

Comparison of Serum Albumin Separation by Ammonium Sulfate Precipitation and Cibacron Blue Affinity Chromatography

Running Head: Albumin Separation by Precipitation and Affinity

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ABSTRACT: Serum albumin, which is synthesized in the liver and has many physiological functions, is one of the most abundant proteins in plasma. Albumin is a valuable biomarker of many diseases and has indications in many diseases. The increasing use of albumin in biotechnological applications, its important ligand-carrying properties and its wide range of applications in clinical and basic scientific studies require high purity serum albumin separation. Many methods such as ammonium sulfate precipitation, heat shock method, plasma fractionation with ethanol, chromatographic methods (column, affinity), acetone/trichloroacetic acid precipitation are used for albumin separation from serum. In this study, 2 methods based on ammonium sulfate precipitation and Cibacron Blue affinity chromatography were compared. The purity of the isolated proteins was monitored by SDS-PAGE and densitometrically analyzed. The selected methods were evaluated in terms of time, applicability, purity and cost. It was concluded that ammonium sulfate precipitation provided a more successful separation in terms of albumin yield and purity compared to Cibacron Blue affinity chromatography.

Keywords: *Serum Albumin, Ammonium Sulfate, Cibacron Blue F3GA, Affinity Chromatography, Protein Fractionation*

1 INTRODUCTION

Human serum albumin, which is synthesized in the liver and released into circulation without being stored after synthesis, is one of the most abundant proteins in plasma [1]. Human serum albumin is also found in tissues and body fluids outside the plasma. Serum albumin concentration is 3.5-5 g/dL in adults and 2.9-5.5 g/dL in children [2,3].

Serum albumin has many physiological functions. Albumin is a macromolecule that is

the primary determinant of plasma oncotic pressure and primary regulator of fluid distribution between body compartments [4–6]. It also has an extraordinary ligand binding capacity and is a storage and carrier for many endogenous and exogenous molecules [7]. These molecules include bile acids, bilirubin, amino acids (cysteine, tryptophan, tyrosine), fatty acids, hormones (aldosterone, progesterone, testosterone, cortisol), metal ions (Cu, Ca, Cl, Mg, Zn), and drugs such as

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antibiotics, phenytoin, digoxin [1,5,8].

Serum albumin is a valuable biomarker of many diseases such as cancer, rheumatoid arthritis, ischemia and postmenopausal obesity [7,9,10]. Albumin is widely used for therapeutic purposes in different medical applications. Its uses include hypovolemia, shock, burns, surgical blood loss, trauma, bleeding, cardiopulmonary bypass, hemodialysis, acute liver failure, chronic liver disease, nutritional support and hypoalbuminemia [6,11–14].

Recently, biotechnological applications of serum albumin have been reported, including implantable biomaterials, surgical adhesives and sealants, biochromatography, ligand capture and fusion proteins. Furthermore, albumin has different applications in related research areas such as cryopreservation, stabilization of some proteins and as a supplementation in cell culture [15–18].

Albumin is also important for the body as a ligand carrier. Being a drug carrier affects the therapeutic effect of drugs in the body by affecting their efficacy and half-life. Therefore, isolation of pure serum albumin from serum is important for clinical laboratory applications and basic scientific research [19,20]. Due to its clinical, physiological, and biopharmaceutical function, the need for high-purity albumin isolation has emerged. The annual demand for albumin in the globally is estimated to be approximately 500 tons [21].

Since the last century, albumin has been used in therapeutic and research approaches due to its critical physiological and biopharmaceutical function [21]. Studies have been carried out to obtain high purity and high quality albumin, and many methods have been used and compared for this purpose [3,8,21–23]. Due to the high usage areas of albumin and its high consumption amount, there are studies on albumin separation methods from serum and their comparisons [3,21]. The frequently used methods in the literature for albumin separation from serum can be listed as follows: ammonium sulfate (AS) precipitation [24–26], heat shock method, plasma fractionation with ethanol [27], chromatographic methods (column, affinity) [24,25,27], and acetone/trichloroacetic acid precipitation.

Among the methods of albumin purification from serum, affinity chromatography, ammonium sulfate (AS) precipitation and ethanol fractionation have been evaluated in different studies. Some studies, compared AS precipitation with affinity chromatography [24]. AS precipitation is usually carried out using two-step precipitation method, but there are also studies in the literature that use the four-step precipitation method [26].

Although studies comparing affinity chromatography and two-step AS precipitation have been reported in the literature should be written, no study evaluated both two-step and four-step AS precipitation in combination with

affinity chromatography. Therefore, in this study, three different methods for purifying albumin from serum: Two-step AS precipitation (AS2), Four-step AS precipitation (AS4), and Cibacron blue affinity chromatography (CBAC) were compared in terms of time, applicability, purity, and cost.

As a result, it was concluded that ammonium sulfate precipitation provided a more successful separation in terms of albumin yield and purity compared to the CBAC method.

2 MATERIAL AND METHOD

This study was approved by the Malatya Clinical Research Ethics Committee (2022/101). A total of 6 normolipidemic human sera, three male and three female, aged between 34 and 49 years, were pooled. Albumin separation was performed in sample portions using AS2, AS4, and CBAC methods. Routine biochemical analysis of the pooled serum was also performed. The organization of the study is shown in Figure 1.

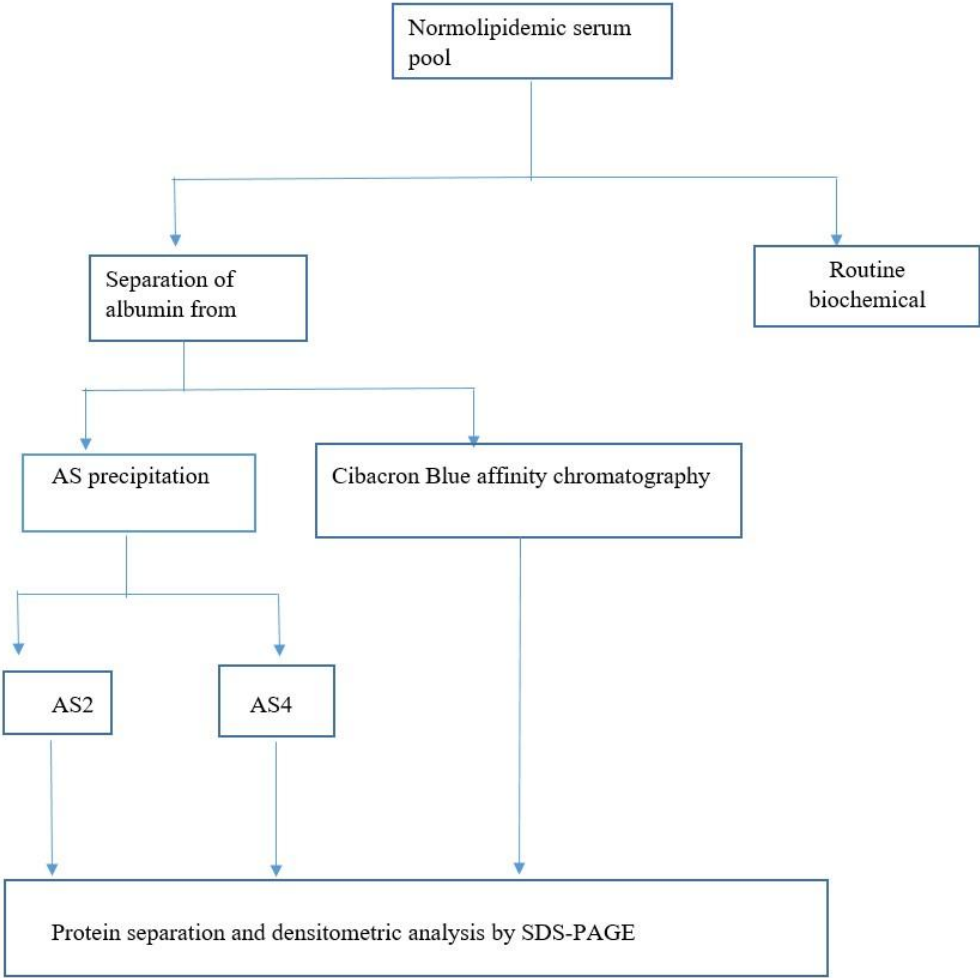


Figure 1: Working diagram.

2.1 Two-stage AS precipitation (AS2)

Ammonium sulfate is frequently preferred in protein precipitation processes. Ammonium sulfate is preferred due to its high solubility, stabilization of protein structure, relatively low density, and cheap and easy availability [28,29]. The basic principle of ammonium sulfate precipitation is to precipitate proteins from the solution with the addition of salt. Proteins are prevented from forming hydrogen bonds with water and the salt facilitates their interaction by forming aggregates that precipitate out of solution [30].

For the AS2 method, a 4.1 M AS solution was prepared and adjusted to pH 7.4

with 0.1 M NaOH. The steps of the method are shown in Figure 2. 1.174 mL AS was added to 1 mL of serum sample in the first step, and room temperature at 10,000 g for 15 min. Globulins were separated from the precipitate. After adding 1.159 mL AS to the supernatant again, the precipitate and supernatant were separated by centrifugation. In the second step, albumin was separated from serum in the precipitate [24,25]. The obtained albumin and globulins were separated by SDS-PAGE electrophoresis. Densitometric spot analysis of the bands present in the gel electrophoresis was performed.

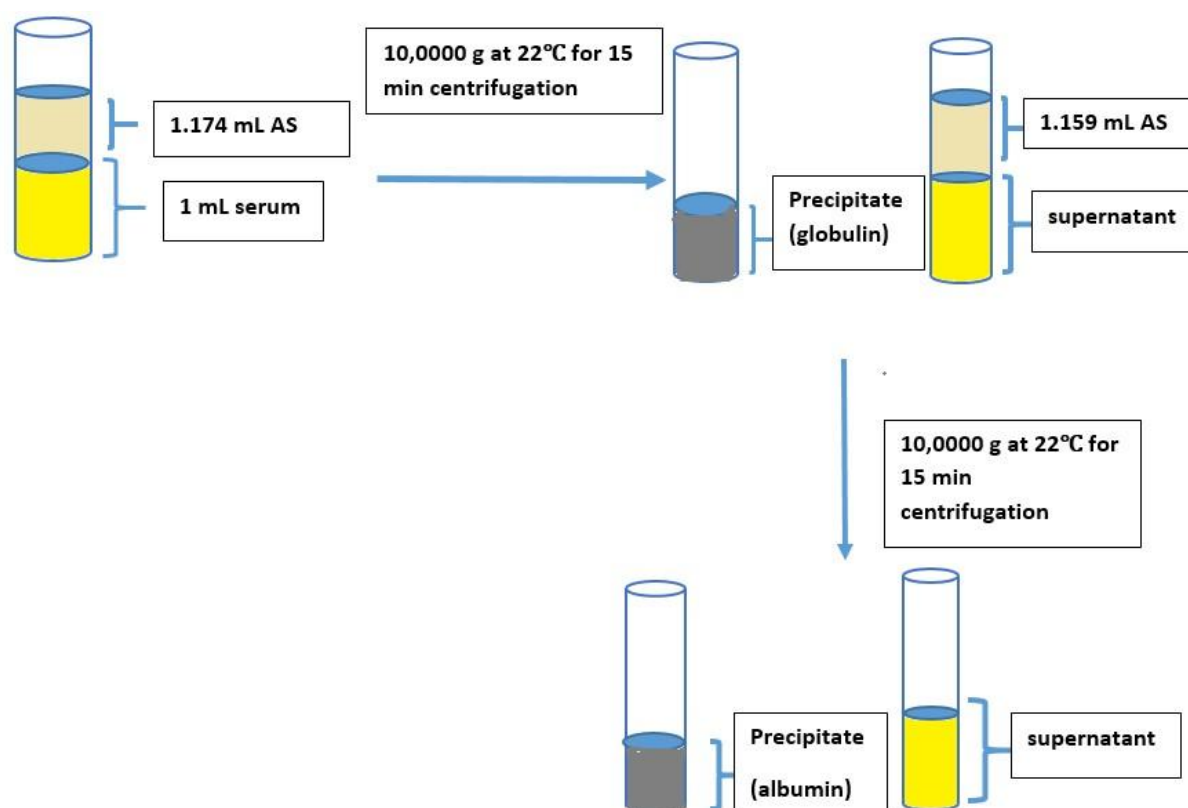


Figure 2: AS2 precipitation method flowchart.

2.2 Four-stage AS precipitation (AS4) method

The AS4 method uses solid AS. It consists of four steps. The amount of solid AS to be added to the serum and supernatant at each stage is indicated in Table 1. After the addition of solid AS, the samples at each stage were left to ice incubation for 2 hours and then centrifuged at 4°C 10,000 g for 15 min. The supernatant and precipitate fractions were separated. At 40% and 50% AS concentrations, mostly globulins were separated in the precipitate fraction. At 70% AS concentration, a fraction with a higher concentration of albumin was obtained because precipitation in the previous steps mostly removed globulins and other proteins in the sample [26]. The precipitates were dissolved in 20 mM Tris (pH 9) buffer and separated by SDS-PAGE electrophoresis. The bands present on gel electrophoresis were spot analyzed.

Table 1: Precipitation of albumin from 100 ml serum with ammonium sulfate.

%AS concentration	Initial volume (ml)	Added AS (g)	Final volume (ml)
%40	100.00	23	83.30
%50	83.30	4.6	76.50
%60	76.50	4.72	66.40
%70	66.40	4.25	60.00

2.3 Cibacron Blue affinity chromatography (CBAC)

In Cibacron Blue affinity chromatography, the stationary phase is a cross-linked agar gel containing covalently

bound Cibacron Blue F3GA dye. It binds to albumin with a high affinity. It is mainly used for the removal of albumin from serum samples. However, albumin retained by Cibacron Blue on the column can be eluted again later [31–33]. This kit used for this purpose has a serum capacity of 0.1 ml and an albumin capacity of 5 mg per column.

The steps in the package insert for this kit were followed, respectively. 20 mM phosphate buffer (pH 7) was prepared for the application. 125 µl serum sample was diluted with 625 µl buffer, room temperature centrifuged at 10,000 g for 20 sec., and washed with 400 µl buffer to obtain albumin-free serum (AFS). To elute column-bound albumin (A), 500 µl of Laemmli buffer without glycine and bromphenol blue was added and centrifuged at 10,000 g for 20 sec. The A and AFS fractions were separated by SDS-PAGE electrophoresis and subjected to densitometric analysis.

2.4 Separation of serum albumin and globulins by SDS-PAGE electrophoresis and densitometric spot analysis

Albumin and globulin isolates obtained from AS2, AS4, and CBAC methods were subjected to SDS-PAGE electrophoresis. SDS-PAGE monitored the purity of the isolated protein. 5% loading and 10% separation acrylamide gels were prepared. 0.2 M glycine, 0.02 M tris, and 0.0035 M SDS were prepared as electrophoresis buffer. Each well was

loaded with 20-25 µg of protein. Electrophoresis at 120 V, 80 mA was continued for 2 hours [34]. The resulting gel was stained with Coomassie Blue. It was then washed 3 times at 3-hour intervals with a washing solution of methanol, acetic acid, and distilled water.

For densitometric analysis of protein bands on the gel, gel images were saved to a computer in sgd format, and protein bands were manually positioned in the images. After background correction, protein bands were analyzed densitometrically, and data were converted to raw volumes by SYNGENE GeneTools quantification software (GeneTools software, version 4.03.05, Syngene, Cambridge, UK). Percentage and ratio analyses of globulins and albumin were performed using the determined densitometric raw volumes.

2.5 Biochemical analysis of serum pool

Routine biochemical analyses were performed on a Beckman Coulter AU5800 device with Beckman Coulter kits specific for each parameter.

3 RESULT

3.1 Biochemical results of the serum pool

The biochemical analysis of the serum pool is presented in Table 2. Biochemical parameter levels were within normal limits except for sodium and magnesium values.

3.2 Protein composition and densitometric analysis of albumin fractionation steps by AS precipitation and Cibacron Blue affinity chromatography by SDS-PAGE

The electrophoretogram of SDS-PAGE analysis of protein compositions of albumin isolation samples from serum by different

Table 2: Biochemical results of the serum pool.

Results			
Glucose	84 mg/dL	K	4.33 mmol/L
Albumin	4.3 g/dL	Fe	64 ng/mL
Total protein	7.3 g/dL	Phosphate	3.46 mg/dL
Total bilirubin	0.27 mg/dL	IgA	229 g/L
Direct bilirubin	0.02 mg/dL	IgM	142 g/L
Creatine Kinase	85 U/L	IgG	1219 g/L
Creatinine	0.84 mg/dL	AST	14 U/L
CK-MB	8 ng/mL	ALT	20 U/L
Uric Acid	5.2 mg/dL	GGT	27 U/L
Urea	29 mg/dL	ALP	79 U/L
Cl	103 mmol/L	HDL	45 mg/dL
Na	133 mmol/L	Triglyceride	173 mg/dL
Mg	1.82 mg/dL	Cholesterol	169 mg/dL
Ca	9.47 mg/dL	LDH	149 U/L

fractionation methods is shown in Figure 3. The analyses were repeated twice to thrice for each separation method. Very similar results were obtained. The electrophoretic separation of the albumin standard showed the presence of minor bands other than the albumin band (Figure 3, Column 1). These multiple minor bands can be considered to be dimers or aggregates present in such commercial preparations [35,36], as inferred from the results of other investigators. A small amount of albumin band was observed in the globulin precipitate of the AS2 method (Figure 3, Column 3), whereas no globulin band was detected in the albumin fraction (Figure 3, Column 4). Therefore, a clearer isolation of

albumin is observed. In the electrophoretic analysis of precipitate isolations of the AS4 method, it was observed that as the ammonium sulfate concentration increased from 40% to 70%, the albumin band intensified.

In contrast, the globulin fraction precipitated in the isolate decreased in parallel, and the purity of the albumin band reached the highest degree at 70% precipitate. (Figure 3, Columns 5-8). When the protein composition of the isolates belonging to the CBAK method was examined, it was seen in Figure 3, Column 9, that there is a significant amount of albumin and globulin in the fraction obtained after passing the serum through the column. In the fraction after-elution of cola-bound albumin,

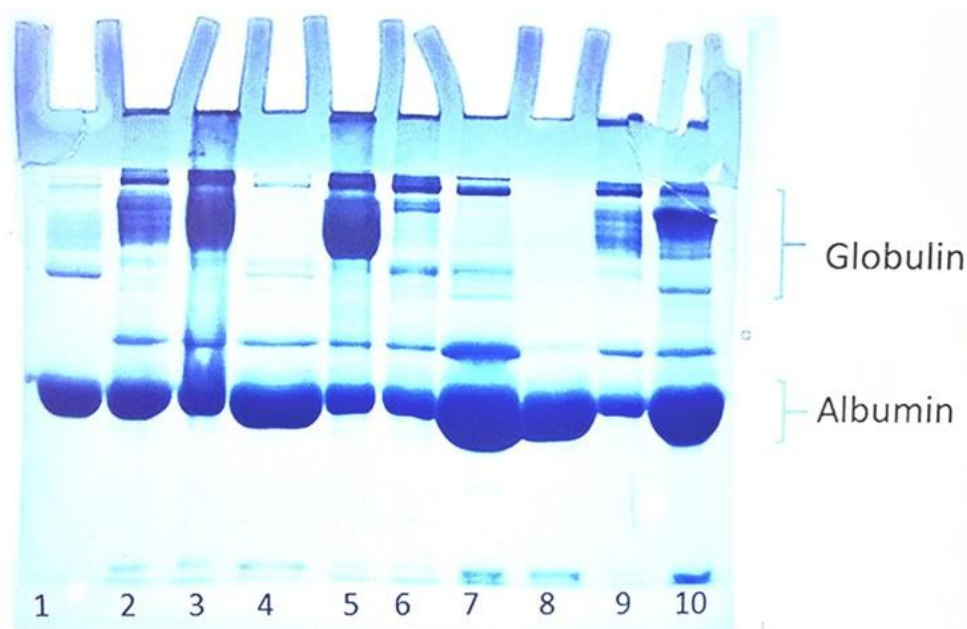


Figure 3: SDS-PAGE electrophoregram of serum albumin fractionation by AS precipitation and Cibacron Blue affinity chromatography 1: Standard albumin 2: Human serum, 3: AS2 globulin, 4: AS2 albumin, 5: 40% AS4 precipitate, 6: 50% AS4 precipitate, 7: 60% AS4 precipitate, 8: 70% AS4 precipitate, 9: CBAC(AFS) 10: CBAC(A) (Approximately 20-25 μ g protein loaded in each lane.)

albumin and globulin bands were present, with albumin predominating (Figure 3, Column 10). It is understood that immobilized Cibacron Blue resin binds globulins and albumin. The lack of specificity of this dye against albumin has also been observed in investigators' studies other [31].

Only albumin and globulin bands in the electrophoretograms were subjected to densitometric analysis. The sum of densitometric data for albumin and globulin was accepted as 100%.

Table 3 shows the percentage ratios of G, A, and A/G. While the A/G ratio was 1.8 in normal serum, this ratio increased to 9.4 in the albumin-rich fraction isolated by the AS2 method. In the AS4 method, the A/G ratio was 21.57 in the albumin-rich fraction, AS4 70%. The CBAC method, the A/G ratio was determined 3.83 in the to be albumin-rich fraction. It is seen that the fraction with the highest albumin purity among the methods used is the 70% fraction in the AS4 method. There is an albumin separation of 11.98 times compared to the A/G ratio of 1.8 before albumin separation in human serum. The albumin-rich fractions obtained by the three methods used contained globulin ranging from 9.6% to 59.60%. Even the albumin standard sample showed a 14.27% globulin band.

In precipitation with ammonium sulfate, globulins precipitated first at low concentrations, while albumin precipitated more predominantly as the concentration of

ammonium sulfate was increased. Even in the albumin-free serum (AFS) fraction isolated by the CBAC method, globulins were together with albumin.

Table 3: Albumin (A) and Globulin (G) % ratios and Albumin/Globulin (A/G) ratios in isolates.

	G(%)	A(%)	A/G(%)
Standard albumin	14,27	85,72	6,00
Serum	35,11	64,88	1,8
AS2 globulin	48,71	51,28	1,05
AS2 albumin	9,6	90,36	9,4
AS4 %40	59,60	40,39	0,67
AS4 %50	42,76	57,23	1,33
AS4 %60	9,32	90,67	9,72
AS4 %70	4,43	95,56	21,57
CBAC(AFS)	84,18	15,81	0,18
CBAC(A)	18,56	71,12	3,83

4 DISCUSSION

Albumin separation was performed using two-step AS2, four-step AS4, and CBAC with samples from the normolipidemic serum pool. SDS-PAGE separated protein bands, and densitometric image analysis was performed to evaluate the separated fractions. The three methods were evaluated in terms of time, cost, yield and purity.

AS2 and AS4 methods are based on ammonium sulfate precipitation in principle. However, in the AS4 method, solid AS is added according to the amount of serum and supernatant and incubated at -20°C for 2 hours before centrifugation [26]. The lack of a stock solution, and weighing at each step carries the risk of causing differences between samples.

The AS2 method can be considered more standardized because it uses 4.1 M saturated AS solution and the volume of the stock solution is constant at each step. CBAK contains the functional group Cibacron Blue and is based on the principle of affinity chromatography.

Approximately 35 minutes is required for albumin separation from 1 sample with the AS2 and the CBAC methods and 9 hours and 30 minutes with the AS4 method. AS2 and CBAC seem more advantageous than AS4 in terms of time and practicality. The AS2 and AS4 methods use ammonium sulfate solution. Preparing and using saturated ammonium sulfate solution is sufficient for the samples, but the number and cost of kits for CBAC are higher. For 1 sample, the kit costs 708 TL, AS2 costs 11.6 TL, and AS4 costs 5.1 TL.

When Table 3 is analyzed, the highest albumin percentage is the 70% fraction in the AS4 method with 95.56. The highest A/G ratio also belongs to this fraction. The gel images in Figure 3 support these data. However, since a significant amount of protein was lost in the lower fractions until the 70% fraction was obtained, this decreased the amount of albumin obtained. The rate of protein loss can be understood from the albumin percentages in the 40%, 50%, and 60% fractions. Gel images in previous studies also support this idea [26].

The percentage of albumin fraction obtained from the AS2 method is 90.36, and the A/G ratio is 9.4. Since the method consists

of two steps, there is not a high rate of protein loss as in AS4 [23,24]. Band 3 in Figure 3 shows that most globulins and other proteins are removed in the first step of the method.

The package insert of the CBAC method states that CBAC(AFS) does not contain albumin and CBAC(A) does not contain globulin. However, as can be seen in Figure 3, neither portion complies with this. Although binding of serum proteins other than albumin cannot be ruled out, the current product appears to bind globulin as well. Its non-specific adsorption is stated to be minimized. The disadvantage of this ligand is its possible lack of specificity. The literature has reported that Cibacron Blue dye has ionic, hydrophobic, and aromatic character and thus shows affinity for many proteins [37]. The kit has a serum capacity of 0.1 ml and an albumin capacity of 5 mg per column. It has been reported that 125 µl of serum sample can be applied to one kit. Sample size that can be applied is quite low. However, it is advantageous that it can be applied quickly and easily; its low cost and low specificity of the functional group to albumin reduce its preferability. Based on gel images and densitometric analysis, AS2 and AS4 are preferable.

A study comparing AS precipitation and affinity chromatography with Cibacron Blue in albumin separation, reported that the albumin purity obtained by AS precipitation was significantly different from that isolated

by affinity chromatography. It was reported that albumin obtained by affinity chromatography had a significant γ -globulin band, but the same band disappeared in the first step of AS2 precipitation. The effect of increasing AS concentration on albumin yield and purity was investigated in the same study. It was reported that the content of other proteins decreased at increasing AS concentrations; the gel images obtained were more pure, but the HSA yield decreased, so the most appropriate precipitation conditions were 1.174 (54% AS) and 1.159 (70% AS) ml in two steps, respectively [24]. In the present study, the same volumes were used in the AS2 method. Another study comparing AS2 precipitation and affinity chromatography, reported that the precipitation of serum albumin with AS2 enabled the purification of albumin with preserved structural and functional properties in terms of antioxidant and enzymatic activity through a rapid and efficient protocol [38].

Albumin is an important protein that is used in different scientific studies as a chemical substance in medicine, including biotechnological studies [18,19]. In this article, albumin was obtained using three methods to be compared. When the parts and band images in the methods are evaluated, it is observed that it is challenging to obtain albumin close to 100% purity. Because the image of 99% commercial albumin preparation, which is claimed to be free of globulin and fatty acids,

does not consist entirely of pure albumin (Figure 3, Line 1). These may result from dimers or aggregates reported to be present in commercial albumin preparations. The presence of two albumin preparations with different molecular weights was also revealed by MS analysis [35,36]. Although many protein impurities are present, the dominant protein appears albumin. Among the three methods, albumin from the albumin portion of AS2 and the 70% portions of AS4 are cleaner than globulins and other proteins. However, since AS4 requires 2 hours of incubation for each part and albumin is lost in three stages, it can be said that the AS2 method provides less loss in time and can be applied more easily. However, there is a need for further research and development of methods combining precipitation methods with chromatographic methods to obtain purer albumin.

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6 CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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