by chromatography. Te Booy *et al.* combined DEAE-Sephadex A-50 anion exchange chromatography with affinity chromatography using a special affinity matrix dimethylamino-propylcarbamylpentyl-Sepharose CL-4B. The first step separated coagulation factors II, IX and X, while factor VIII (also known as anti-hemophilic factor) was isolated by the affinity step yielding good recovery and high specific activity [19].

3.2 Laboratory scale applications for biomedical research

In common laboratory practice, the different plasma proteins are not solely isolated for medicinal reasons but rather for other kind of research use, e.g. enzyme characterization, purification, immunization, structural analysis, biomarker discovery, pilot studies for large scale method development, etc. Plasma fractionation for deep proteomic analysis is discussed in details in section 4. Similar to that of listed under section 3.1., ion exchange chromatography is a frequently used tool in the laboratories.

Hemophilia A and von Willebrand's disease are inherited bleeding disorders caused by the deficiencies of factor VIII and von Willebrand factor, respectively. FVIII/VWF concentrates are used in the therapy of the two diseases [20] but also FVIII alone can be given to hemophiliac patients to restore hemostasis [21]. These two glycoproteins were isolated by the combination of ammonium sulfate precipitation and anion exchange

chromatography and after the two separation steps a fraction containing the complex of the two proteins was obtained in which the stability of FVIII was assured by the high VWF content [22]. The FVIII/VWF complex can be dissociated by the addition of calcium, however the anion exchange chromatography after the addition of calcium was not successful before Josic *et al.* introduced a second anion exchange step to divide the two coagulation factors from each other. The FVIII was stabilized by its isolation with albumin. Another method was recently developed for the purification of coagulation factor VIII in which first a direct plasma anion exchange chromatography step was applied resulting in a fraction containing factor VIII with co-elution of vitamin K dependent factors, factors IX and X, which proteins were later separated by size exclusion chromatography from the factor VIII fraction [23].

Heparin affinity chromatography is another widespread method for the isolation of plasma proteins usually combined with anion exchange chromatography as Josic *et al.* described for the purification of antithrombin III, factor IX and factor X [24]. Hoffer *et al.* combined adsorption chromatography, hydrophobic interaction chromatography and heparin affinity chromatography for the isolation with high purity and specific activity of factor IX [25]. The advent of high performance liquid chromatography (HPLC) offered another possibility to isolate plasma proteins. Vitamin D binding protein was isolated from human plasma both by HPLC and low pressure chromatographic methods. Using high performance liquid chromatography much higher purity and yield were obtained than by traditional methods in significantly shorter processing time [26]. High performance affinity chromatography was developed for the purification of proteins in an efficient manner utilizing the high specificity of the affinity matrix and the fast and high resolution HPLC technique [27].

4 Plasma fractionation for proteomics

Deep proteomic profiling of biological samples can lead to a vast amount of information that can be utilized for the description of biological processes, molecules or the characterization of diseases, e.g. through the identification of disease specific markers. In biomarker discovery proteomic methods complement the ones employed by genomics [28]. With the advent of modern tandem mass spectrometry (MS/MS) instrumentation, proteins can be studied rapidly, precisely with high sensitivity and

reproducibility [29]. The so called ‘top-down’ MS-based proteomics approach works at the protein level, while the ‘bottom-up’ method analyzes peptides derived from proteins after proteolytic digestion. While the latter technique is more widespread the former is getting more and more popular [30]. A third approach if this kind is referred to as a ‘middle-down’ technique, offering the benefits of both the ‘top-down’ and ‘bottom-up’ methods. Proteins are digested with enzymes (e.g. endoproteinase Lys-C, OmpT outer membrane protease) cutting the proteins less frequently than trypsin, thus producing longer peptides that allows comprehensive characterization of large proteins along with their posttranslational modifications [31, 32]. These three approaches are compared in Figure 1.

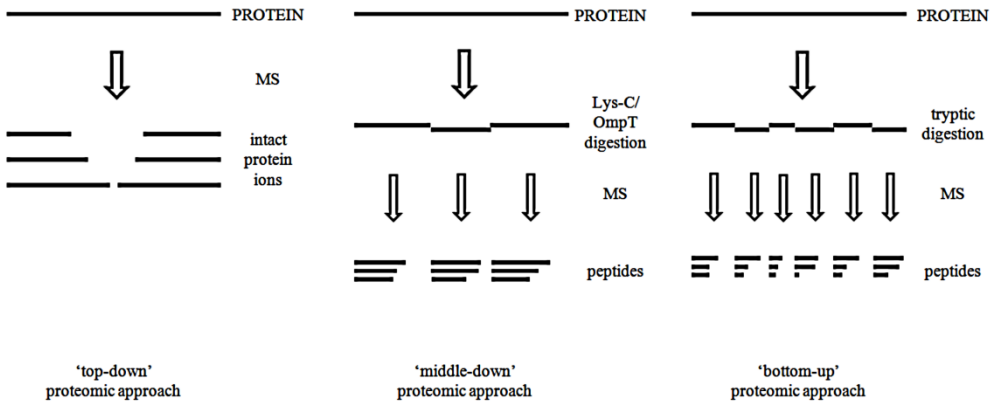

Figure 1. Scheme of the ‘top-down’, ‘middle-down’ and ‘bottom-up’ proteomic approaches.

Comprehensive analysis of plasma proteins is a challenging task due to the high complexity and large dynamic range of the plasma components, thus appropriate sample preparation, such as pre-fractionation is highly advised before the deeper exploration of its proteome for biomarker discovery [33]. The usual workflow of sample preparation for MS or MS/MS-based proteomics analysis starts with the depletion of high abundance proteins that can interfere with the detection of the lower abundant ones. This step also decreases the dynamic concentration range of plasma proteins, enriches the subproteome of interest and provides pre-fractionation before deeper analysis [34]. Application of three or more methods of fractionation definitely increases the

accessibility of lower abundance plasma proteins and the use of different pre-fractionation/separation techniques can significantly raise the number of discoverable proteins. Such separation techniques include two-dimensional gel electrophoresis (2D-GE) that separates proteins first by their isoelectric point (pI) then by molecular mass (MW), although having limitations for the analyses of proteins with extreme pI values and/or MWs (higher than 150 or lower than 10 kDa). This technique usually results in up to hundreds or a few thousands of protein spots that can be excised and analyzed downstream by mass spectrometry methods. Multi-dimensional liquid chromatography (MDLC) is another effective technique for global plasma protein fractionation prior to MS analysis [35, 36]. Besides MDLC, affinity based separation methods are also widespread, mainly for the removal of the most abundant plasma components. Usually multi-dimensional liquid chromatography and the affinity based separation are combined during sample preparation for MS-based proteomic analyses.

4.1 Affinity-based depletion of the high abundant plasma proteins

In proteomics a great variety of methods have been developed for the removal of the most abundant plasma components before downstream processing or analysis. While in industrial scale plasma fractionation albumin is one of the main products, in plasma proteomics it has to be removed to enhance the detection of lower abundant proteins. Travis *et al.* discovered that Sepharose bead conjugated Cibacron Blue (a chlorotriazine dye) bound albumin with high affinity [37]. This traditional depletion method, also called as Blue Sepharose chromatography became a widespread separation technique in analytical scale because it can be performed in relatively low cost. However, problems with non-specific binding were discovered, i.e., the dye not only bound HSA but other, non-albumin-type proteins [38, 39]. Immunoaffinity columns containing immobilized monoclonal anti-albumin antibodies [40] or affibodies [41] are also broadly used to partition this most abundant plasma constituent with very high specificity. Several types of columns containing IgGs from different mammalian species (goat, mouse, rabbit, etc.) are commercially available [42]. Hinerfeld *et al.* reported that chicken anti-human HSA immunoglobulin Y (IgY) antibodies - bound to hydrazide beads - featured high affinity towards human albumin, thus provided highly specific depletion [43]. Akgöl *et al.* [44] synthesized porous polymeric beads by suspension polymerization of 2-

hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA). The dye Reactive Red 120 was attached to these poly(HEMA-EGDMA) beads to adsorb HSA from human plasma with high specificity, offering an alternative method for albumin removal.

The second most abundant constituents of the human plasma are the immunoglobulins. One of the most frequently used immunoglobulin isolation techniques is thiophilic adsorption based, introduced by Porath *et al.* [45]. The method was based on the general affinity of immunoglobulins to a ligand carrying a sulphone group in the immediate vicinity of a thioether group. Utilizing this phenomenon an adsorbent was synthesized comprising of a thioether sulphur and a neighboring sulphone group. This so-called 'T-gel' readily adsorbed immunoglobulin G, M and A from human serum. Their studies also revealed that under optimized conditions the process was capable of preserving the native structure and biological activity of the immunoglobulins [46]. The method was recently applied for deep plasma fractionation [47]. An interesting application was reported by Coffinier *et al.* who immobilized a thiophilic ligand on ethylene-vinyl alcohol copolymer based hollow fiber membranes to remove immunoglobulin G from human plasma [48]. Specific bacterial cell wall proteins, like protein A from *Staphylococcus aureus* [49], protein G from *streptococci* [50] and protein L from *Peptostreptococcus magnus* [51] are known to specifically bind various subsets of immunoglobulins [52]. Since the isolation of these bacterial proteins can be expensive, alternative lower cost solutions were explored. By the help of combinatorial chemistry, stable mimetic ligands were produced mimicking the function of protein A, opening up the possibility to generate synthetic affinity ligands for immunoglobulin depletion in a more cost-effective way [53]. Sulfamethazine (SMZ) was immobilized on poly(glycidyl methacrylate) (PGMA) beads with bisoxirane spacer to yield an affinity medium that could provided an effective tool for the depletion of immunoglobulin G from plasma [54]. New specific-affinity beads with metal chelate ligands and synthetic polymer bead matrices were synthesized by Özkara *et al.* [55] to isolate immunoglobulins from human plasma. Metal-chelated spherical poly(hydroxyethyl methacrylate) (PHEMA) beads were prepared by suspension polymerization and (L)-histidine metal-complexing ligand was immobilized on the gel beads by covalent binding. Zn(II), Ni(II), Co(II), and Cu(II)

were chelated on the gel beads. Application of these metal chelate based affinity gel beads could offer the possibility to deplete IgG from human plasma in one step.

Affinity-based pre-fractionation of plasma components prior to MS or MS/MS-analysis is an important sample preparation tool since it reduces the complexity of the plasma sample, enriches proteins of interest while enhancing the throughput by generating fewer fractions than other pre-fractionation methods [56]. The application of the so-called multiple affinity removal system (MARS) was an important step towards the improved detection of low abundant proteins, compared to the depletion of albumin and/or the immunoglobulins only. Consequently, modern depletion methods are based on this multi-immunoaffinity depletion approach to remove high abundant plasma components using either antibody or affibody based stationary phases. Tu *et al.* used IEF-LC-MS/MS to compare the affectivity of various commercially available immunoaffinity columns for the depletion of 7 and 14 most abundant plasma proteins. For example, the multiple affinity removal system for the depletion of 14 high abundant plasma components deplete the following proteins: HSA, transferrin, haptoglobin, IgG, IgA, alpha-1-antitrypsin, fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, complement component C3, IgM, apolipoprotein A1, apolipoprotein A2 and transthyretin [57]. After the immunodepletion step, 25% more proteins were identified than in the untreated plasma sample [58].

The combination of immunodepletion with reversed-phase liquid chromatography also allowed identification of low abundance. A study showed that the depletion of the six most abundant plasma components with antibody containing spin column removed about 85% of the total amount of the plasma proteins and enhanced the detectability of the low abundance proteins for 2-D gel electrophoretic analysis [59]. An IgY microbead based depletion system was reportedly efficient for the depletion of 12 abundant proteins - albumin, IgG, transferrin, alpha-1-antitrypsin, IgA, IgM, alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, apolipoprotein A2, alpha-1-acid glycoprotein, and fibrinogen - from plasma. One of the most important advantage of using avian IgY antibodies was that they cross-reacted with heterologous human

proteins to a significantly lesser extent than mammalian ones decreasing the number of nonspecific interactions [60].

Another new development in the field is the so-called “ProteoMiner” method that is utilizing a hexapeptide based a combinatorial peptide ligand library with a high variety of sequences. This approach is reportedly capable to equalize low and high abundant plasma protein concentrations. The mode of action of the library is based on solid phase affinity adsorption. The process reportedly captures almost all plasma proteins at more or less the same level and therefore equalizes their concentration therefore significantly decreases the wide dynamic concentration range of plasma proteins for qualitative analysis [61]. Similar normalization technique was recently reported by means of monoclonal antibody technology by Takács and coworkers [62].

Subproteomes, such as the glycoproteome, are distinct groups of proteins within the plasma proteome and affinity based methods are frequently used for partitioning these categories [63]. Technologies were developed for glycoproteome analysis based on lectin and pseudo-lectin affinity fractionation [64, 65]. Multi-lectin affinity chromatography (M-LAC) is a recently developed approach for the fractionation of the human plasma glycoproteome that utilizes a combination of different lectins in one column. M-LAC was combined with a multi affinity removal system immunodepletion column to deplete the most abundant - mainly glycosylated plasma proteins [66] and the automated platform (High Performance Multi-Lectin Affinity Chromatography, HP-M-LAC) was elaborated comprising targeted depletion with fractionation of the glycoproteome. The workflow consisted of depletion with anti-albumin antibody column and the M-LAC column of concanavalin A, jacalin, and wheat germ agglutinin. The platform proved to be stable, highly glycan specific and reproducible providing high recoveries for plasma proteome fractionation [67]. Combining boronic acid (a pseudo lectin) chromatography with lectin affinity chromatography, dubbed as BLAC, were applied to isolate glycoproteins by using both selective and/or combined elution conditions [68]. Boronic acid and concanavalin A (BLAC/Con A) resin-filled micropipette tips were also developed as an automated affinity micropartitioning tool to isolate and enrich various classes of glycoproteins from human serum/plasma for

downstream processing like N-linked glycan profiling [69]. The scheme of boronic acid lectin affinity chromatography is depicted in Figure 2.

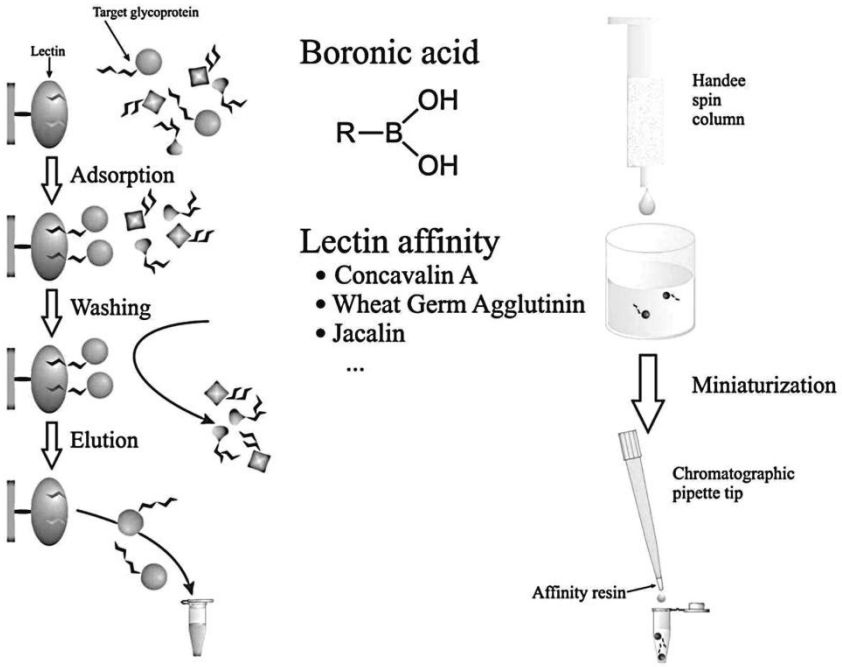

Figure 2. Schematics of boronic acid lectin affinity chromatography (BLAC).

4.2 Non-affinity-based depletion methods

Alternative methods for affinity based albumin and IgG removal are continuously being developed. One of the simple ones was a short monolithic column utilizing anion-exchange chromatography [70]. Multistep processes include the above described salting out method (section 2.1) to first precipitate the immunoglobulins from human plasma (4 M ammonium sulfate) followed by fractionating with anion exchange chromatography using diethylaminoethyl (DEAE)-cellulose column to acquire pure IgG [71]. Mahn *et al.* studied the depletion of the highly abundant plasma proteins with ammonium sulfate (AS) precipitation followed by hydrophobic interaction chromatography (HIC) for proteomics analysis. They found that the combination of the two methods had the potential to remove the highly abundant proteins from plasma, in particular albumin, immunoglobulins, alpha-1-antitrypsin, haptoglobin, and fibrinogen. Because of the simplicity and cost-effectiveness of this process, it could be a good alternative to antibody based methods [72, 73]. Brgles *et al.* utilized the principles of displacement

chromatography for plasma fractionation and the isolation of low abundance proteins. They applied monolithic ion exchange columns under overloading conditions with the consideration that HSA, IgG and other weakly bound plasma proteins were displaced by stronger binding ones in anion and cation exchange modes [74].

4.3 Multi-step plasma proteome fractionation

A 783-fraction containing human plasma proteome library was generated by Kovács *et al.* - referred to as the Analyte Library - by applying a carefully designed workflow with the aim to use the Library for monoclonal antibody proteomics-based antigen identification [47]. First, 500 mL normal pooled human plasma was subject to Blue Sepharose affinity chromatography for HSA depletion followed by the removal of immunoglobulins from the albumin-depleted flowthrough by thiophilic interaction chromatography. Then, a pre-fractionation step of ammonium sulfate precipitation with increasing salt saturations of 35, 45, 65 and 75% was applied. The precipitates were dissolved and consecutively separated by size exclusion, cation and anion exchange chromatography steps. The 20 most concentrated ion exchange chromatography fractions were subject to hydrophobic interaction chromatography. Preservation of the native forms of the intact plasma proteins was top priority throughout the fractionation process. The scheme of the Library generation protocol is delineated in Figure 3. The Library fractions proved to be applicable for mAb proteomics-based antigen identification [75].

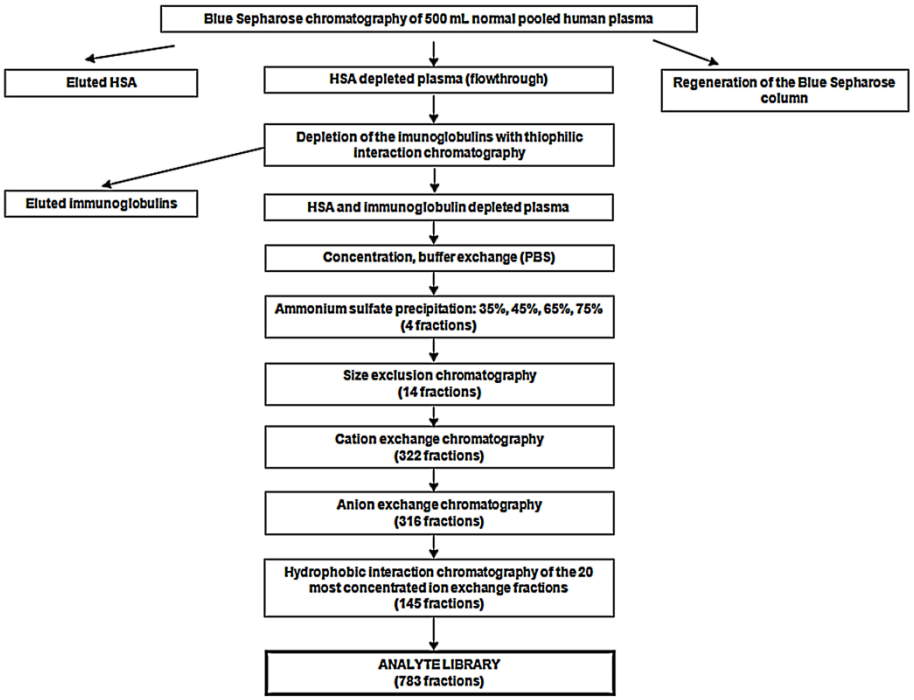

Figure 3. Flowchart of the Analyte Library generation process.

Another large scale fractionation process employed strong cation exchange chromatography as the first dimension followed by reversed-phase HPLC as second dimension to pre-fractionate the human plasma proteome prior to linear ion trap MS analysis [76]. 1292 proteins were identified, several of them below 10 ng/mL (published physiological concentration). Another liquid-based fractionation system utilized ion exchange chromatography followed by reversed-phase chromatography [77, 78]. This platform was able to reveal post-translational modifications (PTMs) as well as to profile basic and hydrophobic proteins. For the time being, Liu *et al.* generated one of the most extensive proteome maps of the human plasma using a multidimensional approach by combining cation exchange chromatography, reversed-phase liquid chromatography, ion-mobility spectrometry, and mass spectrometry (SCX-LC-IMS-MS). Their analysis of 37,842 unique peptide sequences lead to 9087 unique protein entries, 2928 of which met their criteria of high-confidence assignments anticipated to be reproducibly detected with their approach. [79]. A high-resolution three-dimensional intact-protein-based quantitative analysis system (the IPAS) was developed by Wang *et al.* [80]. By using their profiling approach, proteomic changes in allogeneic bone marrow transplantation (BMT) acute graft-*versus*-host disease (GVHD) were detected and identified. The

workflow of immunodepletion, isoelectric focusing, reversed-phase liquid chromatography and SDS-PAGE analysis was applied for the separation of plasma proteins prior to mass spectrometry resulting in uncovering a broader range of proteomic changes than with two-dimensional gel electrophoresis. Moritz *et al.* combined continuous free-flow electrophoresis (first dimension) and reversed-phase HPLC (second dimension) creating a proteomics separation tool and found that in comparison with the gel-based fractionation methods this technique can be applied for the separation of either high molecular weight proteins or small peptides [28].

4.4 Other techniques

Four precipitation methods were studied by Jiang *et al.* prior to deeper proteomic analysis comparing trichloroacetic acid (TCA), acetone, chloroform/methanol, and ammonium sulfate. TCA and acetone precipitation proved to be more efficient in terms of sample concentration and desalting for a proteomic analysis than chloroform/methanol or ammonium sulfate although the latter two yielded better protein recoveries [81].

5 Concluding Remarks and Future Prospective

In this paper we reviewed the large variety of the methods developed to fractionate human plasma both in industrial and laboratory scales. Besides the traditional and most widely used methods of salting out, precipitation, and chromatography, novel approaches such as affinity- and non-affinity-based depletion of the high abundant plasma proteins and multi-step/multi-dimensional proteome fractionation methods were discussed. Large/industrial scale techniques were described as they usually aim the purification of plasma proteins for medicinal purposes, while laboratory scale approaches were associated with a wider range of scientific applications, among them one is proteomic analysis which was demonstrated in a more detailed way. These techniques require continuous improvement to further fractionate this important biological fluid more effectively towards the manufacturing of new therapeutic products and the identification of novel biomarkers. The ideal method would first include a highly specific depletion step without the occurrence of co-depletion (which has to be taken into consideration in the case of e.g. Blue Sepharose chromatography). The more

specific monoclonal antibody based techniques are usually expensive and cannot be used in the case of larger plasma volumes that are frequently needed for the production of adequate amount of analyte. Certain multi-step methods dealing with larger volumes could generate the needed amounts although they sometimes are consumptive thus their automation could help to decrease their labor intensities, but the conditions are not available in every labs. Separation of the proteins in the widest possible molecular weight range (unlike e.g. two-dimensional gelelectrophoresis) would also be favorable.

Acknowledgement

The financial support of the K-81839 OTKA grant of the Hungarian Scientific Research Fund is greatly appreciated. The publication is also supported by the TÁMOP-4.2.2/B-10/1-2010-0024 project.

References

[1] Anderson, N.L.; Anderson, N.G., The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics*, **2002**, *1*, (11), 845-867.
[2] Burnouf, T., Modern plasma fractionation. *Transfus. Med. Rev.*, **2007**, *21*, (2), 101-117.
[3] Denizli, A., Plasma fractionation: conventional and chromatographic methods for albumin purification. *Hacettepe J. Biol. Chem.*, **2011**, *39*, (4), 315-341.
[4] Kunz, W.; Henle, J.; Ninham, B.W., 'Zur Lehre von der Wirkung der Salze' (about the science of the effect of salts): Franz Hofmeister's historical papers. *Curr. Opin. Colloid Interface Sci.*, **2004**, *9*, (1-2), 19-37.
[5] Cohn, E.J.; Strong, L.E.; Hughes, W.L., Jr.; Mulford, D.J.; Ashworth, J.N.; Melin, M.; Taylor, H.L., Preparation and properties of serum and plasma proteins; a system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.*, **1946**, *68*, 459-475.
[6] van Oss, C.J., On the mechanism of the cold ethanol precipitation method of plasma protein fractionation. *J. Protein Chem.*, **1989**, *8*, (5), 661-668.
[7] Kistler, P.; Nitschmann, H., Large scale production of human plasma fractions. Eight years experience with the alcohol fractionation procedure of Nitschmann, Kistler and Lergier. *Vox Sang.*, **1962**, *7*, 414-424.
[8] Matejtschuk, P.; Dash, C.H.; Gascoigne, E.W., Production of human albumin solution: a continually developing colloid. *Br. J. Anaesth.*, **2000**, *85*, (6), 887-895.
[9] de Villiers, V.; Wilson, J.G.S., Ethanol and heath-treated plasma fractionation methods in South Africa. *Vox Sang.*, **1978**, *35*, (6), 405-411.
[10] Kekwick, R.A.; Mackay, M.E.; Nance, M.H.; Record, B.R., The purification of human fibrinogen. *Biochem. J.*, **1955**, *60*, (4), 671-683.
[11] Hořejší, J.; Smetana, R., The isolation of gamma globulin from blood-serum by rivanol. *Acta Med. Scand.*, **1956**, *155*, (1), 65-70.

[12] Polson, A.; Ruiz-Bravo, C., Fractionation of plasma with polyethylene glycol. *Vox Sang.*, **1972**, *23*, (1), 107-118.
[13] Atha, D.H.; Ingham, K.C., Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. *J. Biol. Chem.*, **1981**, *256*, (23), 12108-12117.
[14] Schneider, W.; Lefèvre, H.; Fiedler, H.; McCarty, L.J., An alternative method of large scale plasma fractionation for the isolation of serum albumin. *Blut*, **1975**, *30*, (2), 121-134.
[15] Burnouf, T., Chromatography in plasma fractionation: benefits and future trends. *J. Chromatogr. B Biomed. Appl.*, **1995**, *664*, (1), 3-15.
[16] Vasileva, R.; Jakab, M.; Haskó, F., Application of ion-exchange chromatography for the production of human albumin. *J. Chromatogr.*, **1981**, *216*, 279-284.
[17] Tanaka, K.; Sawatani, E.; Shigueoka, E.M.; Campos, T.C.X.B.; Nakao, H.C.; Dias, G.A.; Fujita, R.K.; Arashiro, F., A chromatographic method for the production of a human immunoglobulin G solution for intravenous use. *Braz. J. Med. Biol. Res.*, **1998**, *31*, (11), 1375-1381.
[18] Kumpalume, P.; Podmore, A.; LePage, C.; Dalton, J., New process for the manufacture of alpha-1 antitrypsin. *J. Chromatogr. A*, **2007**, *1148*, (1), 31-37.
[19] te Booy, M.P.W.M.; Faber, A.; de Jonge, E.; Wolterink, E.P.; Riethorst, W.; Beugeling, T.; Bantjes, A.; Over, J.; König, B.W., Large-scale purification of factor VIII by affinity chromatography: optimization of process parameters. *J. Chromatogr.*, **1990**, *503*, (1), 103-114.
[20] Federici, A.B., The factor VIII/von Willebrand factor complex: basic and clinical issues. *Haematologica*, **2003**, *88*, (6), EREP02.
[21] Lewis, S.J.; Stephens, E.; Florou, G.; Macartney, N.J.; Hathaway, L.S.; Knipping, J.; Collins, P.W., Measurement of global haemostasis in severe haemophilia A following factor VIII infusion. *Br. J. Haematol.*, **2007**, *138*, (6), 775-782.
[22] Josic, D.; Schwinn, H.; Stadler, M.; Strancar, A., Purification of factor VIII and von Willebrand factor from human plasma by anion-exchange chromatography. *J. Chromatogr. B Biomed. Appl.*, **1994**, *662*, (2), 181-190.
[23] Cheng, E.; Jinzenji, D.; Lorthiois, A.P.A.A.; de Carvalho, R.R.; Tanaka-Azevedo, A.M.; Raw, I.; Martins, E.A.L., Purification of coagulation factor VIII using chromatographic methods. Direct chromatography of plasma in anion exchange resins. *Biotechnol. Lett.*, **2010**, *32*, (9), 1207-1214.
[24] Josic, D.; Bal, F.; Schwinn, H., Isolation of plasma proteins from the clotting cascade by heparin affinity chromatography. *J. Chromatogr.*, **1993**, *632*, (1-2), 1-10.
[25] Hoffer, L.; Schwinn, H.; Josić, D., Production of highly purified clotting factor IX by a combination of different chromatographic methods. *J. Chromatogr. A*, **1999**, *844*, (1-2), 119-128.
[26] Taylor, G.A.; Mazhindu, H.N.; Findlay, J.B.C.; Peacock, M., Purification of vitamin D binding protein from human plasma using high performance liquid chromatography. *Clin. Chim. Acta*, **1986**, *155*, (1), 31-41.
[27] Ernst-Cabrera, K.; Wilchek, M., High-performance affinity-chromatography. *Trends Anal. Chem.*, **1988**, *7*, (2), 58-63.
[28] Moritz, R.L.; Skandarajah, A.R.; Ji, H.; Simpson, R.J., Proteomic analysis of colorectal cancer: prefractionation strategies using two-dimensional free-flow electrophoresis. *Comp. Funct. Genomics*, **2005**, *6*, (4), 236-243.

[29] Nilsson, T.; Mann, M.; Aebersold, R.; Yates, J.R.I.; Bairoch, A.; Bergeron, J.J.M., Mass spectrometry in high-throughput proteomics: ready for the big time. *Nat. Methods*, **2010**, *7*, (9), 681-685.
[30] Walsh, G.M.; Rogalski, J.C.; Klockenbusch, C.; Kast, J., Mass spectrometry-based proteomics in biomedical research: emerging technologies and future strategies. *Expert Rev. Mol. Med.*, **2010**, *12*, e30.
[31] Wu, S.L.; Kim, J.; Hancock, W.S.; Karger, B., Extended Range Proteomic Analysis (ERPA): a new and sensitive LC-MS platform for high sequence coverage of complex proteins with extensive post-translational modifications-comprehensive analysis of beta-casein and epidermal growth factor receptor (EGFR). *J. Proteome Res.*, **2005**, *4*, (4), 1155-1170.
[32] Wu, C.; Tran, J.C.; Zamdborg, L.; Durbin, K.R.; Li, M.; Ahlf, D.R.; Early, B.P.; Thomas, P.M.; Sweedler, J.V.; Kelleher, N.L., A protease for 'middle-down' proteomics. *Nat. Methods*, **2012**, *9*, (8), 822-824.
[33] De Bock, M.; de Seny, D.; Meuwis, M.A.; Servais, A.C.; Minh, T.Q.; Closset, J.; Chapelle, J.P.; Louis, E.; Malaise, M.; Merville, M.P.; Fillet, M., Comparison of three methods for fractionation and enrichment of low molecular weight proteins for SELDI-TOF-MS differential analysis. *Talanta*, **2010**, *82*, (1), 245-254.
[34] Selvaraju, S.; El Rassi, Z., Liquid-phase-based separation systems for depletion, prefractionation and enrichment of proteins in biological fluids and matrices for in-depth proteomics analysis--an update covering the period 2008-2011. *Electrophoresis*, **2012**, *33*, (1), 74-88.
[35] Delahunty, C.M.; Yates, J.R.I., MudPIT: multidimensional protein identification technology. *Biotechniques*, **2007**, *43*, (5), 563, 565, 567.
[36] Tang, J.; Gao, M.; Deng, C.; Zhang, X., Recent development of multi-dimensional chromatography strategies in proteome research. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **2008**, *866*, (1-2), 123-132.
[37] Travis, J.; Bowen, J.; Tewksbury, D.; Johnson, D.; Pannell, R., Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochem. J.*, **1976**, *157*, (2), 301-306.
[38] Gianazza, E.; Arnaud, P., A general method for fractionation of plasma proteins. Dye-ligand affinity chromatography on immobilized Cibacron blue F3-GA. *Biochem. J.*, **1982**, *201*, (1), 129-136.
[39] Subramanian, S., Dye-ligand affinity chromatography: the interaction of Cibacron Blue F3GA with proteins and enzymes. *CRC Crit. Rev. Biochem.*, **1984**, *16*, (2), 169-205.
[40] Gong, Y.; Li, X.; Yang, B.; Ying, W.; Li, D.; Zhang, Y.; Dai, S.; Cai, Y.; Wang, J.; He, F.; Qian, X., Different immunoaffinity fractionation strategies to characterize the human plasma proteome. *J. Proteome Res.*, **2006**, *5*, (6), 1379-1387.
[41] Eriksson, C.; Schwenk, J.M.; Sjoberg, A.; Hober, S., Affibody molecule-mediated depletion of HSA and IgG using different buffer compositions: a 15 min protocol for parallel processing of 1-48 samples. *Biotechnol. Appl. Biochem.*, **2010**, *56*, (2), 49-57.
[42] Tam, S.W.; Pirro, J.; Hinerfeld, D., Depletion and fractionation technologies in plasma proteomic analysis. *Expert Rev. Proteomics*, **2004**, *1*, (4), 411-420.
[43] Hinerfeld, D.; Innamorati, D.; Pirro, J.; Tam, S.W., Serum/Plasma depletion with chicken immunoglobulin Y antibodies for proteomic analysis from multiple Mammalian species. *J. Biomol. Tech.*, **2004**, *15*, (3), 184-190.

[44] Akgöl, S.; Tuzmen, N.; Denizli, A., Porous dye affinity beads for albumin separation from human plasma. *J. Appl. Polym. Sci.*, **2007**, *105*, (3), 1251-1260.
[45] Porath, J.; Maisano, F.; Belew, M., Thiophilic adsorption--a new method for protein fractionation. *FEBS Lett.*, **1985**, *185*, (2), 306-310.
[46] Hutchens, T.W.; Porath, J., Thiophilic adsorption of immunoglobulins--analysis of conditions optimal for selective immobilization and purification. *Anal. Biochem.*, **1986**, *159*, (1), 217-226.
[47] Kovács, A.; Sperling, E.; Lázár, J.; Balogh, A.; Kádas, J.; Szekrényes, Á.; Takács, L.; Kurucz, I.; Guttman, A., Fractionation of the human plasma proteome for monoclonal antibody proteomics-based biomarker discovery. *Electrophoresis*, **2011**, *32*, (15), 1916-1925.
[48] Coffinier, Y.; Legallais, C.; Vijayalakshmi, M.A., Separation of IgG from human plasma using thiophilic hollow fiber membranes. *J. Membrane Sci.*, **2002**, *208*, (1-2), 13-22.
[49] Sjöquist, J.; Meloun, B.; Hjelm, H., Protein A isolated from Staphylococcus aureus after digestion with lysostaphin. *Eur. J. Biochem.*, **1972**, *29*, (3), 572-578.
[50] Björck, L.; Kronvall, G., Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J. Immunol.*, **1984**, *133*, (2), 969-974.
[51] Housden, N.G.; Harrison, S.; Roberts, S.E.; Beckingham, J.A.; Graille, M.; Stura, E.; Gore, M.G., Immunoglobulin-binding domains: Protein L from Peptostreptococcus magnus. *Biochem. Soc. Trans.*, **2003**, *31*, (Pt 3), 716-718.
[52] Greenough, C.; Jenkins, R.E.; Kitteringham, N.R.; Pirmohamed, M.; Park, B.K.; Pennington, S.R., A method for the rapid depletion of albumin and immunoglobulin from human plasma. *Proteomics*, **2004**, *4*, (10), 3107-3111.
[53] Kabir, S., Immunoglobulin purification by affinity chromatography using protein A mimetic ligands prepared by combinatorial chemical synthesis. *Immunol. Invest.*, **2002**, *31*, (3-4), 263-278.
[54] Liu, Y.; Zhao, R.; Shangguan, D.; Zhang, H.; Liu, G., Novel sulfamethazine ligand used for one-step purification of immunoglobulin G from human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **2003**, *792*, (2), 177-185.
[55] Özkara, S.; Yavuz, H.; Denizli, A., Purification of immunoglobulin G from human plasma by metal-chelate affinity chromatography. *J. Appl. Polym. Sci.*, **2003**, *89*, (6), 1567-1572.
[56] Pernemalm, M.; Lewensohn, R.; Lehtio, J., Affinity prefractionation for MS-based plasma proteomics. *Proteomics*, **2009**, *9*, (6), 1420-1427.
[57] Zolotarjova, N.; Mrozinski, P.; Chen, H.; Martosella, J., Combination of affinity depletion of abundant proteins and reversed-phase fractionation in proteomic analysis of human plasma/serum. *J. Chromatogr. A*, **2008**, *1189*, (1-2), 332-338.
[58] Tu, C.; Rudnick, P.A.; Martinez, M.Y.; Cheek, K.L.; Stein, S.E.; Slebos, R.J.; Liebler, D.C., Depletion of abundant plasma proteins and limitations of plasma proteomics. *J. Proteome Res.*, **2010**, *9*, (10), 4982-4991.
[59] Echan, L.A.; Tang, H.Y.; Ali-Khan, N.; Lee, K.; Speicher, D.W., Depletion of multiple high-abundance proteins improves protein profiling capacities of human serum and plasma. *Proteomics*, **2005**, *5*, (13), 3292-3303.
[60] Huang, L.; Harvie, G.; Feitelson, J.S.; Gramatikoff, K.; Herold, D.A.; Allen, D.L.; Amunngama, R.; Hagler, R.A.; Pisano, M.R.; Zhang, W.W.; Fang, X., Immunoaffinity separation of plasma proteins by IgY microbeads: meeting the needs of proteomic sample preparation and analysis. *Proteomics*, **2005**, *5*, (13), 3314-3328.

[61] Boschetti, E.; Righetti, P.G., Hexapeptide combinatorial ligand libraries: the march for the detection of the low-abundance proteome continues. *Biotechniques,* **2008**, *44*, (5), 663-665.
[62] Csanky, E.; Olivova, P.; Rajnavolgyi, E.; Hempel, W.; Tardieu, N.; Elesne Toth, K.; Jullien, A.; Malderez-Bloes, C.; Kuras, M.; Duval, M.X.; Nagy, L.; Scholtz, B.; Hancock, W.; Karger, B.; Guttman, A.; Takacs, L., Monoclonal antibody proteomics: discovery and prevalidation of chronic obstructive pulmonary disease biomarkers in a single step. *Electrophoresis,* **2007**, *28*, (23), 4401-4406.
[63] Medvedev, A.; Kopylov, A.; Buneeva, O.; Zgoda, V.; Archakov, A., Affinity-based proteomic profiling: problems and achievements. *Proteomics*, **2012**, *12*, (4-5), 621-637.
[64] Kobata, A.; Endo, T., Immobilized lectin columns: useful tools for the fractionation and structural analysis of oligosaccharides. *J. Chromatogr.*, **1992**, *597*, (1-2), 111-122.
[65] Hageman, J.H.; Kuehn, G.D., Boronic Acid matrices for the affinity purification of glycoproteins and enzymes. *Methods Mol. Biol.*, **1992**, *11*, 45-71.
[66] Dayarathna, M.K.D.R.; Hancock, W.S.; Hincapie, M., A two step fractionation approach for plasma proteomics using immunodepletion of abundant proteins and multi-lectin affinity chromatography: Application to the analysis of obesity, diabetes, and hypertension diseases. *J. Sep. Sci.,* **2008**, *31*, (6-7), 1156-1166.
[67] Kullolli, M.; Hancock, W.S.; Hincapie, M., Automated platform for fractionation of human plasma glycoproteome in clinical proteomics. *Anal. Chem.,* **2010**, *82*, (1), 115-120.
[68] Monzo, A.; Bonn, G.K.; Guttman, A., Boronic acid-lectin affinity chromatography. 1. Simultaneous glycoprotein binding with selective or combined elution. *Anal. Bioanal. Chem.,* **2007**, *389*, (7-8), 2097-2102.
[69] Monzo, A.; Olajos, M.; De Benedictis, L.; Rivera, Z.; Bonn, G.K.; Guttman, A., Boronic acid lectin affinity chromatography (BLAC). 2. Affinity micropartitioning-mediated comparative glycosylation profiling. *Anal. Bioanal. Chem.,* **2008**, *392*, (1-2), 195-201.
[70] Petric, T.C.; Brne, P.; Gabor, B.; Govednik, L.; Barut, M.; Strancar, A.; Kralj, L.Z., Anion-exchange chromatography using short monolithic columns as a complementary technique for human serum albumin depletion prior to human plasma proteome analysis. *J. Pharm. Biomed. Anal.,* **2007**, *43*, (1), 243-249.
[71] Habeeb, A.F.S.A.; Francis, R.D., Preparation of human immunoglobulin by ammonium sulfate precipitation. *Vox Sang.,* **1976**, *31*, (6), 423-434.
[72] Mahn, A.; Reyes, A.; Zamorano, M.; Cifuentes, W.; Ismail, M., Depletion of highly abundant proteins in blood plasma by hydrophobic interaction chromatography for proteomic analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.,* **2010**, *878*, (15-16), 1038-1044.
[73] Mahn, A.; Ismail, M., Depletion of highly abundant proteins in blood plasma by ammonium sulfate precipitation for 2D-PAGE analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.,* **2011**, *879*, (30), 3645-3648.
[74] Brgles, M.; Clifton, J.; Walsh, R.; Huang, F.; Rucevic, M.; Cao, L.; Hixson, D.; Müller, E.; Josic, D., Selectivity of monolithic supports under overloading conditions and their use for separation of human plasma and isolation of low abundance proteins. *J. Chromatogr. A,* **2011**, *1218*, (17), 2389-2395.

[75] Kovács, A.; Patai, Z.; Guttman, A.; Kádas, J.; Takács, L.; Kurucz, I., Fractionation of the human plasma proteome for monoclonal antibody proteomics-based biomarker discovery 2: Antigen identification by dot blot array screening. Manuscipt in preparation.
[76] Jin, W.-H.; Dai, J.; Li, S.J.; Xia, Q.C.; Zou, H.F.; Zeng, R., Human plasma proteome analysis by multidimensional chromatography prefractionation and linear ion trap mass spectrometry identification. *J. Proteome Res.*, **2005**, *4*, (2), 613-619.
[77] Levreri, I.; Musante, L.; Petretto, A.; Bruschi, M.; Candiano, G.; Melioli, G., Separation of human serum proteins using the Beckman-Coulter PF2D system: analysis of ion exchange-based first dimension chromatography. *Clin. Chem. Lab. Med.*, **2005**, *43*, (12), 1327-1333.
[78] Wang, L.; Zhong, N., Application of the ProteomeLab (TM) PF2D protein fractionation system in proteomic analysis for human genetic diseases. *Cent. Eur. J. Chem.*, **2012**, *10*, (3), 836-843.
[79] Liu, X.; Valentine, S.J.; Plasencia, M.D.; Trimpin, S.; Naylor, S.; Clemmer, D.E., Mapping the human plasma proteome by SCX-LC-IMS-MS. *J. Am. Soc. Mass Spectrom.*, **2007**, *18*, (7), 1249-1264.
[80] Wang, H.; Clouthier, S.G.; Galchev, V.; Misek, D.E.; Duffner, U.; Min, C.K.; Zhao, R.; Tra, J.; Omenn, G.S.; Ferrara, J.L.M.; Hanash, S.M., Intact-protein-based high-resolution three-dimensional quantitative analysis system for proteome profiling of biological fluids. *Mol. Cell. Proteomics*, **2005**, *4*, (5), 618-625.
[81] Jiang, L.; He, L.; Fountoulakis, M., Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A*, **2004**, *1023*, (2), 317-320.