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Journal of Chromatography A, 1023 (2004) 317–320
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

# Comparison of protein precipitation methods for sample preparation prior to proteomic analysis

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Received 4 September 2003; accepted 8 October 2003

**Abstract**

Protein samples should be free of salt and other disturbing agents and have an appropriate concentration to be suitable for two-dimensional (2D) electrophoresis, the principal step of proteomics. To find the most efficient method for sample preparation, we used human plasma and compared four widely applied precipitation methods, using trichloroacetic acid (TCA), acetone, chloroform/methanol and ammonium sulfate, as well as ultrafiltration. Precipitation with TCA and acetone and ultrafiltration resulted in an efficient sample concentration and desalting. We also found that ammonium sulfate fractionation can efficiently remove albumin, which represents more than 50% of plasma proteins.


*Keywords:* Sample preparation; Proteomics; Proteins

## 1. Introduction

Appropriate sample preparation is essential for obtaining reliable results in a proteomic analysis [1,2]. Samples should have a high protein concentration (usually higher than 10 mg/ml) and be free of salt and other disturbing factors, such as ionic detergents, nucleic acids, lipids, etc., that could interfere with the two-dimensional (2D) electrophoresis. Precipitation followed by pellet uptake in isoelectric focusing (IEF) compatible sample solution is generally employed to concentrate and selectively separate proteins in the sample from the contaminating species. Protein concentration and desalting methods, however, are associated with artifacts, which can affect the results of the proteomic study, the most common being the incomplete protein recovery. Here we used human plasma and compared the most widely used precipitation methods, using trichloroacetic acid (TCA), acetone, chloroform/methanol and ammonium sulfate, as well as ultrafiltration in order to chose the most reliable one for a high throughput sample analysis. Furthermore, we studied the effect of increasing ammonium sulfate concentration on albumin depletion from plasma.

Plasma is frequently used as an experimental material to start with because it is easy to collect. Plasma and cerebrospinal fluid (CSF), whose composition is similar but the protein concentration is quite different from that of plasma, are body fluids that can be analyzed to monitor the course of a disease. Alterations in the physiological or disease states change levels of key regulatory proteins in body fluids. Information about the magnitude of the change of identified disease-specific proteins and clusters in body fluids thereof may be of diagnostic, prognostic, or therapeutic significance [3].

## 2. Experimental

### 2.1. Plasma

Human plasma was from control individuals and had a protein concentration of about 65 mg/ml. All precipitation methods and ultrafiltration were performed with the same protein sample and amount (0.8 mg), only the fractionation with ammonium sulfate was performed with 2 mg of total protein.

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doi:10.1016/j.chroma.2003.10.029

### 2.2. TCA precipitation

A 12.3 μl of plasma (0.8 mg) were diluted to 200 μl with phosphate-buffered saline (PBS) and 40 μl of 60% TCA (Fluka, Buchs, Switzerland) were added to the diluted solution. The mixture was incubated overnight on ice and centrifuged at 10 000 g, 4 °C for 30 min. The supernatant was removed and 100 μl of 90% ice-cold acetone (Fluka) were added to wash the pellet. The sample was incubated on ice for 15 min and centrifuged as above. The acetone-containing supernatant was removed and the pellet was air dried. For 2D gel electrophoresis, the pellet was suspended in 200 μl of sample buffer, consisting of 7 M urea, 2 M thiourea, 20 mM Tris, pH 7.5, 2% 3-[(cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS), 0.4% 1,4-dithioerythritol and a drop of bromophenol blue and centrifuged at 10 000 g at room temperature for 5 min.

### 2.3. Acetone precipitation

A 12.3 μl of plasma was diluted to 50 μl with PBS. Three volumes of ice-cold acetone (150 μl) were added into the diluted plasma solution and kept overnight on ice. The sample was centrifuged at 10 000 g, 4 °C for 30 min, the supernatant was removed and the pellet was air dried. For 2D gel electrophoresis, the pellet was suspended in 200 μl of sample buffer as above.

### 2.4. Chloroform/methanol precipitation

Plasma (12.3 μl) was diluted to 200 μl with PBS and 0.5 ml of chloroform/methanol (70:30 v/v, both from Merck, Darmstadt, Germany) mixture was added. After staying on ice for 2 h, the lower organic layer was discarded. The mixture in the tube was centrifuged at 10 000 g, 4 °C for 30 min and the supernatant (about 200 μl) was transferred to Ultrafree-4 tubes and concentrated to 50 μl (2000 g, 10 °C). About 1 ml of sample buffer was added and the mixture was centrifuged at 2000 g, 10 °C until the volume of the concentrate reached about 200 μl, as is required for the first dimensional separation.

### 2.5. Ammonium sulfate precipitation

To 12.3 μl of plasma diluted with PBS to 200 μl, 132.4 mg of ammonium sulfate powder (Fluka) was added and the mixture was gently vortexed for 10 min. After 2 h on ice, the mixture was centrifuged at 10 000 g, 4 °C for 30 min. The supernatant was removed, and 90% ice-cold acetone (200 μl) was added to wash the pellet. After staying on ice for 15 min, the mixture was centrifuged again as above. The supernatant was removed and the pellet was air dried. The pellet was suspended in 200 μl of IEF-sample buffer and analyzed by 2D electrophoresis.

### 2.6. Ultrafiltration

A 12.3 μl of plasma and 1 ml of 20 mM Tris HCl, pH 7.5 were placed into an Ultrafree-4 device and centrifuged at 2000 g, 10 °C until the volume of the concentrate reached about 50 μl. Then 1 ml of sample buffer was added and the mixture was centrifuged at 2000 g, 10 °C until the concentrate reached the volume required for the first dimensional separation (about 200 μl).

### 2.7. Fractionation with ammonium sulfate

We further performed a fractionation of the plasma proteins with increased concentrations of ammonium sulfate. Two milligrams (30 μl) of plasma was diluted with PBS to 500 μl and ammonium sulfate (88 mg) was added to reach a 30% saturation. After gentle vortexing for 10 min, the sample was left at room temperature for 1 h and centrifuged at 10 000 g, 20 °C for 30 min. The supernatant was transferred to another tube and ammonium sulfate (72 mg) was added to reach a 50% saturation. The mixture was treated as above. The proteins in the supernatant were further fractionated with 70 and 90% ammonium sulfate saturation (77 and 85 mg of ammonium sulfate were added, respectively). Each of the four pellets was washed with 300 μl of ice-cold 90% acetone, dried in the air and suspended in 200 μl of sample buffer as above.

### 2.8. Two-dimensional electrophoresis

The precipitates recovered from the precipitation steps and the concentrate after ultrafiltration were analyzed by two-dimensional gel electrophoresis essentially as reported [4,5]. After dissolved in sample buffer and centrifuged, the samples were applied on IPG pH 3–10 non-linear strips. Focusing started at 200 V and the voltage was gradually increased to 5000 V at 3 V/min and kept constant for a further 24 h (approximately 180 000 kVh totally). The second-dimensional separation was performed on 12% sodium dodecylsulfate (SDS) polyacrylamide gels. The gels (180 mm × 200 mm × 1.5 mm) were run at 50 mA per gel, in an ISO-DALT apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). After protein fixation for 2 h with 50% methanol, containing 5% phosphoric acid, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 24 h. Dye excess was washed out with $H_2O$ and the gels were scanned in an Agfa DUOSCAN densitometer (resolution 400). Spot identity was deduced by similarity to samples analyzed by MS [6].

## 3. Results and discussion

### 3.1. Precipitation methods

Fig. 1 shows the results of the 2D electrophoresis analysis. In general, the four precipitation methods and

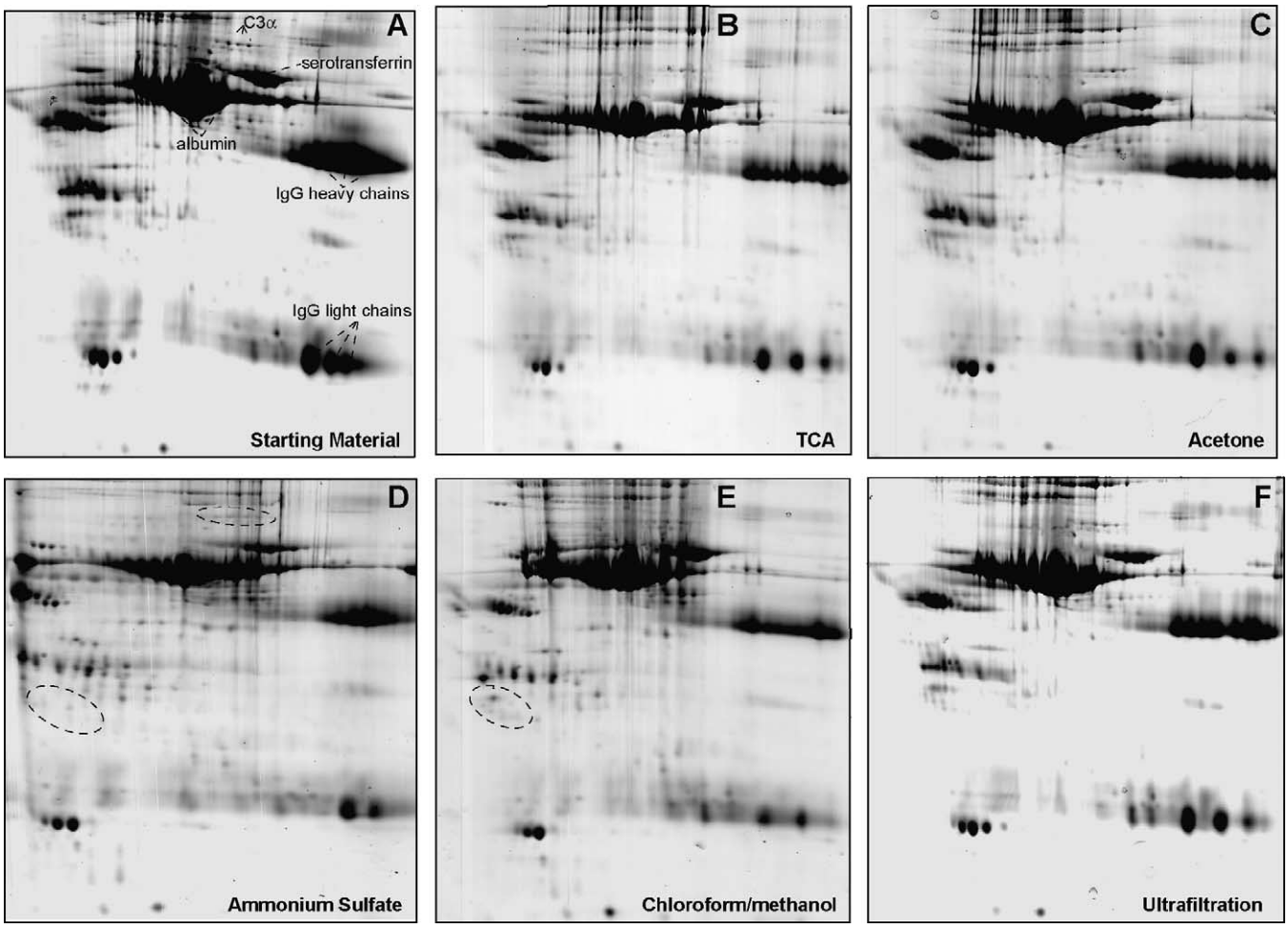


Fig. 1. Two-dimensional electrophoresis analysis of human plasma (A) and of pellets following precipitation with trichroroacetic acid (B), acetone (C), ammonium sulfate (D) and chloroform/methanol (E) and ultrafiltration (F). A 0.8 mg of plasma was used in each case. Analysis was performed on pH 3–10 NL IPG strips, (18 cm) in the first dimension and 12% SDS gels in the second dimension. The gels were stained with Coomassie blue. (D and E) The elliptic areas indicate gel regions in which spots are missing or are very weak compared to the starting material.

ultrafiltration delivered comparable results with no profound deviations. No significant differences were observed between untreated plasma (Fig. 1A) and treated samples (Fig. 1B–F). The recovered protein amount in the sample buffer was not measured because of the small volume and the interference of the solubilizing agents. During TCA precipitation, a protein loss probably took place, most likely on account of the acetone-wash step. Thus, the strong spots representing albumin, heavy and light IgG chains (Fig. 1B) are weaker in comparison with the starting material (Fig. 1A). However, all spots can be detected after TCA precipitation. The acetone precipitation, a relatively easy to perform concentration and desalting step, resulted in a good recovery of all spots (Fig. 1C). Quantitative ammonium sulfate precipitation also resulted in an efficient precipitation of most proteins. The spots representing certain proteins, like complement factor B, C3a and clusterin, are very weak or are missing (the expected position is indicated in Fig. 1D). The chloroform/methanol method yielded satisfactory results as well, although the spots representing C3a and clusterin are missing in the precipitate (Fig. 1E). Ultrafiltration resulted in recovery of practically all proteins.

Thus, TCA precipitation, acetone precipitation and ultrafiltration delivered a higher protein recovery compared to ammonium sulfate and chloroform/methanol steps, which also were satisfactory. From the practical point of view, the easiest method to perform is precipitation with TCA, although it usually requires two steps, precipitation with TCA and removal of TCA traces with acetone. Precipitation with acetone requires larger organic solvent volumes (at least threefold of sample volume) and it is inconvenient to perform if the original sample volume is larger than 300 μl. Ultrafiltration performs very well, however, it is often labor demanding to follow the concentrate volume, the centrifugation steps are usually lengthy, the filters are often blocked and finally the filtration devices are expensive. Thus, the methods of choice are precipitation with TCA or

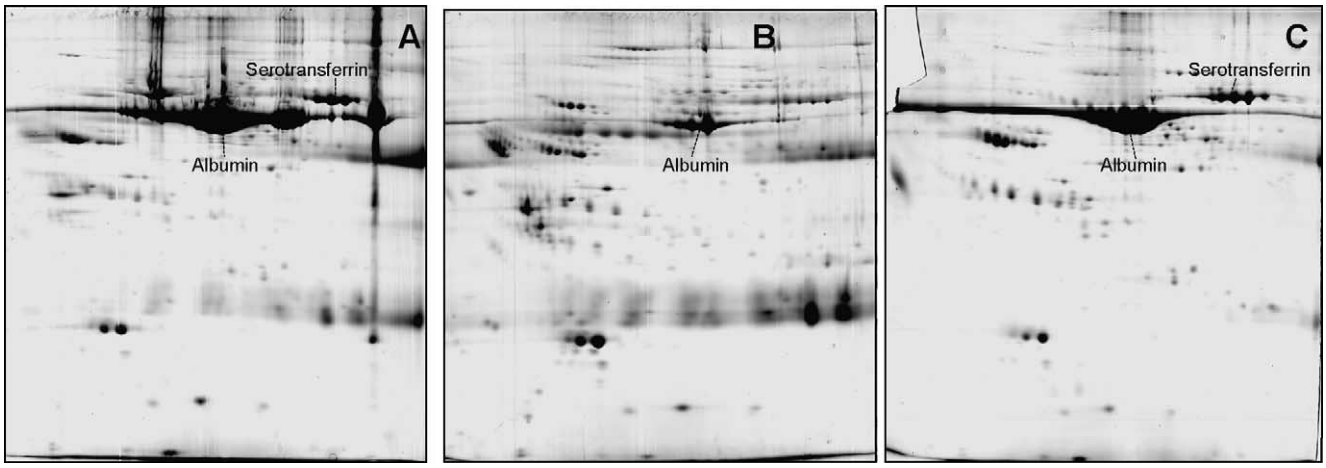


Fig. 2. Two-dimensional electrophoresis analysis of human plasma (A) and of the pellets following precipitation with 50% (B) and 70% ammonium sulfate (C). A 2.0 mg of plasma was used for fractionation. About 0.8 mg protein was applied on each gel. The analysis was performed as stated under legend to Fig. 1.

acetone, where time and costs are important factors. The incubation times used in this work were chosen to be long on purpose in order to achieve a quantitative recovery and can be essentially reduced, in particular for samples with a high protein concentration. Our experience has shown that precipitation with acetone does not perform well with dilute samples, whereas precipitation with TCA yields good results, in particular if the contact time with acid is kept long. This is particularly important for CSF samples, which contain a low protein amount (usually 0.1–0.5 mg/ml). Ultrafiltration may not perform well for CSF which contains small particles and may result in blocking of the filters.

### *3.2. Plasma fractionation with ammonium sulfate*

Furthermore, we studied the effect of ammonium sulfate on the removal of high abundance proteins in plasma. Albumin represents about 50%, heavy IgG chains 19%, serotransferrin 10% and light IgG chains 9% of plasma proteins. The high-abundance components may not allow the signals of the low-abundance proteins to be detected in 2D gels of total plasma proteins. The pellet of the 50% ammonium sulfate precipitation included the majority of plasma proteins and only a small percentage of albumin (Fig. 2B). The 70% ammonium sulfate pellet mainly included albumin, serotransferrin, anti-trypsin and haptoglobin-1 (Fig. 2C). This fraction did not include antibody chains. Starting with 2 mg of plasma, the protein recovery was 0.54 mg (27%) in the 50% and 1.10 mg (55%) in the 70% ammonium sulfate precipitate. Thus, with this simple step most of albumin and serotransferrin can be removed which leads to an increase of the signals of the lower abundant components (compare Fig. 2B with 2A).

## 4. Conclusion

Appropriate sample preparation is essential for obtaining good results in 2D electrophoresis, the principal step of proteomics [1]. The most common methods of sample concentration and desalting are precipitation and ultrafiltration approaches. Our goal was to find the most efficient method for a high throughput sample analysis. We used plasma as a protein sample because a large number of plasma samples are usually analyzed for diagnostic purposes and marker detection and plasma analysis may provide clues for CSF treatment and facilitate the discovery of disease markers in body fluid. We found that precipitation with TCA and acetone and ultrafiltration result in an efficient sample concentration and desalting for a proteomic analysis. Ammonium sulfate fractionation can efficiently remove albumin, which represents more than 50% of plasma proteins.

## Acknowledgements

We thank Dr. S. Evers for critical reading of the manuscript.

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