Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/d5ay02123e

Received 22nd December 2025
Accepted 26th January 2026

DOI: 10.1039/d5ay02123e

rsc.li/methods

Adapting aqueous biphasic systems to solid-phase extraction for bioanalytical applications: high-abundance protein depletion and prostate-specific antigen extraction from human serum

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Aqueous biphasic systems are investigated here, from standard liquid-phase to solid-phase extraction configurations for human serum pretreatment. Using prostate-specific antigen as a model biomarker, this strategy achieves >90% depletion of high-abundance proteins and demonstrates strong potential for bioanalytical applications compared to conventional precipitation methods.

Despite the potential of biomarkers for advancing healthcare and sustainable development, their clinical application is still limited by the challenges posed by sample matrix effects.[1] Blood serum, a routine sample in clinical testing, contains high-abundance proteins, mainly immunoglobulin G (IgG) and human serum albumin (HSA), which often interfere with bioanalysis.[2] Bioanalytical sample pretreatment techniques used to reduce IgG and HSA content in serum include protein precipitation (PP), liquid–liquid extraction (LLE) with volatile organic solvents and solid-phase extraction (SPE) using affinity capture or depletion strategies.[3] Due to superior analytical performance, SPE overcomes some limitations of PP and LLE, such as the use of organic solvents, biomarker losses as well as limited miniaturization and automation capabilities.[3,4] Still, it often relies on multistep protocols and expensive affinity ligands to maximize the depletion of high-abundance molecules or biomarker capture.[5] Regardless of their utility, no single technique can simultaneously provide high selectivity and biomarker recovery yield while also addressing cost-effectiveness and environmental concerns.[3]

Due to their tailored eco-friendly and cost-effective properties, aqueous biphasic systems (ABS) have emerged as key enabling technologies in bioanalysis. ABS are LLE and emerging liquid-phase microextraction (LPME) techniques, in which the use of water-immiscible organic solvents is traditionally replaced by polymers, inorganic or organic salts and water, offering gentle conditions for biomolecules.[6] ABS have demonstrated the potential to transform technical, economic and environmental aspects of biomarker discovery and detection, improving proteome coverage, streamlining cumbersome laboratory analytical setups and enabling point-of-care applications.[7]

The concept of ABS can be transposed to SPE configurations using aqueous biphasic extraction chromatographic (ABEC) resins/materials. ABEC-based processes are adapted from ABS composed of PEG and salts, using high molecular weight monomethylated polyethylene glycols (Me-PEGs) covalently attached to an inert solid support along with high ionic strength salt solutions.[8] These resins allow for adsorption-based separation, where the surface interacts with the surrounding fluid.[8] ABEC resins/materials feature process flow compatibility and selectivity.[8]

While they may replicate technological functionalities of ABS, as shown in the extraction of metal ions, dyes and proteins,[8–10] to the best of our knowledge, there are no previous reports on the use of ABEC resins to improve bioanalysis, particularly to pretreat human fluids.

This work presents, for the first time, the adaptation of ABS to SPE configurations, combining their advantages to tackle the existing challenges in bioanalytical sample pretreatment. Both liquid-phase and solid-phase configurations of ABS are developed and compared for their performance to pretreat human serum samples, focusing on depleting high-abundance proteins and simultaneously extracting a target biomarker. Fig. 1 outlines the workflow steps for both ABS and SPE configurations developed.

To expedite the development of distinct ABS configurations, PEG with a molecular weight of 2000 $g \cdot mol^{-1}$ (PEG 2000) and citrate buffer ($C_6H_5K_3O_7/C_6H_8O_7$, pH $\approx$ 7) were selected as phase-forming components, based on the availability of the corresponding ternary phase diagram.[11,12] ABS were adapted to SPE configurations using Me-PEG with the same molecular weight, as PEG 2000 grafted onto the resin (ABEC-2000), along with the same buffer as the liquid/eluent phase. PEG-2000- and $C_6H_5K_3O_7/C_6H_8O_7$-induced PP were also appraised as

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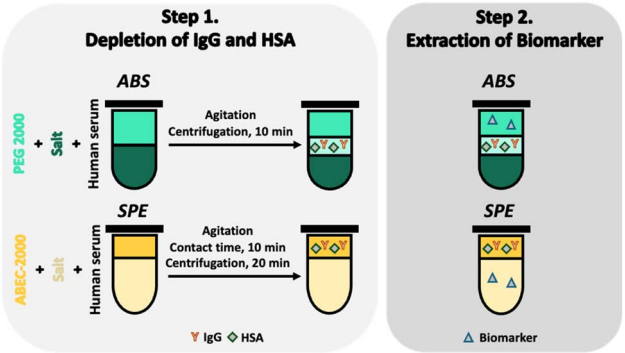

Fig. 1 Workflow applied for the depletion of high-abundance serum proteins and biomarker extraction using liquid-phase and solid-phase configurations of ABS.

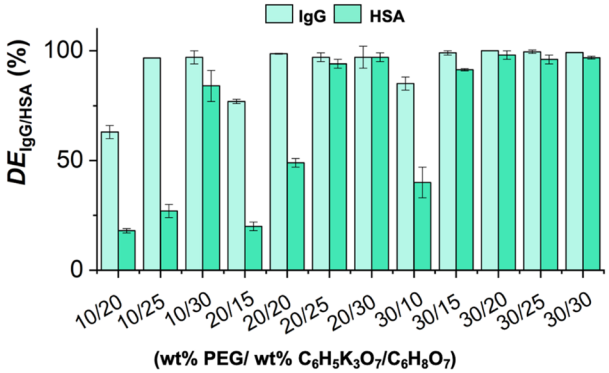

Fig. 3 Depletion efficiencies of IgG and HSA using ABS composed of PEG 2000 and $C_6H_5K_3O_7/C_6H_8O_7$ in the presence of 10 wt% human serum.

benchmarks. For direct comparison, preparation conditions, component composition and serum quantity (10 wt%) were kept consistent or slightly adjusted across different sample pretreatment configurations depending on their specific requirements. Information regarding all materials, experimental procedures and performance parameters of the three serum pretreatment configurations is provided in the SI (Tables S1–S6).

Various aqueous solutions of PEG 2000 and $C_6H_5K_3O_7/C_6H_8O_7$ were initially tested as precipitating agents to assess their individual effects on IgG and HSA depletion. PEG-induced PP operates through volume exclusion,[13] whereas salt-induced PP relies on reducing protein solubility by promoting ions–proteins interactions, particularly at the isoelectric point (pI) proximity.[14] Under the conditions appraised, increasing concentrations of precipitating agent generally improve depletion efficiencies, as shown in Fig. 2.

IgG depletion ranged from 24% to 80% at 10–30 wt% PEG 2000, with the highest efficiency at 20 wt% PEG 2000, while HSA depletion was marginal, between 0% and 5%. IgG has a larger molecular weight (160 KDa *vs.* 66 KDa), being more prone to precipitate.[11] Salt-induced PP of IgG achieved 95% at 30 wt% $C_6H_5K_3O_7/C_6H_8O_7$, while HSA showed negligible depletion throughout the entire concentration range. Aside from its lower molecular weight, HSA has a pI of 4.7 and is more soluble at pH $\approx$ 7, making it less prone to precipitation.[14]

Fig. 3 shows the depletion efficiencies of high-abundance serum proteins obtained with ABS, using various mixture compositions placed at the biphasic region of the ternary phase diagram for the system PEG 2000 + $C_6H_5K_3O_7/C_6H_8O_7$ + water.[12] Upon introduction of the serum sample, these systems form a PEG-rich top phase and a $C_6H_5K_3O_7/C_6H_8O_7$-rich bottom phase, separated by a solid interphase enriched with depleted IgG and HSA. An increase in the weight percentages of both $C_6H_5K_3O_7/C_6H_8O_7$ and PEG 2000 (10–30 wt%) enhances the depletion of both high-abundance serum proteins, exceeding 84% efficiency. This effect is more pronounced for HSA, which is less easily precipitated due to its lower molecular weight (66 KDa *vs.* 160 KDa) and isoelectric point (4.7 *vs.* 7–9.5).[11,14] Overall, high-abundance serum proteins depletion is accomplished by their decreased solubility in the PEG-rich phase, with further assistance from the salting-out effect.

Fig. 4 depicts the depletion of high-abundance serum proteins using SPE, where the ABEC-2000 resin simulates the PEG-rich phase of the ABS. The absence of $C_6H_5K_3O_7/C_6H_8O_7$ prevented any protein depletion at 30 wt% resin, highlighting its essential role in the development of ABEC-based SPE, comparable to its function in ABS. Increasing the concentrations of both resin and $C_6H_5K_3O_7/C_6H_8O_7$ leads to more efficient depletion of both high-abundance serum proteins, with a more pronounced effect on HSA. Thus, the effects observed

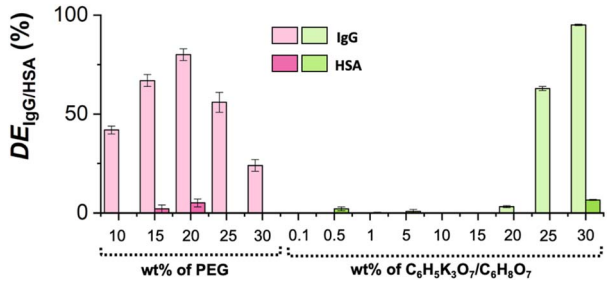

Fig. 2 Depletion efficiencies of IgG and HSA using PEG-2000-induced or $C_6H_5K_3O_7/C_6H_8O_7$-induced PP in the presence of 10 wt% human serum.

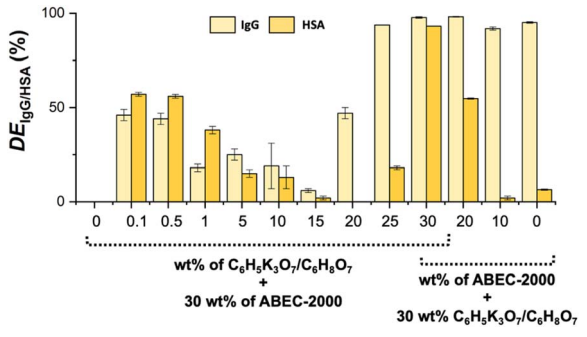

Fig. 4 Depletion efficiencies of IgG and HSA using ABEC-based SPE in the presence of 10 wt% human serum.

with ABS are here transferred to the ABEC-based SPE configuration.

Distinct trends depending on the nature of high-abundance serum protein and the concentration of $C_6H_5K_3O_7/C_6H_8O_7$ were observed in depletion with ABEC-based SPE (*cf.* Fig. 4). Accordingly, it can be either governed by protein-resin interactions, with a significant contribution from the salt effect (at lower concentrations of $C_6H_5K_3O_7/C_6H_8O_7$), or predominantly controlled by the salt effect (at higher concentrations of $C_6H_5K_3O_7/C_6H_8O_7$). On the one hand, IgG depletion is concentration-dependent in the absence of ABEC-2000 (*cf.* Fig. 2), with depletion efficiencies varying from modest to minimal as the concentration of $C_6H_5K_3O_7/C_6H_8O_7$ ranges from 0.1 to 20 wt%. Based on this evidence, it can be inferred that IgG depletion by SPE within this concentration range, when observed, is based on IgG-ABEC interactions. Hydrogen bonding, hydrophobic effects and dispersive interactions appear to govern IgG depletion at $C_6H_5K_3O_7/C_6H_8O_7$ below 20 wt%, as IgG has no overall net charge at pH ≈ 7 (pI 7–9.5).[15] However, at higher $C_6H_5K_3O_7/C_6H_8O_7$ concentrations (25–30 wt%), IgG depletion occurred even in the absence of resin (*cf.* Fig. 2). This suggests that, within the ABEC-based SPE configuration, a salt-induced effect leading to IgG precipitation cannot be ruled out. On the other hand, HSA is negatively charged and more soluble in aqueous medium at pH ≈ 7, flowing more readily through the resin at lower $C_6H_5K_3O_7/C_6H_8O_7$ concentrations (<20 wt%).[14] At higher concentrations of $C_6H_5K_3O_7/C_6H_8O_7$ and ABEC-2000, the increased ionic strength and hydrogen bonding begin to exert a stronger influence on HSA-ABEC interactions. With a higher effective concentration of Me-PEG available for interactions, HSA depletion reaches a maximum of 93% at 30 wt% $C_6H_5K_3O_7/C_6H_8O_7$ + 30 wt% ABEC-2000. Although a fully adsorption-driven process is preferred for future implementation in continuous flow/SPE cartridges to avoid potential clogging, the simultaneous and efficient depletion of both high-abundance serum proteins still depends on a combined precipitation-adsorption mechanism. Further optimization of this configuration is thus required, particularly at lower $C_6H_5K_3O_7/C_6H_8O_7$ concentrations (0.1–0.5 wt%), which seem to promote the depletion of both IgG and HSA to a certain extent, without $C_6H_5K_3O_7/C_6H_8O_7$-induced IgG precipitation.

Fig. 5 compiles the depletion efficiencies across all sample pretreatment configurations under optimal conditions, while also keeping consistent system compositions for direct comparison. Both liquid-phase and solid-phase variants of ABS outperform PP in the single-step depletion of IgG and HSA regardless of the system composition, achieving maximum depletion efficiencies of 99% and 97%, respectively. Under the assessed conditions, depletion is improved by a synergistic effect between the PEG-rich phase (in ABS) or the resin (in SPE) and salt, which is common to both configurations. ABS combines the characteristics of LLE and PP into a water-rich, high-performance variant of sample pretreatment, whereas ABEC-based SPE leverages the properties of ABS to replace costly affinity ligands with more affordable yet efficient ABEC resins. From a Green Sample Preparation perspective,[16] both

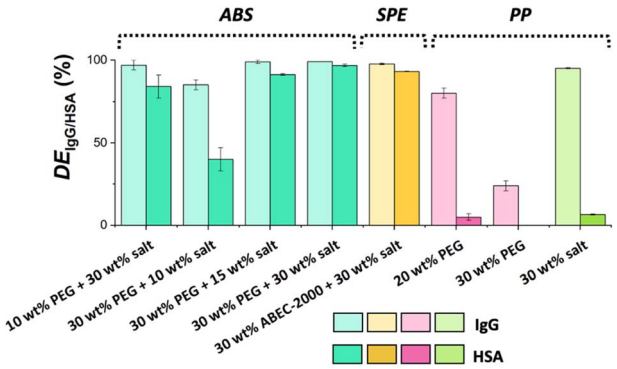

Fig. 5 Comparative assessment of depletion efficiencies of IgG and HSA using ABS, ABEC-based SPE and PP in the presence of 10 wt% human serum.

configurations of sample pretreatment can contribute to the use of safer solvents and sustainable materials, minimize waste and reduce the quantities of sample, chemicals and materials required. They also allow integrating the depletion of both IgG and HSA in a single-step setup, while ensuring safe procedures for laboratory technicians.

After identifying the optimal conditions for high-abundance serum protein depletion, the suitability of standard ABS and adapted SPE configurations for biomarker extraction was confirmed. Prostate-specific antigen (PSA) was used as a model protein, as it is a clinically validated biomarker of prostate cancer, one of the most incident cancers worldwide. It is useful to assist in early detection and disease monitoring, with immunoassays measuring PSA at a cutoff of 4 $ng \cdot mL^{-1}$ in serum serving as the gold standard.[17] However, minimizing sample matrix effects is crucial to increase the reliability of clinical outcomes,[18] further supporting PSA's pertinence as a proof of concept.

Fig. 6 displays the PSA recovery yields obtained for all three sample pretreatment configurations, using serum samples

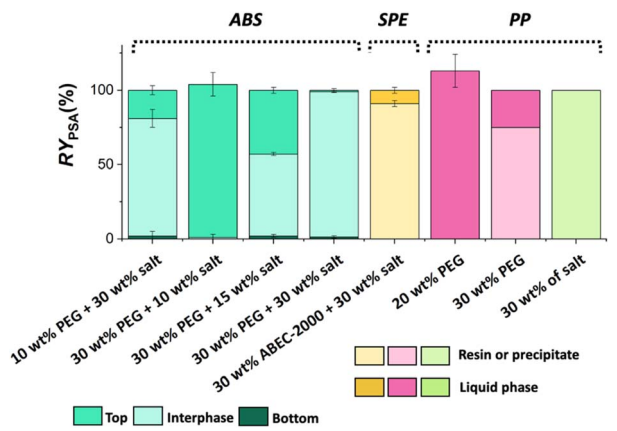

Fig. 6 Recovery yield of PSA in the top phase, interphase, and bottom phase using ABS and in the liquid phase and resin/precipitate using ABEC-based SPE and PP in the presence of 10 wt% human serum spiked with 4 $ng \cdot mL^{-1}$ of PSA.

spiked with PSA at a clinically significant concentration of 4 $ng \cdot mL^{-1}$.[17] At this stage, PSA should be extracted into the top phase of ABS or the liquid phase of ABEC-based SPE or PP for direct analysis. Simultaneously, IgG and HSA should be depleted in the interphase of ABS or the solid phase/precipitate of ABEC-based SPE or PP, enabling a single-step design. This strategy should render serum samples more suitable for reliable analytical detection and quantification, as matrix effects from IgG and HSA are significantly reduced.

Although PP using 20 wt% PEG 2000 achieves complete PSA extraction in the liquid phase while depleting 80% of IgG, it only removes 5% of HSA. Previous reports indicate that protein solubility is higher at 20 wt% PEG and that smaller proteins such as PSA (34 kDa) are more difficult to precipitate.[13,19] While the behavior of all three proteins in the presence of 20 wt% PEG 2000 is explained, this evidence implies a different mechanism at higher PEG concentrations. At 30 wt% PEG 2000, neither IgG nor HSA depletion occurs to a significant extent (*cf.* Fig. 5), while high PSA precipitation takes place. Since PSA undergoes selective precipitation, an extra recovery step should be incorporated into the analytical workflow for further analysis, consequently increasing technical complexity.

A threshold exists at 20 wt% PEG 2000 for both PSA extraction and IgG/HSA depletion using PP, likely due to concentration- and protein-sensitive interactions between PEG, water and high-abundance serum proteins. Regardless of the serum pretreatment configuration, higher concentrations of both $C_6H_5K_3O_7$/$C_6H_8O_7$ and PEG 2000 generally lead to notable PSA loss. This can be due to co-precipitation/co-adsorption events, driven by PSA reduced solubility and affinity for the liquid phase.[14] Overall, these conditions are not suitable for simultaneously depleting IgG and HSA while recovering PSA in the appropriate liquid phase for further analysis.

Fig. 6 also demonstrates the ability of ABS to be tailored for biomarker extraction while maintaining efficient depletion performance. A delicate balance between PEG 2000 and $C_6H_5K_3O_7$/$C_6H_8O_7$ compositions is necessary to control co-precipitation events, primarily through attenuation of salting-out effects. ABS composed of 30 wt% PEG 2000 + 10 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$ grants the greatest potential, achieving 100% extraction of PSA in the top phase, while reducing 85% of IgG and 40% of HSA from human serum at the interphase. When adapting to SPE with 30 wt% ABEC-2000 + 30 wt% $C_6H_5K_3O_7$/$C_6H_8O_7$, 91% of PSA is co-depleted with IgG and HSA in the solid phase.

Performance comparison of ABS and SPE configurations reveals a trade-off between high-abundance protein depletion and PSA extraction (*cf.* Fig. 5 and 6). Both systems achieve comparable IgG and HSA removal and outperform conventional PP. However, in the SPE configuration, PSA is unavoidably co-depleted, as salt concentrations cannot be further reduced without substantially impairing protein removal (Fig. 4). In contrast, ABS compositions can be fine-tuned to preserve PSA with only a minor compromise in depletion efficiency, which is critical for bioanalytical applications. While ABS therefore provide superior selectivity for PSA extraction, the adaptation of the ABS concept to the SPE format using ABEC resin may still be attractive from a practical perspective. In particular, it may facilitate handling and integration into established automated or miniaturized SPE workflows, while also offering easier implementation of material reuse.

Overall, this work expands the application of ABS in bioanalysis, transitioning from traditional liquid-phase to innovative SPE formats. ABS offer flexible configurations for bioanalytical sample pretreatment, in which PEG or PEG-functionalized materials and salt are synergistically combined to enhance performance compared to conventional methods such as PP.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data supporting this article has been included as part of the supplementary information (SI). Supplementary information: detailed materials and methods for IgG and HSA depletion and PSA extraction, along with supporting tables summarizing compositions, depletion efficiencies and PSA recovery yields for the studied systems. See DOI: https://doi.org/10.1039/d5ay02123e.

Acknowledgements

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

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