

## Impact of the depletion of high-abundance proteins in blood plasma/serum on the proteome of these media in growing farm animals

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**Summary:** The aim of the study was to use hexapeptide combinatorial libraries to evaluate the influence of high-abundance protein depletion on low-abundance protein enrichment, protein concentration, and 2D gel quality in plasma and serum proteomes of growing piglets and calves. Depleted and nondepleted samples of plasma/serum were resolved using 2D electrophoresis (2DE). Bioinformatic analysis of the 2D gels showed an increased number of detected spots on each gel after depletion of excess high-abundance proteins in calf plasma (+26.46%) and in piglet serum (+49.40%). Relative expression of selected low-abundance protein spots in the blood plasma/serum samples was higher after high-abundance protein depletion. Both media showed decreased expressions of high-abundance protein spot clusters on 2D gels after treatment. The results of this study confirm both the high efficiency of high-abundance protein depletion and the increase in concentrations of low-abundance proteins on 2D gels after treatment. The method of depletion is reproducible and it may be used as an alternative to immunodepletion for preparing blood plasma/serum samples from young, growing animals for 2DE. Increased content of the low-abundance proteins on the 2D gels enables complete analysis of plasma/serum proteome and thereby allows for the discovery of novel biomarkers that might be useful in veterinary medicine.

**Key words:** Proteome, high-abundance protein depletion, 2-dimensional electrophoresis, farm animals

### 1. Introduction

Both blood plasma and serum are very useful sources for proteomic analysis because they are easy to obtain from animals and they contain vast amounts of substances (e.g., proteins) that may be used as diagnostic indicators of various diseases (1). The circulatory system provides constant blood flow and metabolic exchange between the surrounding tissues, enabling the assessment of the metabolic state of the organism. Additionally, plasma/serum proteins and polypeptides undergo qualitative and quantitative changes in response to various processes (e.g., physiological, pathological), thereby reflecting the actual state of the organism (2,3).

Proteomic techniques are commonly applied for the analysis of plasma/serum protein composition. Two-dimensional electrophoresis (2DE) is predominantly used in body fluid analyses (4). This proteomic tool has been successfully applied in numerous animal studies. Special attention is paid to 2 groups of farm animals, pigs and cattle, which are the most frequently used animal models for proteomic research (5). Farm animal proteomics

research is divided into 2 categories. The first focuses on defining a global view of protein expression pattern in cells, tissues, and biological fluids, including blood plasma/serum. Over the past few years, many studies have been undertaken to establish 2D maps of bovine and porcine plasma/serum proteins (1,6–9). The second category is aimed at assessing the influence of various physiological and/or pathophysiological factors on blood plasma/serum proteome changes. For example, proteomic tools have been successfully employed to determine changes in plasma protein profiles after colostrum and milk intake of neonatal calves (10) and piglets (7). Proteomic studies have also been undertaken to identify novel biomarkers of infection and inflammation in both bovine (11) and porcine (1) blood plasma. A high salt content and a large disproportion between low- and high-abundance proteins are the main factors responsible for the difficulty in fully characterizing the serum/plasma proteome. The wide dynamic range in the abundance of plasma/serum proteins may be greater than 10 orders of magnitude (12–14). These disproportions are more dramatic in

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young animals, and especially in neonates. In this group of animals, increased plasma/serum albumin and IgG concentrations are the result of the lack of an intestinal barrier and feeding with protein-rich colostrum and milk (7,15). It is estimated that the 10 most abundant proteins, such as albumin, transferrin, haptoglobin, IgA, IgG, IgM, and  $\alpha$ -1 antitrypsin, constitute 90% of all proteins present in these biological fluids. The aforementioned proteins dominate in the 2D gels and mask the presence of proteins that may have a similar isoelectric point or molecular weight, thereby lowering protein resolution on the gel and limiting the ability to analyze the entire proteome. The remaining 10% are low-abundance proteins, whose concentration is very low and changes markedly in response to regulatory factors (12–14). Therefore, the most crucial step in the proteomic analysis is proper plasma/serum sample preparation including depletion of high-abundance proteins (13).

Various methods are used to remove abundant proteins from plasma and serum. However, 2 of them are the most frequently used in the proteomic studies. These include affinity chromatography along with immunochromatography (16,17) and techniques based on combinatorial hexapeptide ligand libraries (18,19).

Immunochromatography (immunoaffinity chromatography) is based on a solid stationary phase consisting of antibodies immobilized on the activated chromatographic matrix. This method is based on antigen–antibody interaction (20). Utilization of immobilized antibodies enables selective isolation of target molecules from complex mixtures (21). Quian et al. (22) depleted human serum/plasma proteins with chicken IgY antibodies. Immunoaffinity-based depletion methods are rarely used to prepare samples from animal body fluids for 2DE because commercially available kits are predominantly dedicated to human or laboratory animals serum/plasma proteins (23).

Combinatorial hexapeptide ligand libraries are based on solid phase extraction, enabling selective protein enrichment and concentration. This approach depends on proteins binding to their specific ligand. When the ligand reaches a maximum binding capacity, proteins that exceed the capacity of the beads are removed from the sample. If a high number of ligands are used in a single analysis, all of them will independently bind specific proteins until they reach their maximum capacity. High-abundance proteins that are present in the plasma/serum samples quickly saturate their high-affinity ligand, whereas low-abundance proteins are fully adsorbed on their specific ligands. This technique enables the compression of the dynamic range of the protein concentration, thereby allowing the detection of these proteins present in a very low concentration (24,25). The commercially available ProteoMiner (Bio-

Rad) kit dedicated to enriching and concentrating low-abundance proteins is an example of techniques based on combinatorial hexapeptide ligand libraries (19,26).

The aim of the present study was to assess the influence of the ProteoMiner kit on low-abundance protein enrichment, protein concentration, and 2D gel quality in resolved plasma and serum proteins of growing piglets and calves.

## 2. Materials and methods

### 2.1. Animals

The experiment was carried out on 2 groups of animals: calves and piglets.

#### 2.1.1. Calves

A total of 4 healthy male Polish Holstein-Friesian calves of the black and white variety were used. Blood samples were collected on the seventh day of the calf's life. Blood samples were drawn via jugular venipuncture into tubes pre-coated with  $K_3EDTA$ . Samples were then centrifuged at 3000 rpm for 15 min at 4 °C. Next, plasma samples were stored at –80 °C until analysis. The use and handling of animals was approved by the local ethics committee (no. 03/2010 of 14.01.2010).

#### 2.1.2. Piglets

Blood samples were collected from 4 healthy 50-day-old PIC × Penarlan P76 crossbred male piglets. When the blood clot had formed (after 45 min at 4 °C), the samples were centrifuged at 3000 rpm for 15 min at 4 °C. Achieved serum samples were stored at –80 °C until analysis. The use and handling of animals for this experiment was approved by the local ethics committee (no. 13/2012 of 23.05.2012).

### 2.2. Plasma and serum sample preparation

Plasma and serum samples were prepared in the same manner. Before analysis, all samples were thawed at 0 °C and divided into 2 groups. One group of samples (both plasma and serum) was processed with protein equalizer technology [ProteoMine Protein Enrichment Large-Capacity Kit (Bio-Rad)] to decrease the concentration of high-abundance proteins and to increase the low-abundance protein concentration. The second group consisted of plasma and serum samples without any depletion processing. Samples of both groups were then precipitated with cold acetone at –20 °C for 2 h. Subsequently, all samples were centrifuged (20,000 × g, 4 °C, 30 min) and protein pellets were dissolved in the lysis buffer (5 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris, 0.2% w/v 3–10 ampholytes, 2 mM TBP).

### 2.3. Isoelectric focusing

The first dimension was performed on 3–10 nonlinear (NL) ReadyStrip IPG strips (Bio-Rad) 11 cm in length in the case of piglet serum proteins (150 µg of proteins) and on 4–7 NL ReadyStrip IPG strips 24 cm in length in the

case of calf plasma proteins (800 µg of proteins). Strips were rehydrated first passively (5 h, 0 V, 20 °C) and then actively (12 h, 50 V, 20 °C). Due to different IPG strip lengths used in the present study, 2 different isoelectrofocusing (IEF) programs were run (Protean IEF Cell, Bio-Rad). Serum samples were focused according to the following procedure: (i) 250 V for 125 Vh, (ii) 500 V for 250 Vh, (iii) 1000 V for 500 Vh, (iv) linear increase to 5000 V for 1.5 h, (v) 5000 V for 25,000 Vh. Plasma samples were run according to the following program: (i) 250 V for 300 Vh, (ii) 500 V for 850 Vh, (iii) 1000 V for 1500 Vh, (iv) linear increase to 5000 V for 2.5 h, (v) 5000 V for 90,000 Vh. After IEF, the IPG strips were reduced in equilibration buffer (6 M urea, 0.5 M Tris/HCl, pH 6.8, 2% w/v SDS, 30% v/v glycerol) containing 1% (w/v) DTT for 15 min and then alkylated with equilibration buffer containing iodoacetamide (2.5% w/v) for 20 min.

#### 2.4. SDS-PAGE

The second dimension was performed on 12% SDS polyacrylamide gels (20 × 25 cm) at 40 V for 2.5 h and subsequently at 100 V for 16 h at 10 °C (Protean Plus Dodeca Cell Electrophoretic Chamber, Bio-Rad).

#### 2.5. Gel staining

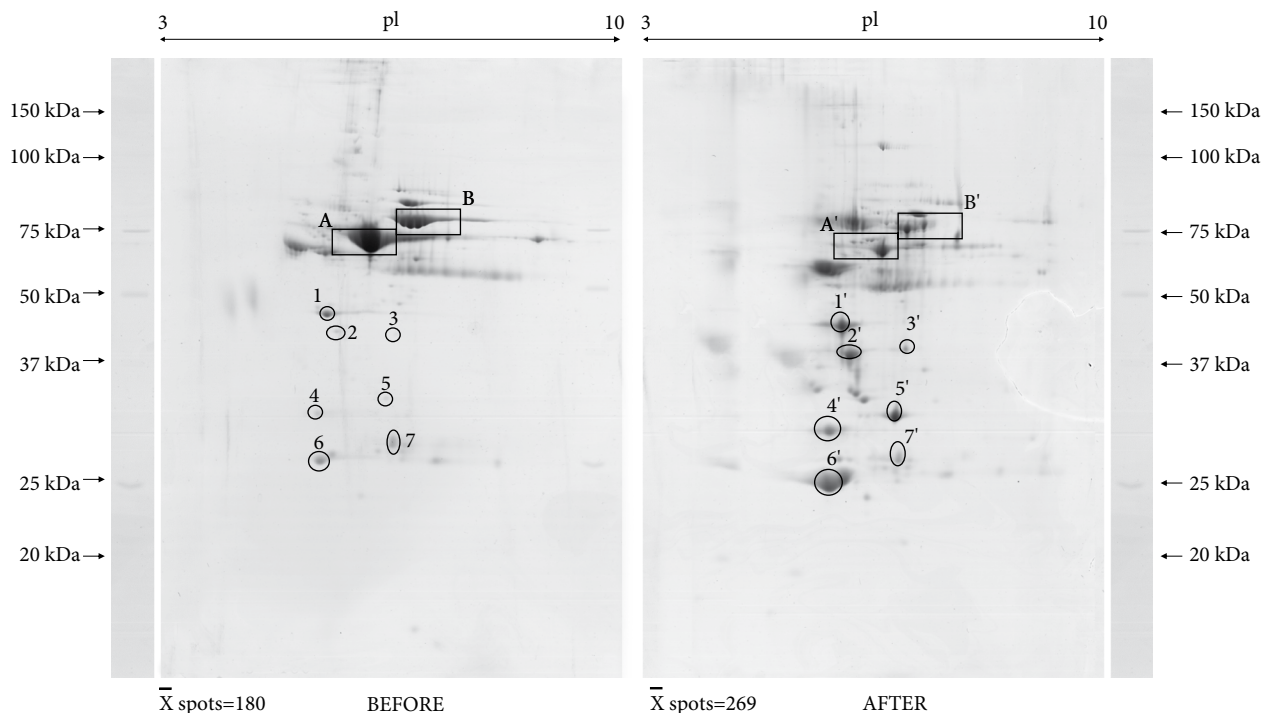
After 2DE separation, gels were stained with colloidal Coomassie Brilliant Blue G-250 according to Pink et al. (27).

#### 2.6. Image analysis

All gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). The 2D image computer analysis was performed using PDQuest Advanced 8.0. (Bio-Rad). Analytical procedures performed on each gel included spot background subtraction and spot detection and matching. The parameters used for between-gel comparisons were the size of the faintest spot, the smallest spot, and the size of the largest spot. Selected master gels represented the highest number of spots and the best protein pattern. Normalization of each individual spot was performed using a local regression model. Analysis was performed in order to determine the quantity of protein spots on each 2D gel and also to assess the efficiency of the ProteoMiner kit. A coefficient of variation (CV) was also estimated to assess the reproducibility of this kit.

#### 3. Results

Piglet serum proteins within the pH range of 3–10 and calf plasma proteins in the pH range of 4–7 before and after ProteoMiner application were separated using 2DE. 2D gel representing porcine serum proteins, before ProteoMiner depletion, resolved on average 180 protein spots. Depletion of high-abundance proteins resulted in the detection of over 269 protein spots on the 2D gels, increasing spot detection by 49.40% (Figure 1). In the case of calf blood plasma



**Figure 1.** Representative 2D images of piglet serum proteins before and after ProteoMiner treatment. Proteins (150 µg) were separated by IEF using 3–10 IPG strips, followed by SDS-PAGE (12% gels). Protein spot clusters in the native serum were marked in frames A and B and in frames A' and B' after ProteoMiner depletion. Spots that displayed expression changes before high-abundance protein depletion were numbered as 1–7 and after protein depletion as 1'–7'. Spot numbers on the gels correspond to those in Table 1.

proteome (Figure 2), before high-abundance proteins were removed, approximately 531 protein spots were detected on the 2D gels. After ProteoMiner treatment, the quantity of the protein spots observed on the gels increased to 798 (+26.46%).

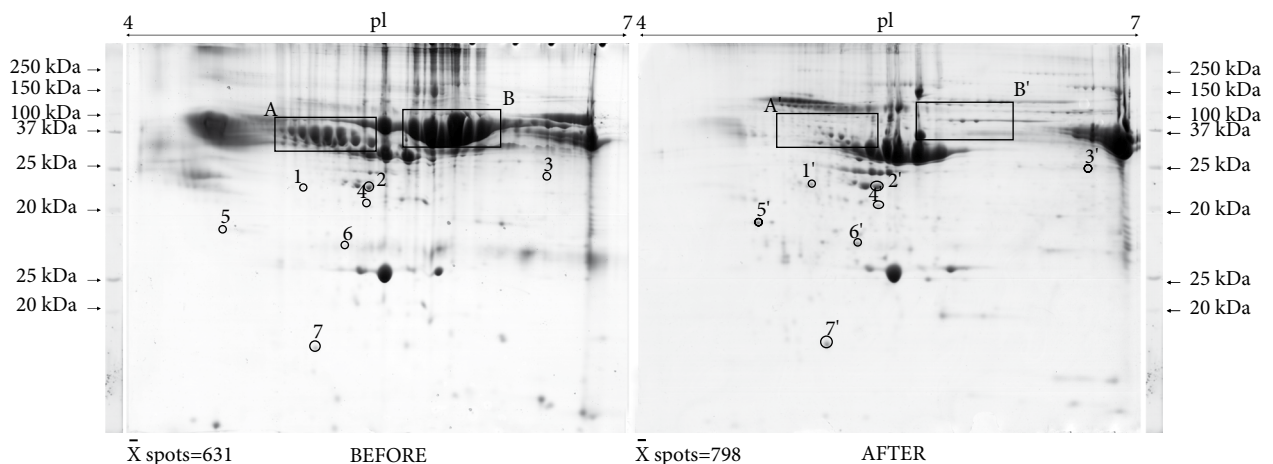
Analysis of 2D gels representing both serum and plasma samples deprived of high-abundance proteins showed a decrease in protein spots cluster formation (Figures 1 and 2, respectively, frames A' and B') when compared to the gels representing plasma and serum proteins before ProteoMiner depletion (Figures 1 and 2, frames A and B). Before removing proteins that were present in high concentrations (frames A and B), large, strongly stained protein spots were observed on the 2D gels.

Additionally, the relative abundance of randomly selected protein spots from porcine serum samples increased considerably after treatment with the ProteoMiner beads in comparison to the native serum sample (Figure 1, spots 1–7; Table 1). Changes in the expression levels of particular spots were between 1.96- and 158.11-fold. Similar changes were also observed in the case of calf plasma samples (Figure 2, spots 1–7; Table 2). Differences in the relative abundance of protein spots were between 1.62- and 10.40-fold. Protein depletion did not influence the gel's reproducibility, which was confirmed by the CV (%). Gels representing proteins of piglet serum had CV values of 36.56% before and 35.38% after high-abundance protein depletion. In the case of neonatal calf plasma proteome samples, the CV values were similar before (33.50%) and after (35.44%) reduction of high-abundance proteins.

#### 4. Discussion

High-abundance plasma/serum proteins mask the presence of proteins that may be present in much smaller concentrations, thereby lowering their resolution on 2D gel. The results of the present study indicate that the ProteoMiner kit successfully enriched low-abundance proteins of both serum and plasma samples and increased spot detection by 49.40% and 26.46%, respectively. In the study of Hagiwara et al. (19), applying the ProteoMiner technique increased the number of plasma proteins detected on the gels. The authors compared nondepleted and depleted protein samples resolved using the 2D-DIGE technique and they observed a higher number of protein spots after treatment (538 before, 697 after). Sennels et al. (26) found that protein spots detected on the 2D gels of native human serum samples increased by a factor of 4 (from 200 to over 800) after protein library beads treatment. Marco et al. (28) also observed an increase in the number of protein spots on the 2D gels representing porcine and bovine serum proteins. High reproducibility of the depletion process after treatment with the hexapeptide library beads has been confirmed by the comparison of 1D electropherograms. Only minor variations in the band intensities within treated samples were observed by the authors (19,28). Li et al. (18) also observed a high level of consistency from sample to sample when processed with similar and variable hexapeptide library bead volumes.

This study confirmed the efficiency of high-abundance protein removal both from piglet serum and calf plasma samples. This is in accordance with the results of Marco-Ramell and Bossols (29). Relative expression of albumin and IgG protein spot clusters on the 2D gels representing



**Figure 2.** Representative 2D images of calf plasma proteins before and after ProteoMiner treatment. Proteins (800 µg) were separated in the first dimension by IEF using 4–7 IPG strips, followed by SDS-PAGE (12% gels). Protein spot clusters in the native serum were marked in frames A and B and in frames A' and B' after ProteoMiner depletion. Spots that displayed expression changes before high-abundance protein depletion were numbered as 1–7 and after protein depletion as 1'–7'. Spot numbers on the gels correspond to those in Table 2.

**Table 1.** Changes in the expression level of selected protein spots from piglet serum samples before and after ProteoMiner treatment. Spot numbers in the table correspond to those shown on the 2D gel in Figure 1.

Spot no.	Average relative expression		Fold change
	Before	After	
1	108.2	701.3	+6.48
2	5.7	379.7	+66.61
3	1.6	15.0	+9.37
4	13.2	172.6	+13,08
5	3.7	585.0	+158.11
6	27.3	294.0	+10.78
7	1.6	15.0	+9.37

bovine and porcine serum proteins decreased after ProteoMiner depletion. Marco-Ramell and Bossols (29) observed a 3.7-fold decrease in albumin abundance in bovine and 2.7-fold decrease in porcine serum samples, and a 3.8-fold reduction of IgG heavy chain in bovine and 2.8-fold reduction in porcine serum samples. Higawara et al. (19) suggested that an increased number of detected spots on the 2D gels with a concomitant decrease in intensity of high-abundance proteins after ProteoMiner treatment are evidence of the selective human plasma proteome enrichment by ProteoMiner. The authors also found that albumin is the most depleted protein fraction of this biological sample. Fold differences of relative expression of the spots representing this protein ranged from 38.4 to 52.6.

In the current study, protein spot expression increased in response to the removal of selected high-abundance proteins based on combinatorial hexapeptide ligand libraries. Similar results were obtained by Marco-Ramell and Bossols (29). The levels of  $\alpha_1$ -glycoprotein and transthyretin on the 2D gels representing porcine and bovine serum proteins increased after treatment with the ProteoMiner kit. Expression of  $\alpha_1$ -glycoprotein increased 3.4-fold (cattle) and 3.2-fold (pigs), whereas abundance of transthyretin increased 3.5-fold (cattle) and 2.8-fold (pigs). Hagiwara et al. (19) reported that the relative expression of vitronectin spots on 2D gels representing

**Table 2.** Changes in the expression level of selected protein spots from calf plasma samples before and after ProteoMiner treatment. Spot numbers in the table correspond to those shown on the 2D gel in Figure 2.

Spot no.	Average relative expression		Fold change
	Before	After	
1	8.2	85.3	+10.40
2	429.0	695.4	+1.62
3	15.8	66.3	+4.20
4	8.3	22.1	+2.66
5	55.1	172.2	+3.12
6	47.5	159.3	+3.35
7	117.9	210.2	+1.78

human plasma proteome increased by 16.5- to 52.9-fold after ProteoMiner treatment. Other proteins showing increased relative expression were complement factors C3 (20.0-fold) and C4-A (71.0-fold). Rasaputra et al. (30) also observed an increased expression of 8 randomly selected protein spots on the 2D gels representing chicken serum samples after protein depletion using a technique based on combinatorial hexapeptide ligand libraries.

In conclusion, the results of our study confirmed both the high efficiency of high-abundance protein depletion and a substantial increase in the concentration of low-abundance proteins on the 2D gels after ProteoMiner treatment. Moreover, this technique based on hexapeptide combinatorial libraries is reproducible and may be used as an alternative to immunodepletion methods for blood plasma and serum samples preparation for 2DE for both adult and young, growing animals. Increased content of the low-abundance proteins on the 2D gels enables complete proteome analysis and thereby allows for the discovery of novel biomarkers that might be useful in veterinary medicine.

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