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# Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets

For the preparation of proteins for proteome analysis, precipitation is frequently used to concentrate proteins and to remove interfering compounds. Various methods for protein precipitation are applied, which rely on different chemical principles. This study compares the changes in the protein composition of human blood platelet extracts after precipitation with ethanol (EtOH) or trichloroacetic acid (TCA). Both methods yielded the same amount of proteins from the platelet preparations. However, the EtOH-precipitated samples had to be dialyzed because of the considerable salt content. To characterize single platelet proteins, samples were analyzed by two-dimensional fluorescence differential gel electrophoresis. More than 90% of all the spots were equally present in the EtOH- and TCA-precipitated samples. However, both precipitation methods showed a smaller correlation with nonprecipitated samples (EtOH 74.9%, TCA 79.2%). Several proteins were either reduced or relatively enriched in the precipitated samples. The proteins varied randomly in molecular weight and isoelectric point. This study shows that protein precipitation leads to specific changes in the protein composition of proteomics samples. This depends more on the specific structure of the protein than on the precipitating agent used in the experiment.

**Keywords:** Ethanol / Platelets / Protein precipitation / Proteomics / Trichloroacetic acid
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## 1 Introduction

Proteome analysis of clinical samples aims at characterizing disease-specific changes in the protein expression profile of an affected tissue. Such changes are potential diagnostic markers or may help to identify drug targets. Blood platelets are small enucleated cellular particles that play a fundamental role in hemostasis, contributing to the formation of vascular plugs. Pathologically, they are involved in thrombosis and atherosclerosis. A detailed analysis of the proteome and signaling cascades in platelets from patients is expected to aid the development of new therapeutic agents that may help to treat thrombotic diseases [1, 2]. Platelets can be isolated from peripheral blood in a few preparative steps to a high cellular purity. To prevent activation of platelets, it is advisable to avoid strong centrifugation and to separate platelets from plasma protein by gel filtration. The gel-filtered platelets are more quiescent, and are generally in better condition than platelets prepared by multiple steps of centrifugation [3]. However, it is impossible to concentrate the platelets using this technique. Therefore, the protein concentration is low and direct proteomic analysis of this platelet isolation is not possible. In addition, cells contain proteases and high levels of non-protein impurities. An appropriate sample preparation is essential for obtaining reliable results in a proteomic analysis. Such a preparation should work by inhibiting protease activities and quantitatively enriching proteins, while leaving behind substances, such as salts, lipids, and nucleic acids, which would interfere with any further proteomic analysis. Protein precipitation followed by dissolving the pellet in IEF compatible sample solution is generally employed to concentrate and selectively separate proteins in the sample from the interfering substances. In addition, the protein denaturation during precipitation leads to an inhibition of proteases [4].

The precipitation of proteins from biological fluids had been observed for hundreds of years (*e.g.,* the precipitation of casein from milk by dilute acid). However, the molecular basis of protein solubility began to receive serious attention only in the middle of the last century. Fractionation of human plasma by precipitation carried forward

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**Abbreviations: DIGE**, differential gel electrophoresis; **EtOH**, ethanol; **GFP**; gel-filtered platelet; **PRP**, platelet-rich plasma; **TM3**, tropomyosin $\alpha$ 3

during World War II made possible the preparation of many protein components of the human plasma [5]. The solubility of proteins is determined by four variables: pH, ionic strength, temperature, and protein concentration. Numerous different techniques have been developed for protein precipitation by modifying one or more of these parameters (for review see [6]). Several methods have been adapted to the needs of proteome analysis [4, 7, 8] and today various protein extraction kits are commercially available which apply precipitation techniques. The present study evaluates the applicability of two methods of protein precipitation for clinical proteomics: ethanol (EtOH) precipitation and TCA precipitation. Both methods are commonly used in the preparation of protein extracts for proteomic analysis, although they rely on different chemical principles. EtOH causes precipitation of proteins mainly because it significantly lowers the dielectric constant of the aqueous solution (relative dielectric constant at 20°C for $H_2O$ is 18 and for $C_2H_5OH$ is 26). In general, ionic compounds are more soluble in solvents with high dielectric constants. Through its polar groups, EtOH interacts with the polar group of the protein in competition with water. In addition, the hydrophobic groups may disrupt the intramolecular hydrophobic interaction. Finally, a large volume of EtOH reduces the effective concentration of water, leaving only a small amount for hydration of the protein. Upon dehydration by EtOH, protein molecules attract each other to a sufficient degree by van der Waals forces and thus become insoluble in the EtOH-water mixtures [9]. TCA, in contrast, leads to a strong decrease in pH, resulting in denaturation and consequently precipitation of the protein. A recent study showed that the three chloro groups in the molecule also play an important role in protein precipitation, which is not clear [10]. TFA, which is a stronger acid than TCA and possesses three fluoro groups instead of chloro groups as in TCA, is not such a potent protein precipitation-inducing agent.

The precipitation efficiency of both EtOH and TCA depends also on the physicochemical characteristics of the protein. Therefore, they are also applied for the fractionation of protein mixtures. Especially EtOH is broadly used for protein fractionation even at industrial scale. Proteomics comprises the analysis of thousands of different protein species simultaneously. Applying EtOH or TCA precipitation in the analysis of such complex protein mixtures may result in depletion of particular protein species and in a relative enrichment of other protein species. The present study investigates qualitative and quantitative changes in the protein composition of platelet extracts after protein precipitation. It describes the protein yield, the linearity, and the protein selectivity of EtOH and TCA precipitation. By comparing these characteristics with nonprecipitated samples, the study evaluates the suitability of protein precipitation in the proteome analysis of clinical samples of this type.

## 2 Materials and methods

### 2.1 Blood sampling and platelet preparation

Peripheral venous blood was drawn without stasis from 60 healthy volunteers aged between 20 and 90 years (30 female/30 male; average age 53 ± 21 years). The study was approved by the local ethics committee. Blood was drawn from the antecubital vein of the subjects into vacutainer tubes containing 0.129 mol/L trisodium citrate (Vacuette system; Greiner, Kremsmuenster, Austria). The first 3 mL of blood was discarded as usual for platelet studies. For exclusion of erythrocytes and leukocytes, the citrated whole blood was centrifuged at 50 × *g* for 20 min at room temperature. The resulting supernatant was the platelet-rich plasma (PRP). A plasma-free platelet suspension was prepared by passing PRP through a size-exclusion chromatography (SEC) column. One milliliter PRP was applied onto 11 mL packed Sepharose 2B (Sigma, Steinheim, Germany) column (BioRad, Hercules, CA, USA; 15 mm diameter) equilibrated in calcium-free Dubecco's PBS (GIBCO, Paisley, Scotland, UK). Platelet fractions (1.5 mL) of each individual were collected after an elution volume of 2.5 mL, and platelet concentration was counted on a MicroDiff 18 Blood Analyzer (Coulter Electronics, Miami, FL, USA).

### 2.2 TCA precipitation

Fifteen-hundred microliters of platelet suspension was mixed with 500 μL of ice-cold 6.1 N TCA solution (Sigma) containing 80 mM DTT (Roche Diagnostics, Mannheim, Germany). The mixture was incubated for 1 h at 4°C to allow the protein precipitation to complete. Then the extract was centrifuged at 10 000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet was washed four times with 1500 μL of ice-cold acetone (p.a. grade; Merck, Darmstadt, Germany) each, containing 20 mM DTT; the pellet was regained in each step by centrifugation at 10 000 × *g*. Thereafter, the centrifuged pellet was dried by air evacuation. For 2-DE, the pellet was resolubilized in denaturing 2-D sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl (pH 8.5) by shaking overnight at 4°C. Seventy microliters of the sample buffer was used *per* $100 \times 10^6$ platelets. Alternatively, the samples were stored in the last wash aliquot of acetone at −70°C until further use.

### 2.3 EtOH precipitation

Nine-hundred microliters of platelet suspension was mixed with 8000 μL EtOH (99.9% Uvasol p.a; Merck). Proteins were allowed to precipitate in this EtOH solution for 3 h at room temperature followed by storage at −20°C overnight. To collect the proteins, the samples were centrifuged at 10 000 × *g* for 20 min at 4°C and the supernatant was removed. The pellet was washed once with pure EtOH before it was dried (as above). For 2-DE, the pellet was resolubilized in 100 μL of 2-D sample buffer as above *per* 100 × $10^6$ platelets. Alternatively, the samples were stored in the 90% EtOH solution at −20°C until further use.

### 2.4 Dialysis

To reduce the salt contamination of 2-D samples from EtOH-precipitated platelet proteins, they were dialyzed against a 40× sample volume of identical 2-D sample buffer for 3 h at room temperature with PlusOne Mini Dialysis Kit (molecular mass cut-off 1 kDa) (Amersham Biosciences,Uppsala, Sweden).

### 2.5 Preparation of proteins from nonprecipitated platelets

Fifteen-hundred microliters of gel-filtered platelet (GFP) suspension was centrifuged at 1500 × *g* for 10 min at room temperature. The supernatant was discarded and the platelet pellet was solubilized in 100 μL of 2-D sample buffer as above *per* 100 × $10^6$ platelets.

### 2.6 Determination of protein concentration

The protein concentration in resolubilized samples was determined in triplicate using a CBB protein assay kit with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA). With appropriate predilution, the 2-D sample buffer components do not interfere with the protein assay. Therefore, the samples were diluted 1:20 with PBS and 5% of the 2-D sample buffer was added in the BSA standards.

### 2.7 Analysis of platelet proteins by 1-DE and 2-DE

IEF was performed loading 120 μg (for gels to be silver-stained) or 150 μg (for gels with CyDye-labeled proteins) of platelet proteins by in-gel rehydration in a volume of 450 μL, denaturating the 2-D buffer (7 M urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, 0.5% Servalyt™ pH 3–10; Serva, Heidelberg, Germany) onto 24 cm IPG DryStrips, pH 3–10 linear and pH 4–7 linear (Amersham Biosciences) and focused for 50 and 30 kVh, respectively, using an Amersham IPGphor unit. Before loading onto SDS-polyacrylamide gels, IPG strips were incubated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% DTT and then for another 15 min in equilibration buffer containing 2.5% iodoacetamide. The SDS-polyacrylamide gels (26 × 20 cm × 1 mm, T = 11%, C = 2.6%) were cast according to Laemmli [11]. The second dimension was performed using an Ettan DALT six System (Amersham Biosciences) according to the manufacturer's instructions. 1-D SDS-gel electrophoresis was performed with 13 × 16 cm gels.

### 2.8 Protein staining and image analysis of silver-stained gels

Silver nitrate staining for analytical gels was performed according to Heukeshoven and Dernick [12], while gels for MS analysis were stained with mass-compatible silver stain according to Shevchenko [13]. The silver-stained gel images were digitized using a Molecular Imager® FX (BioRad). Computer-aided 2-D image analysis was carried out using the MELANIE 3 software (GeneBio, Geneva, Switzerland).

### 2.9 Protein labeling and image analysis of DIGE gels

Fifty micrograms of resolubilized platelet protein preparations was labeled with 333 pmol of CyDye DIGE Fluor minimal dyes (Amersham Biosciences). Pre-electrophoretic labeling was performed according to the manufacturer's instructions. The gels with separated labeled proteins were scanned using the Typhoon™ 9410 imager (Amersham Biosciences), and the protein patterns were displayed with the IQTools software. All sample gel images were processed by the DeCyder DIA (differential in-gel analysis) software (Amersham Biosciences) module to codetect and differentially quantify the protein spots.

### 2.10 In-gel protein digestion

The spots of interest were excised from the gels, chopped into pieces, and transferred into 0.5 mL tubes (Axygen, Union City, CA). The gel pieces were washed twice with 200 μL of 50 mM $NH_4HCO_3$ buffer (pH 8.5) (Sigma) for 10 min and afterwards with 200 μL of 50 mM $NH_4HCO_3$ in 50% ACN (HPLC-grade; Merck) for 10 min. Subsequently, the gel pieces were dehydrated by adding 50 μL ACN and

allowed to reswell in 180 μL of 10 mM DTT in 50 mM $NH_4HCO_3$ buffer in order to perform reduction (56°C, 30 min). After cooling to room temperature, the solution was replaced by 150 μL of 50 mM iodoacetamide in 50 mM $NH_4HCO_3$ buffer and the gel pieces were incubated in the dark for 20 min at room temperature. The gel pieces were washed three times with 200 μL of 50 mM $NH_4HCO_3$ buffer and three times with 50 mM $NH_4HCO_3$ in 50% ACN for 10 min at room temperature, dehydrated with 50 μL ACN, and dried in the Eppendorf Concentrator 5301 (Eppendorf, Hamburg, Germany) for 5 min. The enzymatic digestion of the proteins was carried out on ice by a stepwise addition of 0.5–2 μL of 12.5 ng/μL trypsin (sequenzing-grade unmodified; Roche, Basel, Switzerland) in 50 mM $NH_4HCO_3$ buffer until they were totally rehydrated. Finally, enough 50 mM $NH_4HCO_3$ buffer was added to keep the gel pieces covered during digestion at 37°C overnight. After digestion, the supernatant was removed and the peptides were extracted once with 20 μL 50 mM $NH_4HCO_3$ buffer and twice with 20 μL 5% formic acid (Sigma) by sonification for 5 min at room temperature in an ultrasonic water bath (Sonorex RK 255 H; Bandelin, Berlin, Germany).

### 2.11 NanoHPLC-MS/MS protein sequencing

All nanoHPLC separations were performed on the UltiMate system from LC Packings (Amsterdam, The Netherlands). The in-gel digests were loaded onto a precolumn (PepMap C18 material, 300 μm ID × 5 mm length; LC Packings) by the FAMOS μ-autosampler and the Switchos loading pump operated at 20 μL/min using water with 0.1% TFA (Pierce, Rockford, IL, USA) as mobile phase. The sample was eluted from the precolumn in a back flush mode. The dimensions of the separation column were 0.075 mm ID × 150 mm length, 3 μm particle size. The flow rate of the nanoHPLC system was set at 200 nL/min and the UV detector was operated at 214 nm using the nano UV-Z view flow cell (volume 3 nL). The mobile phases were A = 95% water (HPLC-grade, Supra-Gradient, Biosolve B.V., The Netherlands), 5% ACN (HPLC-grade, Supra-Gradient, Biosolve B.V.), 0.1% formic acid (Fluka, Buchs, Switzerland); and B = 30% water, 70% ACN, 0.1% formic acid. The HPLC gradient for separation was 0–50% B in 30 min and 50–100% B in 2 min. The nanoHPLC system was coupled to an IT mass spectrometer (LCQ Deca XPplus, Thermo Finnigan) *via* a nanoESI source using Pico Tip emitters (New Objective, Cambridge, MA, USA). The following ESI parameters were used: spray voltage, 1.8 kV; capillary temperature, 185°C; capillary voltage, 45 V; tube lens offset voltage, 25 V; and the electron multiplier at −1050 V. The collision energy was set automatically depending on the mass of the parent ion. Gain control was set to $5 \times 10^7$. The data were collected in the centroid mode using Dynamic Exclusion. One MS experiment (full-MS) was followed by three MS/MS experiments of the three most intensive ions (intensity at least $1 \times 10^6$). The analysis of MS/MS spectra with respect to the peptide identity was routinely performed by applying both the MASCOT (Matrix Science) and the SEQUEST (Thermo Finnigan) search engines. A peptide was reliably identified only if the individual peptide scores were >43 (MASCOT) and >3.2 (SEQUEST).

## 3 Results

### 3.1 Sample quality of precipitated platelets and compatibility with 2-DE

To evaluate and compare the applicability of TCA precipitation and EtOH precipitation for clinical proteomics, we used these methods for the preparation of proteins from blood platelets for 2-DE. GFP showed a mean concentration of $117 \pm 42 \times 10^6$ Plt/mL. This suspension was subjected to a precipitation either with TCA or with EtOH, according to the procedures described in Section 2. The 2-D gel of TCA-precipitated proteins showed a clear protein pattern with about 1400 individual spots, which were found with good reproducibility (Fig. 1A). However, EtOH-precipitated proteins were only well separated in the lower p*I* range (Fig. 1B). In the p*I* range above 6.0, the proteins were only seen as horizontal streaks. This indicates a nonsufficient separation of EtOH-precipitated proteins in the first-dimensional IEF. Figures 1D–F show the electrical current and the voltage profile of the IEF separation. The high current peak in the first part of the IEF of EtOH-precipitated proteins indicates that this sample contained high salt concentrations (Fig. 1E, arrow). These salts had to be removed by dialysis in order to get a satisfactory separation by 2-DE (Fig. 1C and F). This dialysis step was included later on in all following EtOH-precipitation experiments.

### 3.2 Protein yield

The protein precipitation methods were evaluated in the GFP preparations of 60 different volunteers. The amount of proteins, which was extracted from $100 \times 10^6$ platelets after TCA precipitation, was equal to that extractable after EtOH precipitation from the same number of platelets (Fig. 2A). The protein amount increased linearly with the number of platelets used in the experiments (Fig. 2B). As an alternative approach for increasing the platelet concentration of GFP, we centrifuged the cell suspension and extracted the proteins from the pellet in the 2-D sample

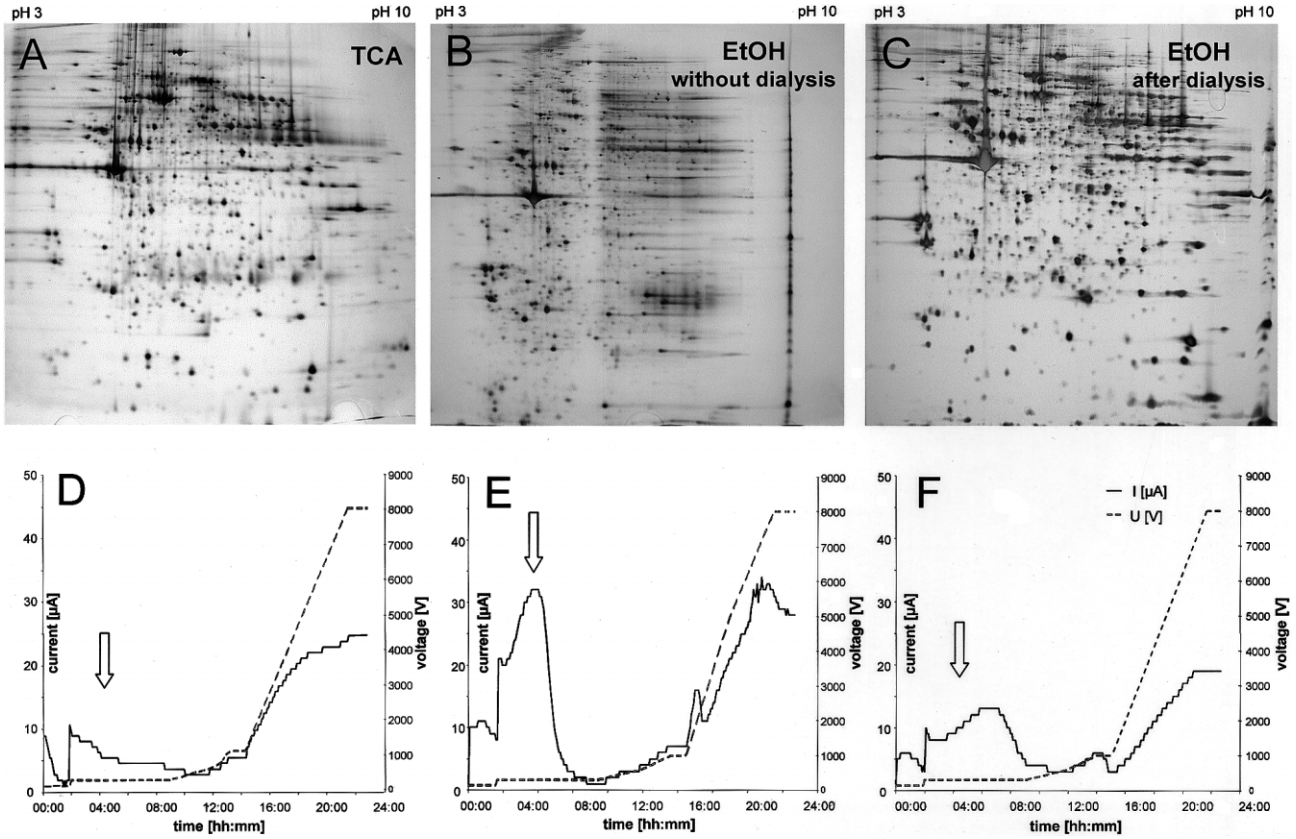


**Figure 1.** 2-D electrophoretic analysis of gel-filtered human platelet suspension following precipitation with (A) TCA and (B) EtOH resolubilized in 2-D sample buffer. In (C) EtOH-precipitated and -solubilized platelet proteins were dialyzed against fresh 2-D sample buffer to remove salt contamination. Twelve-hundred microliters of prepared platelet proteins was loaded by passive rehydration onto 24 cm pH 3–10 IPG strips. Current and voltage profile were recorded of (D) TCA-, (E) EtOH-, and (F) EtOH-precipitated and -dialyzed platelet samples during the 1-D. Gels were stained with analytical silver nitrate stain.

buffer. The supernatant contained almost no protein (2 μg proteins *per* 100 × $10^6$ platelets). The amount of proteins extracted from these nonprecipitated platelets was similar to that found after precipitation (124 ± 10 μg proteins *per* 100 × $10^6$ platelets). These data show that both precipitation methods have a similar protein yield and indicate that precipitation followed by resolubilization is not accompanied by a major protein loss.

### 3.3 Specificity of protein precipitation

To characterize potential protein specific differences in precipitation efficiency, we performed a comparative electrophoretic analysis. Nonprecipitated, TCA-precipitated, and EtOH-precipitated platelet proteins were separated in 1-D SDS-PAGE. The results are shown in Fig. 3A. The protein band pattern is similar in all three samples. Only the nonprecipitated platelets show some slight bands in the upper molecular weight range, which are barely detectable in the precipitated samples. TCA- and EtOH-precipitated proteins showed a nearly identical pattern of protein bands.

For definitive comparison of different precipitation methods on platelet protein pattern, we switched to the differential gel electrophoresis (DIGE) technology. TCA-precipitated proteins, EtOH-precipitated proteins, and nonprecipitated proteins were labeled with three different fluorescent dyes, combined and separated by 2-DE. The overlay of the three signals is shown in Fig. 3B. Most protein spots are equally present in all three samples (black spots). However, some protein spots which are present in the nonprecipitated samples are diminished in the TCA-precipitated and EtOH-precipitated samples (magenta spots). These proteins are equally distributed all over the 2-D gel. Almost no TCA-specific protein spots (blue) or EtOH-specific protein spots (yellow) are visible. However, some green protein spots are present in the 2-D gel, indicating proteins which are enriched by both precipitation

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