Journal Pre-proof

Controlling Serum Protein Depletion in Aqueous Biphasic Systems: Interphase Precipitation versus Bottom Phase Retention for Bisphenol A Monitoring

Maria S.M. Mendes, Inês B. Santana, Mara G. Freire, Francisca A. e Silva

PII: S0003-2670(26)00296-5

DOI: https://doi.org/10.1016/j.aca.2026.345346

Reference: ACA 345346

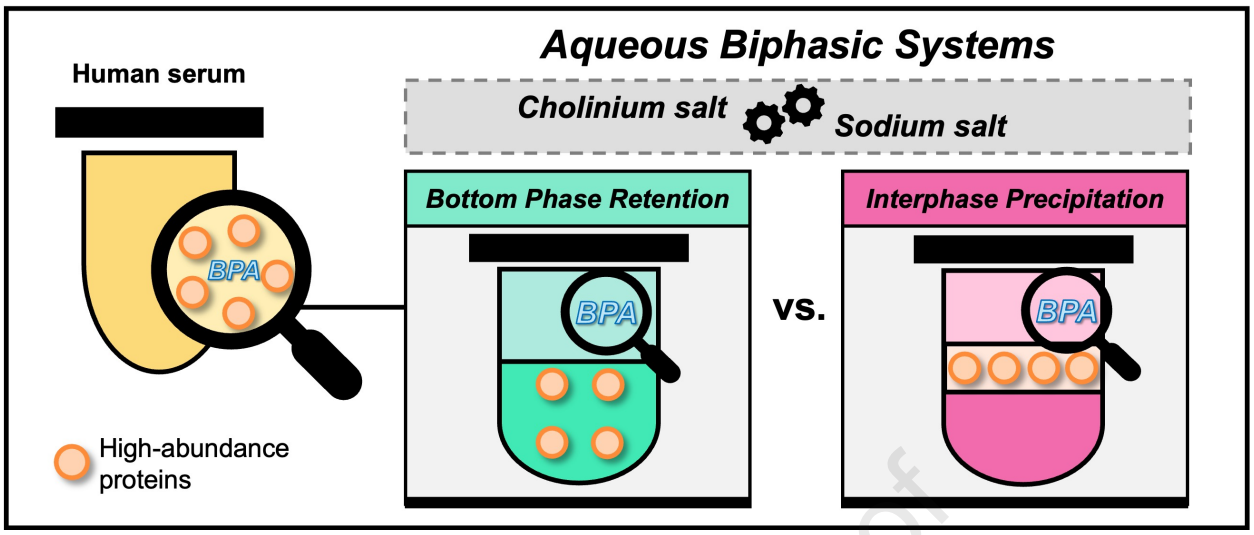
To appear in: *Analytica Chimica Acta*

Received Date: 16 December 2025

Revised Date: 2 March 2026

Accepted Date: 3 March 2026

Please cite this article as: M.S.M. Mendes, I.B. Santana, M.G. Freire, F.A. e Silva, Controlling Serum Protein Depletion in Aqueous Biphasic Systems: Interphase Precipitation versus Bottom Phase Retention for Bisphenol A Monitoring, *Analytica Chimica Acta*, https://doi.org/10.1016/j.aca.2026.345346.



Controlling Serum Protein Depletion in Aqueous Biphasic Systems: Interphase Precipitation versus Bottom Phase Retention for Bisphenol A Monitoring

Maria S. M. Mendes[1], Inês B. Santana[1], Mara G. Freire[1]*, Francisca A. e Silva[1]*

[1]CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

*To whom correspondence may be addressed. E-mail: maragfreire@ua.pt; francisca.silva@ua.pt

Abstract

Background: Human serum is a clinically relevant yet analytically challenging matrix. Its high abundance of proteins interferes with the detection of low-concentration biomarkers and environmental contaminants, including bisphenol A (BPA), an endocrine disruptor monitored as a marker of human exposure. High-abundance proteins introduce matrix effects that can enhance, distort or suppress analytical signals, ultimately compromising assay performance. Aqueous biphasic systems (ABS) provide a promising pretreatment strategy to address these limitations by selectively depleting high-abundance proteins and generating a cleaner matrix that supports effective analyte recovery and improved detection sensitivity.

Results: In this work, a dual-strategy ABS platform was introduced and shown to deliberately direct protein depletion via two complementary mechanisms: (i) *interphase precipitation*, where serum proteins aggregate at a solid interphase, and (ii) *bottom phase retention*, where proteins accumulate in the salt-rich bottom phase. By combining polypropylene glycol (PPG) with either cholinium or sodium salts, the salt composition tunes phase behavior, selectively depletes high-abundance proteins, and enhances BPA recovery. Sodium salts generally outperformed cholinium salts due to stronger hydration and salting-out effects. Between the two implemented strategies, systems promoting *interphase precipitation* achieved up to 94% protein depletion, while simultaneously driving BPA into the largely protein-depleted PPG-rich top phase, minimizing matrix bias and enabling more efficient detection.

Significance: This work demonstrates, for the first time, that rational control of ABS composition can selectively switch between protein precipitation and liquid-liquid partition, establishing a versatile and tunable serum pretreatment platform. This approach enables efficient depletion of high-abundance proteins and sensitive recovery of BPA and potentially other low-concentration analytes. Supported by green and blue analytical chemistry metrics, it represents a significant advance in bioanalytical workflows and environmental exposure assessment, while offering a more sustainable alternative to conventional methods.

Keywords: human serum, sample pretreatment, aqueous biphasic system, bisphenol A

1. Introduction

Human serum is a central matrix in clinical and bioanalytical studies, offering a diverse array of molecular indicators that reflect disease, exposure and physiological

status [1]. This molecular diversity, however, is embedded within a highly complex proteome, where protein concentrations span over ten orders of magnitude [1]. High-abundance proteins, predominantly human serum albumin (HSA), immunoglobulins (IgG and IgA), haptoglobin, α_1 antitrypsin, and transferrin, account for nearly 85% of the total serum protein content, thus shaping the behavior of the matrix [1]. As a result, low-abundance analytes, including clinically and environmentally relevant markers, are susceptible to suppression or masking, reducing their detectability with conventional analytical methods [1].

Bisphenol A (BPA), an endocrine-disrupting chemical widely used in plastics and resins, represents one such low-abundance analyte [2]. Its pervasive use results in routine detection in human fluids, such as serum, plasma, and urine, where BPA serves as a marker of exposure [2]. This concern calls for effective monitoring to assess human exposure and associated health risks, including potential links to hormone-sensitive cancers [2]. A range of analytical techniques, including high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and enzyme-linked immunosorbent assays (ELISA), have been employed for BPA quantification [3]. However, because BPA typically occurs at trace concentrations ($ng \cdot mL^{-1}$ or $pg \cdot mL^{-1}$), high-abundance proteins and other matrix constituents can strongly interfere with its detection by introducing effects that compromise the integrity and reliability of the analytical signal [3]. Consequently, efficient pretreatment is essential to deplete/remove these proteins and reduce matrix bias, enabling reliable quantification [3].

Common pretreatment strategies to support BPA detection include protein precipitation (PP), solid-phase extraction (SPE) and liquid-liquid extraction (LLE) [4]. PP involves the denaturation and removal of proteins using organic solvents, polymers, salts, acids, bases, or heat, followed by centrifugation [4]. For instance, acetonitrile has been applied as a precipitating agent, improving BPA quantification in human plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS), with a reported limit of detection (LOD) of about 5 $ng \cdot mL^{-1}$ and a limit of quantification (LOQ) of 10 $ng \cdot mL^{-1}$ [5]. SPE enables the isolation and concentration of target analytes from complex samples through retention-elution or direct elution using a solid stationary phase, based on interactions such as ion exchange, polarity, or immunoaffinity [4]. For example, SPE has been successfully applied to urinary and peritoneal fluids, efficiently removing proteins and enabling accurate BPA quantification by GC-MS/MS, with LODs ranging from 0.015-0.05 $ng \cdot mL^{-1}$ [6]. Finally, LLE separates compounds based on their differential solubility in two immiscible phases, typically aqueous and organic [4]. BPA quantification in serum by LC-MS/MS using acetonitrile as the organic phase achieved 100% recovery, with a LOD of 0.009 $ng \cdot mL^{-1}$ and a LOQ of 0.028 $ng \cdot mL^{-1}$ [7]. Despite their effectiveness, conventional methods often rely on multistep workflows and volatile or toxic solvents, limiting their overall simplicity, biocompatibility, and cost-effectiveness [4]. These constraints motivate the development of alternative pretreatment strategies that integrate efficiency, sustainability, and analytical performance.

As versatile aqueous LLE platforms composed of polymers, salts, or other hydrophilic compounds such as amino acids, carbohydrates and ionic liquids, aqueous biphasic systems (ABS) fulfill these criteria. Their adjustable composition, combined with mild and biocompatible operating conditions, enables the selective separation of diagnostic and environmental markers, ultimately improving detection limits and simplifying analytical workflows [8]. In urine, ABS have been used for the extraction and concentration of

proteins, vitamins, drugs, and metals [9–12]. When applied to blood, these systems proved compatible with various analytical techniques such as chromatography, immunoassays and spectrophotometry [13–16]. In saliva, a particularly challenging matrix due to analyte dilution, ABS allowed the sensitive detection of phenolic endocrine disruptors, with extraction efficiencies approaching 100%, alongside substantial protein depletion via interfacial precipitation [17].

Depending on the phase-forming components and sample matrix, ABS can be tuned to operate through liquid-liquid distribution or interfacial precipitation, enabling distinct separation routes for proteins and small molecules [18]. By adjusting the composition and physicochemical environment, ABS can be directed toward protein solubilization or aggregation, thus providing versatile and controllable tools for biological sample pretreatment [19–21]. Although both mechanisms have been individually described [10,13], their systematic comparison and rational exploitation as complementary routes for protein depletion in ABS remain underexplored. Herein, the deliberate control of ABS composition is demonstrated to selectively induce either protein precipitation or liquid-liquid distribution, establishing a dual-strategy platform for improved BPA analysis in human serum: (i) *bottom phase retention*, where high-abundance proteins (HSA and IgG) are removed through selective accumulation in a liquid phase, and (ii) *interphase precipitation*, where these proteins are efficiently depleted via solid-phase formation at the interphase. In both strategies, BPA is preferentially extracted into the top aqueous phase, ensuring compatibility with analytical detection and enabling accurate quantification in protein-rich conditions. ABS were tailored by combining polypropylene glycol (PPG) with either cholinium or sodium salts, where differences in hydration, ionic character and interaction with proteins define phase behavior and direct the system toward *interphase precipitation* or *bottom phase retention*. In addition, the analytical performance and sustainability of the proposed platform were systematically evaluated using green and blue analytical chemistry metrics.

2. Materials and methods

2.1. Chemicals, analytical standards, and biological samples

Polypropylene glycol with an average molecular weight of 400 $g \cdot mol^{-1}$ (PPG 400; Sigma-Aldrich) was used to prepare ABS in combination with selected salts. Cholinium salts included cholinium dihydrogen phosphate ([Ch][DHP], purity = 98 wt%; Iolitec), cholinium acetate ([Ch][Ac], purity = 98 wt%; Iolitec), cholinium dihydrogencitrate ([Ch][DHCit], purity = 99 wt%; Sigma-Aldrich), cholinium bitartrate ([Ch][Bit], purity = 97 wt%; Acros Organics), cholinium chloride ([Ch]Cl, purity = 98 wt%; Acros Organics) and cholinium bromide ([Ch]Br, purity = 98 wt%; TCI). Sodium salts comprised sodium dihydrogen phosphate 1-hydrate (Na[DHP], purity = 99 wt%; PanReac AppliChem), sodium acetate (Na[Ac], purity = 100 wt%; Prolabo), sodium chloride (NaCl, purity = 99.5 wt%; Fisher Scientific), and sodium bromide (NaBr, purity = 99 wt%; Sigma-Aldrich). Chemical structures of all compounds are presented in Figure 1. Phosphate-buffered saline (PBS) pellets were purchased from Sigma-Aldrich and used to prepare an aqueous solution yielding 0.01 M phosphate buffer, 0.0027 M potassium chloride (KCl) and 0.137 M sodium chloride (NaCl), with a pH 7.4 at 25 °C, according to the manufacturer's instructions.

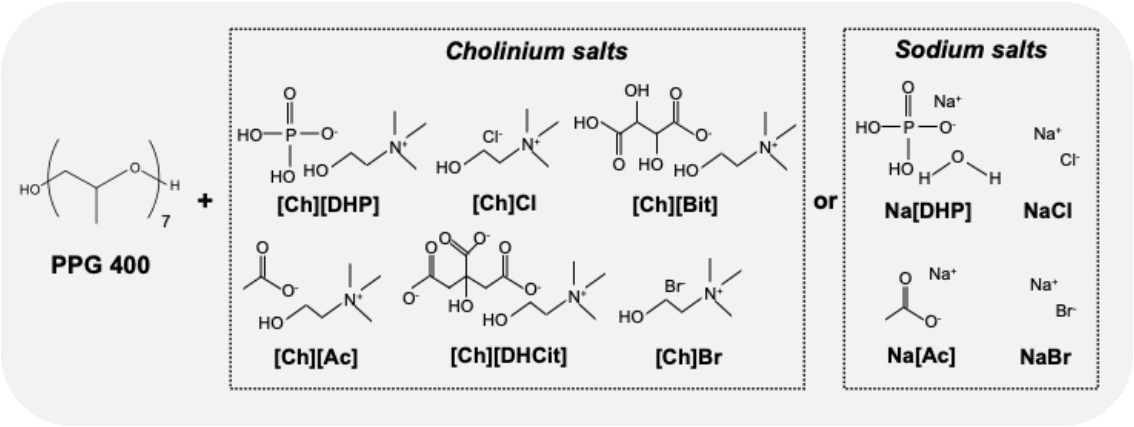


Figure 1. Chemical structures of ABS components, highlighting the polymer (PPG 400) and the two salt families.

Human serum (H4522 – Lot # SLBX6353) was obtained from Sigma-Aldrich and stored at -20 °C until use. The mobile phase for Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC) used in the quantification of IgG and HSA was prepared with doubled distilled water purified by a reverse osmosis system followed by a treatment with a Milli-Q plus 185 water purification system, as well as sodium dihydrogen phosphate 1-hydrate ($NaH_2PO_4 \cdot H_2O$, purity = 99%), disodium hydrogen phosphate 7-hydrate ($Na_2HPO_4 \cdot 7H_2O$, purity = 99%) and NaCl (purity = 99.5 %) from PanReac AppliChem. Calibration curves were established using lyophilized HSA (purity > 97 wt%; stored at 4 °C until use; Sigma-Aldrich) and purified human IgG (29.4 $mg \cdot mL^{-1}$; stored at -80 °C until use; Innovative Research, inc.).

For BPA quantification, the HPLC mobile phase consisted of an aqueous solution of formic acid (purity = 99 wt%; Carlo Erba) and acetonitrile HPLC-grade (purity = 99.99 wt%; Fisher Chemical). BPA standard (purity > 99 wt%) was obtained from Sigma-Aldrich.

2.2. ABS binodal curves

Binodal curves of ABS composed of PPG 400 and [Ch]Br, Na[DHP], NaBr, NaCl or Na[Ac] were determined to identify mixture compositions within biphasic regions. These results were complemented with binodal curves previously reported for PPG 400 ABS containing [Ch][DHP], [Ch][Ac], [Ch][Bit], [Ch][DHCit], or [Ch]Cl [22].

Binodal curves were experimentally obtained using the cloud point titration method at atmospheric pressure and (25 ± 1) °C [22]. Aqueous stock solutions of PPG 400 (90 wt%) and salt solutions (25-60 wt%) were prepared for this purpose. The titration consisted of gradually adding the salt solution dropwise to the PPG 400 solution under continuous stirring until turbidity indicated phase separation. Water was then added dropwise until the mixture became clear again, corresponding to a monophasic region. Repeating this procedure while weighing all added components enabled calculation of mixture compositions (wt%) used to construct binodal curves.

2.3. HSA and IgG depletion

Specific compositions within ABS biphasic regions were selected to evaluate the ability of different ABS to deplete IgG and HSA through either *bottom phase retention* or

interphase precipitation, as shown in Figure 2. For direct comparison, most systems were prepared with 30 wt% PPG 400, 30 wt% cholinium or sodium salt, 38 wt% PBS aqueous solution, and 2 wt% human serum. However, for ABS containing sodium salts with lower aqueous solubility (NaBr, Na[Ac], NaCl, Na[DHP]), the composition was adjusted to 30 wt% PPG 400, 15 wt% sodium salt, 53 wt% PBS aqueous solution, and 2 wt% human serum. It should be noted that PBS solution was included as the aqueous component to complete 100 wt% of each ABS composition. Consequently, its content varies between 38 and 53 wt%, depending on the specific polymer-salt pair. PBS aqueous solution also provides a consistent ionic background and physiologically relevant conditions, reducing uncontrolled effects on system behavior.

All components except human serum were weighed and vortex-mixed until completely dissolved. Human serum was then added to reach a final ABS mass of 1.00 g, comprising 0.30 g of PPG 400, 0.02 g of human serum, and either 0.30 g or 0.15 g of salt combined with 0.38 g or 0.53 g of PBS aqueous solution, depending on the composition. The mixtures were centrifuged at 3500 rpm for 10 min, and phases were carefully separated using syringes. All ABS assays were performed in triplicate to allow reporting of mean values and standard deviations. In all cases, the top phase was enriched in PPG 400, whereas the bottom phase was enriched in the cholinium or sodium salt. When present, the solid interphase corresponded to the precipitated high-abundance proteins.

Each aqueous phase and the initial human serum samples were analyzed by SE-HPLC using a Chromaster HPLC system (Hitachi) equipped with a Shodex Protein KW-802.5 analytical column (8 mm × 300 mm) and a Shodex Protein KW-G 6B guard column. The mobile phase (50 mM of $NaH_2PO_4 \cdot H_2O/Na_2HPO_4 \cdot 7H_2O$ buffer and 0.3 M NaCl) was run isocratically at 0.5 $mL \cdot min^{-1}$ for 40 min. The injection volume was 25 µL, with protein detection at 280 nm using a diode-array detector (DAD). Column oven and autosampler temperatures were maintained at 25 °C and 10 °C, respectively. HSA and IgG concentrations in the top and bottom phases were quantified using calibration curves previously established in the range 0.01-1.0 $mg \cdot mL^{-1}$. The corresponding calibration curves, including the coefficient of determination (R^2), limits of detection (LOD), limits of quantification (LOQ), and relative standard deviations (RSD, %), are shown in Figure S1 in the Supplementary Material. The LOD was calculated from calibration curves analyzed in duplicate using the ratio between the standard error of the regression (sy/x) and the slope of the calibration curve, multiplied by a factor of 3.3. The LOQ was subsequently estimated as three times the corresponding LOD value. The RSD (%) was calculated as the ratio of the standard deviation to the mean of replicate measurements at each concentration, multiplied by 100, and is reported as a concentration-dependent interval.

The direct isolation and analysis of the solid interphase were technically challenging with the type of systems here studied, increasing the risk of cross-contamination and analytical errors. Therefore, protein content at the interphase was calculated by mass balance from the measured protein amounts in the two liquid phases and the initial serum content.

Because the two ABS depletion strategies rely on distinct separation mechanisms, i.e., protein precipitation at a solid interphase or protein retention in the bottom liquid phase, the efficiency of protein depletion was expressed uniformly as recovery yield ($RY_{IgG/HSA}$%; Equation 1), defined as:

$$RY_{IgG/HSA}\% = \frac{m_{IgG/HSA}^{Top/Inter/Bottom}}{m_{IgG/HSA}^{Serum}} \times 100 \quad (1)$$

where $m_{IgG/HSA}^{Top/Inter/Bottom}$ corresponds to the mass of IgG or HSA quantified in the respective phase (top, interphase or bottom), and $m_{IgG/HSA}^{Serum}$ is the initial mass of each protein in the commercial serum. This approach allows direct comparison of protein distribution across all phases, regardless of whether depletion occurs via *interphase precipitation* or *bottom phase retention*.

2.4. BPA recovery and analytical performance

ABS that exhibited the best performance in depleting high-abundance proteins were subsequently evaluated for their ability to extract BPA into the protein-depleted top phase. This phase contains markedly reduced levels of HSA and IgG due to their depletion either by *interphase precipitation* or by *bottom phase retention* (Figure 2). Spiked human serum was prepared by adding BPA to a final concentration of 0.3 mg·mL^{-1}, a value selected according to the sensitivity range of the HPLC-DAD. It should be noted that testing BPA levels higher than typical biological concentrations is useful, as it is representative of BPA partition in ABS, while allowing verification that the PPG-rich phase is not saturated. Spiked samples were further incorporated into ABS following the same preparation and phase-separation procedure used for protein depletion assays.

Each top phase was analyzed by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) using a Shimadzu HPLC system equipped with a Kinetex® C18 LC column (250 x 4.6 mm) and a DAD detector. The mobile phase, consisting of 0.002% v/v formic acid in water (A) and acetonitrile (B) was delivered at 1 mL·min^{-1} for 45 min under the following gradient: 0-3 min, 80% A / 20% B; 4-6 min, 65% A / 35% B; 20-28 min, 45% A / 55% B; 33-45 min, 80% A / 20% B. The injection volume was 25 µL, the detection wavelength was set at 230 nm and the column oven was maintained at 30 °C. BPA quantification was performed using a calibration curve constructed from standard solutions (0.01-1.0 mg·mL^{-1}) prepared under the initial conditions of the gradient elution method, i.e., acetonitrile/water with 0.002% v/v formic acid (v/v, 20/80). The corresponding calibration curve, including the R^2 (0.9994), LOD (0.049 mg·mL^{-1}), LOQ (0.15 mg·mL^{-1}) and RSD (0-4%), is shown in Figure S1 in the Supplementary Material.

To assess potential matrix effects arising from the presence of PPG 400 during BPA quantification, a calibration curve was prepared in the PPG-rich top phase of the ABS composed of 30 wt% PPG 400, 30 wt% NaBr and 40 wt% PBS aqueous solution (cf. Figure S2 in the Supplementary Material). Comparison of the calibration curves yielded a slope ratio of 1.04, confirming negligible matrix interference across the working concentration range.

To evaluate analytical performance, recovery yields of BPA (RY_{BPA}%, Equation 2) were calculated as:

$$RY_{BPA}\% = \frac{m_{BPA}^{Top}}{m_{BPA}^{Serum}} \times 100 \quad (2)$$

where m_{BPA}^{Top} is the mass of BPA in the top phase, and m_{BPA}^{Serum} is the initial mass added to the serum sample.

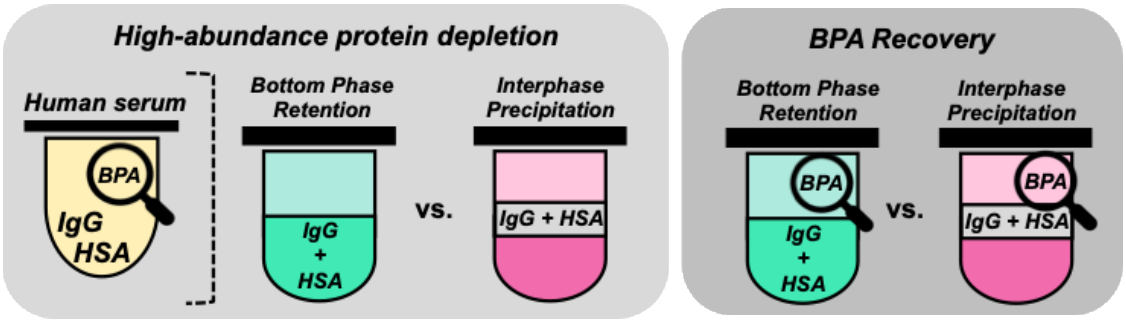


Figure 2. Control of ABS composition to achieve either *interphase precipitation* or *bottom phase retention* of high-abundance proteins, enabling protein depletion in human serum and BPA recovery from spiked samples.

3. Results and Discussion

This work establishes an ABS-design framework for protein depletion in human serum, demonstrating a dual-strategy approach in which phase behavior is strategically manipulated to induce either liquid-liquid protein distribution or interfacial protein precipitation. These behaviors were explored to enhance analytical detection of low-abundance analytes, here represented by BPA, by selectively removing high-abundance serum proteins that compromise sensitivity and accuracy. BPA was selected as a model analyte due to its health and environmental relevance, as well as the analytical challenges it poses in protein-rich matrices. The ABS pretreatment strategy, though developed for BPA, is anticipated to be applicable to other low-concentration analytes.

To implement this design strategy, cholinium and sodium salts were systematically compared as phase-forming agents in combination with PPG 400. PPG 400 was selected due to its well-balanced physicochemical properties, namely its intermediate hydrophilic-hydrophobic character and high-water miscibility, which together promote efficient phase formation with both sodium and cholinium salts [22]. These features enable rapid phase separation and efficient mass transfer, which are particularly beneficial for analytical workflows [23]. Moreover, the combination of PPG 400 with different salts allows for deliberate tuning of ABS hydrophilicity, salting-out strength and molecular interactions. These parameters are critical for driving protein depletion toward either *bottom phase retention* or *interphase precipitation*: the two complementary serum pretreatment strategies identified in this work (Figure 2). Specifically, cholinium salts were chosen for their biocompatibility, tunable hydrophilicity, and capacity to tailor molecular interactions, while sodium salts were included as classical agents capable of promoting efficient phase separation through well-defined ion-water interactions [24,25]. For the present work, these compounds are referred to as “cholinium salts” to maintain consistency with the designation of sodium salts, while they are commonly featured in ionic liquids studies. Using salts systematically varied in both anion and cation type enabled a mechanistic evaluation of the contributions of each ionic component to phase behavior and sample pretreatment. By comparing systems in which either the anion or the cation was held constant, it became possible to separate anion-driven from cation-driven effects, enabling a more rational design of phase formation and protein depletion strategies.

3.1. Controlling ABS phase separation via ionic selection

With ABS-design rationale now delineated, the influence of ionic composition on ABS formation and the resulting binodal curves, which define the operational window for inducing protein depletion, was first examined. Cholinium salts shared a common cation, but incorporated different anions (viz. [Ch][DHP], [Ch][Ac], [Ch]Cl, [Ch][Bit], [Ch][DHCit], and [Ch]Br), whereas the sodium salts included matched anions (viz. Na[DHP], Na[Ac], NaBr, and NaCl) to enable direct comparison. With most systems containing cholinium salts previously reported [22], ABS containing [Ch]Br and sodium salts were experimentally characterized here to extend the understanding of ion specific effects. The corresponding binodal curves are presented in Figure 3, demarcating the boundary between monophasic and biphasic regions, with compositions above the curve forming two immiscible aqueous phases. The closer the curve is to the origin, the more efficient the ionic species in promoting phase separation, and the broader the compositional window available for designing protein depletion strategies. Numerical binodal data for the newly characterized systems are detailed in the Supplementary Material (Table S1).

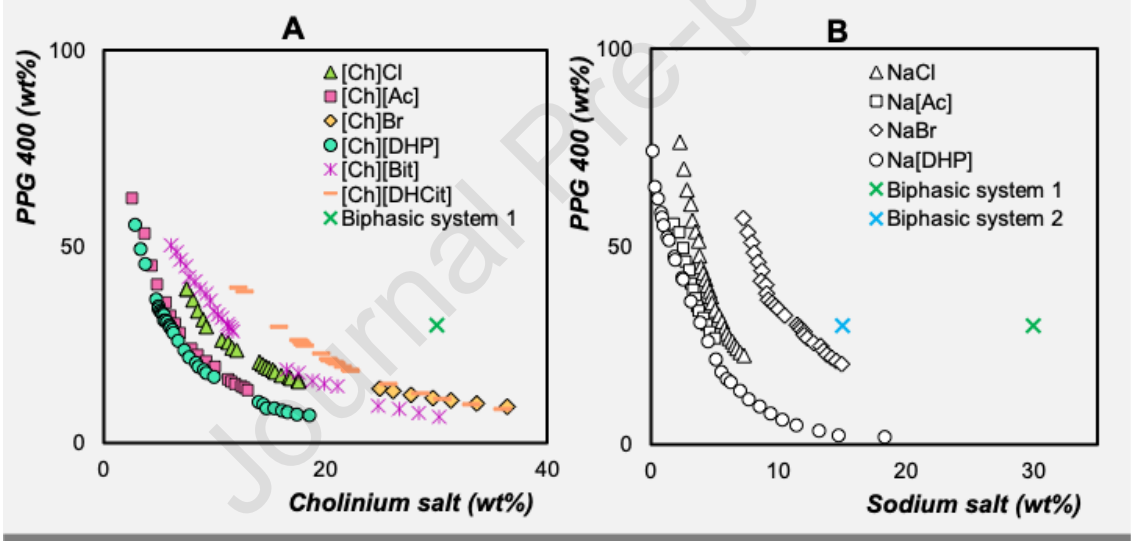


Figure 3. Binodal curves for ABS comprising PPG 400 with various (A) cholinium and (B) sodium salts, showing biphasic system compositions selected for protein depletion and BPA recovery studies. Data obtained experimentally or from literature [22].

Among cholinium salts (Figure 3A), ABS formation is strongly anion-dependent, with higher water-affinity anions more effectively excluding PPG 400 and promoting phase separation. This behavior, well-documented in previous studies [22], is exemplified by [Ch][DHP], followed by [Ch][Ac], [Ch]Cl, [Ch][Bit] and [Ch]Br. $[DHP]^-$ anion possessing the highest polar surface, accounts for [Ch][DHP]'s superior phase-separation ability. Both $[Ac]^-$ and Cl^- act as strong hydrogen bond acceptors, efficiently interacting with water and favoring phase separation in the presence of relatively hydrophobic polymers such as PPG 400 [22,26]. [Ch][Bit], derived from a dicarboxylic acid, contains additional hydroxyl groups and a longer alkyl chain compared to $[Ac]^-$, which modulates water interactions and slightly reduces its ability to induce phase separation [22]. [Ch]Br, experimentally characterized in this work for the first time, exhibits lower water affinity than [Ch]Cl, resulting in reduced capacity to exclude PPG

400 and form ABS [22,26]. [Ch][DHCit], despite its high-water affinity, behaves as an outlier, likely due to self-aggregation events that impede homogeneous phase separation [22]. Overall, water-cholinium salt interactions dominate these systems, with additional contributions from cholinium salt-polymer interactions in specific cases.

According to Figure 3B, sodium salts present an analogous anion-mediated profile, with ABS formation efficiency following the order Na[DHP] > Na[Ac] > NaCl > NaBr. In polymer-salt systems, phase separation is predominantly governed by the salting-out effect, whereby ion hydration promotes the exclusion of PPG 400 into a distinct aqueous phase [27–29]. Sodium salts generally form ABS more efficiently than cholinium salts, due to smaller cations, higher charge density, and stronger ionic interactions [30]. High-charge-density ions drive liquid-liquid demixing by displacing PPG, reducing its solubility and enhancing phase separation [31]. In contrast, cholinium-salt ABS depend on a broader interplay among PPG 400, the salt, and water, with the cholinium cation forming additional interactions, especially hydrogen bonds, that may moderate phase separation compared with sodium salts [22]

3.2. Tailoring protein depletion in human serum via ABS: *interphase precipitation* vs. *bottom phase retention*

Supported by each ABS binodal curves, representative biphasic mixture compositions were selected to investigate HSA and IgG depletion from human serum. In Figure 3, biphasic system 1 (30 wt% PPG 400, 30 wt% cholinium or sodium salt, 38 wt% PBS aqueous solution, and 2 wt% human serum) represents the standard mixture used for most salts, enabling direct comparison. Biphasic system 2 (15 wt% NaBr, Na[Ac] or NaCl) was selected for salts with lower aqueous solubility to ensure stable two-phase formation while maintaining 30 wt% PPG 400 and 2 wt% serum, with the salt and PBS aqueous solution contents adjusted accordingly. These formulations allowed the assessment of protein depletion under consistent preparation conditions while accounting for the solubility limitations of each salt.

Protein recovery yields in the phase of interest are shown in Figure 4 for PPG 400 ABS tuned with cholinium or sodium salts, revealing the combined effects of ion hydration and specific interactions with the proteins. The results further highlight the two complementary depletion strategies: *interphase precipitation*, where high-abundance proteins aggregate at the interface between the phases, and *bottom phase retention*, where proteins preferentially accumulate in the salt-rich liquid phase. Representative photographs of both behaviors are provided in the Supplementary Material (Figure S3), and detailed numerical data for all systems, including recoveries in the other phases, are provided in Table S2. Depending on ionic composition and ABS formation capacity, these results demonstrate how ABS can be tailored to control serum fractionation and reduce matrix complexity.

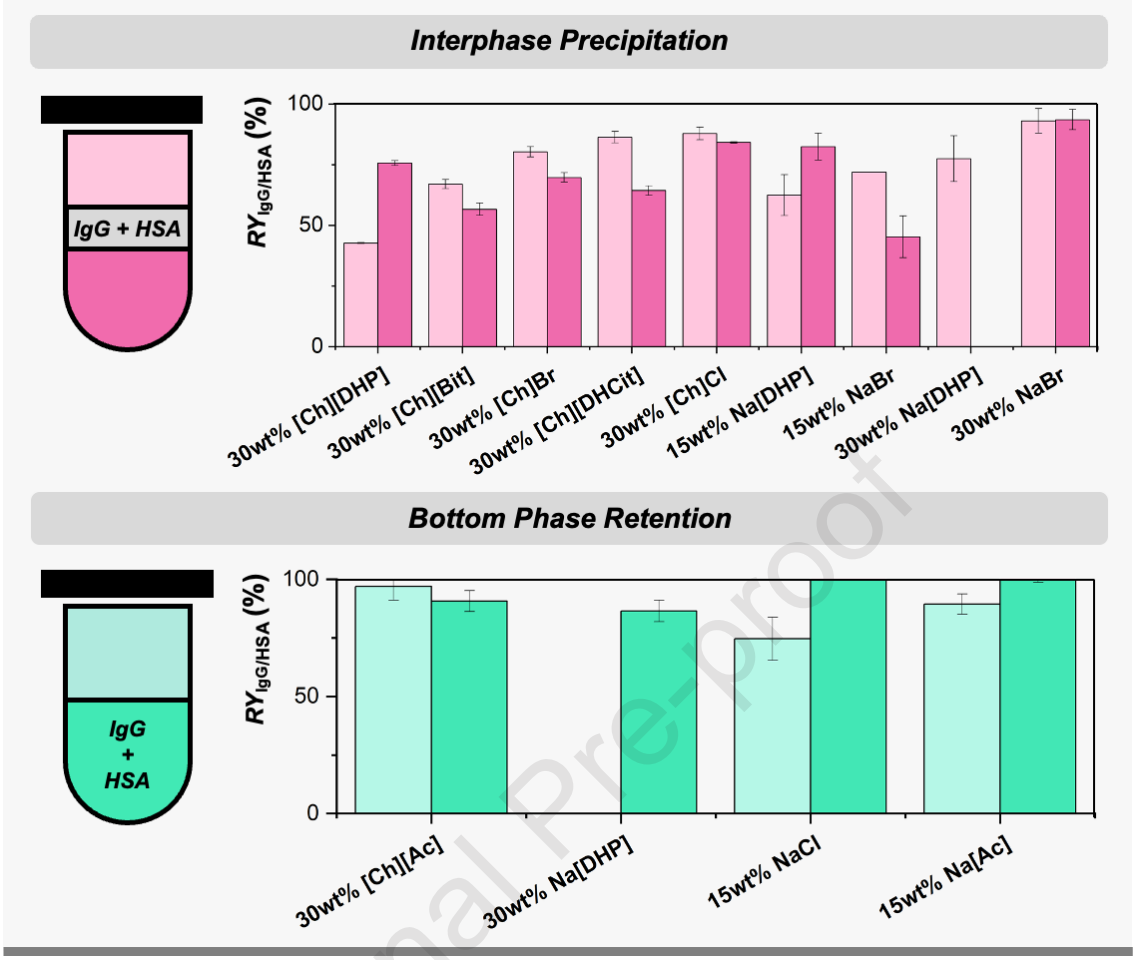


Figure 4. Recovery yields of IgG (RY_{IgG}, light bars) and HSA (RY_{HSA}, dark bars) in the interphase (pink bars), and bottom phase (green bars) of ABS comprising 30 wt% PPG 400, 15-30 wt% cholinium or sodium salt, 53-38 wt% PBS aqueous solution and 2 wt% human serum. Green bars indicate *bottom phase retention*, pink bars denote *interphase precipitation*, and the left-side schematic illustrates the corresponding protein depletion strategy visually. Values are expressed as mean ± standard deviation (n = 3).

Interphase precipitation was predominant in systems containing PPG 400 combined with 30 wt% [Ch][DHP], [Ch][Bit], [Ch]Br, [Ch][DHCit], [Ch]Cl, and 15-30 wt% Na[DHP] or NaBr (Figure 4, pink bars). These systems readily form ABS, facilitating protein destabilization and aggregation at the interphase, with IgG depletion ranging from 43% to 93% and HSA depletion from 13% to 94%. The high concentration and relative hydrophobicity of PPG in the top phase exclude water, destabilizing proteins and promoting aggregation [32]. The salts further enhance this effect by modulating protein hydration and solubility through ionic interactions and water-structuring forces [33]. This combined effect ultimately promotes protein precipitation at the interphase, with the salting-out effect particularly relevant for the sodium salts. Moreover, comparisons between cholinium and sodium salts with the same anion reveal protein-specific behaviors. In the Na[DHP]/[Ch][DHP] pair, the sodium salt showed higher efficiency in IgG depletion (77% vs. 43%), whereas the cholinium salt was more effective for HSA depletion (76% vs. 13%). For the NaBr/[Ch]Br pair, the sodium salt outperformed the cholinium salt in both IgG (93% vs. 80%) and HSA (94% vs. 70%) depletion. These differences arise from the higher charge density, smaller size, and stronger hydration of

Na^+, promoting aggregation via salting-out, while cholinium cations form stabilizing interactions that maintain protein solubility [9,22,34,35]. Additionally, increasing the salt concentration enhances the salting-out effect, as observed for NaBr, where IgG and HSA depletion improved significantly at 30 wt% compared to 15 wt% [34]. Protein-specific interactions, including electrostatic forces and hydrophobic effects, also modulate retention and precipitation [34], as exemplified by the contrasting HSA response to Na[DHP] concentration (15 wt%: 82% vs. 30 wt%: 13%).

Bottom phase retention was observed in systems containing NaCl, Na[Ac], and [Ch][Ac] in combination with PPG 400 (Figure 4, green bars). In these systems, proteins accumulate in the salt-rich bottom phase due to strong hydrogen bond accepting ability of $[Ac]^-$ and Cl^- anions, reducing protein solubility in the polymer-rich top phase [29,36]. Among these, the [Ch][Ac] system achieved depletion of 97% for IgG and 91% for HSA, revealing the strong hydrophilic interactions between the cholinium salt and water that enhance phase separation [36]. Sodium salts also demonstrated effective protein depletion, with Na[Ac] achieving 89% IgG and 100% HSA depletion, and NaCl achieving 75% IgG and 100% HSA depletion, closely matching the performance of the [Ch][Ac] system.

Of note, at 30 wt% Na[DHP], HSA mainly accumulates into the bottom phase, whereas IgG precipitates at the interphase (both behaviors are reflected in Figure 4, with bottom phase and interphase recoveries indicated by the green and pink bars, respectively). This observation demonstrates that adjusting ABS composition and the ionic component allows different proteins to undergo either *interphase precipitation* or *bottom phase retention* within the same system. Under these conditions, the larger size and multimeric structure of IgG likely render it more susceptible to salting-out and interphase precipitation, whereas the smaller size of HSA could favor its retention in solution [37]. This highlights the potential of ABS as a versatile platform that could be tailored to the characteristics of diverse biological matrices and molecular targets as well as to specific analytical requirements of the intended applications.

Overall, the observed protein depletion behaviors can be rationalized based on the specific ionic properties of each system. *Interphase precipitation* is driven by polymer-induced protein destabilization, amplified by ionic interactions and salting-out effects. In turn, *bottom phase retention* relies on ion hydration and reduced protein solubility in the polymer-rich phase. Protein depletion was most efficient in systems containing 30 wt% [Ch][DHCit], [Ch]Cl, [Ch]Br, or NaBr for *interphase precipitation*, and in systems containing 30 wt% of [Ch][Ac], 15 wt% Na[Ac], or 15 wt% NaCl for *bottom phase retention*. The best-performing systems were comparable for IgG removal between *interphase precipitation* (93% IgG and 94% HSA with 30 wt% NaBr) and *bottom phase retention* (97% IgG and 91% HSA with 30 wt% [Ch][Ac]), whereas *bottom phase retention* proved superior for HSA depletion, achieving complete removal with 15 wt% Na[Ac] or NaCl. The resulting protein-depleted matrices provide an ideal environment to evaluate the recovery and analytical performance in BPA analysis.

3.3. Assessing BPA in protein-depleted ABS phases

Following the targeted removal of high-abundance serum proteins, the most efficient ABS from each depletion strategy were evaluated for their ability to recover BPA. This approach enables assessment of analytical performance in a simplified, protein-depleted matrix, minimizing interference from HSA and IgG and providing a clearer

insight into BPA recovery and detection accuracy. Figure 5 presents the recovery yields of BPA in the PPG-rich top phase for each system; detailed data is given in the Supplementary Material (Table S3).

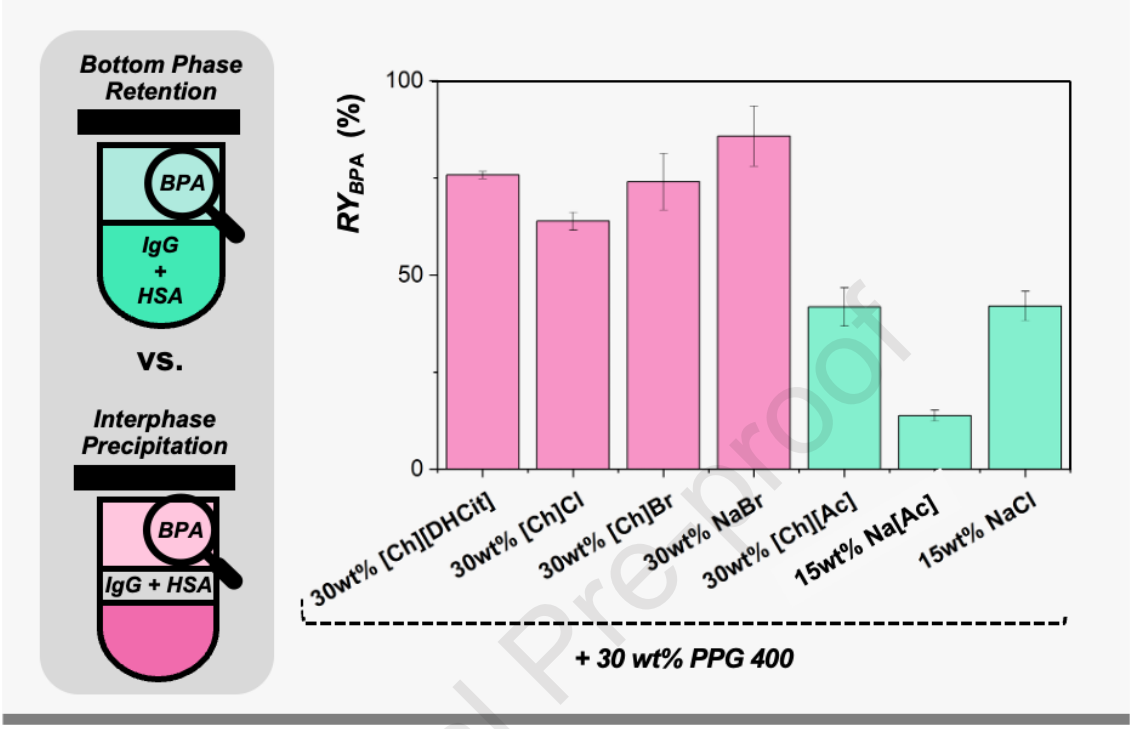


Figure 5. Recovery yield of BPA (RY_{BPA}) in the top phase of ABS comprising 30 wt% PPG 400, 15-30 wt% cholinium or sodium salt, 53-38 wt% PBS aqueous solution and 2 wt% human serum spiked with 0.3 mg·mL^{-1} of BPA. Green bars indicate *bottom phase retention*, pink bars denote *interphase precipitation*, and the left-side schematic illustrates the corresponding protein depletion strategy visually. Values are expressed as mean ± standard deviation (n = 3).

In systems where proteins were predominantly removed via *interphase precipitation*, BPA was recovered in the top, polymer-rich phase. This preferential migration is facilitated by the hydrophobic characteristics of BPA (logarithm of the octanol-water partition coefficient, log $K_{o/w}$ = 3.32), favoring its partition to the less polar, PPG-rich phase [38]. The effective precipitation of high-abundance proteins at the interphase further reduces potential interactions with BPA, creating a favorable migration environment for analyte enrichment. Among these systems, the recovery of BPA reached up to 86%, highlighting the synergy between protein depletion and analyte recovery. Among tested salts, the superior performance of NaBr can be attributed to its strong ionic strength [39], which promotes efficient salting-out.

Conversely, in systems dominated by *bottom phase retention*, BPA recovery in the top phase was markedly lower (14-42%), irrespective of the salt. The retention of proteins in the bottom phase suggests a competitive migration environment that disfavors BPA migration toward the polymer-rich top phase. The association of BPA with the protein-rich bottom phase may be driven by hydrophobic effects or other non-covalent forces, which stabilize its retention alongside the proteins [40]. These findings suggest that macromolecular composition and intermolecular interactions critically influence BPA behavior in ABS.

Remarkably, ABS protein depletion not only simplifies the serum matrix but also critically influences the subsequent recovery of BPA. The *interphase precipitation* strategy demonstrates superior efficiency, simultaneously enabling selective protein depletion (93% of IgG and 94% of HSA) and preferential BPA top phase recovery (86%) in a single step, with ABS composed of 30 wt% PPG 400 and 30 wt% NaBr. In RP-HPLC coupled with UV detection, high-abundance proteins can interfere with analyte signals, causing peak broadening, baseline noise, and co-elution [41], ultimately compromising quantification accuracy and sensitivity. Accordingly, the chromatographic profiles obtained after ABS pretreatment, together with the blank PPG 400 top phase chromatogram with no BPA, support the clean-up efficiency and confirm that the polymer-rich phase does not interfere with BPA determination (cf. Figure S4 in the Supplementary Material). By efficiently removing high-abundance proteins, ABS could reduce matrix interference, sharpen analyte peaks, and enhance detection reliability, representing a clear advantage over untreated serum samples.

In addition to matrix clean-up, the possibility of analyte preconcentration contributes to the analytical versatility of the ABS platform. In this context, BPA concentration in the PPG-rich phase provides insight into its enrichment capability. To this aim, Table S4 in the Supplementary Material reports the masses of the ABS phases, measured BPA concentrations, and the calculated maximum enrichment (EF_{max}) and concentration (*CF*) factors for the *interphase precipitation* strategy, which provided the highest BPA recovery. In an initial assessment, EF_{max}, expressed as the ratio of total ABS mass to PPG-rich phase mass, ranged from 3.35 to 5.19, reflecting the theoretical concentration achievable from phase mass reduction. However, *CF*, the ratio of actual BPA concentration in the PPG-rich phase to the initial serum concentration, remained below 0.1, despite the high recovery yield (64-86%). This behavior reflects phase volume effects related to the PPG-rich phase relative to the amount of serum incorporated into the ABS. While the system could be technically adjusted for preconcentration, in its current form it primarily serves for matrix clean-up through protein depletion rather than analyte enrichment.

Beyond methodological benefits, BPA recovery holds practical potential for a variety of applications. In environmental biomonitoring, trace BPA levels in human serum could be concentrated to detectable levels, assisting epidemiological studies and exposure assessments [42]. In clinical toxicology, where low-level BPA exposure may impact endocrine function, reliable quantification could be achieved even in complex matrices, facilitating patient monitoring [43]. In this context, future efforts could explore the application of this approach to real human serum samples, further reinforcing its translational potential. Finally, ABS could also be applied in food safety and pharmacokinetic studies, where the accurate measurement of trace BPA in diverse types of biological samples is required [44]. Such versatility enables more sensitive and reliable monitoring of BPA exposure across protein-rich matrices and varied real-world scenarios, with clear implications for human health and environmental safety.

3.4. Sustainability assessment of the ABS-HPLC-DAD method for BPA monitoring

To evaluate the environmental sustainability of the proposed ABS-HPLC-DAD method for human serum pretreatment and BPA recovery, complementary green and

blue metrics were applied. The Analytical GREEnness Metric (AGREE) [45] and AGREEprep [46] were used to assess the greenness of the overall analytical procedure and the sample preparation step, respectively, based on the principles of “Green Analytical Chemistry” and “Green Sample Preparation”. Both metrics are presented as color-coded circular diagrams, where each segment represents a criterion and is colored from green (most sustainable) to red (least sustainable), with a central numerical score ranging from 0 to 1. A more detailed evaluation of sample pretreatment sustainability was assisted by the Sample Preparation Metric of Sustainability (SPMS), considering key parameters related to reagent consumption, procedural complexity, energy demand, and waste generation [47]. SPMS results are displayed as a clock-style diagram with color-coded squares and a central numeric score from 0 to 10, allowing rapid visual assessment of performance. Method applicability and practicality were further assessed using the Blue Applicability Grade Index (BAGI), which evaluates attributes associated with throughput, instrumentation, automation, and operational simplicity [48]. BAGI results are shown as an asteroid pictogram with a central score, enabling straightforward comparison of method practicality. The results of the applied metrics are compiled in Figure 6.

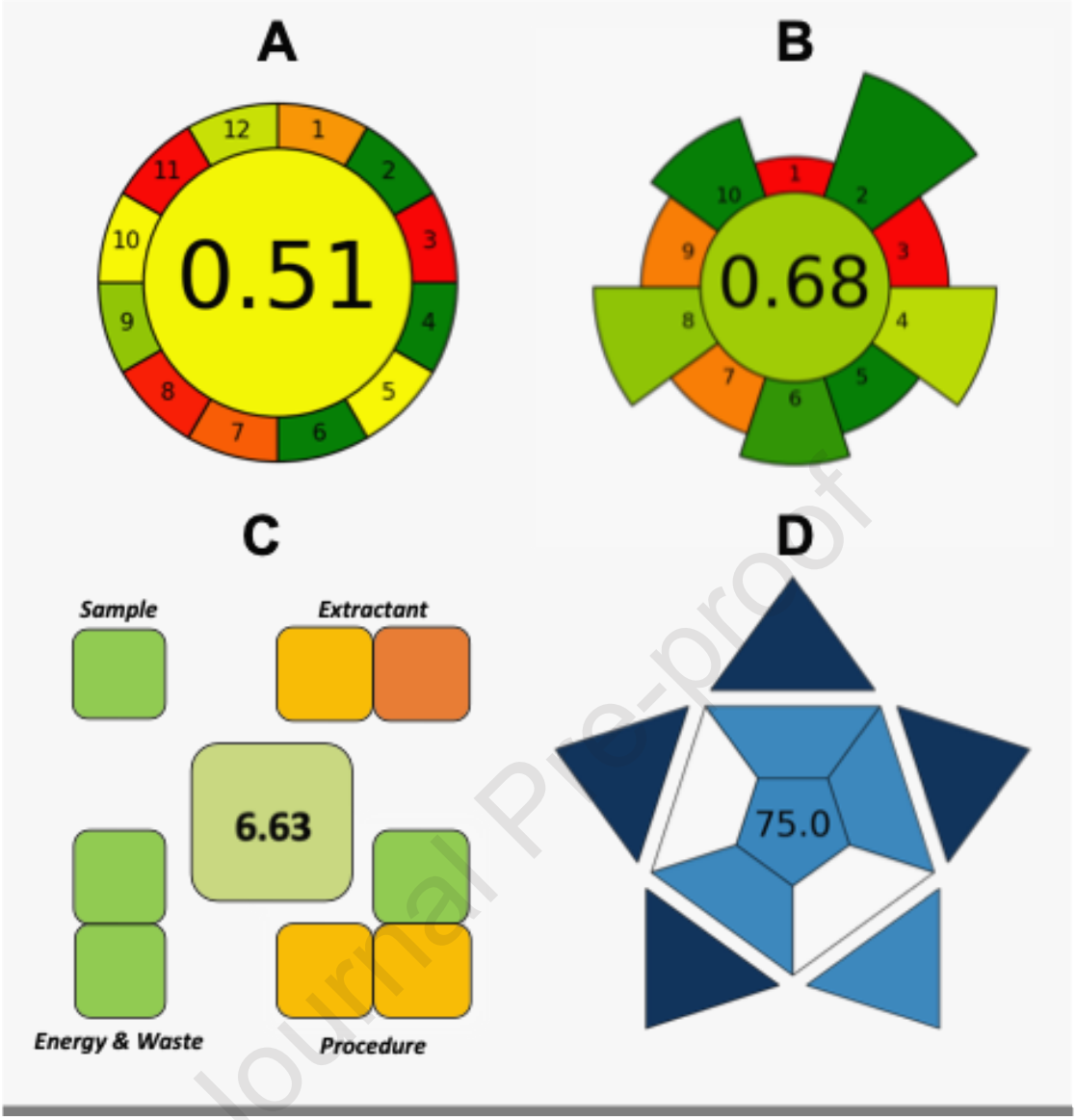


Figure 6. Evaluation of the proposed ABS-HPLC-DAD method by green and blue analytical metrics: (A) AGREE, (B) AGREEprep, (C) SPMS and (D) BAGI.

According to AGREE (Figure 6A), the proposed method achieved a global score of 0.51, indicating a moderate overall greenness. Favorable scores were obtained for criteria related to integration (protein depletion and BPA extraction in a single ABS step), avoidance of derivatization and the use of a miniaturized sample preparation system (1 g total mass) and minimal sample amount (~0.02 g). Additional positive contributions arise from operation at room temperature, and the partial use of safe, low-toxicity reagents (PPG 400 and cholinium or sodium salts). In contrast, lower scores were assigned to criteria related to direct analysis and *in situ* measurement, highlighting the need for off-line sample pretreatment, as well as to analytical waste generation and reagent toxicity, mainly resulting from the chromatographic analysis step, which requires extended HPLC run times and the use of acetonitrile.

The AGREEprep evaluation (Figure 6B) yielded an overall score of 0.68 for the ABS pretreatment step. High scores were observed for the use of safer, non-volatile aqueous solutions of PPG and salts, minimal serum consumption (~0.02 g per ABS), process integration (simultaneous protein depletion and BPA extraction), and operator

safety. Intermediate scores were assigned to sample throughput and energy consumption, reflecting single-sample processing and the centrifugation step, while waste minimization received a moderate score due to aqueous/polymer streams. The post-preparation chromatographic analysis contributed to a lower score, consistent with the inherent energy and resource demands of HPLC.

To further refine the evaluation of the sample preparation step, the SPMS was applied (Figure 6C), yielding a score of 6.63. High performance was associated with low sample and extractant volumes, a limited number of steps, room-temperature operation, and the absence of derivatization. However, SPMS penalizes the lack of extractant reusability, centrifugation-based separation, and limited throughput, preventing the method from achieving higher scores. Finally, the practical applicability assessed by BAGI (Figure 6D) resulted in a score of 75. Strengths include standard instrumentation, commercially available reagents, and straightforward operations, while lower scores arise from the ABS pretreatment involving centrifugation, manual handling, and limited automation, which constrain throughput.

Overall, the ABS-HPLC-DAD method achieves a balanced performance, combining moderate-to-good greenness, efficient and integrated sample preparation, and practical applicability, while highlighting areas for further optimization such as throughput, extractant reusability, and automation.

3.5. Benchmarking the ABS-HPLC-DAD method against reported BPA analysis approaches

To contextualize the performance of the proposed ABS-HPLC-DAD method, its analytical performance and sustainability aspects were compared with reported methods for BPA quantification in biological samples. Comparison criteria are provided in Table S5 in the Supplementary Material, including sample pretreatment strategies, analytical techniques, extraction times, and limits of detection [5–7,49,50]. The corresponding AGREE, AGREEprep, SPMS, and BAGI scores are summarized in Figure S5 in the Supplementary Material.

Conventional sample pretreatment strategies for BPA extraction, such as PP, SPE, and LLE using volatile organic solvents, have been widely reported. Examples include acetonitrile-induced PP in human plasma with an LOD of 5 $ng \cdot mL^{-1}$ [5], SPE followed by GC-MS/MS achieving LODs as low as 0.05 $ng \cdot mL^{-1}$ in urine [6], and acetonitrile-based LLE coupled to LC-MS/MS providing an LOD of 0.009 $ng \cdot mL^{-1}$ in human serum [7]. While these approaches deliver excellent analytical sensitivity, their sustainability is limited by multistep workflows, extensive use of volatile organic solvents, derivatization, increased waste generation and energy-intensive procedures, such as centrifugation, evaporation, and filtration, as indicated by AGREE and AGREEprep scores (Figure S5 in the Supplementary Material). These factors are also reflected in lower SPMS values and only moderate BAGI scores, indicating reduced green credentials and limited applicability for high-throughput or routine analysis.

More recent approaches based on ABS composed of ionic liquids (IL-ABS) [49] and LLE comprising hydrophobic eutectic solvents (HES) [50] represent advances toward greener sample pretreatment. IL-ABS applied to urine samples allowed BPA extraction with UV-Vis detection, but prolonged equilibration times (up to 720 min) restricted sample throughput and reduced AGREEprep and SPMS scores [49]. HES-based pretreatment of whole blood, coupled to LC-MS/MS, decreased volatile organic

solvent use and shortened preparation to ~30 min [49]. Despite improved solvent sustainability, reliance on sonication, centrifugation and energy-intensive LC-MS/MS instrumentation limits overall practicality and negatively impacted AGREE and BAGI scores.

Remarkably, the proposed ABS-HPLC-DAD method achieves intermediate-to-high scores across AGREE (0.51), AGREEprep (0.68), SPMS (6.63), and BAGI (75), reflecting a deliberate compromise between analytical sensitivity, environmental sustainability, and operational practicality. However, the reported LOD of 49000 $ng \cdot mL^{-1}$ is higher than those obtained by MS-based methods [5–7], which is expected due to the use of DAD instead of MS/MS. Even so, the method avoids derivatization, reduces solvent toxicity, minimizes energy consumption, and relies on widely available HPLC equipment, enhancing accessibility for routine analysis. Compared with conventional pretreatment strategies, ABS offer a favorable balance of features: cost-effectiveness, small reagent volumes, energy efficiency (no repeated centrifugation or evaporation), eco-friendliness (aqueous phases instead of volatile solvents), and analytical performance, combining efficient protein depletion with BPA recovery in a single step (up to 86%). While previously reported methods may excel in analytical sensitivity, the ABS strategy provides a holistic improvement across key “Green Sample Preparation” metrics, highlighting its potential for sustainable analytical workflows [51].

4. Conclusions

This work establishes a dual-strategy ABS serum pretreatment platform, exploiting cholinium and sodium salts with PPG 400 to selectively deplete high-abundance proteins. Two complementary strategies were identified using ABS, with the choice of salt determining which approach is implemented: *interphase precipitation*, in which proteins aggregate at the interphase, and *bottom phase retention*, where proteins accumulate in the salt-rich bottom phase. Following protein depletion, the PPG-rich top phase served as the primary medium for BPA recovery and accurate detection, benefiting from reduced protein interference.

Protein depletion was strongly dependent on ABS ionic composition. *Interphase precipitation*, driven by polymer-induced protein destabilization and enhanced by ionic interactions and salting-out effects, achieved up to 94% depletion of IgG and HSA in systems containing [Ch][DHCit], [Ch]Cl, [Ch]Br, or NaBr. *Bottom phase retention*, relying on ion hydration and the reduced solubility of proteins in the polymer-rich phase, reached 97% IgG and 100% HSA depletion in systems with [Ch][Ac], Na[Ac], or NaCl. These optimized systems yielded a simplified, protein-depleted matrix, allowing assessment of BPA recovery and analytical performance, which were markedly influenced by the protein depletion strategy. The *interphase precipitation* strategy clearly excelled, as the effective depletion of high-abundance proteins from the bulk phases (93% of IgG and 94% of HSA) allowed BPA to be predominantly recovered into the PPG-rich top phase, with minimal losses (86% recovery).

The environmental sustainability and practical applicability of the proposed ABS-HPLC platform were critically assessed using complementary green and blue analytical metrics. The intermediate-to-high scores obtained confirm that the method achieves a balanced compromise between analytical performance, environmental sustainability, and operational feasibility, outperforming previously reported approaches.

By exploring mechanistically distinct protein depletion routes in ABS, this work demonstrates how ionic composition and phase design can be tailored to control protein distribution, minimize matrix interferences, and maximize analyte recovery. This dual-strategy platform provides an adaptable and streamlined framework for serum pretreatment and the selective enrichment of exposure markers and potentially other biomarkers. Ultimately, it offers a sustainable and efficient route to improve analytical accuracy in exposure assessment and environmental monitoring, while also holding relevance for broader bioanalytical applications, including clinical analysis.

CRediT authorship contribution statement

Maria S. M. Mendes: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-original draft, Visualization; **Inês B. Santana:** Formal analysis, Investigation, Data Curation; **Mara G. Freire:** Conceptualization, Funding Acquisition, Supervision, Writing – review & editing; **Francisca A. e Silva:** Conceptualization, Writing – review & editing, Supervision, Funding Acquisition, Project Administration.

Acknowledgements

This work was developed within the scope of the project CICECO – Aveiro Institute of Materials, UID/50011/2025 (DOI 10.54499/UID/50011/2025) & LA/P/0006/2020 (DOI 10.54499/LA/P/0006/2020), financed by national funds through the FCT/MCTES (PIDDAC). This work was supported by national funds (OE) through FCT/MCTES under the project ILSurvive, PTDC/EMD-TLM/3253/2020 (DOI 10.54499/PTDC/EMD-TLM/3253/2020). M.S.M.M. acknowledges FCT for the doctoral grant 2022.11229.BD (DOI 10.54499/2022.11229.BD). F.A.eS. acknowledges FCT for the researcher contract CEECIND/03076/2018/CP1559/CT0024 (DOI 10.54499/CEECIND/03076/2018/CP1559/CT0024) under the Scientific Employment Stimulus – Individual Call 2018.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

[1] E. Bellei, S. Bergamini, E. Monari, L.I. Fantoni, A. Cuoghi, T. Ozben, A. Tomasi, High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins, Amino Acids 40 (2011) 145–156. https://doi.org/10.1007/s00726-010-0628-x.

[2] L.N. Vandenberg, M. V. Maffini, C. Sonnenschein, B.S. Rubin, A.M. Soto, Bisphenol-A and the Great Divide: A Review of Controversies in the Field of

Endocrine Disruption, Endocr. Rev. 30 (2009) 75–95. https://doi.org/10.1210/er.2008-0021.

[3] N. Caballero-Casero, L. Lunar, S. Rubio, Analytical methods for the determination of mixtures of bisphenols and derivatives in human and environmental exposure sources and biological fluids. A review, Anal. Chim. Acta 908 (2016) 22–53. https://doi.org/10.1016/j.aca.2015.12.034.

[4] P.L. Kole, G. Venkatesh, J. Kotecha, R. Sheshala, Recent advances in sample preparation techniques for effective bioanalytical methods, Biomedical Chromatography 25 (2011) 199–217. https://doi.org/10.1002/bmc.1560.

[5] I.A. Wiraagni, M.A. Mohd, R. bin Abd Rashid, D.E. bin M. Haron, Validation of a simple extraction procedure for bisphenol A identification from human plasma, PLoS One 14 (2019) e0221774. https://doi.org/10.1371/journal.pone.0221774.

[6] A. Simonelli, R. Guadagni, P. De Franciscis, N. Colacurci, M. Pieri, P. Basilicata, P. Pedata, M. Lamberti, N. Sannolo, N. Miraglia, Environmental and occupational exposure to bisphenol A and endometriosis: urinary and peritoneal fluid concentration levels, Int. Arch. Occup. Environ. Health 90 (2017) 49–61. https://doi.org/10.1007/s00420-016-1171-1.

[7] K. Owczarek, P. Kubica, B. Kudłak, A. Rutkowska, A. Konieczna, D. Rachoń, J. Namieśnik, A. Wasik, Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography–tandem mass spectrometry, Science of The Total Environment 628–629 (2018) 1362–1368. https://doi.org/10.1016/j.scitotenv.2018.02.148.

[8] M.S.M. Mendes, M.E. Rosa, F. Ramalho, M.G. Freire, F.A. e Silva, Aqueous two-phase systems as multipurpose tools to improve biomarker analysis, Sep. Purif. Technol. 317 (2023) 123875. https://doi.org/10.1016/j.seppur.2023.123875.

[9] T. Trtić-Petrović, A. Dimitrijević, N. Zdolšek, J. Đorđević, A. Tot, M. Vraneš, S. Gadžurić, New sample preparation method based on task-specific ionic liquids for extraction and determination of copper in urine and wastewater, Anal. Bioanal. Chem. 410 (2018) 155–166. https://doi.org/10.1007/s00216-017-0705-z.

[10] Z. Du, Y.L. Yu, J.H. Wang, Extraction of proteins from biological fluids by use of an ionic liquid/aqueous two-phase system, Chemistry - A European Journal 13 (2007) 2130–2137. https://doi.org/10.1002/chem.200601234.

[11] M.G. Bogdanov, I. Svinyarov, Analysis of acetylcholinesterase inhibitors by extraction in choline saccharinate aqueous biphasic systems, J. Chromatogr. A 1559 (2018) 62–68. https://doi.org/10.1016/j.chroma.2018.01.007.

[12] P. Berton, R.P. Monasterio, R.G. Wuilloud, Selective extraction and determination of vitamin B12 in urine by ionic liquid-based aqueous two-phase system prior to high-performance liquid chromatography, Talanta 97 (2012) 521–526. https://doi.org/10.1016/j.talanta.2012.05.008.

[13] M.E. Rosa, M.S.M. Mendes, E. Carmo, J.P. Conde, J.A.P. Coutinho, M.G. Freire, F.A. e Silva, Tailored pretreatment of serum samples and biomarker extraction afforded by ionic liquids as constituents of aqueous biphasic systems, Sep. Purif. Technol. 322 (2023) 124248. https://doi.org/10.1016/j.seppur.2023.124248.

[14] W. Yu, K. Li, Z. Liu, H. Zhang, X. Jin, Novelty aqueous two-phase extraction system based on ionic liquid for determination of sulfonamides in blood coupled with high-performance liquid chromatography, Microchemical Journal 136 (2018) 263–269. https://doi.org/10.1016/j.microc.2017.03.053.

[15] A. Gong, X. Zhu, Surfactant/ionic liquid aqueous two-phase system extraction coupled with spectrofluorimetry for the determination of dutasteride in pharmaceutical formulation and biological samples, Fluid Phase Equilib. 374 (2014) 70–78. https://doi.org/10.1016/j.fluid.2014.04.022.

[16] J. Flieger, A. Czajkowska-Żelazko, Aqueous two phase system based on ionic liquid for isolation of quinine from human plasma sample, Food Chem. 166 (2015) 150–157. https://doi.org/10.1016/j.foodchem.2014.06.037.

[17] R. González-Martín, F.A. e Silva, M.J. Trujillo-Rodríguez, D. Díaz Díaz, J. Lorenzo-Morales, M.G. Freire, V. Pino, Ionic liquid-based aqueous biphasic systems as one-step clean-up, microextraction and preconcentration platforms for the improved determination of salivary biomarkers, Green Chemistry 25 (2023) 8544–8557. https://doi.org/10.1039/d3gc02046k.

[18] J.A. Asenjo, B.A. Andrews, Aqueous two-phase systems for protein separation: A perspective, J. Chromatogr. A 1218 (2011) 8826–8835. https://doi.org/10.1016/j.chroma.2011.06.051.

[19] M. Iqbal, Y. Tao, S. Xie, Y. Zhu, D. Chen, X. Wang, L. Huang, D. Peng, A. Sattar, M.A.B. Shabbir, H.I. Hussain, S. Ahmed, Z. Yuan, Aqueous two-phase system (ATPS): an overview and advances in its applications, Biol. Proced. Online 18 (2016) 1–18. https://doi.org/10.1186/s12575-016-0048-8.

[20] E.C. Antunes, H. Temmink, B. Schuur, Polymer and alcohol-based three-phase partitioning systems for separation of polysaccharide and protein, Journal of Chemical Technology & Biotechnology 99 (2024) 259–269. https://doi.org/10.1002/jctb.7534.

[21] G.M. Rather, M.N. Gupta, Three phase partitioning leads to subtle structural changes in proteins, Int. J. Biol. Macromol. 60 (2013) 134–140. https://doi.org/10.1016/j.ijbiomac.2013.05.018.

[22] M. V. Quental, M. Caban, M.M. Pereira, P. Stepnowski, J.A.P. Coutinho, M.G. Freire, Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems, Biotechnol. J. 10 (2015) 1457–1466. https://doi.org/10.1002/biot.201500003.

[23] C.A. Suarez Ruiz, O. Cabau-Peinado, C. van den Berg, R.H. Wijffels, M.H.M. Eppink, Efficient Fractionation of Lipids in a Multiproduct Microalgal Biorefinery by Polymers and Ionic Liquid-Based Aqueous Two-Phase Systems, ACS Sustain. Chem. Eng. 10 (2022) 789–799. https://doi.org/10.1021/acssuschemeng.1c06017.

[24] P.P. Madeira, V.N. Uversky, B.Y. Zaslavsky, Effects of Inorganic Salts on Phase Separation in Aqueous Solutions of Poly(ethylene glycol), Int. J. Mol. Sci. 26 (2025) 4545. https://doi.org/10.3390/ijms26104545.

[25] M. Petkovic, J.L. Ferguson, H.Q.N. Gunaratne, R. Ferreira, M.C. Leitão, K.R. Seddon, L.P.N. Rebelo, C.S. Pereira, Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability, Green Chemistry 12 (2010) 643. https://doi.org/10.1039/b922247b.

[26] A.F.M. Cláudio, L. Swift, J.P. Hallett, T. Welton, J.A.P. Coutinho, M.G. Freire, Extended scale for the hydrogen-bond basicity of ionic liquids, Physical Chemistry Chemical Physics 16 (2014) 6593. https://doi.org/10.1039/c3cp55285c.

[27] X. Zhao, X. Xie, Y. Yan, Liquid–liquid equilibrium of aqueous two-phase systems containing poly(propylene glycol) and salt ((NH4)2SO4, MgSO4, KCl, and KAc):

experiment and correlation, Thermochim. Acta 516 (2011) 46–51. https://doi.org/10.1016/j.tca.2011.01.010.

[28] S.C. Silvério, O. Rodríguez, J.A. Teixeira, E.A. Macedo, The Effect of Salts on the Liquid–Liquid Phase Equilibria of PEG600 + Salt Aqueous Two-Phase Systems, J. Chem. Eng. Data 58 (2013) 3528–3535. https://doi.org/10.1021/je400825w.

[29] R. Sadeghi, F. Jahani, Salting-In and Salting-Out of Water-Soluble Polymers in Aqueous Salt Solutions, J. Phys. Chem. B 116 (2012) 5234–5241. https://doi.org/10.1021/jp300665b.

[30] F.A. e Silva, J.F.B. Pereira, K.A. Kurnia, S.P.M. Ventura, A.M.S. Silva, R.D. Rogers, J.A.P. Coutinho, M.G. Freire, Temperature dependency of aqueous biphasic systems: an alternative approach for exploring the differences between Coulombic-dominated salts and ionic liquids, Chemical Communications 53 (2017) 7298–7301. https://doi.org/10.1039/C7CC02294H.

[31] Y. Chen, J. Ryu, J.D. Bazak, D.T. Nguyen, K.S. Han, Z. Li, J.Z. Hu, K.T. Mueller, V. Murugesan, Ion Solvation-Driven Liquid–Liquid Phase Separation in Divalent Electrolytes with Miscible Organic Solvents, The Journal of Physical Chemistry C 127 (2023) 15443–15453. https://doi.org/10.1021/acs.jpcc.3c01701.

[32] S. Großhans, G. Wang, J. Hubbuch, Water on hydrophobic surfaces: mechanistic modeling of polyethylene glycol-induced protein precipitation, Bioprocess Biosyst. Eng. 42 (2019) 513–520. https://doi.org/10.1007/s00449-018-2054-5.

[33] A.M. Azevedo, A.G. Gomes, P.A.J. Rosa, I.F. Ferreira, A.M.M.O. Pisco, M.R. Aires-Barros, Partitioning of human antibodies in polyethylene glycol–sodium citrate aqueous two-phase systems, Sep. Purif. Technol. 65 (2009) 14–21. https://doi.org/10.1016/j.seppur.2007.12.010.

[34] N. Saha, B. Sarkar, K. Sen, Aqueous biphasic systems: A robust platform for green extraction of biomolecules, J. Mol. Liq. 363 (2022) 119882. https://doi.org/10.1016/j.molliq.2022.119882.

[35] X. Wang, T. Guo, Y. Shu, J. Wang, The anion of choline-based ionic liquids tailored interactions between ionic liquids and bovine serum albumin, MCF-7 cells, and bacteria, Colloids Surf. B Biointerfaces 206 (2021) 111971. https://doi.org/10.1016/j.colsurfb.2021.111971.

[36] L. Xue, Y. Zhao, L. Yu, Y. Sun, K. Yan, Y. Li, X. Huang, Y. Qu, Choline acetate enhanced the catalytic performance of Candida rogusa lipase in AOT reverse micelles, Colloids Surf. B Biointerfaces 105 (2013) 81–86. https://doi.org/10.1016/j.colsurfb.2012.12.050.

[37] R. Raoufinia, A. Mota, S. Nozari, L. Aghebati Maleki, S. Balkani, J. Abdolalizadeh, A methodological approach for purification and characterization of human serum albumin, J. Immunoassay Immunochem. 37 (2016) 623–635. https://doi.org/10.1080/15321819.2016.1184163.

[38] J. Michałowicz, Bisphenol A – Sources, toxicity and biotransformation, Environ. Toxicol. Pharmacol. 37 (2014) 738–758. https://doi.org/10.1016/j.etap.2014.02.003.

[39] X. Liu, L.-R. Zhao, S.-H. Sang, Y.-Y. Gao, Q. Ge, Mean Ionic Activity Coefficients of NaBr in the Ternary System NaBr–SrBr2–H2O at 288.15 K using the Cell Potential Method, J. Chem. Eng. Data 65 (2020) 5083–5089. https://doi.org/10.1021/acs.jced.9b01137.

[40] X. Xie, X. Wang, X. Xu, H. Sun, X. Chen, Investigation of the interaction between endocrine disruptor bisphenol A and human serum albumin, Chemosphere 80 (2010) 1075–1080. https://doi.org/10.1016/j.chemosphere.2010.04.076.

[41] J. Martosella, N. Zolotarjova, H. Liu, G. Nicol, B.E. Boyes, Reversed-Phase High-Performance Liquid Chromatographic Prefractionation of Immunodepleted Human Serum Proteins to Enhance Mass Spectrometry Identification of Lower-Abundant Proteins, J. Proteome Res. 4 (2005) 1522–1537. https://doi.org/10.1021/pr050088l.

[42] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J.R. Paumgartten, G. Schoenfelder, Urinary, Circulating, and Tissue Biomonitoring Studies Indicate Widespread Exposure to Bisphenol A, Environ. Health Perspect. 118 (2010) 1055–1070. https://doi.org/10.1289/ehp.0901716.

[43] Dhanjai, A. Sinha, L. Wu, X. Lu, J. Chen, R. Jain, Advances in sensing and biosensing of bisphenols: A review, Anal. Chim. Acta 998 (2018) 1–27. https://doi.org/10.1016/j.aca.2017.09.048.

[44] K.A. Thayer, D.R. Doerge, D. Hunt, S.H. Schurman, N.C. Twaddle, M.I. Churchwell, S. Garantziotis, G.E. Kissling, M.R. Easterling, J.R. Bucher, L.S. Birnbaum, Pharmacokinetics of bisphenol A in humans following a single oral administration, Environ. Int. 83 (2015) 107–115. https://doi.org/10.1016/j.envint.2015.06.008.

[45] F. Pena-Pereira, W. Wojnowski, M. Tobiszewski, AGREE—Analytical GREEnness Metric Approach and Software, Anal. Chem. 92 (2020) 10076–10082. https://doi.org/10.1021/acs.analchem.0c01887.

[46] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep – Analytical greenness metric for sample preparation, TrAC Trends in Analytical Chemistry 149 (2022) 116553. https://doi.org/10.1016/j.trac.2022.116553.

[47] R. González-Martín, A. Gutiérrez-Serpa, V. Pino, M. Sajid, A tool to assess analytical sample preparation procedures: Sample preparation metric of sustainability, J. Chromatogr. A 1707 (2023) 464291. https://doi.org/10.1016/j.chroma.2023.464291.

[48] N. Manousi, W. Wojnowski, J. Płotka-Wasylka, V. Samanidou, Blue applicability grade index (BAGI) and software: a new tool for the evaluation of method practicality, Green Chemistry 25 (2023) 7598–7604. https://doi.org/10.1039/D3GC02347H.

[49] H. Passos, A.C.A. Sousa, M.R. Pastorinho, A.J.A. Nogueira, L.P.N. Rebelo, J.A.P. Coutinho, M.G. Freire, Ionic-liquid-based aqueous biphasic systems for improved detection of bisphenol A in human fluids, Analytical Methods 4 (2012) 2664–2667. https://doi.org/10.1039/c2ay25536g.

[50] C. Polesca, H. Passos, A.C.A. Sousa, N.M. Tue, J.A.P. Coutinho, T. Kunisue, M.G. Freire, Sustainable pretreatment of blood samples using hydrophobic eutectic solvents to improve the detection of bisphenol A, Green Chemistry 27 (2025) 200–208. https://doi.org/10.1039/D4GC03396E.

[51] Á.I. López-Lorente, F. Pena-Pereira, S. Pedersen-Bjergaard, V.G. Zuin, S.A. Ozkan, E. Psillakis, The ten principles of green sample preparation, TrAC Trends in Analytical Chemistry 148 (2022) 116530. https://doi.org/10.1016/j.trac.2022.116530.

Highlights

- Polypropylene glycol with cholinium or sodium salts forms ABS for serum protein depletion.
- Salt selection tunes ABS phase behavior, protein partition, and BPA recovery.
- Protein depletion is achieved via *interphase precipitation* or *bottom phase retention*.
- *Interphase precipitation* delivers optimal protein depletion and BPA recovery.
- ABS enables accurate detection for exposure and bioanalytical applications.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: