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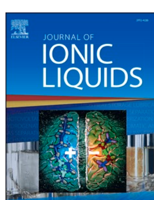

Unlocking chromatographic quantification of serum lactate dehydrogenase via ionic-liquid-based aqueous biphasic system pretreatment

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ABSTRACT

Lactate Dehydrogenase (LDH) is a key biomarker for various diseases, but its conventional analysis using colorimetric assays frequently faces reliability issues due to variations in enzyme activity. Additionally, its low serum concentration relative to high-abundance proteins hinders accurate quantification due to matrix effects. This work pioneers the integration of ionic-liquid-based aqueous biphasic systems (IL-ABS) as a sample pretreatment step for the chromatographic quantification of LDH in human serum. ABS composed of phosphonium-based ILs and citrate buffer ($K_3C_6H_5O_7/C_6H_8O_7$, pH 7) enable LDH quantification based on concentration rather than enzyme activity determined through colorimetric assays. Proper design, focusing on the IL anion and mixture composition, ensures that IL-ABS completely deplete high-abundance proteins, while extracting LDH from human serum with minimal loss in a single step. Under optimal conditions, LDH levels can be determined using size-exclusion high-performance liquid chromatography with diode array detection (SE-HPLC-DAD), with a relative error of 8%. Remarkably, IL-ABS overcome the interfering effects of high-abundance serum proteins, unlocking LDH analysis via chromatography and potentially improving accuracy and practicality over colorimetric assays. This analytical strategy could enhance the clinical significance of LDH as a biomarker, aiding in diagnosis, prognosis, and medical decisions.

1. Introduction

Lactate dehydrogenase (LDH) is a clinically significant biomarker for the diagnosis, prognosis and monitoring of a wide range of medical conditions (Huijgen et al., 1997; Wu et al., 2021). LDH mediates the glycolytic process by converting pyruvate to lactate. It is a widespread cellular enzyme, with its concentration increasing after tissue damage. The normal range of LDH serum levels can vary but is conventionally defined as 100–225 $U.L^{-1}$. High serum LDH levels, i.e., >225 $U.L^{-1}$, are detected in several clinical conditions, including hemolysis, severe infections, heart and liver diseases, infarcts, and cancer, among others (Farhana and Lappin, 2025; Panteghini, 2020). Aberrantly high expression of this enzyme (≥300 $U.L^{-1}$) has been observed in various cancers, including small cells tumours of bone, lymphoma and lung cancer, being usually associated with malignant progression and poor prognosis (Claps et al., 2022; Feng et al., 2018).

Several colorimetric methods based on the reversible lactate-to-pyruvate reaction have been successfully implemented for LDH quantification (Vanderlinde, 1985; Kiianitsa et al., 2003). However, the reading must occur within the first few seconds of the reaction after sample addition, as NADH is often fully consumed before the first reading (Sapan et al., 1999). Furthermore, interferences can significantly affect LDH activity measurement, particularly in complex biological samples like human serum, leading to imprecise results (de los Santos-Álvarez et al., 2002; Buonocore et al., 2016). To cope with matrix effects, LDH colorimetric assays commonly involve multiple steps, including sample pretreatment, followed by enzymatic reactions that can take up to one hour and require several reagents, which may affect enzyme activity (Bisswanger, 2014).

Rather than measuring enzymatic activity, quantifying serum LDH levels using chromatography provides a more reliable and streamlined strategy for bioanalytical applications. Nevertheless, the assessment of protein biomarkers like LDH is often limited by their relatively lower abundance in comparison with other serum proteins, mainly human serum albumin (HSA) and immunoglobulin G (IgG), as well as their levels falling below the detection limits of conventional analytical

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techniques (Lee et al., 2019; Sun et al., 2024). Therefore, serum samples must undergo appropriate processing for protein depletion, fractionation and enrichment to reduce sample complexity while ensuring compatibility with routine analytical instruments (Issaq and Veenstra, 2020; Li et al., 2021; Kim et al., 2015). To establish LDH as a reliable biomarker, the development of advanced sample pretreatment strategies is essential to expand the range of methods available for accurate and expedited analysis.

The use of ionic liquids (ILs) as components of aqueous biphasic systems (ABS, IL-ABS) has been recognized as a gentle and efficient strategy for the extraction and purification of proteins, including enzymes, due to their tunable properties (Lee et al., 2017). IL-ABS have been reported for the efficient extraction of albumin, hemoglobin, trypsin, papain, lysozyme, rubisco, among other proteins, while keeping their stability, activity and structural integrity (Li et al., 2012; Carvalho et al., 2024; Desai et al., 2014; Lin et al., 2013). It has also been demonstrated that, depending on the ABS composition, IL-ABS can either preserve proteins in one of the liquid phases or induce their interfacial precipitation (Pereira et al., 2015). This preliminary evidence unveiled the potential of IL-ABS to be tailored for the selective precipitation and extraction of target proteins from complex samples, such as saliva and urine (Pereira et al., 2020; González-Martín et al., 2023).

Considering the advantages of IL-ABS for protein separation, this work proposes a new analytical method designed to overcome the existing limitations in serum LDH analysis by colorimetric enzymatic assays. ABS composed of phosphonium-based ILs and citrate buffer ($K_3C_6H_5O_7/C_6H_8O_7$, pH 7) were investigated and designed for sample pretreatment through the simultaneous depletion of high-abundance serum proteins, namely human serum albumin (HSA) and immunoglobulin G (IgG), and extraction of the target biomarker LDH into the IL-rich phase. After pretreating human serum samples with the most effective IL-ABS, direct LDH analysis became possible using size-exclusion high-performance liquid chromatography coupled with a diode array detector (SE-HPLC-DAD), thus avoiding the need for more labile colorimetric enzyme activity assays currently employed.

2. Experimental

2.1. ABS components

Potassium citrate buffer ($C_6H_5K_3O_7/C_6H_8O_7$) at pH 7 was prepared using potassium citrate monohydrate, $C_6H_5K_3O_7•H_2O$ (>99 wt% pure), from Sigma-Aldrich, and citric acid, $C_6H_8O_7$ (100 wt% pure), from Fisher Scientific, in a ~25:1 weight-to-weight ratio (w/w), calculated based on anhydrous components. The phosphonium-based ILs tetrabutylphosphonium bromide, $[P_{4444}]Br$ (>96 wt% pure), and tetrabutylphosphonium chloride, $[P_{4444}]Cl$ (>96 wt% pure), were both supplied by Cytec Ind.

2.2. Biological samples and protein standards

Sterile-filtered human serum samples from male individuals with AB-type plasma were commercially supplied by Sigma-Aldrich in pooled form, providing a representative matrix that reflects average serum composition and captures typical biological variability. High-abundance serum proteins standards, namely pure IgG (≥95 wt% pure) and HSA (96 wt% pure) were obtained from Sigma-Aldrich and Alfa Aesar, respectively.

For methodological development purposes, bovine LDH was used as a calibration standard and is not intended as a clinical surrogate for human serum LDH. LDH from bovine heart was acquired at Sigma-Aldrich as an aqueous solution at 1000 $U.L^{-1}$. To enable the assessment of LDH concentration using SE-HPLC-DAD, the total protein concentration was first measured using the BCA assay, following the manufacturer's instructions (BCA Protein Assay Kit – Thermo Scientific™ Pierce™). A calibration curve was initially developed for bovine serum albumin, which served as the reference standard for total protein quantification. The LDH solution was then diluted to different concentrations in phosphate-buffered saline (PBS, 100 wt% pure, Sigma-Aldrich), and the total protein content ($\mu g.mL^{-1}$) was measured to construct the SE-HPLC-DAD calibration curve. LDH absolute mass concentrations were estimated experimentally and used only for internal calibration purposes, not for clinical interpretation or diagnosis. As the main goal of this work was to evaluate the performance of the IL-ABS pretreatment, results are expressed in terms of extraction efficiencies, recovery yields, and relative errors, as described below.

LDH levels in serum typically range from 100 to 225 $U.L^{-1}$, while levels exceeding 300 $U.L^{-1}$ are associated with cancer and poor prognosis (Hermes et al., 2010). Based on these values, IL-ABS were optimized to enable quantification of LDH at the lower end of the serum range (100 $U.L^{-1}$), representing the most challenging scenario for reliable detection. To adapt the concept of IL-ABS for serum pretreatment development, three distinct types of samples were introduced into the systems: (i) serum samples 10-fold diluted to assess the ability to precipitate/deplete high-abundance serum proteins at the interphase; (ii) LDH aqueous solution to investigate LDH extraction and recovery; and (iii) serum samples spiked with LDH.

2.3. Human serum pretreatment with IL-ABS

Mixture compositions prepared and tested were selected based on the ABS ternary phase diagrams determined in a previous work (Pereira et al., 2015), each with a final weight of 1 g: (i) 30 wt% IL + 20, 40 and 60 wt% $C_6H_5K_3O_7/C_6H_8O_7$ buffer solution, plus the respective wt% of an aqueous solution containing LDH or a serum sample diluted 10-fold to achieve a final 100 wt% composition, in order to address the influence of the salt composition; and (ii) 15, 30 and 45 wt% IL + 40 wt% $C_6H_5K_3O_7/C_6H_8O_7$ buffer solution, plus the respective wt% of an aqueous solution containing LDH or a serum sample diluted 10-fold to achieve a final 100 wt% composition, in order to appraise the impact of IL composition.

After weighing the ABS components, each mixture was vigorously stirred and then centrifuged for 10 min at 3500 rpm and at 25 (±1)°C to ensure the complete depletion of high-abundance proteins at the interphase or the full partition of LDH between the coexisting phases. In all systems the top phase corresponds to the IL-rich phase, whereas the bottom phase is mainly composed of $C_6H_5K_3O_7/C_6H_8O_7$ and water. In cases where protein precipitation occurs, the interphase predominantly consists of high-abundance serum proteins, specifically HSA and IgG. All experiments were performed in duplicate, and the reported values correspond to mean values.

The concentration of LDH and high-abundance serum proteins in each phase was quantified using a Chromaster HPLC (VWR, Hitachi) comprising a size-exclusion analytical column (Shodex Protein KW-802.5, 8 mm × 300 mm), coupled to a diode array detector (DAD). A 100 mM phosphate buffer solution at pH 7 prepared using sodium phosphate monobasic (NaH_2PO_4, 99–100.5 wt% pure and sodium phosphate dibasic heptahydrate ($Na_2HPO_4•7H_2O$, 98.2–102.0 wt% pure), also containing 0.3 M of sodium chloride (NaCl, 99.9 wt% pure), was used as the mobile phase. The chromatographic system operated in isocratic mode at a flow rate of 0.5 $mL.min^{-1}$, with the column and autosampler maintained at 25 °C. A 25 μL aliquot of each phase, previously diluted at a 1:10 volume-to-volume ratio (v/v) in phosphate buffer, was injected and analyzed at 280 nm. The retention times of IgG, LDH and HSA were about 15.7, 16.2 and 17.1 min, within a total analysis time of 40 min. Interferences caused by $C_6H_5K_3O_7/C_6H_8O_7$ and the IL within the SE-HPLC-DAD method were regularly monitored using control samples. The quantification of LDH, HSA and IgG in each phase was performed using an external standard calibration method. The concentration range for each calibration curve was as follows: 5–500 $U.L^{-1}$ (1.8–262.9 $\mu g.mL^{-1}$ as determined by the BCA assay) for LDH; 0.5–5 $g \cdot L^{-1}$ for HSA; and 0.1–1 $g \cdot L^{-1}$ for IgG. To evaluate the efficiency of the sample pretreatment

procedure, the following performance parameters were determined: the extraction efficiency of LDH (EE_{LDH}%), the depletion efficiency of high-abundance serum proteins (DE_{Prot}%), the recovery yield of LDH (RY_{LDH}%) and the relative error (*RE*%).

EE_{LDH}% represents the percentage ratio between the weight of LDH in the IL-rich aqueous phase to the total weight of LDH quantified in the mixture, as defined by Eq. (1):

$$EE_{LDH}\% = \frac{[LDH]_{IL} \times w_{IL}}{[LDH]_{IL} \times w_{IL} + [LDH]_{Salt} \times w_{Salt}} \times 100 \quad (1)$$

where $[LDH]_{IL}$ and $[LDH]_{salt}$ is the LDH concentration determined in the IL-rich and the salt-rich phase, while w_{IL} *and* w_{salt} are the total weight of the IL-rich and the salt-rich phase.

DE_{Prot}% is the percentage ratio of the weight of depleted high-abundance proteins to the initial weight of these proteins in serum, according to Eq. (2):

$$DE_{Prot}\% = \frac{w_{Prot}^{Int}}{w_{Prot}^{Serum}} \times 100 \quad (2)$$

where w_{Prot}^{Int} and w_{Prot}^{Serum} correspond to the weight of high-abundance proteins present in the interphase and serum sample, respectively. The amount of high-abundance serum proteins in the interphase was determined by mass balance from the initial protein load and the quantities measured in the IL-rich and salt-rich phases. When no protein was detected in either phase, this calculation yields depletion efficiencies of 100%, supporting complete depletion within the analytical limits of the method.

RY_{LDH}% is the percentage ratio of the mass of LDH in the IL-rich phase to the mass of LDH in the initial aqueous solution, as described in Eq. (3):

$$RY_{LDH}\% = \frac{w_{LDH}^{IL}}{w_{LDH}^{Aqueous\ solution}} \times 100 \quad (3)$$

where $w_{LDH}^{Aqueous\ solution}$ and w_{LDH}^{IL} are the total weight of LDH in the LDH aqueous solution and in the IL-rich phase, respectively.

RE% provides the accuracy of LDH concentration measurement after pretreatment as compared to the LDH concentration initially present in the aqueous solution, according to Eq. (4):

$$RE\% = \left| \frac{w_{LDH}^{IL} - w_{LDH}^{Aqueous\ solution}}{w_{LDH}^{Aqueous\ solution}} \right| \times 100 \quad (4)$$

2.4. Analysis of depleted proteins

Human serum proteins precipitated (depleted) at the interphase of the IL-ABS were analysed by SDS-PAGE. Due to the high protein content of the sample, serum proteins were diluted 2:1 (v/v) in PBS buffer before SDS-PAGE application. The aqueous phase samples from each ABS, containing albumin, were diluted 2:1 (v/v) in a dissociation buffer composed of 2.5 mL of 0.5 M Tris-HCl (pH 6.8), 4.0 mL of 10% (w/v) SDS solution, 2.0 mL of glycerol, 2.0 mg of bromophenol blue and 310 mg of dithiothreitol (DTT). This mixture was heated at 95 °C for 5 min to denature the proteins by reducing disulfide linkages, overcoming some forms of the tertiary protein folding and breaking up the quaternary protein structure. Electrophoresis was performed on polyacrylamide gels (stacking: 4% and resolving: 20%) using a running buffer containing 250 mM Tris-HCl, 1.92 M glycine, and 1% SDS. Proteins were stained with Coomassie Brilliant Blue G-250 (0.1% w/v) in a solution of 50% (v/v) methanol, 7% (v/v) acetic acid and 42.9% (v/v) water. Gels were incubated in an orbital shaker at moderate speed for 2–3 h at room temperature, followed by destaining in a solution containing 7% (v/v) acetic acid, 20% (v/v) methanol and 73% (v/v) water, with moderate shaking for 3–4 h at room temperature. SDS-PAGE Molecular Weight Standards, specifically the full-range marker from VWR, were used as protein standards.

3. Results and discussion

3.1. IL-ABS for LDH extraction and protein depletion

ABS comprising two phosphonium-based ILs, both sharing the common $[P_{4444}]^+$ cation but varying in anion hydrogen-bond basicity (Br^- versus Cl^-) to adjust the polarity of the systems, were investigated for serum pretreatment. ILs were structurally designed based on the excellent ability of those containing the $[P_{4444}]^+$ cation for liquid-liquid demixing in the presence of aqueous buffered salts, thereby facilitating protein depletion in ABS (Pereira et al., 2015; Rosa et al., 2023). To perform an initial screening of IL structures, a biphasic mixture was prepared with the following composition: 60 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution + 30 wt% $[P_{4444}]Br$ or $[P_{4444}]Cl$ + 10 wt% aqueous solution containing LDH (100 $U.L^{-1}$, corresponding to an estimated 53.5 $\mu g.mL^{-1}$ by the BCA method) or a serum sample diluted 10-fold. The reported mass concentration of LDH is provided solely as an internal reference for methodological development and comparative purposes and does not correspond to a clinically established value. All details on the related data are provided in Supplementary Material (Table S1).

Fig. 1 shows the effect of the IL anion on LDH extraction and the simultaneous depletion of high-abundance serum proteins in a single-step procedure. For the two IL-ABS, LDH is fully extracted into the IL-rich phase. However, only the ABS composed of $[P_{4444}]Br$ was able to simultaneously precipitate high-abundance serum proteins at the interphase, with depletion efficiencies of 100%. These protein profiles were further supported by the SE-HPLC-DAD chromatograms of samples sourced from extraction systems carried out with LDH and human serum, performed separately (cf. Fig. 2). The chromatographic peak of LDH appears between 15 and 17 min for both IL-ABS, attesting for their ability to completely extract LDH into the IL-rich phase. No high-abundance proteins were detected in either the IL-rich or salt-rich phases of the $[P_{4444}]Br$-based ABS, suggesting complete precipitation/depletion. However, a peak between 17 and 18 min is observed in the IL-rich phase of the $[P_{4444}]Cl$-based ABS, corresponding to HSA.

When dealing with serum analysis, the removal of interfering proteins is an essential step in sample preparation (de los Santos-Álvarez et al., 2002; Lee et al., 2019; Issaq and Veenstra, 2020; Li et al., 2013). Protein precipitation followed by centrifugation is among the most used techniques (Blanchard, 1981). However, the selection of a suitable precipitating agent and the optimization of the precipitation process for

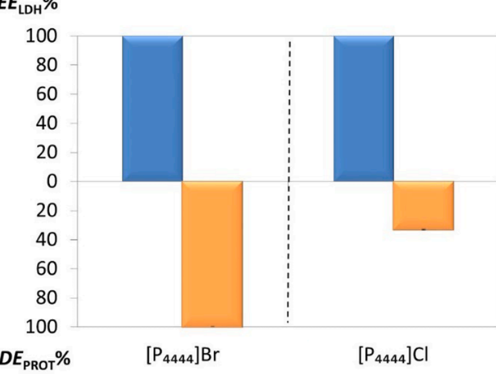

Fig. 1. Extraction efficiency of LDH (EE_{LDH}%, blue bars) and simultaneous depletion efficiency of high-abundance serum proteins (DE_{PROT}%, orange bars) of ABS composed of 30 wt% IL + 60 wt% $K_3C_6H_5O_7/C_6H_8O_7$ + 10 wt% aqueous solution containing either LDH (100 $U.L^{-1}$) or serum sample diluted 10-fold. Error bars represent the standard deviation of independent replicates. In cases of 100% extraction efficiency or complete depletion, error bars are not visible due to zero standard deviation.

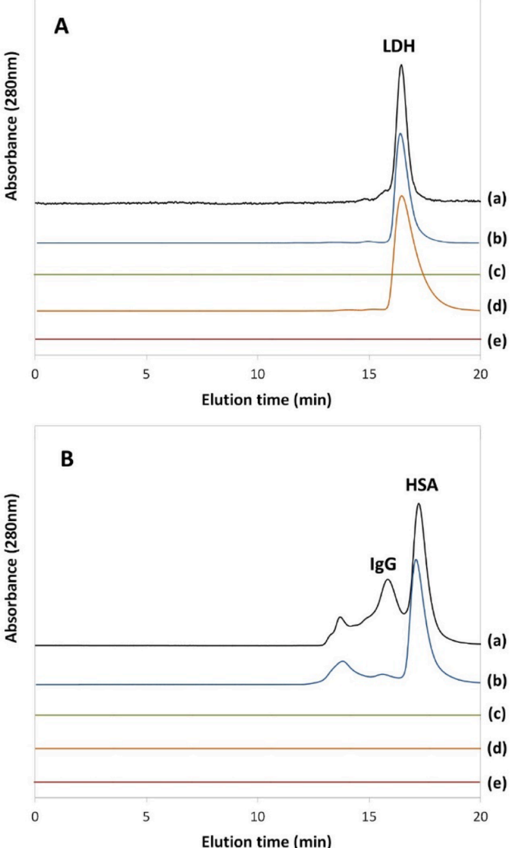

Fig. 2. SE-HPLC-DAD chromatograms of the extraction of LDH (A) and the simultaneous depletion of high-abundance serum proteins (B) of IL-ABS composed of 30 wt% IL + 60 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution + 10 wt % aqueous solution containing either LDH (100 U.L^{-1}) or serum diluted 10-fold: (a) standards; (b) [P_{4444}]Cl-rich phase; (c) [P_{4444}]Cl-respective salt-rich phase; (d) [P_{4444}]Br-rich phase; (e) [P_{4444}]Br-respective salt-rich phase.

the target protein typically require extensive development time (Martinez et al., 2019; Matulis, 2016). Some precipitation agents may cause protein denaturation or loss of bioactivity, while others can bind irreversibly to the protein or lack selectivity for the target species (Martinez et al., 2019; Matulis, 2016). Protein abundance in human serum varies drastically by a factor of 10^{11}, with 97–99% of the total protein mass attributed to the 20 most abundant species (Anderson and Anderson, 2002). Despite advances in proteomics, no single analytical method can provide full coverage of the serum proteome, with current detection technologies limited to 4-5 orders of magnitude (Paulus et al., 2009). On the other hand, the removal of cellular proteins and other extraneous materials is a fundamental step in laboratory-scale protein fractionation workflows (Issaq and Veenstra, 2020). By achieving 100% selective extraction of the LDH biomarker into the IL-rich phase while simultaneously depleting high-abundance proteins, the ABS composed of [P_{4444}]Br proved to be an effective single-step approach for serum pretreatment.

Hydrophobic interactions have been widely reported for IL-protein systems, with stability and bioactivity being strongly influenced by the IL (Schröder, 2017; Schindl et al., 2019). Particularly in ABS composed of ILs and salts, the selective interfacial precipitation of proteins is governed by the combined contribution of IL hydrogen-bond basicity and salt salting-out strength (Pereira et al., 2015; Rosa et al., 2023). Despite differences in phase composition (Pereira et al., 2015), the anion-dependent behavior observed between [P_{4444}]Br- and [P_{4444}]Cl-based ABS can be reasonably explained by differences in hydrogen-bonding ability. Compared to [P_{4444}]Cl, [P_{4444}]Br is expected to exhibit lower hydrogen-bond basicity (β), in agreement with the reported β values for analogous 1-butyl-3-methylimidazolium ([C_4mim]$^+$) ILs (0.87 for [C_4mim]Br and 0.95 for [C_4mim]Cl (Cláudio et al., 2014)). This trend is further reinforced by ABS formation behavior experimentally observed in our previous work (Pereira et al., 2015), which follows the expected anion-dependent hydrogen-bonding strength. The weaker hydrogen-bonding capacity of Br^- is consistent with a reduced stabilization of high-abundance serum proteins in the IL-rich phase, favoring their selective precipitation, as previously reported for IL-ABS (Pereira et al., 2015; Rosa et al., 2023). In addition to the IL hydrogen-bond basicity and salting-out effects, protein solubility is also influenced by their molecular properties and relative abundance. High-abundance serum proteins such as HSA and IgG are present at concentrations several orders of magnitude higher than LDH, promoting intermolecular interactions and aggregation. In contrast, LDH remains below the concentration threshold required for precipitation and is retained in the IL-rich phase.

Given its ability to efficiently extract LDH into the IL-rich phase while depleting high-abundance proteins from serum samples, the ABS composed of [P_{4444}]Br and $K_3C_6H_5O_7/C_6H_8O_7$ (pH 7) was selected for further optimization studies. Various concentrations of [P_{4444}]Br (from 15 to 45 wt%) and $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution (from 20 to 60 wt %) were investigated. All details on the related data are provided in the Supplementary Material (Table S1).

Fig. 3 shows the impact of ABS composition on the extraction of LDH and the depletion of high-abundance serum proteins. Independently of the salt or IL concentration, all compositions studied achieved the complete extraction of LDH to the IL-rich phase and the full depletion of high-abundance serum proteins at the interphase. LDH is preferentially extracted into the IL-rich phase, independent of the system composition, indicating a substantial influence of protein-IL interactions and salting-out effects. To minimize the consumption of samples and reagents for LDH quantification, process optimization was conducted with a target final weight down to 1 g. This is a must when processing samples derived from blood collection. However, due to reduced volume, ABS composed of 30 wt% [P_{4444}]Br + 20 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution or 15 wt% [P_{4444}]Br + 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution formed a relatively small IL-rich phase that could not be accurately separated from the interphase (depleted serum proteins). Under these conditions, no proteins were detected in the opposite phase, and it was assumed that IgG and HSA were fully precipitated. These compositions could only be feasible on a larger scale. For the 1 g scale, more suitable compositions are 30 wt% [P_{4444}]Br + 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution, 30 wt% [P_{4444}]Br + 60 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution and 45 wt% [P_{4444}]Br + 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution.

3.2. LDH quantitative recovery and detection after IL-ABS pretreatment

A critical factor in the development of efficient serum pretreatment methods is minimizing biomarker losses, which could result in false negatives and compromise analytical accuracy. Fig. 4 presents the recovery yields of LDH in [P_{4444}]Br-based ABS, along with the associated relative error of analysis. The system composed of 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution + 45 wt% [P_{4444}]Br is the one leading to the lowest LDH loss (and thus higher recovery yield, RY_{LDH} 92%), resulting in an expected relative error in quantification of about 8%. This value is comparable to the analytical variability typically reported for routine LDH assays, which commonly exhibit inter-assay and inter-laboratory variations within a similar range (Zhao et al., 2025; Chua et al., 2018; Herzum et al., 2003), and aligns with the accuracy generally accepted in routine clinical biochemical analyses (FDA, 2018). Moreover, this system is easy to handle due to the larger volume of the top LDH-enriched phase and the formation of a well-defined, solid protein-rich interphase.

The feasibility of the ABS serum pretreatment method for direct LDH analysis using SE-HPLC-DAD – based on enzyme concentration and not

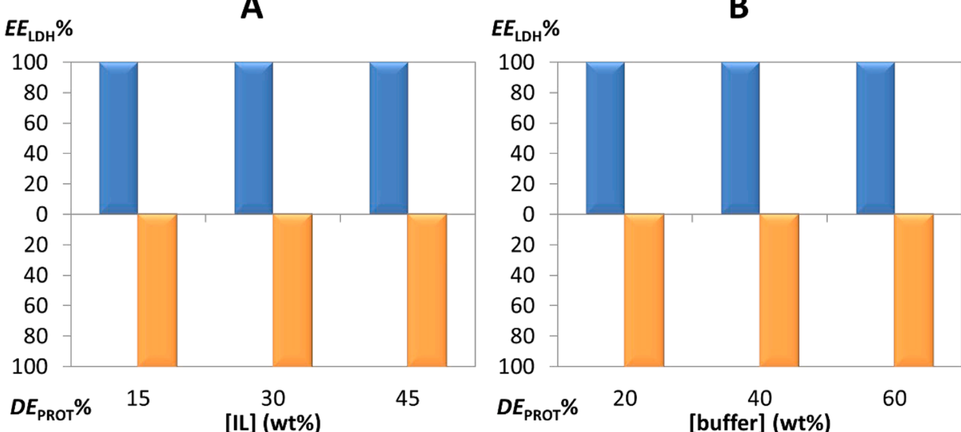

Fig. 3. Extraction efficiency of LDH (EE_{LDH}%, blue bars) and simultaneous depletion efficiency of high-abundance serum proteins (DE_{Prot}%, orange bars) of ABS composed of 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution + different concentrations of $[P_{4444}]Br$ (A) or 30 wt% $[P_{4444}]Br$ + different concentrations of $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution (B). An aqueous solution containing LDH (100 $U.L^{-1}$) or a serum sample diluted 10-fold was added in appropriate amounts to achieve a final composition of 100 wt%. Error bars represent the standard deviation of independent replicates. All values correspond to 100% extraction efficiency and complete depletion, resulting in zero standard deviation and therefore no visible error bars.

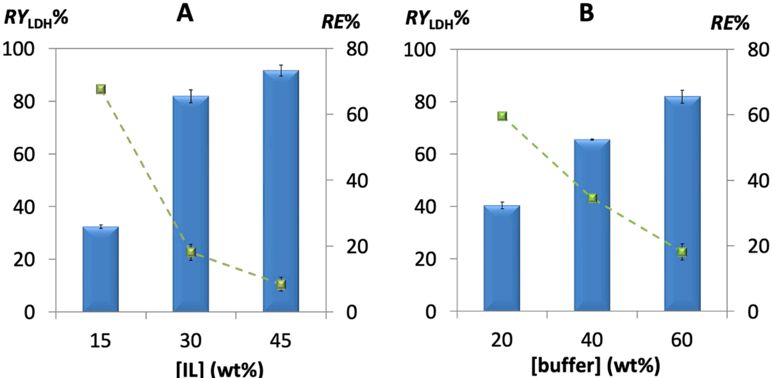

Fig. 4. Recovery yield of LDH (RY_{LDH}, blue bars) and relative error (RE%, green squares) in ABS composed of 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution + different concentrations of $[P_{4444}]Br$ (A) or 30 wt% $[P_{4444}]Br$ + different concentrations of $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution (B), with both sets comprising an aqueous solution containing LDH (100 $U.L^{-1}$) added in appropriate amounts to achieve a final composition of 100 wt%. Error bars represent the standard deviation of independent replicates.

just on its activity – was further confirmed by spiking commercial serum with LDH at 100 $U.L^{-1}$, representing the lower limit for clinical applicability (Hermes et al., 2010). SE-HPLC-DAD was selected as the size-exclusion mechanism separates proteins based on hydrodynamic size, while DAD detection at 280 nm allows sensitive monitoring of protein absorbance in an expedited way.

Fig. 5 displays the SE-HPLC-DAD chromatograms for non-pretreated serum, standard LDH, and the IL-rich and salt-rich phases of ABS, allowing direct assessment of method selectivity. In non-pretreated serum, LDH could not be detected due to interference from high-abundance proteins. After IL-ABS pretreatment, only LDH is observed in the IL-rich phase, with no detectable peaks corresponding to HSA, IgG, aggregates, or protein fragments, confirming that co-elution of residual serum components does not compromise LDH quantification. This comparison with the LDH standard demonstrates that SE-HPLC-DAD, together with prior IL-ABS pretreatment, provides specific and accurate detection of LDH in pretreated serum samples.

The LDH peak, appearing around 16.2 min, is observed both in the LDH standard aqueous solution and in the IL-rich phase after sample pretreatment. The retention of LDH solubility in the IL-rich phase and its chromatographic profile, consistent with the expected molecular weight, suggest that the enzyme is likely preserved under the applied IL-ABS conditions, which employ $[P_{4444}]Br$ in citrate buffer at physiological pH and room temperature. Evidence on ILs' ability to preserve protein and enzyme structure and activity shows that they can remain intact under mild conditions, depending on IL properties and operating parameters (Veríssimo et al., 2022). The potential of similar ILs for biocompatible sample pretreatment has also been demonstrated in our previous work with prostate-specific antigen, further highlighting their practical applicability in analytical workflows (Rosa et al., 2023).

Overall, chromatographic protein profiles indicate 100% LDH extraction efficiency and corroborate the results previously discussed. Neither IgG nor HSA were detected in the IL-rich phase chromatograms, confirming their successful removal. These results were further supported by SDS-PAGE analysis of the precipitated proteins. In addition to HSA and IgG, other serum proteins also precipitate at the interphase, as evidenced by SDS-PAGE gel spots from 150 to 17 kDa (cf. Fig. S1 in the Supplementary Material) (Pieper et al., 2003; Schneider and Pechtl, 2013). These findings confirm that $[P_{4444}]Br$-based ABS can efficiently deplete high-abundance serum proteins, enabling LDH analysis using SE-HPLC-DAD with no matrix interference.

3.3. *Analytical performance of the IL-ABS/SE-HPLC-DAD method*

To critically assess the analytical performance of the proposed IL-ABS/SE-HPLC-DAD method, the main features were compared with

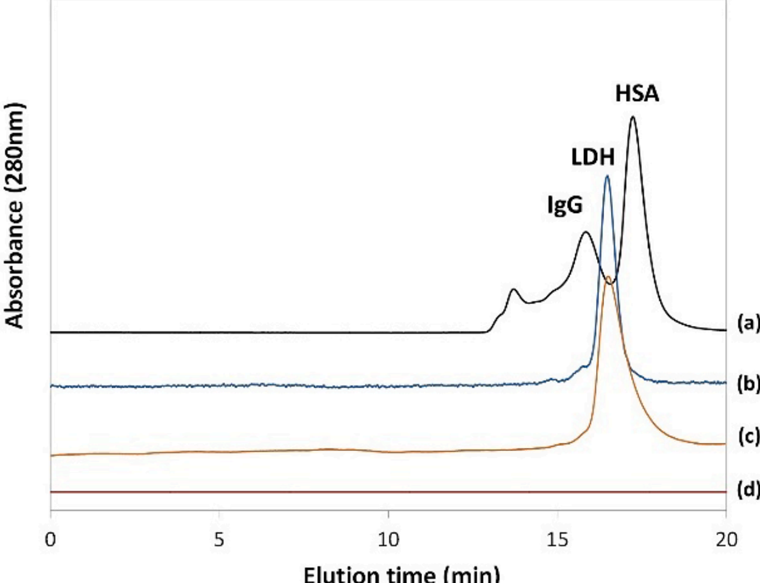

Fig. 5. SE-HPLC-DAD chromatograms of human serum without pretreatment (a), standard LDH (b), IL-rich phase (c), and salt-rich phase (d) after serum pretreatment with an ABS composed of 45 wt% $[P_{4444}]Br$ + 40 wt% $K_3C_6H_5O_7/C_6H_8O_7$ buffer solution + 15 wt% commercial serum spiked with LDH (100 $U.L^{-1}$).

established LDH quantification approaches, namely colorimetric enzymatic assays and immunoassays (ELISA), as summarized in Table 1 (Zhao et al., 2025; Kaja et al., 2017; MyBioSource 2026).

Colorimetric assays that measure enzymatic activity are generally fast per sample but can be influenced by enzyme variability, inhibitors, and assay conditions, affecting reliability and resulting in high susceptibility to matrix interference (Zhao et al., 2025; Kaja et al., 2017). In turn, immunoassays provide high specificity and sensitivity due to antibody-antigen recognition, with lower matrix interference (MyBioSource 2026). However, they require multi-step protocols, specialized reagents, longer assay times, and higher operational costs, which can limit throughput and flexibility in routine laboratory workflows. Remarkably, the IL-ABS/SE-HPLC-DAD workflow quantifies LDH based on protein concentration following size-based separation, offering a balance of accuracy (~8% relative error) and specificity after sample pretreatment. Although the throughput is moderate, including the IL-ABS step, the operational complexity is significantly lower than immunoassays, and the per-sample cost is moderate to low, relying only on standard HPLC instrumentation and minimal consumables. These features make the IL-ABS/SE-HPLC-DAD method a practical and cost-effective solution for routine analytical laboratories. It provides reliable LDH quantification in complex serum matrices while overcoming the main limitations of activity- and antibody-based assays.

Finally, it is important to consider challenges related to biological variability that can affect clinical applicability. Although serum composition varies between individuals and may be altered under pathological conditions, the IL-ABS pretreatment relies on well-established physicochemical mechanisms. Accordingly, salting-out effects and differential IL-protein interactions selectively remove high-abundance proteins while preserving LDH in solution. These mechanisms depend on general protein properties rather than disease-specific features, suggesting the method is likely effective across typical variations in serum composition and highlighting its potential applicability to clinically diverse sera.

4. Conclusions

This work proposes an alternative analytical approach for LDH quantification, focusing on concentration rather than enzymatic activity, as currently appraised by colorimetric assays. In this setup, ABS composed of phosphonium-based ILs and buffered aqueous solutions of $K_3C_6H_5O_7/C_6H_8O_7$ were used for human serum pretreatment prior to

Table 1
IL-ABS/SE-HPLC-DAD versus conventional LDH methods. Performance descriptors for colorimetric (Zhao et al., 2025, Kaja et al., 2017) and immunoassays (MyBioSource 2026) are based on literature reports and are provided for comparative purposes.

Parameter	Colorimetric assays	Immunoassays (ELISA)	IL-ABS/SE-HPLC-DAD (this work)
Analytical principle	Measurement of enzymatic activity	Antibody-antigen recognition	Protein concentration after size-based separation
Reliability / variability	~5–11% coefficient of variation; influenced by enzymatic factors	≤8% intra-assay precision; ≤12% inter-assay precision; influenced by assay conditions and antibody performance	~8% relative error
Limit of detection	~10–50 $U.L^{-1}$	$ng.mL^{-1}$ range	100 $U.L^{-1}$ (demonstrated after pretreatment)
Specificity / matrix interference	Moderate specificity; prone to matrix interference	High specificity; minimal matrix interference	High specificity after pretreatment; negligible matrix interference
Throughput	Moderate-high (minutes to ~1 h per sample)	Moderate-low (2–4 h per sample)	Moderate (~40 min per sample plus pretreatment)
Operational complexity	Moderate (multi-reagent, activity-dependent reaction)	High (multi-step protocol, antibody-based reagents)	Low (single pretreatment step followed by chromatographic run)
Cost	Moderate-low per analysis (kit-based reagents)	High per analysis (antibody-based kits and consumables)	Moderate-low per analysis (standard HPLC; low consumable cost)
Practical implementation	Routine clinical chemistry laboratories	Specialized immunoassay laboratories	Standard analytical laboratories

LDH analysis by SE-HPLC-DAD.

Simultaneous and complete depletion of high-abundance proteins and extraction of LDH from human serum into the IL-rich phase was achieved in a single step using $[P_{4444}]Br$, but not with $[P_{4444}]Cl$, highlighting the dependence on the IL anion and respective hydrogen-bond basicity for optimal depletion performance. Based on these results, the preferential precipitation of high-abundance serum proteins at the interphase appears to be governed by a combination of the IL-rich phase environment, which restricts protein solubility, and the salting-out effect of the salt buffer. Furthermore, carefully selecting the ABS composition is essential to minimize LDH losses and prevent inaccurate quantification that could trigger false negative results. After optimizing both salt and IL composition, the ABS composed of 40 wt% $K_3C_6H_5O_7$/$C_6H_8O_7$ buffer solution + 45 wt% $[P_{4444}]Br$ could be used to accurately quantify LDH in the IL-rich phase using SE-HPLC-DAD, with a low relative error. With this IL-ABS, the interfering effects of HSA and IgG were successfully overcome, unlocking LDH analysis by chromatography.

Overall, the use of IL-ABS for serum pretreatment prior to chromatographic detection represents a promising route to improve the accuracy and practicality of LDH quantification. The proposed analytical setup has the potential to enhance the clinical utility of LDH as a biomarker, improving risk profiling, diagnosis, prognosis, and informed decision-making in medical practice.

CRediT authorship contribution statement

Matheus M. Pereira: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sónia N. Pedro:** Investigation, Formal analysis, Data curation. **Francisca A. e Silva:** Writing – review & editing, Methodology, Funding acquisition. **João A.P. Coutinho:** Writing – review & editing, Supervision, Conceptualization. **Mara G. Freire:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jil.2026.100197.

Data availability

Data will be made available on request.

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