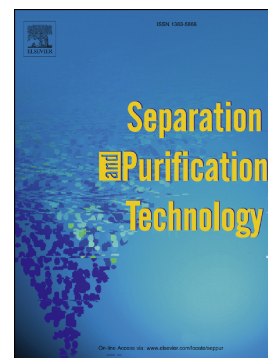


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PEG-BSA aqueous biphasic systems for selective interleukin extraction: introducing the Matrix-Mimetic Phase Strategy

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Abstract

Even though interleukins (ILs) are promising clinical biomarkers, effective pretreatment strategies for their extraction from human samples are yet to be established. Here, we introduce the “Matrix-Mimetic Phase Strategy”, in which aqueous biphasic systems (ABS) composed of polyethylene glycol (PEG) and bovine serum albumin (BSA) exploit BSA as a functional surrogate of the native serum and plasma matrices. This approach confines high-abundance proteins in a BSA-rich bottom phase, while selectively extracting target ILs (i.e., IL-1 β and IL-10) in the PEG-rich top phase. Phase diagrams were first established for systems containing PEG of varying molecular weights, from 5000 to 35000 g·mol⁻¹, confirming the strong influence of polymer size on biphasic formation and guiding the selection of suitable mixtures. IL partition in model systems showed a preferential migration to the PEG-rich top phase, with PEG 5000 achieving the highest recovery yields (93% for IL-1 β and 98% for IL-10). By adjusting PEG molecular weight, the strategy ensures robust and reproducible IL extraction across diverse human samples, yielding up to 91% of IL-1 β and 87% of IL-10 in serum, and 91% and 72% in plasma. Overall, the Matrix-Mimetic Phase Strategy provides an efficient and straightforward pretreatment method that may enhance analytical performance, paving the way for broader biomarker analysis in clinical workflows.

Keywords

Aqueous biphasic systems; Polyethylene glycol; Bovine serum albumin; Interleukins; Biomarker extraction; Human biofluids

1. Introduction

The selective extraction and enrichment of cytokines from human samples is a critical step in biomarker analysis, with implications for both research and clinical diagnostics [1]. Interleukins (ILs), such as IL-1 β and IL-10, are a group of cytokines, which are small, secreted signaling proteins that play essential roles in regulating immune responses, inflammation, and cell communication [1]. Due to their involvement in numerous

pathological processes, including cancer, autoimmune disorders, and infectious diseases, ILs are potential biomarkers for diagnosis, prognosis, and monitoring of disease progression [1]. Cytokines play central and complementary roles in immune regulation. IL-1 β is a key proinflammatory mediator involved in fever, tissue injury, and infection responses, whereas IL-10 functions as a potent anti-inflammatory cytokine that suppresses excessive immune activation and promotes tissue repair [2,3]. Dysregulation of either cytokine, whether through overexpression as observed in autoimmune and inflammatory disorders or suppression as seen in chronic diseases and certain metabolic conditions, can serve as a sensitive indicator of immune imbalance and disease progression [4–8]. However, ILs in serum and plasma can be difficult to detect in some samples, as their concentrations can fall in the low picogram per milliliter range, near or below the sensitivity limits of conventional immunoassays [9]. Moreover, their measurement is affected by interactions with serum and plasma constituents, including other proteins, lipids, soluble receptors, and inhibitors. These components may bind ILs, block antibody access, or produce nonspecific signals, compromising analytical accuracy [9].

Before reaching the detection stage, serum and plasma require pretreatment to minimize matrix interference. Common approaches, such as solid-phase extraction (SPE) and protein precipitation, offer only partial solutions [10]. SPE provides high selectivity through affinity interactions but demands costly reagents and laborious workflows, whereas precipitation is simple and inexpensive yet poorly discriminates between molecules, often leading to critical analyte losses [10]. In this context, aqueous biphasic systems (ABS) or aqueous two-phase systems (ATPS) emerge as alternative approaches, bridging the gap between conventional techniques by combining tunable selectivity with straightforward operation [11]. Composed of two immiscible aqueous phases, typically formed by two polymers, two salts or a polymer and a salt, ABS create a gentle environment in which analytes partition according to their affinities [12]. By adjusting parameters such as pH, ionic strength, component concentration, or molecular weight, these systems can be tailored to favor the selective enrichment of clinically relevant targets without the need for immobilized ligands or harsh precipitation conditions [13]. Depending on their design, their high-water content, low toxicity, and potential for reuse align with the principles of green sample preparation, reinforcing their suitability for clinical analysis [14]. As a result, ABS have attracted increasing attention in clinical contexts, assisting the efficient analysis of tumor-associated biomarkers, including proteins and extracellular vesicles relevant to lung and prostate cancers [13].

In addition to sample enrichment, recent advances have highlighted the use of ABS as confinement strategies for high-sensitivity IL analysis through immunoassays, particularly when working with human plasma or cell culture samples [13]. Traditional polymer-polymer ABS, such as those based on polyethylene glycol (PEG) and dextran (DEX), have been widely studied; however, despite being safe for biological use, their high viscosity and slow phase separation compromise long-term cell confinement and downstream handling [15]. These limitations have motivated the search for alternative phase-forming components and ABS formulations, such as polymers paired with proteins [16]. Combinations of PEG and bovine serum albumin (BSA) were employed by Teixeira et al. [16] to confine suspension-cultured immune cells. PEG-BSA systems preserved cell viability, while enabling controlled IL secretion and improved confinement of IL compared to traditional PEG-DEX systems. Remarkably, the BSA-rich phase reduced nonspecific binding and enhanced immunoassay sensitivity, providing a simple,

equipment-free alternative for multiplexed cell-based screening and immunotherapy development [16].

In clinical chemistry, the term matrix refers to the complex composition of a biological sample, such as serum or plasma, that surrounds the analyte of interest and can significantly influence its detection and quantification [17]. Taking advantage of this concept, the present work introduces a “Matrix-Mimetic Phase Strategy” for the pretreatment of human biofluids and the extraction of IL-1 β and IL-10. Unlike conventional ABS, which rely on the thermodynamic incompatibility of the phase-forming components and their differential affinities toward target biomolecules, the proposed strategy deliberately incorporates one phase-forming component as a functional surrogate of the biological matrix. Although PEG-BSA ABS have previously been reported as biocompatible media for immune cell confinement and cytokine detection [16], the present work extends the application of these systems towards sample pretreatment by exploiting BSA as a surrogate of the albumin-rich protein environment of serum and plasma. This biomimetic design promotes the partition of high-abundance proteins into the BSA-rich bottom phase while selectively recovering IL-1 β and IL-10 in the PEG-rich top phase. To evaluate this strategy, the phase behavior of PEG-BSA systems was characterized, the influence of PEG molecular weight on IL partition and recovery was investigated, and the selected systems were subsequently applied to spiked human serum and plasma samples. To the best of our knowledge, this represents the first proof-of-concept demonstration of PEG-BSA ABS for the selective extraction and quantitative recovery of ILs from human biofluids.

2. Materials and Methods

2.1. Materials

PEGs of varying molecular weights, namely 5000, 8000, and 35000 g·mol⁻¹ (herein abbreviated as PEG 5000, PEG 8000, and PEG 35000, respectively), were used in this work, with PEG 5000 and PEG 35000 purchased from Sigma-Aldrich and PEG 8000 obtained from Alfa Aesar. BSA was acquired from VWR and stored at 4 °C. Two types of human serum, gender pooled (HUMANSRM 9982 D) and male pooled (HUMANSRM 9915 D), as well as human plasma K2EDTA gender pooled (HUMANSRM 9981 D), were acquired from BioIVT. The quantification of IL-1 β and IL-10 was performed using commercially available enzyme-linked immunosorbent assay (ELISA) kits, purchased from Sigma-Aldrich and stored at -20 °C until use. Each kit contained a pre-coated plate, capture and detection antibodies, buffers, diluents, substrates, and a lyophilized IL standard. Spiked serum samples were prepared using the IL standards provided with the respective ELISA kit, which had detection limits of 0.3 pg·mL⁻¹ for IL-1 β and 1 pg·mL⁻¹ for IL-10. The HPLC mobile-phase buffer was prepared using sodium dihydrogen phosphate monohydrate (NaH₂PO₄·H₂O, 99 wt% purity) and disodium hydrogen phosphate heptahydrate (Na₂HPO₄·7H₂O, 99 wt% purity), both purchased from PanReac, and extra-pure sodium chloride (NaCl), obtained from Fisher Scientific.

2.2. Determination of binodal curves for PEG-BSA ABS

Binodal curves for ABS composed of PEG 5000, PEG 8000, or PEG 35000 and BSA were established using the cloud point titration method at 25 $\pm$ 1 °C and atmospheric

pressure [18]. Aqueous BSA solutions (30 wt%) were prepared, along with PEG 5000 and PEG 8000 solutions at 30 wt%, and PEG 35000 solutions at 20 wt%. Under continuous stirring, the BSA solution was added dropwise to the PEG solution until turbidity indicated the biphasic region, followed by dropwise addition of water until the mixture returned to a clear monophasic state. The compositions of the ternary systems after each addition were determined gravimetrically, with an uncertainty of $\pm 10^{-4}$ g. The experimental binodal data were fitted using the three-parameter Merchuk equation [19] (Equation 1):

$$[PEG] = A \exp(B[BSA]^{0.5} - C[BSA]^3) \quad (1)$$

where [PEG] and [BSA] denote the mass percentage of PEG and BSA, respectively, while A , B , and C are the fitted parameters obtained through least-squares regression.

2.3. IL extraction with PEG-BSA ABS: model studies and Matrix-Mimetic Phase Strategy

ABS were prepared by combining PEG stock solutions in phosphate buffered saline (PBS, pH 7.4), namely 20 wt% PEG 35000, 30 wt% PEG 5000 and 8000, with 30 wt% BSA solutions in PBS, serum, or plasma. Three types of ABS were investigated: (i) PEG with BSA in PBS, (ii) PEG with BSA in serum, and (iii) PEG with BSA in plasma. IL-1 β and IL-10 were directly spiked into the stock solutions containing BSA at the desired concentration (20, 30 and 50 pg·mL⁻¹). From these stock solutions, ABS were prepared as 1 g mixtures containing ILs at concentrations of 10, 15 or 25 pg·mL⁻¹. Specifically, for PEG 35000, 0.35 g of the PEG stock solution was weighed together with 0.5 g of the BSA solution (in PBS, serum, or plasma) and 0.15 g of PBS. For PEG 5000 or 8000, 0.23 g of the PEG stock solution was combined with 0.5 g of the BSA solution (in PBS, serum, or plasma) and 0.27 g of PBS. Accordingly, the final compositions of the ABS were 7 wt% PEG, 15 wt% BSA, and 78 wt% PBS for systems without human samples, and 7 wt% PEG, 15 wt% BSA, 35 wt% serum or plasma, and 43 wt% PBS for systems containing human samples. Each mixture was stirred and centrifuged for 5 min at 3000 g and 25 °C. These conditions were adopted to minimize the potential for cytokine degradation or loss of immunoreactivity during phase formation. After two-phase formation, the two phases were carefully separated and collected for analysis. All experiments were performed using three independently prepared ABS mixtures. Results are expressed as mean $\pm$ standard deviation ($n = 3$), providing an indication of the reproducibility of the extraction procedure.

To evaluate the distribution of the main serum proteins between the phases, the PEG-rich top phase and the BSA-rich bottom phase obtained from the ABS containing human serum were analyzed by size-exclusion high-performance liquid chromatography (SE-HPLC). Analyses were performed using a Shodex KW-802.5 column equipped with a KW-G 6B guard column. The mobile phase consisted of 50 mM phosphate buffer containing 0.3 M NaCl and was delivered at a flow rate of 0.5 mL·min⁻¹. The column and autosampler temperatures were maintained at 25 °C and 10 °C, respectively. Samples were injected using a volume of 25 μ L, and protein elution was monitored at 280 nm. The chromatographic profiles of the PEG-rich top and BSA-rich bottom phases were compared to assess the retention of the main serum proteins in each phase.

ELISA was used to quantify the recovered ILs and assess the extraction performance of the PEG-BSA systems. According to the instructions of the manufacturer, standards were reconstituted with the supplied buffer to prepare stock solutions, which were subsequently diluted to generate the calibration standards over the ranges of 0.41-100 pg·mL⁻¹ for IL-1 β and 2.34-150 pg·mL⁻¹ for IL-10. Potential interference from the ABS components was consistently assessed through blank controls prepared under identical conditions but without IL spiking. A separate blank was analyzed for each PEG-rich top and BSA-rich bottom phase. Low residual absorbance values were obtained for all blank phases, and the corresponding blank absorbance was subtracted from the absorbance measured for each IL-containing sample before calculating the recovery yields.

The recovery yields of IL-1 β and IL-10 (RY_{IL} , %) in the PEG-rich top and BSA-rich bottom phases of the different ABS were calculated using Equation 2:

$$RY_{IL} \% = \frac{m_{IL}^{Top/Bottom}}{m_{IL}^{Initial}} \times 100 \quad (2)$$

where m_{IL}^{Top} and m_{IL}^{Bottom} correspond to the mass of each IL in the PEG-rich top phase and in the BSA-rich bottom phase, respectively, while $m_{IL}^{Initial}$ represents the total mass of each IL in the initial mixture.

The theoretical maximum enrichment factor (EF_{max}) was calculated as the ratio between the total ABS mass ($m_{ABS} = 1$ g) and the mass of the PEG-rich top phase (m_{Top}):

$$EF_{max} = \frac{m_{ABS}}{m_{Top}} \quad (3)$$

The experimental concentration factor (CF_{IL}) was calculated as:

$$CF_{IL} = \frac{C_{IL}^{Top}}{C_{IL}^{Initial}} \quad (4)$$

where C_{IL}^{Top} and $C_{IL}^{Initial}$ correspond to the IL concentration in the PEG-rich top phase and in the initial mixture, respectively.

Figure 1 provides an overview of the PEG-BSA ABS protocol, illustrating both the Matrix-Mimetic Phase Strategy applied to serum and plasma alongside the corresponding model studies without human matrix.

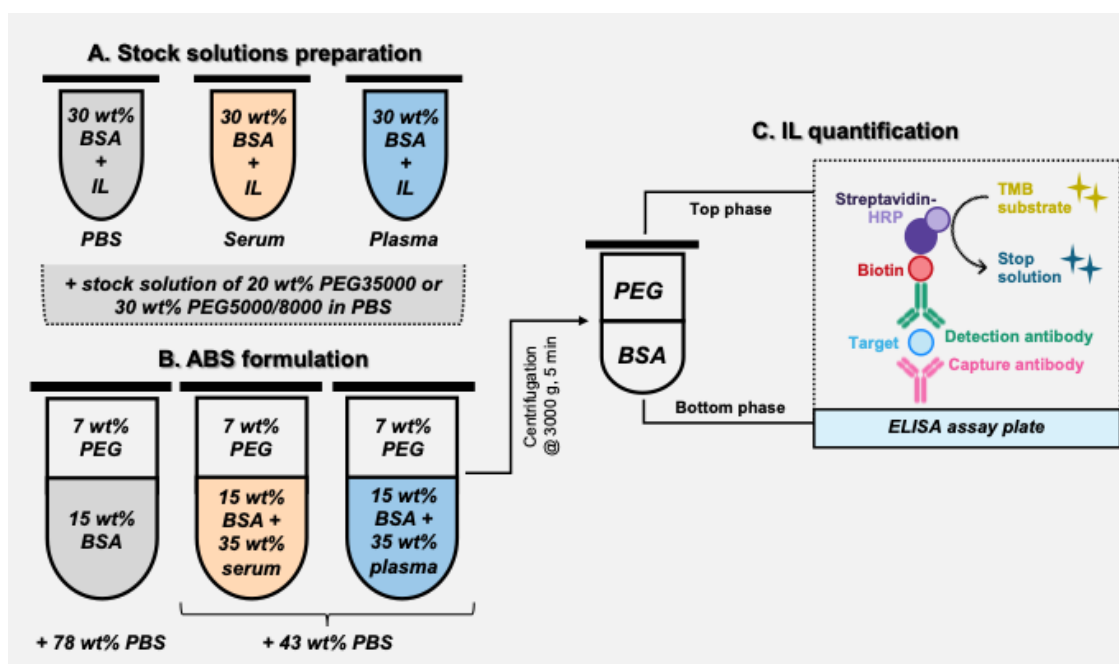


Figure 1. Matrix-Mimetic Phase Strategy for the selective extraction and quantification of ILs from human samples, showing both model PEG-BSA systems and their application to serum and plasma.

3. Results and Discussion

The Matrix-Mimetic Phase Strategy is based on the deliberate selection of at least one phase-forming component to mimic a key feature of the biological matrix, promoting the partition of matrix components and potential interferents into one phase while enriching the target analyte in a cleaner phase more amenable to downstream detection.

To design a selective and biocompatible medium for IL extraction, ABS formed by PEG and BSA were investigated as part of the Matrix-Mimetic Phase Strategy. The use of PEG-BSA ABS for IL extraction, while promising, remains largely underexplored compared to their application in cell confinement [16]. BSA was chosen as a functional surrogate of the dominant serum protein (human serum albumin, HSA), offering structural and functional similarity along with wider availability and lower cost [20]. Within the ABS, BSA forms the bottom phase, mimicking the physicochemical environment of the biological matrix and confining high-abundance proteins. PEG served as the complementary polymeric phase component, providing tunable molecular weight, protein-stabilizing properties and compatibility with downstream immunoassays [21]. PEG molecular weights of 5000, 8000, and 35000 were selected to investigate the influence of polymer chain length on IL partition. PEG 35000 was included based on the previously reported PEG-BSA ABS [16], enabling comparison with the literature, whereas PEG 5000 and PEG 8000 were selected as lower-molecular-weight alternatives expected to provide more hydrated PEG-rich phases, potentially favoring IL recovery.

In human serum and plasma, BSA supplementation triggers phase separation, generating a PEG-rich top phase and a BSA-rich bottom phase containing the bulk of the sample. This configuration allows selective extraction and quantitative recovery of ILs into the PEG-rich top phase, while reducing their contact with high-abundance proteins and providing a matrix environment more amenable to subsequent analysis.

The phase behavior of ABS follows a binodal curve that delineates the boundary between the monophasic region below and the biphasic region above, serving as a reference for selecting compositions suitable for IL extraction. To this end, initial studies focused on the determination of binodal curves for systems composed of PEG of varying molecular weights (PEG 5000, PEG 8000, and PEG 35000) and BSA. Binodal curves were determined at 25 °C and atmospheric pressure using the cloud point titration method [18]. The results for all PEG-BSA systems are presented in Figure 2A. Representative photographs of ABS prepared at three different mixture compositions are shown in Figure 2B, providing a visual demonstration of the phase separation behavior across the studied systems. The corresponding experimental binodal data are summarized in Table S1.

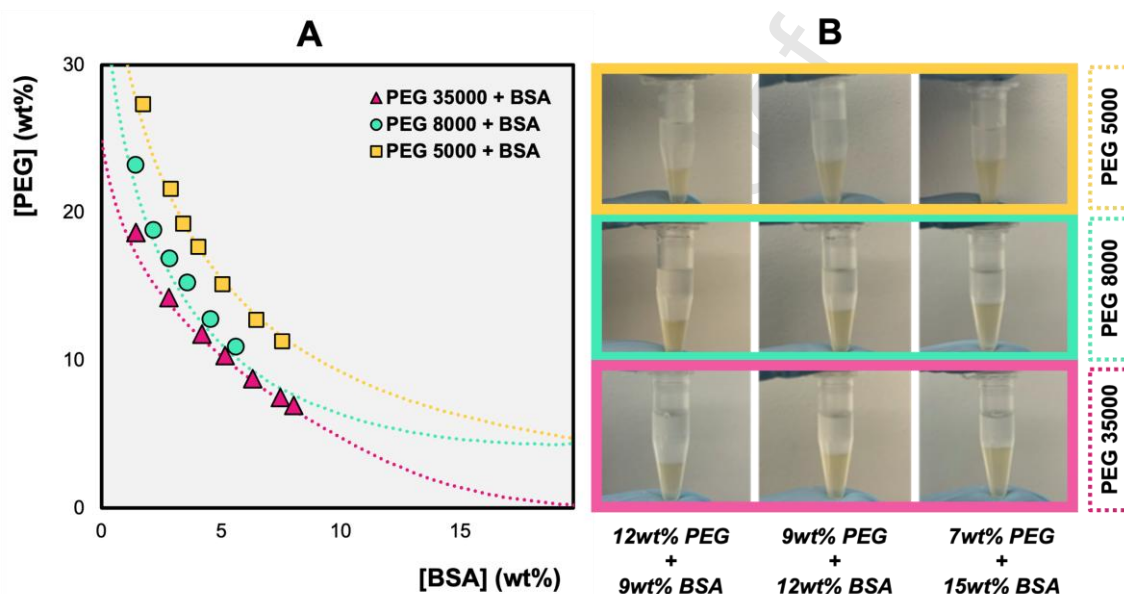


Figure 2. Phase behavior and visual appearance of PEG-BSA ABS. Binodal curves at 25 °C and atmospheric pressure, determined by the cloud point titration method [18], with dashed lines representing the fitting of the experimental data obtained using the equation proposed by Merchuk et al. [19] (A). Representative photographs of ABS, color-coded as follows: yellow - PEG 5000; green - PEG 8000; pink - PEG 35000 corresponding to three mixture compositions within the biphasic region (B).

The binodal curves revealed that using PEG of higher molecular weight lowers the concentration required for phase separation to occur, with the PEG 35000-BSA ABS separating at the lowest concentrations of the three systems. This behavior indicates enhanced polymer-protein incompatibility and more pronounced excluded volume effects associated with larger PEG chains [22,23]. In contrast, PEGs of lower molecular weight exhibit higher affinity for water molecules, thus requiring higher amounts of BSA to induce phase separation [22]. Figure 2B shows that as PEG concentration decreases and BSA concentration increases, the PEG-rich (top) phase becomes smaller while the BSA-rich (bottom) phase expands, reflecting the compositional shift along the binodal curve. This trend is relevant when foreseeing the enrichment of ILs in the PEG-rich phase.

Having characterized the phase separation of each ABS, biphasic mixtures were selected and used for partition experiments, in which IL-1 β and IL-10 were spiked into

the BSA-containing solution prior to ABS formation (final concentration in the ABS of 25 $\text{pg}\cdot\text{mL}^{-1}$). The extraction procedure was carried out under mild aqueous conditions, using PBS at pH 7.4 and a short centrifugation step of 5 min at 3000 g and 25 °C. No prolonged incubation or exposure to extreme pH, temperature, or organic solvents was required. The high recovery yields obtained by ELISA suggest that the epitopes recognized by the capture and detection antibodies remained accessible after phase separation, indicating that the immunoreactivity of IL-1 β and IL-10 was preserved throughout the extraction process.

Figure 3 shows the recovery yields of IL-1 β and IL-10 in both phases of the ABS, namely the PEG-rich top phase and the BSA-rich bottom phase. All data are provided in Table S2. Both IL-1 β and IL-10 exhibited a strong propensity to partition into the PEG-rich top phase. This behavior may result from the combined influence of phase composition, protein-protein interactions, polymer excluded-volume effects, and nonspecific interactions between the ILs and the phase-forming components [24–27]. The protein-rich environment of the BSA-rich bottom phase may limit the partition of the smaller ILs [24], while the PEG-rich top phase may provide a more favorable environment for their solubilization. The relative contribution of these factors to the observed IL partition, however, remains to be elucidated.

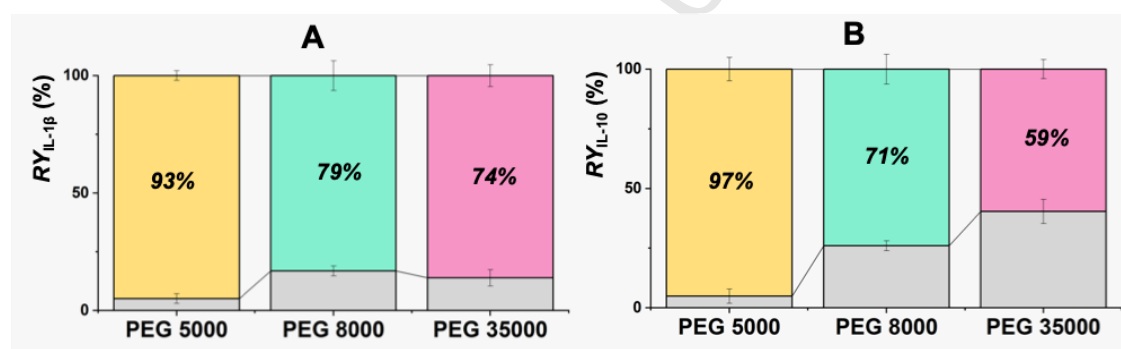


Figure 3. Recovery yields of IL-1 β (A) and IL-10 (B) in the top PEG-rich top phase (yellow: PEG 5000, green: PEG 8000, pink: PEG 35000) and BSA-rich bottom phase (grey bars) of ABS composed of 7 wt% of PEG, 15 wt% of BSA and 78 wt% of PBS. IL-1 β and IL-10 were spiked into the BSA/PBS stock solution, resulting in a final concentration of 25 $\text{pg}\cdot\text{mL}^{-1}$ in the ABS. Values are expressed as mean $\pm$ standard deviation of three independently prepared ABS mixtures ($n = 3$).

The PEG-BSA ABS exhibited distinct recovery efficiencies for the two target ILs, with performance being strongly influenced by the molecular weight of PEG. Both IL-1 β (Figure 3A) and IL-10 (Figure 3B) displayed the highest recovery yields in the PEG 5000-BSA ABS, followed by PEG 8000-BSA and PEG 35000-BSA ABS. The superior performance of the ABS composed of PEG 5000 may be attributed to its lower molecular weight, resulting in enhanced polymer-protein compatibility due to its higher water content [28–30]. This favorable environment facilitates solubilization and transfer of IL into the PEG-rich top phase. Conversely, PEG 35000, with its high excluded volume effect, promotes stronger segregation and lower IL recoveries [31]. Overall, increasing PEG molecular weight negatively affected IL recovery. Among the tested systems, PEG 5000 provided near-quantitative recovery of both ILs, reaching 93% for IL-1 β and 98% for IL-10. In addition to its superior recovery performance, the lower molecular weight of PEG 5000 provides a more hydrated and less viscous PEG-rich phase, facilitating phase

handling and sample collection during pretreatment. The present results demonstrate that PEG 5000 provides an effective formulation for implementing the Matrix-Mimetic Phase Strategy and was therefore selected for subsequent experiments.

The more pronounced decline in recovery yields in the PEG-rich top phase for IL-10 compared to IL-1 β with increasing PEG molecular weight suggests that IL-10 may be more sensitive to water content in the phases. While IL-1 β is active as a monomer (~17 kDa), IL-10 is a dimer of larger size (~36 kDa) [32,33]. PEG-BSA ABS were buffered at pH 7.4 with PBS, preserving protein structure and function [34]. Consequently, differences in electrostatic interactions could influence partition. However, the larger size and dimeric structure of IL-10, along with its reduced solubility in the phases composed of higher molecular weight PEG, likely dominate its lower partition into the PEG-rich top phase compared to IL-1 β . In addition, protein-protein interactions between the ILs and BSA may influence their partition, as differences in molecular structure and surface properties can affect their affinity for the PEG-rich top phase [26]. The observed partition behavior likely results from the combined effects of molecular crowding, polarity, and hydration. Although further studies are needed to clarify how these factors contribute to IL partition, the results highlight the importance of PEG molecular weight in controlling IL recovery. For the PEG molecular weights yielding the highest recoveries, IL losses remained below 15%, consistent with the commonly accepted accuracy criterion for bioanalytical methods [35,36].

After identifying PEG 5000-BSA as the most effective system, the effect of IL concentration on recovery yields was appraised (Figure 4, Table S3). The selected concentrations, ranging from 10 to 25 pg·mL⁻¹, were chosen to remain above the detection limits of the ELISA kits and to enable reliable recovery calculations. Varying IL concentrations revealed robust system performance across a range of analyte levels relevant to quantitative recovery. This evaluation is critical to guarantee consistent recovery in both lower- and higher-concentration scenarios, ensuring accurate and sensitive quantification in clinical samples. The PEG 5000-BSA system maintained high recovery yields across the tested concentration for both IL-1 β (Figure 4A) and IL-10 (Figure 4B), demonstrating its capacity to efficiently extract ILs at varying levels while preserving qualitative performance, within regulatory limits [35,36]. This represents an important advantage in complex clinical scenarios where patient IL concentrations can differ substantially.

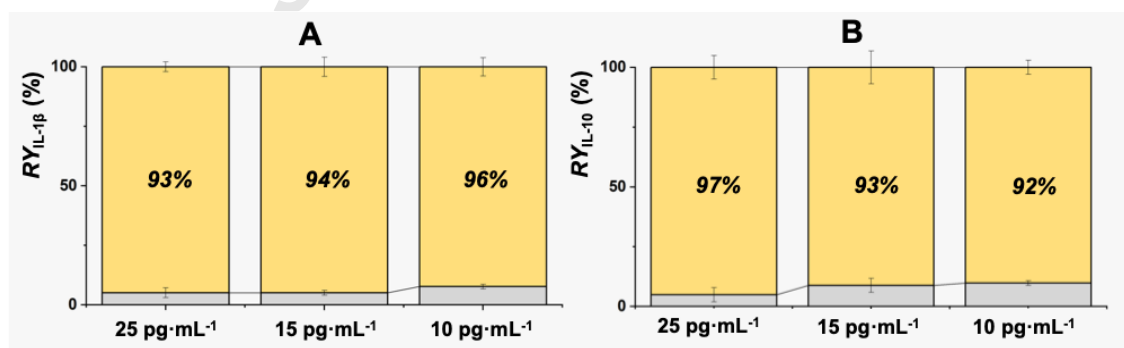


Figure 4. Recovery yields of IL-1 β (A) and IL-10 (B) in ABS composed of 7 wt% of PEG 5000, 15 wt% of BSA and 78 wt% of PBS. Different concentrations of IL-1 β and IL-10 were spiked into the BSA stock solution, resulting in final concentrations of 25, 15, and 10 pg·mL⁻¹ in the ABS. Values are expressed as mean $\pm$ standard deviation of three independently prepared ABS mixtures ($n = 3$).

Since ILs are typically present in complex matrices, the applicability of PEG-BSA ABS was further investigated using physiologically relevant fluids (human serum and plasma). As shown in Figure 5, the direct combination of PEG with such samples did not result in phase separation, regardless of the PEG molecular weight. Attempts to use the sample itself as a phase component did not result in phase separation, as its intrinsic protein composition does not provide the necessary conditions for PEG-based ABS formation. Beyond this, the complex composition of serum and plasma could also alter the phase equilibrium by disrupting intra- and intermolecular interactions among PEG, BSA and water that are essential for ABS formation [37].

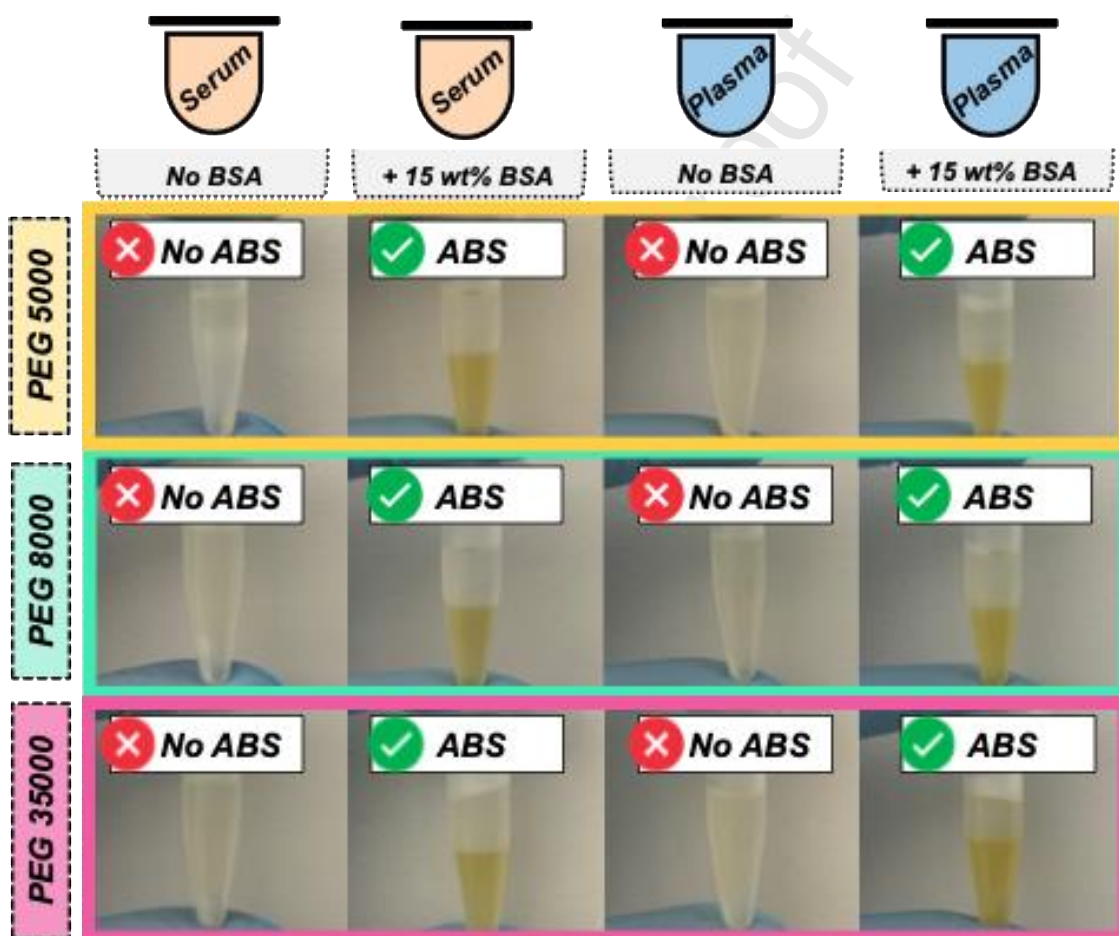


Figure 5. Phase separation behavior of PEG-BSA ABS in complex biological samples. Human serum and plasma were tested with PEG of different molecular weights (5000, 8000, and 35000), in both the absence and presence of BSA.

To ensure phase formation in human serum and plasma, samples were supplemented with BSA prior to PEG addition, guided by the binodal data in Figure 2A. ABS were successfully formed across all tested PEG molecular weights when 15 wt% BSA was added. This principle of using matrix-mimicking supplementation forms the basis of the Matrix-Mimetic Phase Strategy. The applicability of the Matrix-Mimetic Phase Strategy for IL extraction was evaluated by preparing PEG-BSA ABS in the presence of human serum. In this configuration, the ABS consisted of a PEG-rich top phase and a BSA-rich bottom phase, which contained the bulk of the serum proteins, as shown by

SE-HPLC analysis (Figure S1). The chromatograms showed that high-abundance serum proteins, such as HSA (~66 kDa), are predominantly retained in the hydrated, protein-dense BSA-rich bottom phase through macromolecular crowding and hydrophilic interactions. Only negligible protein signals were detected in the PEG-rich top phase, indicating effective separation from the lower molecular weight ILs [25,38,39]. Figure 6 summarizes the recovery yields of ILs across PEG-BSA systems using PEGs of different molecular weights, with detailed data provided in Table S4.

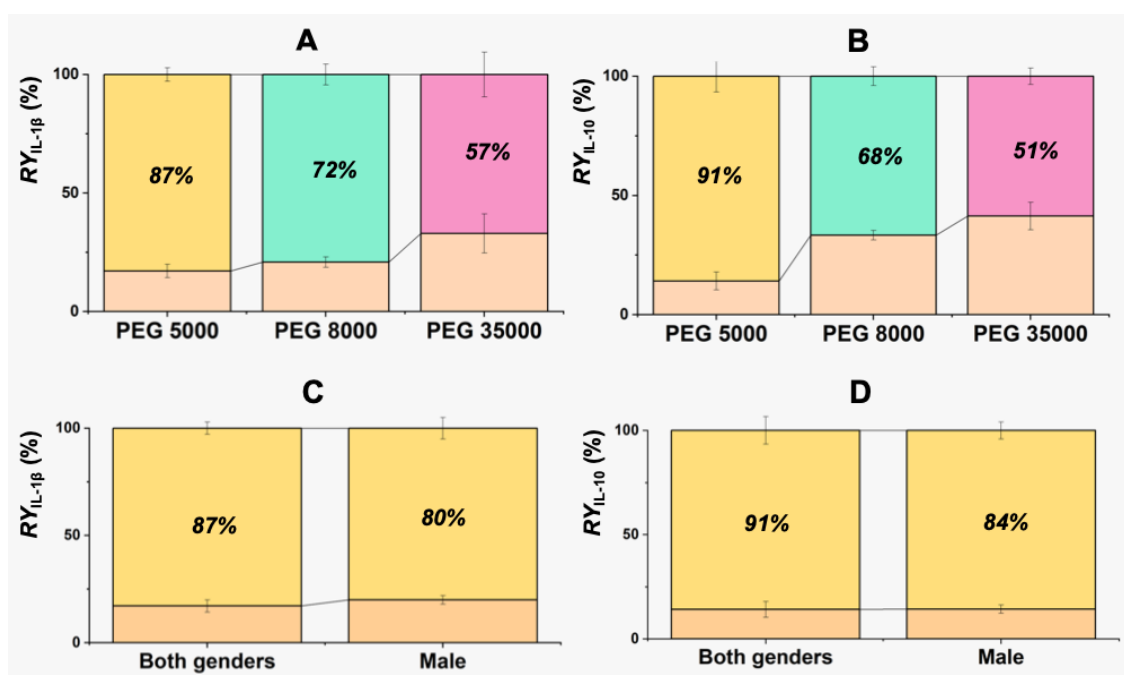


Figure 6. Recovery yields of IL-1 β (A) and IL-10 (B) in the top PEG-rich top phase (yellow: PEG 5000, green: PEG 8000, pink: PEG 35000) and BSA-rich bottom phase (orange bars) of ABS composed of 7 wt% of PEG, 15 wt% of BSA, 35 wt% of human serum pooled from both genders and 43 wt% of PBS. (C) and (D) compare the recovery yields of IL-1 β and IL-10, respectively, obtained for PEG 5000 systems using human serum pooled from both genders and from males only. IL-1 β and IL-10 were spiked into the BSA/serum stock solution, resulting in a final concentration of 25 pg·mL⁻¹ in the ABS. Values are expressed as mean $\pm$ standard deviation of three independently prepared ABS mixtures ($n = 3$).

For both IL-1 β (Figure 6A) and IL-10 (Figure 6B), the ABS composed of PEG 5000 provided the highest recovery yields, followed by PEG 8000 and PEG 35000, indicating a consistent trend of decreasing recovery yields with increasing PEG molecular weight. These results agree with those obtained using model PEG-BSA ABS, although the influence of the IL monomeric vs. dimeric nature was less pronounced (Figure 3). The higher recovery yields obtained with PEG 5000 can be attributed to its lower molecular weight and high-water content, which facilitates the transfer of ILs into the PEG-rich top phase. By contrast, PEG 35000, despite enhancing phase separation, likely generates an overly crowded polymer environment that restricts IL migration, leading to diminished recoveries, while PEG 8000 provides an intermediate balance [28–31].

The recovery yields obtained for human serum pooled from both genders were comparable to those observed for serum from males only (Figures 6C and 6D). This

suggests that gender-related differences in serum composition do not significantly influence IL extraction under the tested conditions provided that sample collection and storage are standardized. In general, IL recovery yields are similar both in the presence and absence of human serum, remaining within the accuracy limits established by regulatory guidelines ($\pm 15\%$) [35,36], and are minimally affected by complex protein and metabolite background.

Extending the study from serum, the Matrix-Mimetic Phase Strategy was applied to human plasma to validate its robustness and performance across different biological matrices. Figure 7 depicts the recovery of IL-10 and IL-1 β in the PEG-rich top and BSA-rich bottom phases; detailed data is given in Table S5. IL-1 β (Figure 7A) and IL-10 (Figure 7B) recovery yields followed the same PEG molecular weight-dependent trend as in model systems and human serum, with PEG 5000 consistently providing the best performance. In these systems, the ABS consisted of a PEG-rich top phase and a BSA-rich bottom phase encompassing the plasma matrix. The lower recoveries compared to serum are consistent with the higher protein and clotting factor content of plasma, including fibrinogen, globulins, and lipoproteins [40], which increase solution viscosity and may compete with ILs for interactions with the PEG-rich top phase. While some improvements could still be explored, the Matrix-Mimetic Phase Strategy achieved high recoveries, particularly for IL-10, meeting regulatory requirements and demonstrating ability to cope with the compositional complexity of plasma.

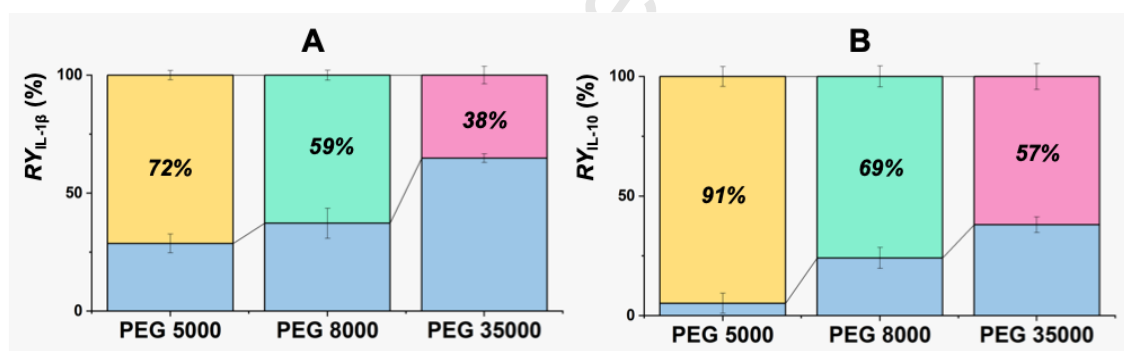


Figure 7. Recovery yields of IL-1 β (A) and IL-10 (B) in the top PEG-rich top phase (yellow: PEG 5000, green: PEG 8000, pink: PEG 35000) and BSA-rich bottom phase (blue bars) of ABS composed of 7 wt% of PEG, 15 wt% of BSA, 35 wt% of human plasma and 43 wt% of PBS. IL-1 β and IL-10 were spiked into the BSA/plasma stock solution, resulting in a final concentration of 25 pg·mL⁻¹ in the ABS. Values are expressed as mean $\pm$ standard deviation of three independently prepared ABS mixtures ($n = 3$).

The successful recovery of IL-1 β and IL-10 from serum and plasma demonstrates the applicability of the Matrix-Mimetic Phase Strategy to complex human biofluids. However, the use of pooled samples spiked with known IL concentrations provided a controlled setting for assessing extraction performance and may not fully reflect the behavior of endogenous ILs. Endogenous cytokines can interact with soluble receptors, carrier proteins, antibodies, and other components of the biological matrix, while their concentrations may vary considerably among individuals and approach or fall below the detection limits of conventional immunoassays. Further evaluation using individual clinical samples containing endogenous ILs will therefore be necessary to establish the applicability of the strategy to clinically relevant samples.

To provide a more comprehensive assessment of the proposed pretreatment strategy, the $EF_{\max}$ and CF_{IL} were also determined (Tables S2-S5). These parameters complement recovery yields by assessing the extent to which the extracted ILs are enriched in the PEG-rich top phase, an important consideration for low-abundance biomarker analysis. The $EF_{\max}$ values ranged from approximately 1.62 to 1.82, with slightly higher values generally observed for PEG 35000 because of the smaller PEG-rich top phase. However, the experimental CF_{IL} depended on both phase-volume reduction and IL recovery. Accordingly, PEG 5000 provided the highest CF_{IL} values, reaching 1.57 for IL-1 β and 1.65 for IL-10 in the model systems, while maintaining similar CF_{IL} across the screened IL concentrations. In human serum and plasma, CF_{IL} values remained above unity despite the lower recoveries, indicating that the proposed pretreatment strategy provides both IL recovery and moderate analyte enrichment. Overall, PEG 5000 provided the most favorable combination of IL recovery and enrichment, further supporting its suitability for the proposed Matrix-Mimetic Phase Strategy.

Compared to conventional cytokine pretreatment approaches, the PEG-BSA Matrix-Mimetic Phase Strategy combines selective IL recovery and moderate enrichment with a simple aqueous workflow involving only mixing, a 5 min centrifugation step, and phase collection, without organic solvents or immobilized affinity ligands. Protein precipitation is simple and inexpensive, but may involve salt co-precipitation, poor recovery at low protein concentrations, protein denaturation, and difficulties in pellet resuspension [41]. Solid-phase extraction and immunoaffinity-based approaches can provide greater selectivity but require specific sorbents or target-specific antibodies and involve additional loading, washing, and elution steps [42]. Membrane-based filtration offers rapid processing and low sample consumption, although analyte adsorption to the membrane can result in variable recoveries, as reported for IL-6, IL-8, IL-10, and TNF- α [43]. Together with the high IL recoveries achieved, these features make the Matrix-Mimetic Phase Strategy an attractive alternative for rapid and cost-effective pretreatment.

These considerations are particularly relevant in analytical settings involving less sensitive analytical methods or platforms that are more susceptible to matrix effects, where low cytokine abundance, complex biological matrices, or limited sample availability can compromise reliable detection. In this work, the applicability of the Matrix-Mimetic Phase Strategy was demonstrated across two physiologically relevant fluids, including serum samples from multiple sources and human plasma. The selective IL extraction shows potential to enhance their recovery to detectable concentrations, addressing one of the major challenges in cytokine quantification. In diseases where IL-1 β and IL-10 are elevated, conventional immunoassays like ELISA can typically detect these biomarkers; however, at the point of care, the limits of detection are often significantly higher, making preanalytical enrichment crucial [44]. Likewise, in clinical contexts where cytokine levels decrease, such as chronic diseases and certain metabolic conditions [6–8], the Matrix Mimetic Phase Strategy can recover ILs to measurable levels, facilitating the establishment of reliable physiological baselines and enabling detection of subnormal cytokine profiles. Beyond disease diagnostics, this strategy holds promise for monitoring therapeutic responses in patients receiving anti-IL biologics, where sensitive companion diagnostics are required to track residual cytokine levels [45,46]. It could also prove valuable in sports medicine, where small fluctuations around baseline cytokine concentrations are biologically meaningful [47], and in neonatal

testing, where scarce blood volumes require analyte concentration prior to analysis [48]. With appropriate design and validation, this approach could serve as a versatile platform for IL extraction with minimal losses, holding promise as a broadly applicable sample pretreatment method across diverse analytical and clinical settings.

The potential translation of these applications into routine analytical settings depends on the adaptability of the Matrix-Mimetic Phase Strategy to higher-throughput and automated workflows. It relies on simple operations, including reagent addition, mixing, centrifugation, phase separation, and collection of the PEG-rich top phase, which are compatible with equipment commonly available in clinical laboratories. Although the present work was performed using 1 g systems, the procedure could potentially be miniaturized to microtube- or microplate-based formats, reducing sample and reagent consumption while enabling parallel processing of multiple samples. The workflow may also be amenable to automation using liquid-handling platforms, particularly for reagent dispensing and collection of the PEG-rich top phase. However, automated implementation would require precise control of phase volumes and interface position to avoid cross-contamination between phases. The strategy could also be integrated with multiplex immunoassays, potentially enabling the extraction of multiple cytokines from the same sample, with analyte-specific partition and possible interference from residual phase-forming components requiring investigation for each assay panel. For liquid chromatography-mass spectrometry (LC-MS) applications, depletion of high-abundance proteins may also benefit downstream analysis, while compatibility with the PEG-BSA system and its impact on MS performance remain to be evaluated. Overall, further development should focus on miniaturization, automation, sample throughput, turnaround time, and compatibility with different downstream analytical platforms to assess the feasibility of routine clinical implementation.

4. Conclusions

This work demonstrates the proof-of-concept development of the Matrix-Mimetic Phase Strategy for the selective and quantitative extraction of ILs from diverse biological samples. The strategy explores ABS composed of PEG and BSA, where BSA functions as a biomimetic agent replicating the native protein composition of serum and plasma. This design enables the formation of a BSA-rich bottom phase that retains high-abundance proteins, while selectively extracting target ILs in the PEG-rich top phase, minimizing interference from the complex matrix.

Phase diagram analysis revealed that PEG molecular weight (PEG 5000, PEG 8000, and PEG 35000) affects biphasic formation, with higher molecular weight PEGs inducing phase separation at lower polymer concentrations. Partition studies in model systems identified PEG 5000-BSA ABS as the most efficient, achieving recovery yields of 93-98% for both L-1 β and IL-10. When applied to human serum and plasma, BSA supplementation proved essential to ensure reliable phase separation. PEG 5000-BSA ABS maintained high recovery yields (up to 91% for IL-1 β and 87% for IL-10), highlighting the robustness across multiple human samples and compliance with regulatory requirements.

Overall, the Matrix-Mimetic Phase Strategy provides a straightforward and effective pretreatment tool for the analysis of ILs in clinically relevant samples. Its reproducibility and compatibility with complex biological fluids, together with its suitability for combination with downstream immunoassays, supports its strong potential to

enhance analytical sensitivity and broaden applicability across clinical and biomedical domains. This strategy could also be extended to other low-abundance biomarkers and adapted for integration into automated or high-throughput diagnostic workflows, contributing to the development of sensitive and widely accessible clinical assays.

CRedit authorship contribution statement

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

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Declaration of competing interest

Maria S. M. Mendes, Mara G. Freire, Francisca A. e Silva, John P. Frampton have patent pending to Dalhousie University and University of Aveiro. If there are other authors, they 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

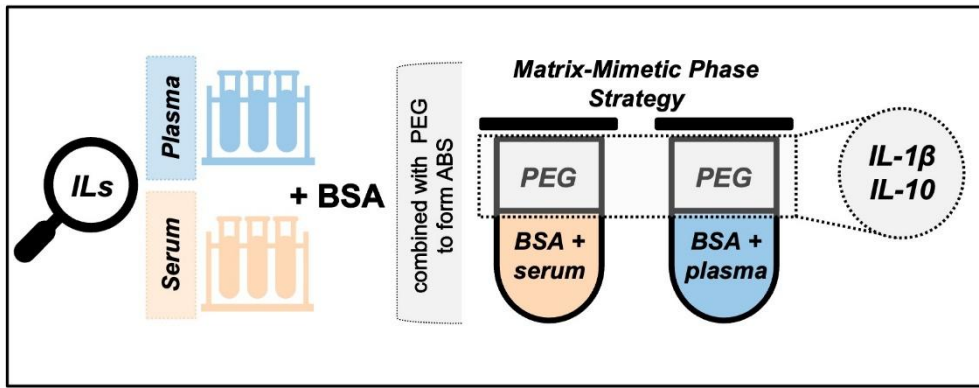
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Highlights

- The “Matrix-Mimetic Phase Strategy” exploits PEG-BSA ABS to extract interleukins.
- BSA acts as matrix surrogate and phase-forming agent, confining abundant proteins.
- PEG-BSA ABS enable selective separation of interleukins from protein-rich matrices.
- PEG molecular weight governs phase formation and interleukin extraction efficiency.
- Robust interleukin recoveries in serum and plasma support translational potential.

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:

Maria S. M. Mendes, Mara G. Freire, Francisca A. e Silva, John P. Frampton have patent pending to Dalhousie University and University of Aveiro. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.