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Depletion of highly abundant proteins in blood plasma by hydrophobic interaction chromatography for proteomic analysis

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ABSTRACT

The proteomic analysis of plasma is extremely complex due to the presence of few highly abundant proteins. These proteins have to be depleted in order to detect low abundance proteins, which are likely to be of biomedical interest. In this work it was investigated the applicability of hydrophobic interaction chromatography (HIC) as a plasma fractionation method prior to two-dimensional gel electrophoresis (2DGE). The average hydrophobicity of the 56 main plasma proteins was calculated. Plasma proteins were classified as low, medium and highly hydrophobic through a cluster analysis. The highly abundant proteins showed a medium hydrophobicity, and therefore a HIC step was designed to deplete them from plasma. HIC performance was assessed by 2DGE, and it was compared to that obtained by a commercial immuno-affinity (IA) column for albumin depletion. Both methods showed similar reproducibility. HIC allowed partially depleting α-1-antitrypsin and albumin, and permitted to detect twice the number of spots than IA. Since albumin depletion by HIC was incomplete, it should be further optimized for its use as a complementary or alternative method to IA.

1. Introduction

Proteomics is a fast growing field, relevant in the task of discovering new biomarkers that reflect the health/disease status of living organisms. The development of this field has been limited due to technical hurdles related to the high complexity of the biological samples to be analyzed, usually blood serum or plasma. These samples show a wide dynamic range of protein concentration, exceeding 10^{10}. Two-dimensional gel electrophoresis (2DGE) can resolve a range of up to 10^4, then 2DGE maps of blood plasma are dominated by the highly abundant proteins, and do not allow detecting low abundance proteins [1]. Mass spectrometry can resolve a range of 10^3 in a single spectrum, but combined with separation steps it can resolve a range of 10^4–10^6 [2]. This range is still wide, and thus many proteins cannot be detected. Then, fractionation steps should be used before 2DGE in order to reduce the dynamic range of proteins concentration.

The most abundant proteins in blood plasma are serum albumin, immunoglobulin, serotransferrin, haptoglobin, fibrinogen and α-1-antitrypsin, which amount to approximately 90% of total protein mass [3]. Different methods can be used to deplete these proteins in plasma samples, being liquid chromatography the most popular one [4–7]. A variety of stationary phases are available for this purpose, including dye-based resins for albumin depletion, protein A and G for immunoglobulin depletion, and specific antibody-affinity columns [3,8]. Besides, other affinity columns containing lectins, peptides or inorganic ligands have been used for this purpose [9]. These methods are easy to use and to scale-up, but are relatively expensive. Additionally, they have some disadvantages, such as non-specific interactions that lead to the loss of some proteins [10,11].

Several complementary strategies have been tested for fractionating plasma samples [8,12], such as sequential anion and cation exchange chromatography followed by 2DGE [4,13]; and strong cation exchange chromatography followed by liquid-phase isoelectric focusing [5]. These approaches considerably improve the capacity to detect low abundance proteins. Then, the optimization of combinatorial processes by coupling immuno-affinity depletion with other conventional separation methods such as ion exchange or hydrophobic interaction chromatography will probably lead to significant advances in proteomics.

The applicability of hydrophobic interaction chromatography (HIC) as a plasma fractionation method has been poorly studied. Geng et al. [14] developed a two-dimensional liquid chromatography column that functions in two retention modes: cation exchange and hydrophobic interaction. The authors propose that this method could be applied to the fast fractionation of intact proteins before

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mass spectrometry analysis. Liu et al. [15] used HIC followed by size exclusion chromatography for purifying protein complexes for the analysis of protein interacting networks. The results obtained by HIC were similar to those obtained by ion exchange chromatography. On the other hand, a HIC matrix consisting of highly acetylated agarose has been used for the isolation of immunoglobulin from porcine serum, with a relative success [16].

The aim of this work was to investigate if fractionating blood plasma by HIC could improve the performance of 2DGE, by reducing the relative concentration of some highly abundant proteins in plasma. First, the hydrophobicity of the main plasma proteins was determined. Then, a cluster analysis was performed in order to classify them as low, medium or high hydrophobicity proteins. A HIC step was designed to deplete highly abundant proteins from rat plasma samples. Finally, the depleted samples were analyzed by 2DGE and the performance of the HIC method was compared with that of a commercial immuno-affinity column.

2. Experimental

2.1. Hydrophobicity of the plasma proteins

The main proteins found in blood plasma were characterized based on their amino acid composition. The amino acid sequences were retrieved from the blood plasma map available in the two-dimensional polyacrylamide gel electrophoresis database (Swiss-2DPAGE, http://www.expasy.ch/ch2d/). The average hydrophobicity (AH) was calculated based on the method proposed by Salgado et al. [17] by means of Eq. (1):

$$\mathrm{AH} = \sum_{i=1}^{20} \left(\phi_i \frac{n_i s_{i,\max}}{\sum_{j \in A} n_j s_{j,\max}} \right) \quad (1)$$

Here AH is the average hydrophobicity for a given protein, ϕ_i is the hydrophobicity value assigned to amino acid i by the Cowan–Whittaker hydrophobicity scale [18] normalized to take values ranging from 0 to 1 [19], n_i is the number of amino acids of type i in the protein, $s_{i,\max}$ is the maximum solvent accessible area that an amino acid X can have when forming part of the G–X–G tripeptide in extended conformation [20], and i (i = 1, ..., 20) indicates the different standard amino acids. Finally, a cluster analysis was performed in order to classify the plasma proteins in low, medium or high hydrophobicity ones.

2.2. Preparation of the plasma samples

Six Wistar rats, 21 days old, were fed a Torula yeast-based diet (Dyets Inc., Bethlehem, USA) during 10 weeks. Animals were maintained at 20 °C, 12 h/12 h day/night cycles in stainless steel cages with free access to deionised water and the diet. After the breeding period, blood from each one of the rats was collected by cardiac puncture, using standard protocols [21]. Collection was made in heparin tubes (BD Biosciences, USA). The tubes were inverted up and down 10 times and immersed in an ice bath. The tubes were centrifuged at 1300 × *g* for 10 min at 4 °C. Plasma was separated from erythrocytes and buffy coat. Supernatant was transferred to a new centrifuge tube and centrifuged at 2400 × *g* for 15 min at 4 °C, in order to discard micro platelets. Plasma samples were kept at −80 °C until analysis.

2.3. Depletion by immuno-affinity column

Plasma samples collected from the six rats were pooled, in order to normalize the variations among the animals. Three 25 μL samples of the pooled plasma were albumin depleted by means of the Qproteome™ Murine Albumin Depletion Kit (QIAGEN GmbH, Germany), following the instructions of the manufacturer. Protein concentration in the protein solutions was determined according to the Bradford method using BSA as protein standard. The albumin-depleted protein solutions were freeze-dried and kept at −20 °C until electrophoresis analysis.

2.4. Hydrophobic interaction chromatography

Lab-made HIC columns were prepared by filling a 1-mL syringe (stoppered with glass wool) with 200 μL slurry of Phenyl Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden), allowing settling to the glass wool at the bottom. A final bed size of 100 μL was achieved. The column was drained and equilibrated with Tris–HCl 20 mM pH 7.0 added with 2 M $(NH_4)_2SO_4$ (loading buffer). The salt concentration necessary for eluting high, medium or low hydrophobicity proteins was determined experimentally. For this, a representative protein belonging to each cluster was chosen: α-1-antitrypsin (high hydrophobicity), serum albumin (medium hydrophobicity), and fibrinogen (low hydrophobicity). The pure proteins were purchased from Sigma Co. (St. Louis, MO, USA). After equilibration with loading buffer, 50 μL of a solution containing 1 mg/mL pure protein was loaded. The column was washed five times with 1 mL loading buffer, and then gradient elution was performed, using a steepness of −0.2 M/mL. 0.5 mL fractions were collected and A_{280} was registered.

Once the salt concentration of the three elution buffers was determined, pooled plasma samples were fractionated by HIC. After equilibration, 125 μL of plasma (containing approximately 4.5 mg protein) diluted 4-fold in buffer Tris–HCl 20 mM pH 7.0 added with 2.4 M $(NH_4)_2SO_4$ was loaded. Then the column was washed five times with 1 mL loading buffer. Elution was performed stepwise, using a different ionic strength for eluting each group of proteins (high, medium or low hydrophobicity). In this way, three elution fractions were obtained.

The HIC fractions were subjected to dialysis against Tris–HCl 20 mM pH 7.0 overnight, and concentrated by acetone precipitation [22]. Pellets were air dried and kept at −20 °C until electrophoresis analysis.

2.5. Two-dimensional gel electrophoresis

Proteins in the depleted and untreated plasma samples were analyzed by 2DGE using the method of Toledo et al. [23] that is based on that described by O'Farrell [24]. Briefly, 300 μg of protein were resuspended in 50 μL of lysis buffer (9.5 M urea, 2% Triton X-100, 1.6% ampholytes 4–7 range, 0.4% ampholytes 3–10 range, and 5% β-mercaptoethanol), incubated at room temperature for 15 min and loaded onto lab-made first dimension gels (115 mm height and 3 mm internal diameter capillary tubes). A 4.0–7.0 pH gradient was used. Gel prefocusing was carried out according to the following program: 200 V for 15 min, 300 V for 15 min and 400 V for 15 min. Isoelectric focusing (IEF) was performed at 400 V for 20 h, to complete 8000 V h. After IEF, the gels were extruded from the glass capillary tubes and equilibrated immediately in 2 mL of equilibration solution (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.0625 M Tris–HCl pH 6.8) for 10 min. A control first dimension gel was run in order to determine the resulting pH scale. Vertical SDS-PAGE was run with lab-made homogeneous acrylamide gel (11.5% acrylamide; 180 mm height and 120 mm wide), at a constant voltage of 50 V during 16 h. In some gels, a molecular weight marker was loaded in order to determine the resulting MW scale. Gels were soaked in a solution of 25% methanol and 7.5% acetic acid for 30 min, stained in Coomassie Brilliant Blue R-250 for 12 h (0.1% Coomassie Blue R-250, 25% methanol, 7.5% acetic acid) and destained in a solution of 25% methanol and 7.5% acetic acid. Coomassie staining is the most reliable quantitative protein staining method and it is widely

Table 1
Cluster analysis of plasma proteins based on their hydrophobicity.

Cluster 1: high hydrophobicity[a]	Cluster 2: medium hydrophobicity[a]	Cluster 3: low hydrophobicity[a]
α-1-Antitrypsin (0.588)	Prothrombin (0.569)	Plasminogen (0.553)
α-1-Antichymosin (0.610)	Haptoglobin (0.566)	Kininogen-1 (0.529)
Angiotensin (0.617)	Complement factor B (0.564)	Apolipoprotein A-I (0.546)
α-2-Macroglobulin (0.589)	Immunoglobulin J (0.573)	Apolipoprotein E (0.552)
Antithrombin III (0.588)	Fibrinogen γ (0.569)	Fibrinogen α (0.537)
Apolipoprotein A-II (0.596)	Serum albumin (0.561)	Fibrinogen β (0.550)
Apolipoprotein C-II (0.615)	Vitamin D-binding protein (0.572)	Histidine-rich glycoprotein (0.500)
Apolipoprotein C-III (0.596)	Serotransferrin (0.567)	Apolipoprotein A-IV (0.531)
C-reactive protein (0.627)	Immunoglobulin heavy chain γ-2 (0.571)	Platelet basic protein (0.552)
Serum amyloid P-component (0.626)	Immunoglobulin heavy chain γ-3 (0.570)	Clusterin (0.555)
Leucine-rich α-2-glycoprotein (0.615)	Immunoglobulin heavy chain γ-1 (0.566)	
Plasma retinol-binding protein (0.581)	Immunoglobulin δ (0.566)	
AMBP protein (0.592)	Immunoglobulin heavy chain μ (0.580)	
Transthyretin (0.600)	Ceruloplasmin (0.575)	
Hemopexin (0.591)	Complement C3 (0.581)	
α-1B-glycoprotein (0.603)	α-1-Acid glycoprotein-1 (0.582)	
Apolipoprotein δ (0.630)	α-1-Acid glycoprotein 2 (0.581)	
α-1-Antiplasmin (0.620)	Hemoglobin subunit α (0.574)	
Complement C1S	Complement factor I (0.574)	
Subcomponent (0.600)	Vitronectin (0.567)	
Complement c4-B (0.587)	Zinc-α-2-glycoprotein (0.575)	
β-Actin (0.598)		
Hemoglobin subunit β (0.588)		
Serum paraoxonase/arylesterase 1 (0.618)		
α-2-HS-glycoprotein (0.591)		
Immunoglobulin α chain C (0.597)		

[a] The average hydrophobicity of each protein (calculated by Eq. (1)) is given in brackets.

used in proteomics studies [25]. All chemicals were analytical grade and were purchased from Sigma Co. (St. Louis, MO, USA).

2.6. Image analysis

Image acquisition was performed with an ImageScanner II device (GE Healthcare, Uppsala, Sweden). Intensity calibration was carried out using an intensity step wedge prior to the image capture. The TotalLab™ v2.01 software (Nonlinear Dynamics Ltd., NC, USA) was used for image analysis. Spots were automatically detected and matched. Each spot volume was determined densitometrically and processed by background subtraction. Spot volumes of all gels were normalized by dividing the raw quantity of each spot by the total quantity of all valid spots in that gel, as recommended in literature [26,27]. Finally, gel images were manually annotated.

2.7. Protein identification

Most protein spots detected by 2DGE were identified through mass spectrometry. They were excised directly from the gels and analyzed by MALDI-TOF at the University of Colorado Health Sciences Centre (UCHSC), USA. Gel bands were cut into small pieces to enhance cleaning of bands and trypsin absorption. Bands were manually digested using the standard Proteomics Core protocol [28,29]. Samples were digested overnight with modified porcine trypsin at room temperature. Digest solution was spotted on a MALDI target with α-cyano-4-hydroxycinnamic acid for sample co-crystallization. Samples were analyzed using MALDI-TOF mass spectrometry in a Voyager DE-STR system (PerSeptive Biosystems Inc., MA, USA). Data was calibrated, deisotoped and centroided and a peak list was generated. Peak lists were searched using the MASCOT search engine (http://www.matrixscience.com) against the nrNCBI database v20070204/Rodent subset.

The other spots were identified by comparison with those shown in the human blood plasma map available in the two-dimensional polyacrylamide gel electrophoresis database (Swiss-2DPAGE, http://www.expasy.ch/ch2d/). A grid was built on the gel image using the pH and the molecular weight scales obtained experimentally by the 2DGE runs. The experimental isoelectric point and molecular weight of each spot to be identified by this way were used to locate the corresponding spot on the reference map. This identification strategy is frequently used in proteomics [30].

2.8. Statistical analyses

The cluster analysis was performed by using the k-means method and Euclidean distance. The data set was divided in 3 hydrophobicity clusters: high, medium and low.

The results of the 2DGE analysis were expressed as mean ± standard deviation of three replicates for each experimental run. Statistically significant differences between the depletion methods and with respect to the untreated plasma were determined by a Student's *t*-test at a 95% confidence level. Statgraphics™ Plus 5.1 (Statistical Graphics Corp., USA, 2000) was used in all statistical analyses.

3. Results and discussion

3.1. Characterization of the plasma proteins

The average hydrophobicity of the 56 main proteins found in blood plasma was determined based on their amino acid composition. The proteins were classified in three clusters: high (cluster 1), medium (cluster 2) and low (cluster 3) hydrophobicity. The results of the cluster analysis are shown in Table 1, as well as proteins hydrophobicity calculated by Eq. (1). The average hydrophobicity in cluster 1 was 0.603 ± 0.015, in cluster 2 was 0.572 ± 0.006, and in cluster 3 was 0.541 ± 0.017. Most proteins were classified as highly hydrophobic (25 proteins), while 21 proteins resulted of medium hydrophobicity, and only 10 were classified as lowly hydrophobic. The highly abundant proteins in plasma were classified mainly in cluster 2: albumin, Ig heavy chain γ-1, Ig heavy chain γ-2, Ig heavy chain γ-3, Ig δ, Ig heavy chain μ, serotransferrin, haptoglobin, and fibrinogen γ. This represents 70% of the proteins considered as highly abundant. Despite the high number of proteins

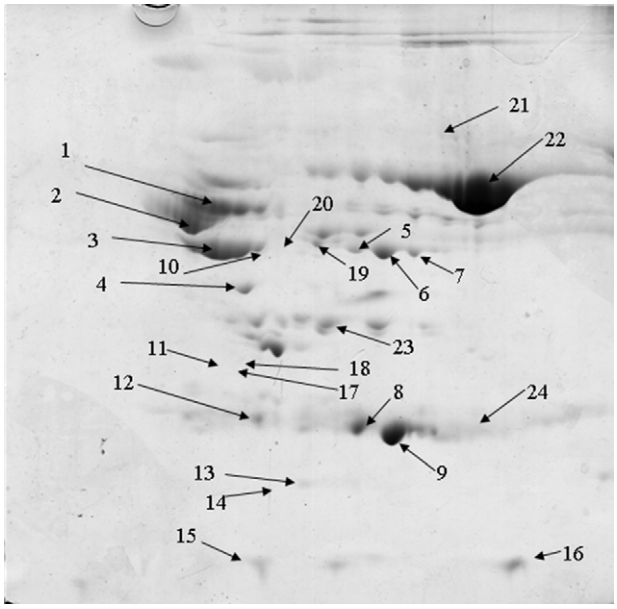

Fig. 1. Reference image of a two-dimensional electrophoresis gel of rat blood plasma. The highlighted spots were identified either by mass spectrometry or by comparison with a reference map. Protein identities are given in Table 2.

Table 2
Identities of the protein spots highlighted in Fig. 1.

Spot nr.	Protein	Identification method
1	Hepatic nuclear factor 6	Mass spectrometry
2	Contrapsin-like protease inhibitor	Mass spectrometry
3	α-1-Antitrypsin	Mass spectrometry
4	Apolipoprotein A-IV	Mass spectrometry
5	Fibrinogen	Mass spectrometry
6	Fibrinogen	Mass spectrometry
7	Fibrinogen	Mass spectrometry
8	Apolipoprotein A-I	Mass spectrometry
9	Apolipoprotein A-I	Mass spectrometry
10	α-1-Antitrypsin precursor	Mass spectrometry
11	Cyclin H	Mass spectrometry
12	Immunoglobulin light chain	Mass spectrometry
13	Apolipoprotein E	Mass spectrometry
14	Apolipoprotein E	Mass spectrometry
15	Haptoglobin	Mass spectrometry
16	Transthyretin	Mass spectrometry
17	Apolipoprotein A-IV	Mass spectrometry
18	Zinc finger protein 108	Mass spectrometry
19	α-1-Antitrypsin	Mass spectrometry
20	α-1-Antitrypsin	Mass spectrometry
21	Gelsolin	Mass spectrometry
22	Albumin	Swiss-2D database plasma map
23	Haptoglobin	Swiss-2D database plasma map
24	Immunoglobulin light chain	Swiss-2D database plasma map

classified as highly hydrophobic (cluster 1), it has to be noted that albumin represents 75% of total protein mass in blood plasma, and immunoglobulin amounts around 10%, both considered of medium hydrophobicity (cluster 2). Then, more than 85% of total protein mass in blood plasma corresponds to medium hydrophobicity proteins. However, considering the individual proteins, 37% of plasma proteins were classified in cluster 2; and of them, 38% corresponds to highly abundant proteins (9 proteins).

The highly abundant proteins exhibited similar average hydrophobicities, varying between 0.561 (albumin) and 0.588 (α-1-antitrypsin). This constitutes a relatively narrow range, considering that usually a protein hydrophobicity (calculated by Eq. (1)) can take values from 0.500 for a very hydrophilic protein (such as cytochrome c), up to 0.700 for a hydrophobic protein (such as membrane proteins). Since most highly abundant proteins show a similar hydrophobicity, it should be possible to use a HIC step to deplete a plasma sample not only from albumin, but also from immunoglobulin and other highly abundant proteins. Even further, other separation techniques that exploit hydrophobicity, such as aqueous two-phase systems or salt precipitation, could probably be useful to deplete plasma from these proteins. In this sense, it was reported that ammonium sulfate fractionation can efficiently remove albumin from human plasma samples [22].

Since the experimental methodology for 2DGE available at our laboratory differs from that used to build the 2DGE plasma map (http://www.expasy.ch/ch2d/), a reference image was constructed using the protocol described in Sections 2.5–2.7. This image is shown in Fig. 1, where protein spots identified by mass spectrometry are indicated, as well as those identified by comparison with databases. Table 2 shows the identity corresponding to each protein spot.

3.2. Plasma fractionation by hydrophobic interaction chromatography

Plasma samples were subjected to HIC with the aim to deplete some highly abundant proteins and thus to increase the relative abundance of less abundant plasmatic proteins. Elution was performed in three steps: (1) 0.6 M $(NH_4)_2SO_4$ elution buffer, since fibrinogen eluted with this salt concentration in the preliminary runs. This protein shows one of the highest hydrophobicities in cluster 3 (low hydrophobicity proteins); (2) 0.5 M $(NH_4)_2SO_4$ elution buffer, since serum albumin eluted at 0.55 M $(NH_4)_2SO_4$ (albumin shows the lowest hydrophobicity in cluster 2), and α-1-antitrypsin eluted at 0.45 M $(NH_4)_2SO_4$ (which shows the lowest hydrophobicity in cluster 1); (3) 0.0 M $(NH_4)_2SO_4$, to completely elute the highly hydrophobic proteins (cluster 1). Since the first and the last fractions were depleted from some highly abundant proteins (because the highly abundant proteins eluted in the second fraction), they were pooled and analyzed by 2DGE. In this way, HIC depleted plasma was analyzed in single 2DGEs, in triplicate.

In HIC runs, 85% total loaded protein was retained by the column (which corresponds to approximately 3.83 mg protein); fractions 1 and 3 (which were the depleted fractions) contained 31% of the retained protein (approximately 1.0–1.2 mg protein) and the discarded fraction (fraction 2), which contained some of the highly abundant proteins in blood plasma, had 68% of the retained protein (approximately 2.6 mg). The eluted fractions had a total protein concentration lower than that found in the loaded sample (approximately 9 mg/mL). However, for analysis purposes, this was not relevant because the fractions were subjected to acetone precipitation before 2DGE, and thus the dilution effect was cancelled.

Fig. 2 shows representative 2DGE images of untreated plasma (Fig. 2(a)), compared to immuno-affinity depleted (Fig. 2(b)) and HIC depleted (Fig. 2(c)) plasma. The zones where the highly abundant proteins are found are highlighted in Fig. 2(d)–(f). In these figures, zone 1 corresponds to albumin, zone 2 to α-1-antitrypsin, zone 3 to fibrinogen, zone 4 to haptoglobin, and zone 5 to immunoglobulin. Both depletion strategies accomplished a reduction in the relative abundance of albumin, as compared to the untreated plasma. Besides, it results apparent that the abundance of other highly abundant proteins was affected too. In addition, the total number of spots detected was also modified.

Table 3 shows the quantification of the highly abundant proteins after each treatment, as well as the statistical analysis. Since many spots in a specific zone corresponded to isoforms of one protein, the relative abundance of each protein was calculated considering the entire zone of the 2DGE where it was found (see Fig. 2(d)–(f)). The relative abundance of each protein was expressed as normalized volume (V_n), i.e. after background subtraction, the raw volume of

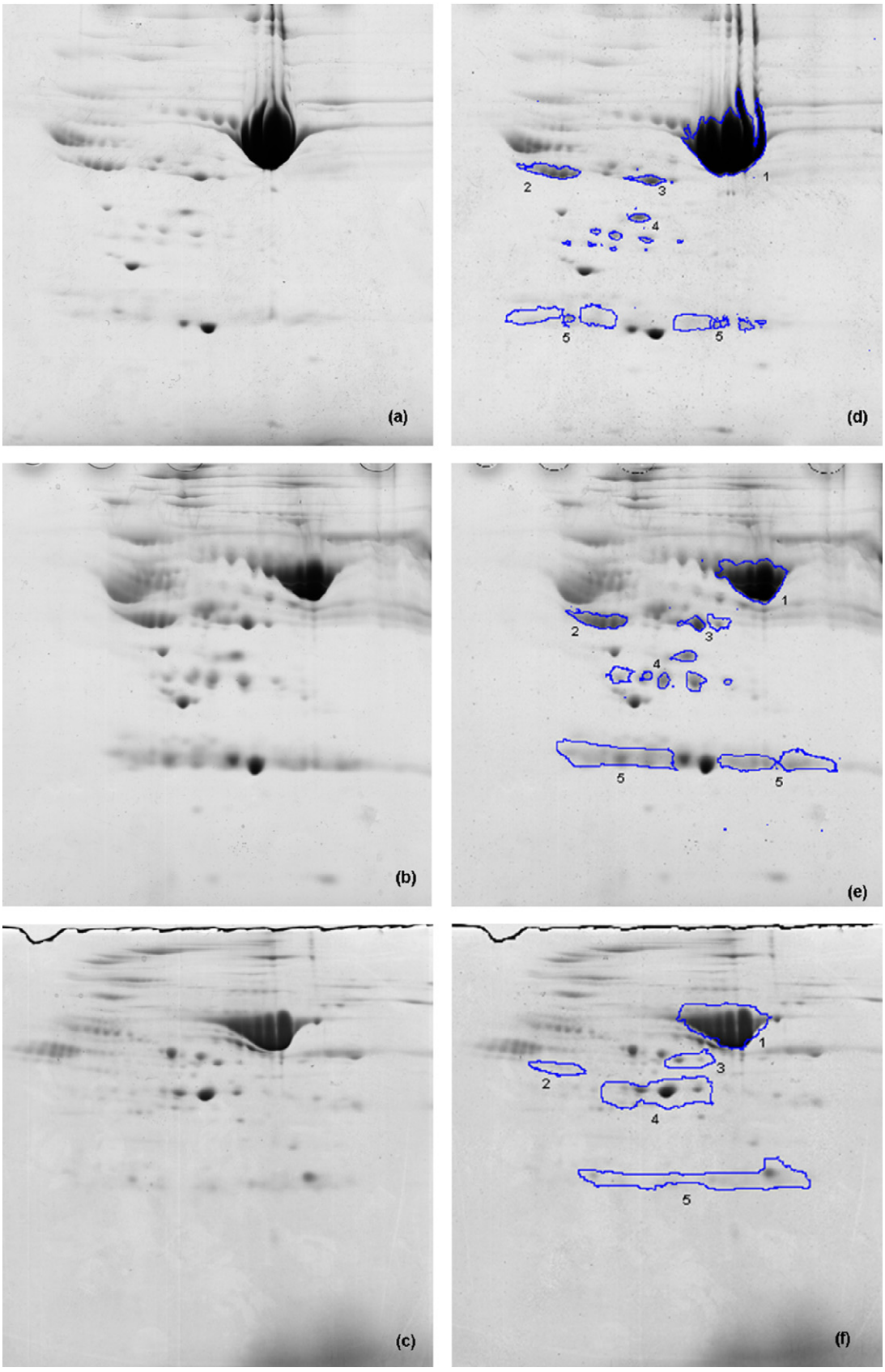

Fig. 2. Two-dimensional gel images of the untreated (a and d), immuno-affinity depleted (b and e) and hydrophobic interaction depleted (c and f) plasma. In the annotated images, the spots found in the five zones corresponding to the highly abundant proteins are highlighted: albumin (1), α-1-antitrypsin (2), fibrinogen-γ (3), haptoglobin (4), and immunoglobulin (5).

Table 3
Relative abundance of the highly abundant proteins using the different depletion treatments. Statistically significant differences are given in bold.

Protein	Untreated	Immuno-affinity depleted		Hydrophobic interaction depleted		Depletion methods[a]
	$V_n \pm SD$[b]	$V_n \pm SD$[b]	p-Value[c]	$V_n \pm SD$[b]	p-Value[c]	p-Value[c]
Albumin	7064 ± 266	3568 ± 117	**0.0034**	6187 ± 780	0.2714	**0.0420**
Immunoglobulin	215 ± 17	1699 ± 449	**0.0433**	463 ± 225	**0.0045**	**0.0429**
α-1-Antitrypsin	344 ± 65	635 ± 41	**0.0121**	62 ± 2	**0.0102**	**0.0026**
Haptoglobin	94 ± 13	363 ± 91	**0.0117**	175 ± 3	**0.0041**	0.0991
Fibrinogen	180 ± 4	278 ± 92	0.1359	156 ± 24	0.1528	0.2092

[a] Comparison between the two depletion methods.
[b] The relative abundance of the proteins was expressed as normalized spot volume (V_n). The reported values correspond to the average of three experiments. SD is the standard deviation.
[c] Statistically significant differences were determined by a Student's t-test with a 95% confidence level.

each spot in that zone were summed and then this number was divided by the sum of the raw volume of all the spots in that gel. In this way, the relatively low reproducibility of the technique is overcome, thus reducing the high variability usually found in 2DGE analysis [26,31].

Immuno-affinity depletion of albumin significantly reduced the relative abundance of that protein, but it significantly increased that of immunoglobulin, α-1-antitrypsin and haptoglobin, compared to the untreated plasma. Since these three are considered highly abundant proteins, it is desirable to keep their concentration as low as possible. In this way, the detection of lower abundance proteins, which are likely to be of biomedical interest, is facilitated. Besides, the abundance of fibrinogen-γ was also increased, but this change was not statistically significant (p-value > 0.05). Considerably better results were obtained by Linke et al. [3], who depleted seven highly abundant rat plasma proteins with an immuno-affinity column with coupled antibodies directed against albumin, IgG, transferrin, IgM, haptoglobin, fibrinogen and α-1-antitrypsin. It has to be noted that the use of antibodies increases considerably the process cost.

HIC depletion significantly reduced the relative abundance of α-1-antitrypsin, and it increased that of immunoglobulin and haptoglobin, compared to the untreated plasma. However, the relative abundance of immunoglobulin in the HIC depleted samples was significantly lower than that found in the immuno-affinity depleted plasma, resulting in a 3.7-fold reduction. A similar situation was observed for haptoglobin, whose abundance was considerably lowered (2-fold) in the HIC depleted plasma, as compared to the immuno-affinity depleted one.

Despite the relative abundances of albumin and fibrinogen-γ were also reduced, these changes were not significant, compared to the untreated plasma. The abundance of fibrinogen-γ was slightly lower than that found in the untreated plasma and 2-fold lower than that obtained in the albumin immuno-affinity depleted plasma. As a consequence, HIC allowed the partial depletion of albumin and α-1-antitrypsin, and a much lower increase in the relative abundance of immunoglobulin, haptoglobin and fibrinogen-γ, compared to the immuno-affinity column.

Fig. 3 shows a comparison between the immuno-affinity and the HIC depletion treatments, for the most abundant proteins in plasma. For comparison purposes, the relative abundance of the proteins was expressed as percentage with respect to the abundance (as normalized volume) found for each one in the untreated plasma. In this way, a 100% was assigned for every protein in the untreated plasma, and the respective abundance found in the plasma samples subjected to the different depletion treatments was expressed with respect to it. The relative abundance of immunoglobulin and haptoglobin were dramatically reduced when plasma samples were subjected to HIC fractionation, as compared to albumin immuno-affinity depletion. The abundance of immunoglobulin was lowered 3.7-fold, while that of haptoglobin was reduced 2-fold, when HIC was used. Additionally, the abundance of α-1-antitrypsin was reduced in the HIC depleted samples by 5-fold compared to the untreated plasma, and 10-fold compared to the immuno-affinity depleted samples. The effect of the treatments on albumin was not so dramatic; probably because this is by far the most abundant protein in plasma (~75% of total protein mass [22]). Then a relatively low percentage reduction may represent an important reduction in terms of mass. The depletion level of albumin reached by HIC was clearly lower than that obtained by means of the immuno-affinity column. This could be related to a competition between albumin and other proteins having a similar hydrophobicity, mainly immunoglobulin, α-1-antitrypsin and haptoglobin, thus leading to a partial saturation of the HIC resin. The maximum capacity of 100 μL HIC resin is 4.5 mg (45 mg/mL); besides it is estimated that 125 μL plasma contain a total of 4.4 mg protein, and then the column could have been near its saturation (considering that all protein was adsorbed to the resin). On the other hand, the capacity of the immuno-affinity column used in this study is 0.6 mg albumin/column. One column was used to deplete 25 μL plasma, as recommended by the manufacturer. Considering that albumin amounts approximately 75% of total protein mass, 25 μL plasma contains ~0.7 mg albumin, and then it was expected that an important amount of albumin still remained in the depleted plasma samples.

The performance of both depletion methods was also compared based on the total number of spots that could be detected in each

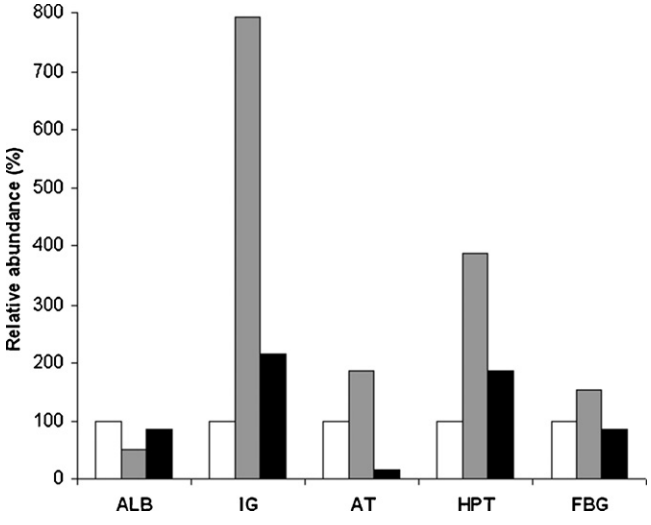

Fig. 3. Comparison of the depletion level achieved with the different treatments. The relative abundance of the highly abundant proteins is expressed as percentage with respect to the abundance (as normalized volume) found in the untreated plasma. White bars correspond to the untreated plasma, gray bars correspond to immuno-affinity depleted plasma, and black bars correspond to HIC depleted plasma. ALB, albumin; IG, immunoglobulin; AT, α-1-antitrypsin; HPT, haptoglobin; FBG, fibrinogen-γ.

case (data not shown). In the untreated plasma, an average of 113 spots was detected, while in the immuno-affinity depleted plasma this number was significantly reduced to approximately half (61 spots, p-value = 0.0287). Probably the missing spots correspond to different isoforms of albumin and other proteins dragged by it. The HIC depletion method allowed to detect 110 spots, a significantly higher number than in the immuno-affinity depleted plasma (p-value = 0.0497). Then, HIC was able to both reduce the relative abundance of some highly abundant proteins in blood plasma, as well as to increase the number of protein spots that were possible to detect, as compared to immuno-affinity column. The number of spots detected in the HIC depleted plasma equals that observed in the untreated plasma; however, in the first case the concentration of some of the most abundant proteins was considerably reduced. Since the most abundant proteins were depleted, the maintenance of the same number of spots necessarily occurred due to an increase in the relative concentration of some proteins found in relatively low concentrations in the untreated plasma. These low abundance proteins would probably be impossible to detect without subjecting plasma samples to depletion.

Linke et al. [3] reported that depleting the seven most abundant proteins in plasma by immuno-affinity did not increase significantly the number of spots that could be detected in 2DGE, compared to the untreated plasma. The HIC depletion method showed a similar performance. However, HIC increased in 80% the number of detected spots, compared to the albumin immuno-affinity depletion used in the present study.

4. Conclusions

The reproducibility of 2DGE from HIC depleted plasma was similar to that obtained from immuno-affinity depleted plasma. HIC exhibited some advantages since it allowed depleting albumin and α-1-antitrypsin, and additionally it resulted in a much lower increment of immunoglobulin and haptoglobin abundances than those obtained by the immuno-affinity column used in this study. In this way, HIC depletion allowed detecting twice the number of protein spots than immuno-affinity depletion did. For these reasons, HIC could be proposed as a depletion method complementary to affinity columns. However, the operating conditions in HIC should be optimized in order to maintain the high number of spots that are detected if HIC is used as the sole depletion method.

Finally, since HIC is relatively inexpensive, its use could be proposed as an economic alternative to affinity columns for depleting highly abundant proteins in plasma samples prior to 2DGE.

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