A comparison of albumin removal procedures for proteomic analysis of blood plasma

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Abstract. Blood biomarkers are usually present in low concentration and can be masked by the high-abundance proteins, of which albumin is the predominant one. The purpose of this study was to compare four different albumin removal methods compatible with in-gel based proteomics, applicable for plasma, without requiring specific techniques and high financial input. Plasma underwent albumin depletion with ultrafiltration device Amicon Ultra, commercial ProteoPrep Blue Albumin and IgG Depletion Kit, acetonitrile precipitation method and precipitation with acetonitrile-methanol protocol. All samples were evaluated by 1-D and 2-D gel electrophoresis with subsequent mass spectrometry protein identification. Two of the tested methods (ProteoPrep BlueKit and acetonitrile-methanol precipitation) maintained sufficient protein content for further in-gel analyses. Their 2-D protein profiles were distinctively separated and overlapped with protein profile of crude plasma. Protein spot count showed significant increase in protein spots, compared to crude plasma, only with acetonitrile-methanol precipitation method. Precipitation with acetonitrile-methanol method significantly increased number of protein spots on 2-D protein profile and improved score of mass spectrometry identification. However, albumin was still present and found in number of protein spots.

Key words: Albumin removal — Mass spectrometry — Biomarkers — Blood plasma

Abbreviations: 1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; ABC, ammonium bicarbonate; ACN, acetonitrile; AMW, acetonitrile-methanol-water; BSA, bovine serum albumin; HAPs, high-abundance proteins; IEF, isoelectric focusing; LAPs, low-abundance proteins; MS, mass spectrometry; MW, molecular weight; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Introduction

In clinical practice, blood samples are routinely used for wide range of diagnostic purposes as they are easily obtained and can be repetitively sampled. Proteins in circulatory system reflect person´s physiology. Changes in concentration of certain proteins or their post-translational modification reflect particular disease state and could be used as diagnostic and prognostic biomarkers (Anderson and Anderson 2002).

However, discovery of new blood protein biomarkers faces difficulties, as approx. 95% of bulk mass of proteins is made up of few high-abundance proteins (HAPs), predominantly albumin (Fang and Zhang 2008). They produce large signals that can mask or interfere with the detection of low abundant biomarkers (Roche et al. 2009). Around half of the protein mass in blood serum and plasma consists of albumin, and low-abundance proteins (LAPs) are likely to be in concentration of five to ten orders of magnitude

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lower (<<10 ng/ml). Moreover, albumin is a known carrier and transporter of physiologically important proteins and peptides, so it is important to note that its removal can result in loss of potential biomarkers (Tirumalai 2003).

Rodents represent highly controllable model and an important part of biomarker discovery. Particularly rats and mice are commonly used in various medical research areas (Hood et al. 2005; Linke et al. 2007). Rodent blood proteome has its own specific profile but similar wide dynamic range in protein concentrations as human blood and therefor presents the same challenges in proteomic research (Haudenschild et al. 2014). Rat blood profiles show that albumin and IgG make up about 80% of total plasma protein and their depletion could result in significant increase of concentration of medium-abundance proteins and LAPs (Sharp and LaRegina 1998).

Removal of albumin and other HAPs from blood plasma or serum is necessary step prior implementing proteomic methods for biomarker discovery. There is a number of different approaches to remove albumin including immunoaffinity-mediated proteomic separation, centrifugal ultrafiltration, commercial kits based on Cibacron blue or precipitation in organic solvents (Tucholska et al. 2007; Holewinski et al. 2013; Qui et al. 2015). However, removal methods are often expensive and time consuming with limited sample capacity (Kay et al. 2008).

In present work, four different methods of albumin removal have been studied: centrifugal ultrafiltration device Amicon Ultra with protein cut-off limit of 50 kDa (Merck Millipore), commonly used commercial ProteoPrep Blue Albumin and IgG Depletion Kit (Sigma-Aldrich) based on Cibacron Blue dye and two methods based on protein precipitation in organic solvents. These methods were compared using one-dimensional and two-dimensional gel electrophoresis (1-DE and 2-DE) with MALDI-TOF mass spectrometry (MS) protein identification. 2-DE coupled with MS identification is a common proteomic approach and was already successfully employed in blood biomarker studies of e.g. cervical, hepatocellular or colorectal carcinoma (Henkel et al. 2012; Guo et al. 2015; Lim et al. 2016). Our main goal was to see if commonly available and inexpensive organic solvent methods are comparable to those commercial ones and applicable for in-gel proteomic analyses.

Materials and Methods

Animals

A total number of 6 adult male Wistar rats (Velaz, Czech Republic) at the age of 3 months with mean weight of 335 ± 42.7 g were used in this study. They were housed in a temperature-controlled room 22 ± 2°C on a 12-h light/dark cycle

with access to food and water *ad libitum*. All procedures on animals were performed in accordance with ethical and moral principles and approved by the State Veterinary and Food Department of the Slovak Republic and by Ethical Committee of Comenius University in Bratislava, Jessenius Faculty in Martin (# 1647/2015).

Sample collection

Rat whole blood was collected in 6 ml lithium heparin-coated tubes (Vacutest Kima). Blood was centrifuged at 4°C, 720 × *g* for 20 min. Plasma was aliquoted and stored at −80°C until analysis.

Methods of albumin removal

We compared four different methods that remove albumin to determine the most suitable one for in-gel methods. All samples were kept on ice and manipulations were performed at room temperature if not stated otherwise.

Albumin removal with Amicon Ultra-0.5 centrifugal filter 50K device

The Amicon Ultra-0.5 Centrifugal Filter Device (UFC505096, Millipore) with cut-off limit of 50 kDa was used according to manufacturer's instructions with 400 µl of plasma samples. Spin time of 15 min was used after testing 15, 20 and 30 min spin time with no significant difference in concentration yield. Depleted plasma filtrate was used for further analyses. The cut-off limit (device capturing molecules ≥ 50 kDa), was picked in compliance with the molecular weight (MW) of rat albumin of 70.7 kDa.

Albumin removal with ProteoPrep Blue Albumin and IgG Depletion Kit

Albumin removal with ProteoPrep Blue Albumin and IgG Depletion Kit (PROTBA, Sigma-Aldrich) was performed according to manufacturer´s instructions with 50 µl of plasma. Both fractions, depleted plasma and albumin-enriched protein extract were used for further analyses.

Albumin removal with acetonitrile protocol (ACN Protocol)

Albumin removal was carried out according to protocol described by Kay et al. (2008) and Fernandez et al. (2011). A total volume of 150 µl of each plasma sample was divided into three aliquots. In 1.5 ml disposable microcentrifuge tubes 50 µl of plasma was diluted with 100 µl LC-MS grade H_2O and briefly vortexed. To each aliquot 225 µl of 100% acetonitrile (ACN) was added and the samples were sonicated in ultrasonic bath for 10 min, vortexed briefly and

sonicated for another 10 min. Samples were centrifuged at 14,000 × g for 10 min at room temperature. Supernatants from the aliquots were pooled in clean microcentrifuge tubes and evaporated to dryness in vacuum concentrator centrifuge (Concentrator plus/Vacufuge plus, Eppendorff), reconstituted in 150 μl of sample buffer (0.2 mol/l Tris-HCl pH 6.8, 2% SDS 20% glycerol) and used for further analyses. This protocol was repeated several times to reach necessary protein content for electrophoresis. The pellets containing removed albumin and other high-abundance proteins did not dissolve in buffer solution and hence were impossible to analyse with in-gel methods.

Albumin removal with acetonitrile-methanol-water protocol (AMW Protocol)

Albumin removal was performed according to slightly adjusted protocol previously described by Tucholska et al. (2010). In 1.5 ml disposable microcentrifuge tubes 25 μl of plasma samples were precipitated with five volumes of 100% ACN, vortexed and incubated on ice for 15 min. Samples were centrifuged at 16,000 × g for 15 min and supernatants were transferred to clean microcentrifuge tubes. Pellets were resuspended in 50 μl of AMW solution (12.5 μl of 100% acetonitrile, 12.5 μl of 100% methanol and 25 μl of LC-MS grade H_2O), incubated on ice for 15 min and centrifuged at 16,000 × g for 15 min. Supernatants were added to their previous fractions. Pellets with depleted plasma proteins were left to dry for 20 min at room temperature, reconstituted with 200 μl of acidified water with 1% formic acid and mixed on shaker (20°C, 2 hours, shaking slightly). Supernatants with removed albumin fraction were evaporated to dryness in vacuum concentrator centrifuge and also reconstituted with acidified water. Both fractions were used in further analyses.

Protein assay

To determine total protein content, DC Protein Assay (500-0111, Bio-Rad) in a microplate format was performed according to manufacturer´s instructions.

One-dimensional electrophoresis (1-DE)

Samples (30 μg of protein content) were precipitated with ice-cold acetone and centrifuged at 16,000 × g for 10 min. After protein reconstitution in 30 μl of loading buffer (0.2 M Tris-HCl pH 6.8, 2% SDS 20% glycerol, 0.1% bromophenol blue) samples were boiled for 4 min at 96°C. Proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gels under denaturing conditions using a Bio-Rad mini electrophoresis system in tricine electrophoresis buffer. SDS-PAGE was carried out at 15 mA until the bromophenol blue dye reached the

bottom of the gel. Gel was stained using Bio-Safe Coomassie G-250 stain (161-0786, Bio-Rad) and scanned by GS-800 Calibrated Densitometer (Bio-Rad).

Two-dimensional electrophoresis (2-DE)

Samples (200 μg of protein content) were precipitated with ice-cold acetone and pelleted by centrifugation (16,000 × g for 10 min). After protein reconstitution in Rehydration/ Sample buffer (163-2106, Bio-Rad), they were applied to 7 cm linear ReadyStrip IPG strips with pH 3-10 (163-2000, Bio-Rad) and let to rehydrate overnight. Isoelectric focusing (IEF) was performed using Protean IEF system (Bio-Rad) in three-step protocol (250 V for 20 min, 4000 V for 2 hours and 4000 V until the total voltage reached 10,000 Vh). Prior to the second dimension separation, the strips were equilibrated in Equilibration Buffer I (163-2107, Bio-Rad) for 15 min and in Equilibration Buffer II (163-2108, Bio-Rad) with added 0.03 g/ml iodoacetamide for 15 min. Following equilibration, the IPG strips were placed on 12% polyacrylamide gels and covered with overlay agarose (163-2111, Bio-Rad). SDS-PAGE ran for 20 min at 15 mA and at 25 mA until the bromophenol blue dye reached the bottom of the gel using a Bio-rad mini electrophoresis system in tricine electrophoresis buffer. Gels were stained using Bio-Safe Coomassie G-250 stain (161-0786, Bio-Rad), scanned by GS-800 Calibrated Densitometer (Bio-Rad) and analysed using PDQuest 8 software (Bio-Rad).

Protein identification by mass spectrometry

Protein spots/bands were excised from gels, washed with 50% ACN at room temperature until Coomassie stain was removed and dehydrated in 100% ACN. Spots were then incubated with 10 mM dithiotreitol at 56°C for 45 min to reduce disulphide bridges and in 55 mM iodoacetamide for 30 min in dark to alkylate cysteine residues. Next, spots/ bands were washed two times with 25 mM ammonium bicarbonate (ABC) and 100% ACN alternatively. For protein digestion, 3 μl of trypsin solution (20 ng/μl in ABC, Promega) was added directly on the spot/band and incubated for 30 min on ice. Subsequently 20 μl of ABC were added and incubated overnight at 37°C on shaker. Peptides were extracted with 10% trifluoroacetic acid, dehydrated with 100% ACN and collected samples were concentrated in vacuum concentrator centrifuge until final volume reached approx. 10 μl. Next, 0.75 μl of sample was applied onto AnchorChip target (Bruker Daltonics) and when dried down, 0.75 μl of matrix solution (α-cyano-4 hydroxy cinnamic acid, 1 mg/ml) was added and left to dry. For calibration purposes, Peptide Calibration Standard (8206195, Bruker Daltonics) was applied onto AnchorChip in the same manner as the samples.
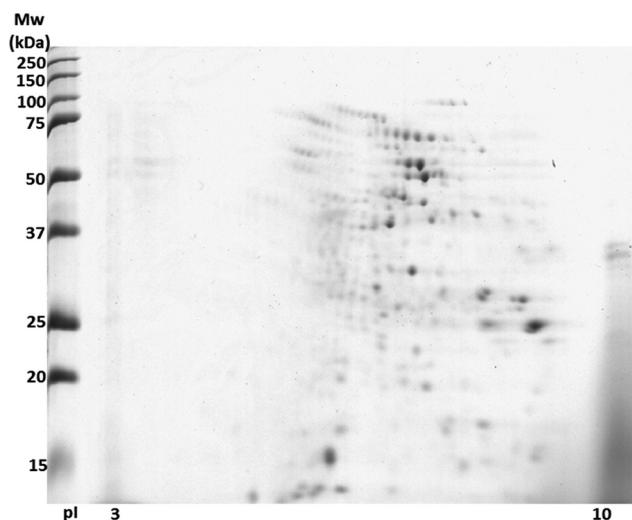
Figure 1. Representative 2-DE SDS-PAGE map of plasma depleted with Amicon filter device. Result is representative of three independent experiments.

The mass spectra were recorded on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) across m/z range 700–3500 kDa. MS/MS fragmentation spectra were obtained by selecting three strongest peaks of each peptide mass map. The SwissProt database was searched using MASCOT database search engine (Matrix Science: www.matrixscience.com/help/scoring_help.html) with following parameters: enzyme trypsin; taxonomy *Rattus norvegicus*, mass tolerance for PMF 50 ppm and one missing cleavage, MS/MS tolerance 0.5 Da, global modification carbamidomethylation (C) and variable modification oxidation: methionine oxidation (M).

Results

Protein content

Total protein content in crude plasma was 69.10 ± 8.46 mg ml. Measured protein concentration of plasma after undergoing individual albumin removal protocols is summarized in Tab. 1. Using ProteoPrep kit and AMW protocol, depleted plasma

maintained nearly 50% of its protein content. After depletion with Amicon filter device protein content in depleted fraction was reduced nearly 600-fold and after ACN protocol about 200-fold. Loss in total protein content (depleted fraction + albumin-enriched fraction) might result from numerous manipulations.

Albumin removal with Amicon Ultra-0.5 Centrifugal Filter 50K Device

Due to low total protein yield, Amicon filter device was excluded from further experiments. A representative 2-DE protein map with protein load of 90 µg is shown on Fig. 1.

After decapitation, 4–6 ml of blood from a single animal is obtained, leading to less than half volume of plasma. For one 2-DE map with standard protein load of 200 µg, approx. 1600 µl of crude plasma would be needed, making this method unsuitable for in-gel proteomics.

One-dimensional electrophoresis

Efficiency of removal methods was evaluated using 1-D electrophoresis. The SDS-PAGE visualised the most abundant proteins in both, depleted and albumin-enriched fractions (Fig. 2). The gel bands corresponding to albumin were less dense in samples that underwent the removal methods (Fig. 2B, 2C, 2D) compared to the crude serum (Fig. 2A).

ProteoPrep kit (Fig. 2E) captured mostly albumin and in part hemopexin and serotransferrin. In albumin-enriched fraction from AMW protocol can be found except from high albumin concentration, hemopexin, serotransferrin, vitamin D-binding protein and apolipoproteins. During acetonitrile washing steps hydrophobic proteins, mainly apolipoproteins can be removed from the plasma together with albumin. Proteins representing the bands were characterised using MALDI-TOF/TOF (for mass spectrometry identification details see Tab. S1 in Supplementary Material).

Two-dimensional electrophoresis

Proteins from crude plasma and different albumin removal methods (ProteoPrep kit depletion, AMW and ACN removal protocols) were analysed using 2-DE (Fig. 3). When compared to crude plasma, protein spot count revealed significant increase in number of visualised spots after applying

Table 1. Protein content measured in individual fractions in plasma after undergoing different albumin depletion methods

| | Removal method | | | |
Fraction	Amicon filter device (mg/ml)	ProteoPrep kit (mg/ml)	AMW protocol (mg/ml)	ACN protocol (mg/ml)
Depleted fraction	0.12 ± 0.03	28.33 ± 1.97	32.93 ± 8.17	0.34 ± 0.02
Albumin-enriched fraction	28.65 ± 5.51	7.68 ± 2.63	3.79 ± 0.36	–

AMW protocol and surprisingly decrease after ProteoPrep and ACN depletion. Spot count for each method is summarised in Tab. 2.

In region with high occurrence of albumin, that often covers other proteins (MW 50–75 kDa), 15 protein spots were selected for identification with MALDI-TOF/TOF (Fig. 3, see zoomed region). Results from protein identifications are summarized in Tab. 3 (for mass spectrometry identification details see Tab. S2–S4 in Supplementary Material). Success of protein identification was determined by Mascot score which is a probability-based scoring to judge whether a result is significant or not. In general, scores greater than 67 are considered significant ($p < 0.05$) (Matrix Science 2016).

In crude plasma (Fig. 3A), we identified albumin (ALBU_RAT) in 7 spots, one of them (spot no. 6) in a mixture with T-kininogen (KNT1_RAT). Highly prevalent were also fibrinogen isoforms (FIBA_RAT and FIBB_RAT), together in 6 spots. Out of the selected spots, we were not able to identify proteins in spot no. 5 due to insufficient number of matched peptides. Protein in spot no. 8, actin-related protein 2/3 complex subunit 1A (ARC1A_RAT), was identified with low Mascot score (see Tab. 3) and its MW and pI (42.1 kDa and 8.46) does not correlate with its position on the protein map making the result unreliable.

After applying ProteoPrep kit (Fig. 3B), albumin was found only in two spots and spots with fibrinogen isoforms decreased to three. Spot no. 4 was not identified. Previously not identified spot no. 5 was discerned as tubulin beta-3 chain (TBB3_RAT) but with low Mascot score. Only 8 spots reached Mascot score above 67 (Tab. 3). Most of the proteins

Table 2. Number of protein spots detected in the crude plasma sample and after different albumin removal methods

Removal method	Number of spots
Crude plasma	213 ± 9
ProteoPrep kit	181 ± 21
AMW protocol	261 ± 8
ACN protocol	149 ± 14

with low Mascot score were not concentrated in the supposed position of the identified proteins.

When using AMW protocol (Fig. 3C), all 15 protein spots were identified with Mascot score above 67. We identified albumin in six spots and fibrinogen isoforms in four spots. Albumin was removed from spot no. 4 and no. 6. Spot no. 4 was after AMW removal identified as transferrin (TRFE_RAT). Spot no. 6 was identified as KNT1_RAT as well as spot no. 5, previously unidentified in crude plasma. Moreover, protein in spot no. 8, which was in crude plasma identified with very low Mascot score, was depicted as fibrinogen gamma (FIBG_RAT) with sufficient score (Tab. 3).

We were not able to find all 15 selected protein spots in ACN protein maps (Fig. 3D). Overall protein profile of depleted sample was significantly changed and did not overlap with that of crude plasma. Furthermore, only the spot identified as albumin (ALBU_RAT) reached Mascot score over 67. The rest of excised protein spots demonstrated low Mascot score (Tab. 3).

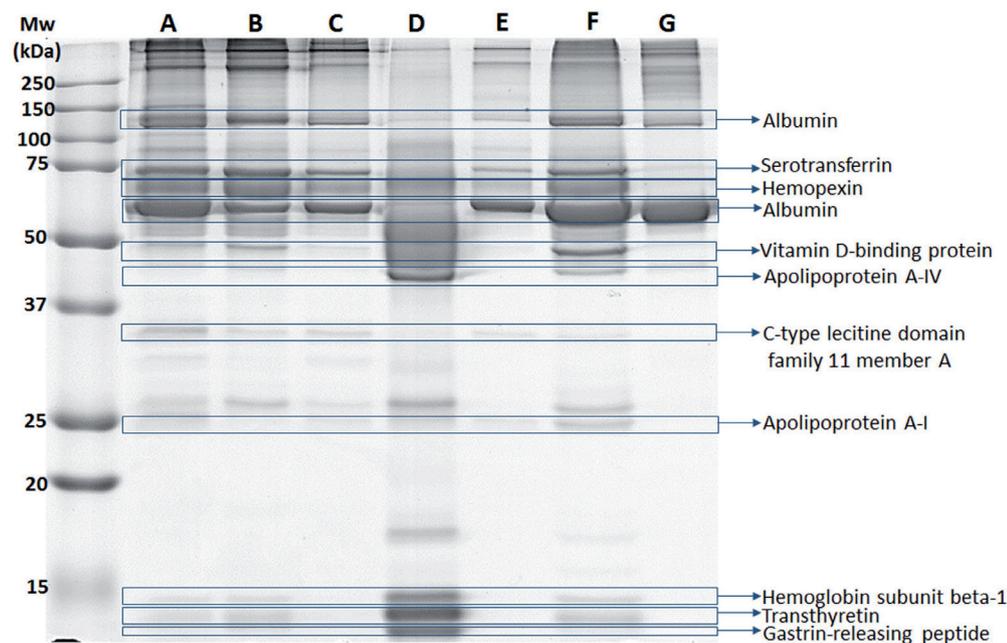Proteins in albumin-enriched fractions from ProteoPrep kit (Fig. 4A) and AMW protocol (Fig. 4B) were also visu-

Figure 2. 1-DE SDS-PAGE analysis of the different albumin removal methods: A, crude plasma; B, ProteoPrep kit depletion; C, AMW protocol depletion; D, ACN protocol depletion; E, albumin-enriched fraction from ProteoPrep kit; F, albumin-enriched fraction from AMW protocol; G, albumin positive control (BSA). AMW, acetonitrile-methanol-water; ACN, acetonitrile; BSA, bovine serum albumin.
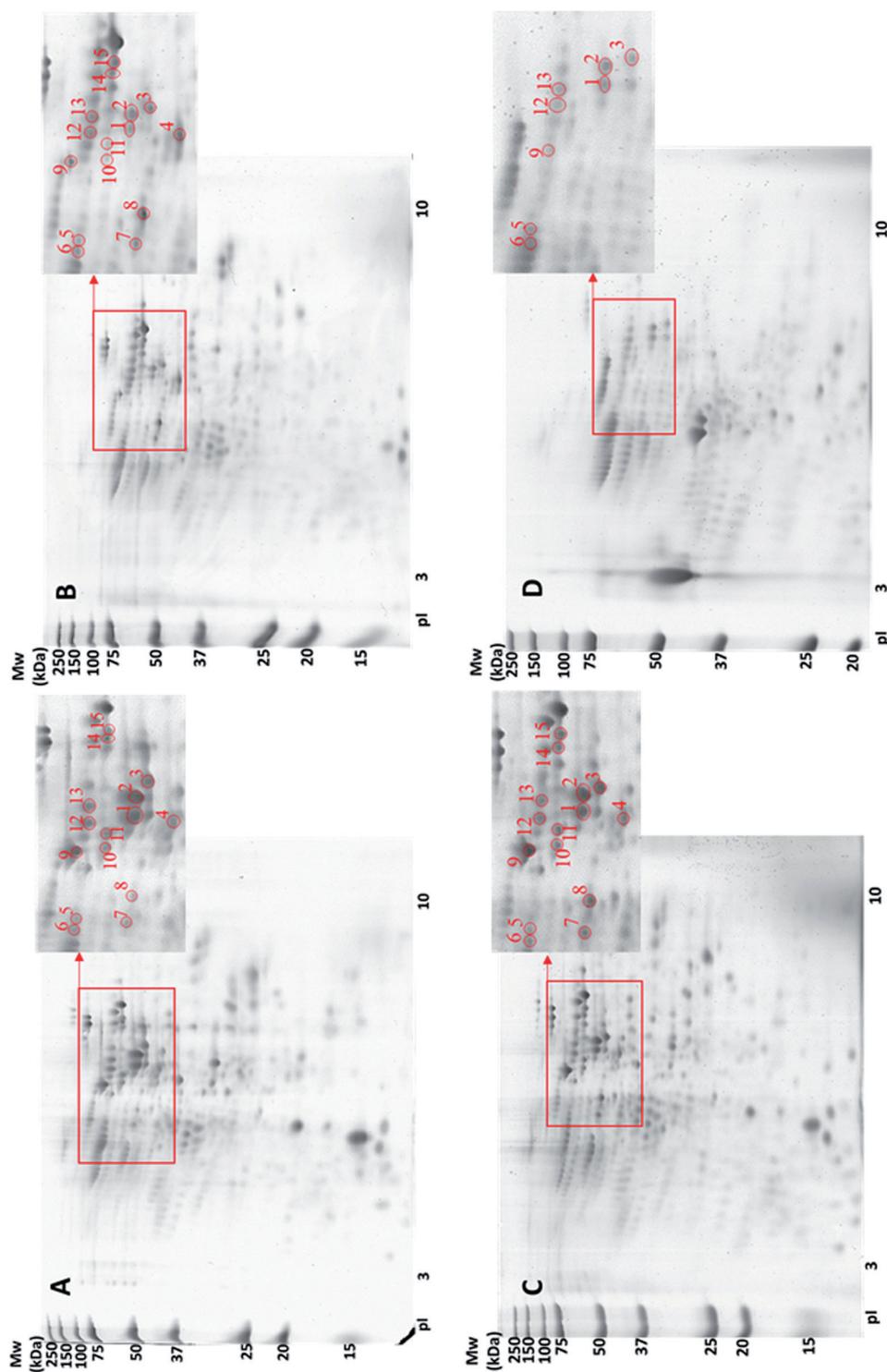
Figure 3. Representative 2-DE SDS-PAGE maps to compare different albumin removal methods: crude plasma (**A**), ProteoPrep kit depletion (**B**), AMW protocol depletion (**C**), ACN protocol depletion (**D**). Results are representative of four independent experiments. Representative gels show the same sample undergoing different methods of albumin removal. Square – zoomed region with high abundance of albumin. Excised protein spots for MALDI MS/MS identification are labelled with number that corresponding to Tab. 3. For abbreviations, see Fig. 2.
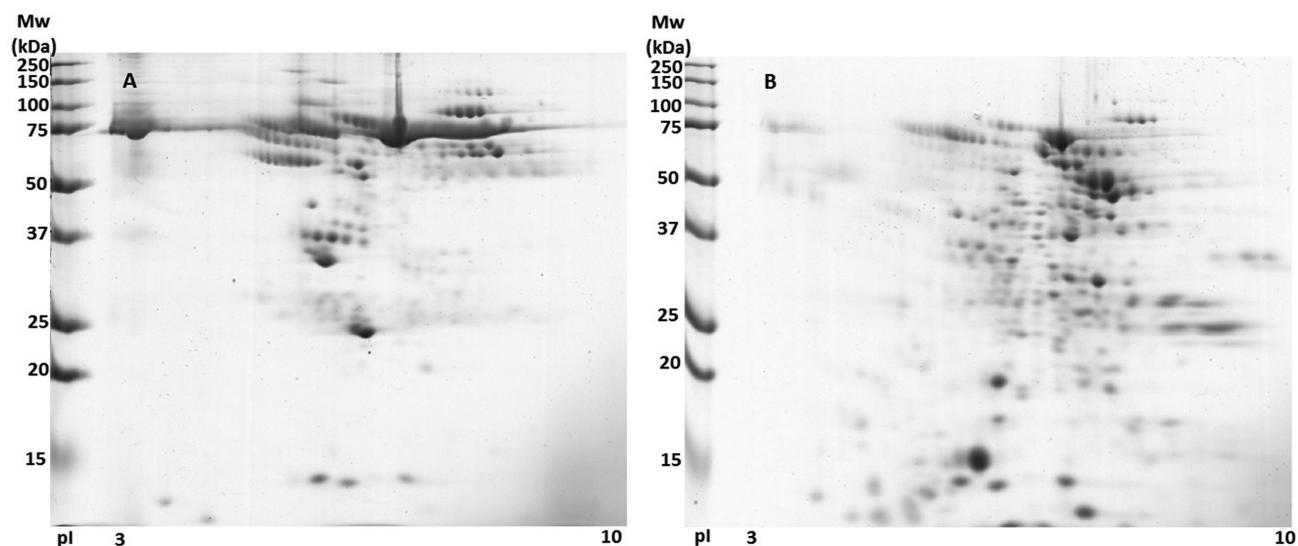
Figure 4. Albumin-enriched representative 2-DE maps: albumin-enriched fraction from ProteoPrep kit (**A**) and albumin-enriched fraction from AMW protocol (**B**). Results are representative of four independent experiments.

alised. Albumin-enriched fraction from ACN protocol was not analysed, as it was not possible to dissolve the resulting pellet in sample buffer. Protein map of ProteoPrep kit retention (albumin-enriched fraction) shows high abundance of proteins with Mw over 50 kDa with extensive cluster in place with expected albumin. Visualisation of proteins from

Table 3. Identified proteins from protein maps

Spot No.	Crude plasma		Commercial KIT		AMW protocol		ACN protocol	
	UniProt ID	Total Mascot score	UniProt ID	Total Mascot score	UniProt ID	Total Mascot score	UniProt ID	Total Mascot score
1	ALBU_RAT	358	RPN1_RAT Δ	26	ALBU_RAT	419	SP20H_RAT	30
2	ALBU_RAT	185	ALBU_RAT	277	ALBU_RAT	337	ALBU_RAT	258
3	ALBU_RAT	231	ALBU_RAT	357	ALBU_RAT	288	DO1L1_RAT*Δ	33
4	ALBU_RAT	196	X	X	TRFE_RAT*	373	KNT1_RAT*	32
5	X	X	TBB3_RAT*°	29	KNT1_RAT*	111	KNT1_RAT*	48
6	Mixture KNT1_RAT* ALBU_RAT	206 254	KNT1_RAT*	326	KNT1_RAT*	123	KNT1_RAT*	38
7	FIBG_RAT	134	FIBG_RAT	98	FIBG_RAT	169	—	—
8	ARC1A_RAT*Δ	27	SCO2_RAT Δ	32	FIBG_RAT	237	—	—
9	ALBU_RAT	239	VIME_RAT	35	ALBU_RAT	462	BABA2_RAT*Δ	33
10	FIBA_RAT Δ	181	BRMS1_RAT*Δ	34	ALBU_RAT	278	—	—
11	ALBU_RAT	426	NSF1_RAT Δ	27	ALBU_RAT	357	—	—
12	FIBA_RAT Δ	220	HEMO_RAT	241	HEMO_RAT	331	BABA2_RAT*Δ	27
13	FIBA_RAT Δ	336	HEMO_RAT	117	HEMO_RAT	375	AMZ2_RAT*	35
14	Mixture FIBB_RAT FIBA_RAT Δ	353 190	FIBB_RAT	200	FIBB_RAT	249	—	—
15	FIBA_RAT Δ	246	FIBA_RAT Δ	322	FIBA_RAT Δ	132	—	—

Spot numbers correspond to those on Fig. 2. * MW of identified protein does not correspond with its MW in gel map; Δ, pI of identified protein does not correspond with its pI in gel map; X, spot was excised but protein was not identified with MS; — spot was not found on the gel.
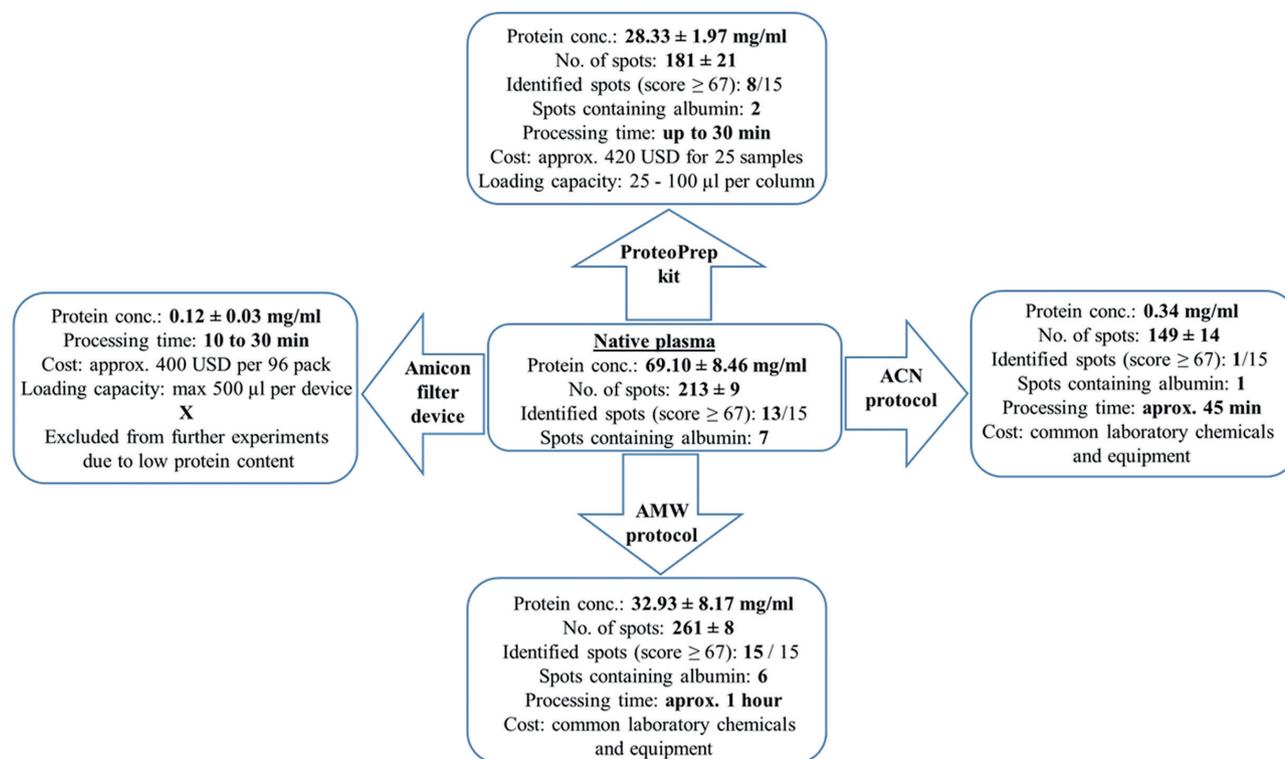
Figure 5. Scheme representing effectivity of different albumin removal methods. Highlighted by bold are important outcomes for evaluation of effectivity of the dealbumination method. ACN, acetonitrile; AMW, acetonitrile-methanol-water; conc, concentration.

AMW albumin-enriched fraction shows that high number of proteins was removed from the crude sample together with albumin.

Discussion

Using crude plasma for 2-DE leads to overloading of abundant proteins and poor spot resolution. Thus, albumin removal is a crucial preparation step for plasma proteome investigation with in-gel methods (Olver et al. 2010). In present work we tested four different albumin removal strategies for in-gel proteomic approaches. These methods were selected due to their applicability for 2-DE, high sample capacity, minimal preparation steps with short preparation time and convenient price. The above points are illustrated in Fig. 5.

The Amicon filter device was ruled out after initial protein concentration measurements. To achieve sufficient protein load for a single 2-DE nearly whole rat plasma sample would be needed, disabling further investigation of the sample.

Out of the remaining strategies the most sufficient one proved to be albumin removal with AMW protocol (incubation with acetonitrile and methanol) (Fig. 5). This depletion strategy significantly increased the number of

spots and highlighted spots with molecular mass under 50 kDa. The spots on protein map were well focused and distinctively separated. Furthermore, all selected spots for mass spectrometry were identified with high Mascot score, thus improving the protein identification compared to crude plasma. On the other hand, albumin was still present in the depleted plasma sample but its density decreased (Fig. 2). Also, albumin was identified in several protein spots and 2-DE gel map of albumin-enriched fraction suggests that together with albumin part of protein content gets washed away (Fig. 4B). However, the 2-DE gel map from albumin-enriched fraction overlaps with the one of depleted plasma and we assume that there is decrease in concentration of individual proteins and not in total number of proteins. Adding ACN to a sample weakens the hydrophobic interactions leading to protein denaturation and subsequent precipitation (Tucholska et al. 2010; Fernandez et al. 2011). The addition of ACN in a volume of at least 20% disrupts association of proteins with albumin, decreasing the chance of possible biomarker loss (Tirumalai et al. 2003; Kay et al. 2008). It was also shown that methanol precipitation leaves large amount of albumin in solution. Moreover, organic solvent precipitation removes organic-soluble contaminants such as salts and lipids which could interfere

with IEF (Seong et al. 2017). Tucholska et al. (2010) previously reported successful implementation of organic solvent precipitation with ACN and methanol for low abundance blood proteins detection with liquid chromatography (LC) MS. Furthermore, also a usage of 70% and 50% cold acetone shows similar removal efficiency of albumin/IgG to that of two commercial kits, ProteoExtract and ProteoPrep (Seong et al. 2017). Albumin removal using organic solvents as acetonitrile and methanol removes albumin to some extent and can be used for biomarker discovery using proteomic in-gel methods.

Another tested organic precipitation strategy, ACN protocol, resulted in significant decrease in number of spots and the overall protein profile did not correspond to that of crude plasma. Out of all 15 proteins selected for identification, only one spot identified as albumin possessed significant Mascot score. This method selectively enriches proteins of hydrophobic nature and was developed mainly for LC-MS and multiple reaction monitoring (Kay et al. 2008; Fernandez et al. 2011) and seems unfit for 2-DE followed by MALDI-TOF MS.

The tested commercial ProteoPrep Blue Albumin and IgG Depletion Kit is based on Cibacron Blue stationary phase. Cibacron blue is a polycyclic anionic ligand that acts as a mimic of NAD^+ and $NADP^+$ but also through electrostatic, hydrophobic and other forces and it has been long used for protein purification (Riske et al. 2007; Fang and Zhang 2008; Pisanu et al. 2018). Successful use of Cibacron blue for biomarker discovery was already published (Ahmed et al. 2004). Its disadvantage is that this method also depletes albumin associated proteins and peptides. In present study we observed a slight decrease in number of spots in protein map of plasma depleted with PreoteoPrep Blue kit even though the spots were well separated with good resolution. The removal of albumin was sufficient with visible decrease of density on 1-DE gel (Fig. 2B) despite albumin still being in 2 spots. However, only about half of the proteins selected for identification reached significant Mascot score.

Furthermore, Pisanu et al. (2018), who compared Cibacron Blue based kit and six other commercial depletion kits (four based on immunoaffinity with antibody directed against specific proteins, one based on G protein with human serum albumin antibodies and last one based on the affinity for combinatorial peptide ligand library) on human serum found, that out of the tested depletion methods, the Cibacron Blue kit showed lowest increase in number of proteins and worst outcome in removal of albumin content. Highest increase in number of proteins was reached using peptide library approach, ProteoMiner and albumin was in human serum most completely removed after immunoaffinity SpinTrap kit.

It was shown that even removal of one major protein, like albumin, clearly allows the detection of new proteins. Also, there is limited beneficial impact increased drawbacks in removing associated proteins when depleting more than 12 HAPs (Roche et al. 2009). Interestingly enough, Grubbs et al. (2015), who tested albumin depletion methods on porcine serum reported that it is preferable for 2-DE to use crude plasma or serum with narrow pH range that isolate the albumin toward the edges of the gel.

It is important to note that studies focused on characterizing the albuminome, a subproteome consisting of the proteins and peptides associated with albumin were published and albuminome could also contain some potential disease biomarkers (Camaggi et al. 2010; Holewinski et al. 2013).

Conclusion

Removing albumin and other HAPs from blood plasma/serum remains challenging task not only in our laboratory but for many research groups worldwide in different areas from medical, through veterinary to basic animal research.

Out of the four tested albumin depletion methods, precipitation with AMW protocol showed the best results. The protein spots were distinctively separated and the protein spot count after application of this method significantly increased. Also, AMW protocol improved protein identification with MALDI-TOF/TOF MS. However, the protein profile of albumin-enriched fraction shows that the method depletes also fraction of proteins with lower molecular mass.

Albumin depletion using organic solvents as acetonitrile and methanol removes albumin to some extent and can be used for biomarker discovery using proteomic in-gel methods. Simultaneously, it is advisable to pay attention to the albumin-enriched fraction and look for possible improvements to decrease the removal of proteins with lower molecular mass.

Conflict of interest. The authors report no conflict of interest.

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Supplementary Material

A comparison of albumin removal procedures for proteomic analysis of blood plasma

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Supplementary Tables

Table S1. Mass spectrometry identification details for identified proteins from 1-DE

Protein	UniProt ID	MW (kDa)	pI	Protein sequence coverage (%)	Total Mascot score	Expected MS
Albumin	ALBU_RAT	70.68	6.09	20	233	4.00E-20
Apolipoprotein A-I	APOA1_RAT	30.1	5.52	57	309	1.00E-27
Apolipoprotein A-IV	APOA4_RAT	44.43	5.15	16	211	6.40E-18
C-type lectine domain family 11 member A	CLC11_RAT	36.71	5.28	1	21	6.4
Gastrin-releasing peptide	GRP_RAT	15.75	6.28	20	26	2.3
Hemoglobin subunit beta-1	HBB1_RAT	16.08	7.88	32	132	5.10E-10
Hemopexin	HEMO_RAT	52.06	7.58	19	102	5.10E-07
Serotransferrin	TRFE_RAT	78.51	7.14	16	164	3.20E-13
Transthyretin	TTHY_RAT	15.82	5.77	39	92	4.60E-06
Vitamin D-binding protein	VTDB_RAT	55.11	5.65	20	169	1.00E-13

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Table S2. Mass spectrometry identification details for crude plasma protein map

Spot no.	UniProt ID	MW (kDa)	pI	Protein sequence coverage (%)	Total Mascot score	Expected MS
1	ALBU_RAT	70.68	6.09	21	358	1.30E-32
2	ALBU_RAT	70.68	6.09	26	185	2.50E-15
3	ALBU_RAT	70.68	6.09	11	231	6.40E-20
4	ALBU_RAT	70.68	6.09	11	196	2.00E-16
5	SPRYZ_RAT	22.12	6.22	15	24	33
	mix.					
6	KNT1_RAT	48.83	6.08	28	206	2E-17
	ALBU_RAT	70.68	6.09	23	254	3.2E-22
7	FIBG_RAT	51.23	5.62	31	134	3.20E-10
8	ARC1A_RAT	42.14	8.46	18	-	18
9	ALBU_RAT	70.68	6.09	15	239	1.00E-20
10	FIBA_RAT	87.37	5.51	15	181	6.40E-15
11	ALBU_RAT	70.68	6.09	27	426	2.00E-39
12	FIBA_RAT	87.37	5.51	17	220	8.00E-19
13	FIBA_RAT	87.37	5.51	27	336	2.00E-30
	mix.					
14	FIBB_RAT	54.83	7.9	52	353	4e-32
	FIBA_RAT	87.37	5.51	23	190	8e-16
15	FIBA_RAT	87.37	5.51	26	246	2.00E-21

Spot numbers correspond to those on Fig. 2A.

Table S3. Mass spectrometry identification details for ProteoPrep kit plasma protein map

Spot no.	UniProt ID	MW (kDa)	pI	Protein sequence coverage (%)	Total Mascot score	Expected MS
1	RPN1_RAT	68.376	6.05	9	20	20
2	ALBU_RAT	70.682	6.09	28	277	1.60E-24
3	ALBU_RAT	70.682	6.09	25	357	1.60E-32
4	-	-	-	-	-	-
5	TBB3_RAT	50.842	9.4	12	29	4.82
6	KNT1_RAT	48.828	6.08	40	326	2.00E-29
7	FIBG_RAT	51.228	5.62	10	98	1.30E-06
8	SCO2_RAT	57.491	9.35	10	32	4.8
9	VIME_RAT	53.757	5.06	10	35	2.7
10	BRMS1_RAT	28.459	4.67	13	34	3.1
11	NSF1_RAT	50.38	8.92	9	27	1.7
12	HEMO_RAT	52.06	7.58	24	241	6.40E-21
13	HEMO_RAT	52.06	7.58	25	117	1.60E-08
14	FIBB_RAT	54.828	7.9	40	200	8.00E-17
15	FIBA_RAT	87.373	5.51	25	322	5.10E-29

Spot numbers correspond to those on Fig. 2B.

Table S4. Mass spectrometry identification details for AMW plasma protein map

Spot no.	UniProt ID	MW (kDa)	pI	Protein sequence coverage (%)	Total Mascot score	Expected MS
1	ALBU_RAT	70.682	6.09	31	419	1.00E-38
2	ALBU_RAT	70.682	6.09	16	337	1.60E-30
3	ALBU_RAT	70.682	6.09	22	288	1.30E-25
4	TRFE_RAT	78.512	7.14	21	373	4.00E-34
5	KNT1_RAT	48.826	6.08	19	111	6.40E-08
6	KNT1_RAT	48.826	6.08	22	123	4.00E-09
7	FIBG_RAT	51.228	5.62	21	169	1.00E-13
8	FIBG_RAT	51.228	5.62	35	237	1.60E-20
9	ALBU_RAT	70.682	6.09	46	462	5.10E-43
10	ALBU_RAT	70.682	6.09	31	278	1.30E-24
11	ALBU_RAT	70.682	6.09	40	357	1.60E-32
12	HEMO_RAT	52.06	7.58	30	331	6.40E-30
13	HEMO_RAT	52.06	7.58	33	375	2.50E-34
14	FIBB_RAT	54.828	7.9	41	249	1.00E-21

Spot numbers correspond to those on Fig. 2C.