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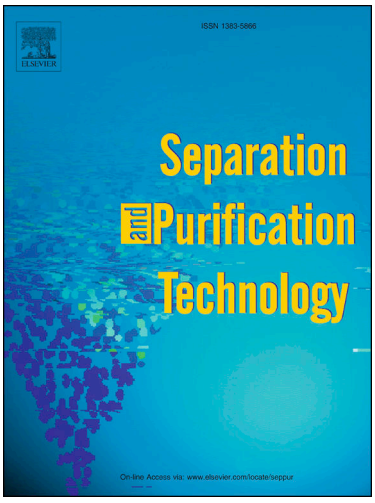

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Towards the improved monitoring of bacterial infections by the isolation of DNA from human serum using ionic-liquid-based aqueous biphasic systems

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Abstract

Early infection diagnosis is crucial to decrease morbidity and mortality rates. However, complex biological samples, like human blood or serum, contain high abundance proteins and metabolites that reduce the sensitivity of methods used to identify and quantify nucleic acids in bacterial infections diagnosis. To address this issue, we investigated aqueous biphasic systems (ABS) composed of polypropylene glycol 400 and cholinium-based ionic liquids (ILs) at different pH values for the pre-treatment of human serum, aiming the separation of DNA from human serum albumin (HSA) to reduce the interference on the DNA quantification by real-time PCR (qPCR). Remarkable extraction efficiencies of DNA to the IL-rich phase were obtained with all investigated systems, ranging between 90 and 100% in a single-step, with no significant losses of DNA observed (yield at the IL-rich phase > 90%). At low pH values HSA precipitates, whereas at neutral pH no HSA precipitation is observed. This trend suggests that IL-based ABS can be tuned to selectively isolate DNA from HSA by adjusting the pH. The most effective ABS identified is composed of cholinium glycolate at pH 5, allowing to completely precipitate HSA at the ABS interface and leading to an IL-rich phase enriched in DNA with high purity (> 98%) that can be quantified by qPCR. Finally, it is shown that the IL-rich phase is able to maintain the DNA's structural integrity at room temperature, for up to six months, implying that the IL-rich phase of the selected ABS could also be a suitable DNA storage medium. In summary, designed IL-based ABS can be applied as a pretreatment strategy of human serum, allowing to isolate bacterial DNA and opening new perspectives in the monitoring of bacterial infections.

Keywords: DNA, human serum albumin, purification, ionic liquids, two-phase systems, PCR.

1. Introduction

Infectious diseases are currently one of the leading causes of death in developing countries [1,2]. Such diseases are caused by pathogenic organisms, such as bacteria, viruses and parasites. The World Health Organization reported that infectious diseases contributed to one-quarter of all deaths worldwide in 2019 [1]. These numbers and the recent pandemic scenario show that infectious diseases continue to pose a significant health and economic burden to the world and society.

Most current molecular diagnostic techniques (gram stain, classical culture, monoclonal antibodies, proteomic standards, mass spectrometry, among others) are time-consuming, thus preventing contact tracing and delaying an effective treatment, ultimately contributing to the high incidence and mortality rates of infectious diseases [3,4]. Furthermore, some of these techniques still require considerable blood volume for analysis, which can be a major challenge, particularly for elderly and neonatal patients [4]. The COVID pandemics showed the need for rapid diagnostic methods for infectious diseases. Particularly, this has led to the development of nucleic-acid-based amplification technologies (NAATs), such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP), allowing a rapid detection of pathogens and capable of selectively detecting negligible amounts of nucleic acids [4]. Despite the advances in the diagnosis of bacterial infections achieved using these techniques, the presence of inhibitory compounds in complex biological matrices, like human blood or plasma, considerably reduces the sensitivity of these assays due to the lack of purity of the DNA being detected [5]. Therefore, it is highly required to develop effective pretreatment methods of biological samples to improve the diagnosis of infectious diseases. In the case of human serum samples, it is highly desirable to eliminate the interference of the most abundant protein, namely human serum albumin (HSA).

In clinical diagnostics various methods have been developed to pre-treat biological samples and isolate nucleic acids, particularly DNA [6]. Traditionally, the purification of nucleic acids is based on extraction techniques, often comprising hazardous organic solvents, such as phenol, chloroform, formamide and dimethyl sulfoxide (DMSO), which may additionally affect the structural integrity of nucleic acids [7]. Large efforts have been carried out to find sustainable and efficient isolation or purification processes for biomolecules, such as by the use of aqueous biphasic systems (ABS) [8], which can be applied to nucleic acids if properly designed.

ABS are liquid-liquid systems composed of two immiscible water-rich phases, formed by polymer-polymer, polymer-salt or salt-salt mixtures dissolved in aqueous media [9]. They were first described in the 1950s as benign liquid-liquid separation processes [10] for the purification of antioxidant compounds from crude extracts of biomass [11] or proteins/enzymes from fermentation media [12,13] due to their water-rich environment. However, conventional polymer-based ABS have a limited polarity range of the coexisting phases, hindering high extraction efficiencies and enhanced selectivity from being achieved in a single-step. This drawback may be overcome by employing ionic liquids (ILs) as phase-forming components of ABS [14]. In this way, ABS can be tailored by taking advantage of ILs as “designer solvents”, allowing low viscosity of the phases, rapid phase separation and high extraction efficiencies and selectivity [14–17]. ILs are becoming increasingly popular as alternative solvents in extraction processes, with several startups, such as Calagua Innovations, 525 Solutions, Wyonics and Marisignum, already using them at a large scale. However, the potential

environmental risks associated with ILs cannot be ignored, and ways must be sought to mitigate negative impacts. For example, cholinium-based ILs are a promising alternative to imidazolium-based ILs as phase-forming components of ABS, as they can be designed to be non-toxic and biodegradable [18,19].

MacFarlane and co-workers [20] showed that hydrated cholinium-based ILs are able to maintain the structure and chemical stability of the DNA over 6 months at room temperature, while Prasad and co-workers [21,22] demonstrated that cholinium-based ILs could preserve the structural integrity of DNA up to one year. These results suggest that cholinium-based ILs are appropriate solvents for DNA processing, and thus promising options to be included in ABS to act as pretreatment strategies of biological samples.

Given the relevance of isolating DNA to improve the diagnosis of bacterial infections, while considering the described advantages of cholinium-based ILs and ABS, in this work we investigated ABS formed by water, PPG 400 (polypropylene glycol with a molecular weight of 400 $g \cdot mol^{-1}$) and cholinium alkanoates to selectively (one-step) separate DNA and has. HSA is the most abundant protein in human plasma and could interfere with the DNA analysis. HSA is a non-glycosylated globulin that accounts for more than 50% of plasma proteins [23]. After an initial screening on the separation performance, the most promising ABS was used to isolate *Escherichia coli* (*E. coli*) genomic DNA (gDNA) from human serum, with gDNA being analyzed by quantitative real-time PCR (qPCR) to demonstrate the application of the developed strategy in clinical diagnosis.

2. Experimental

2.1. Chemicals

Cholinium-based salts studied in this work were cholinium ((2-hydroxyethyl)trimethylammonium) chloride ([Ch]Cl, purity ≈ 99%) acquired from Sigma-Aldrich, cholinium acetate ([Ch][C_1CO_2], purity ≈ 98%) purchased from Iolitec, and cholinium propanoate ([Ch][C_2CO_2]) and cholinium glycolate ([Ch][Gly]) synthesized by us according to standard protocols [24]. It should be remarked that [Ch]Cl does not fall within the IL category due to its higher melting point. However, it is here described as part of the cholinium-based ILs group for comparison purposes. The polymer polypropylene glycol (PPG) of average molecular weight 400 $g \cdot mol^{-1}$, PPG 400, was supplied by Sigma-Aldrich and used as received. The chemical structures of the investigated ILs and of PPG 400 are depicted in Figure 1.

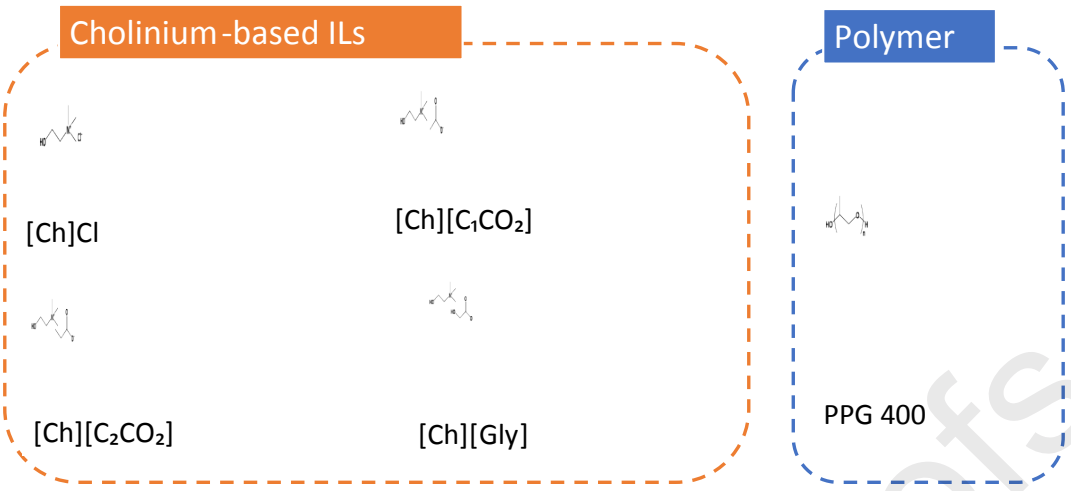

Figure 1. Chemical structures of the ILs and polymer investigated.

The ABS studied at different pH values were established using hydrochloric acid (37% in aqueous solution) and glycolic acid (purity ≈ 99%), both purchased from Sigma-Aldrich, acetic acid (purity ≥ 99.5%) purchased from José Manuel Gomes dos Santos, propanoic acid (purity ≈ 99%) acquired from Merck, and butanoic acid (purity ≥ 99%) and lactic acid (purity 88%-92%) acquired from Riedel-de-Haën.

For the initial extraction assays, deoxyribonucleic acid (DNA) from Salmon supplied by Sigma-Aldrich, and human serum albumin (HSA, 96%) acquired from Alfa Aesar, were used. The solutions of standard DNA to appraise by circular dichroism (CD) were prepared in a buffer solution composed of phosphate salts (PBS, pH=7.4) pellets acquired from Sigma-Aldrich.

For the isolation of DNA from more complex matrices, human serum was acquired from Sigma-Aldrich, which was spiked with *Escherichia coli* (*E. coli*) genomic DNA (gDNA) extracted using the Wizard SV gDNA purification kit (Promega). Real-time qPCR was employed to quantify the content of genomic DNA by measuring changes in fluorescence of the DNA binding dye Maxima® SYBR Green/Fluorescein qPCR Master Mix bought from ThermoFisher Scientific Inc. The potassium hydrogen phosphate (K_2HPO4) was acquired from Panreac and potassium dihydrogen phosphate (KH_2PO4) from Sigma-Aldrich.

2.2. Separation of DNA and HSA

In the studied ABS, the top phase corresponds to the PPG-rich aqueous phase, while the bottom phase is mainly composed of IL and water. The ternary mixture compositions used in the separation experiments were chosen based on the phase diagrams determined for each ABS. In particular, a common mixture composition was prepared for all systems investigated: 30 wt% of PPG 400, 20 wt% of IL/Acid and 50 wt% of water. The separation studies were performed at different pH values, for which each pH value was reached by adding the acid corresponding to the precursor of the IL anion studied. Only for three systems, namely the one composed of $[Ch][C_1CO_2]$ at pH 5

and [Ch][C_2CO_2] at pH 5 (50 wt% of PPG, 20 wt% of IL and 30 wt% of water at pH 5), and [Ch][Gly] at pH 4 (50 wt% of PPG, 20 wt% of IL and 30 wt% of water at pH 4), different mixture compositions were used since it was not possible to obtain a biphasic region with these ILs at lower pH values. DNA and HSA were introduced at the concentrations of 0.5 $g \cdot L^{-1}$ and 1.0 $g \cdot L^{-1}$, respectively, as part of the water added to each ABS.

The separation of DNA and HSA in each ABS was first addressed by carrying out experiments with each individual biomolecule. Each ABS mixture was stirred, centrifuged for 30 min at 3500 rpm, and left to equilibrate for at least 10 min at (25 ± 1) °C to achieve the complete separation on both biomolecules. After equilibrium, both phases were carefully separated, and the HSA and DNA in each phase were quantified by UV-spectroscopy, using a BioTeck Synergy HT microplate reader, at a wavelength of 280 and 260 nm, respectively. Previously established calibration curves were used. At least three individual experiments were performed, allowing to determine the average extraction efficiency, as well as the respective standard deviations. The interference of the PPG 400 and ILs with the quantification method was also taken into account, and blank control samples were always employed.

The percentage extraction efficiency of DNA (EE_{DNA}) and HSA (EE_{HSA}) corresponds to the percentage ratio between the total weight of DNA or HSA in the IL-rich phase to that in the two aqueous phases (Detailed equations given in the Supplementary Material, Eq. S1 and Eq. S2). The recovery yield of DNA (Y_{DNA}) and HSA (Y_{HSA}) corresponds to the percentage ratio between the total weight of DNA or HSA in the IL-rich phase to that added in the initial mixture (Detailed equations given in the Supplementary Material, Eq. S3 and Eq. S4).

2.3. Purification of DNA using [Ch][Gly]-based ABS

Standard aqueous mixtures of DNA and HSA in different weight ratio (1:1 to 1:5) were prepared in 1 mL milli-Q water, and vortex for 30 min for complete solubilization. The studied weight ratio of DNA to HSA corresponds to concentrations of 0.5 $g \cdot L^{-1}$ and 1.0 $g \cdot L^{-1}$ of HSA and DNA, respectively, in the water added to create each ABS. After mixing, each system was left at (25 ± 1) °C for 24 h, allowing the precipitation of HSA and the purification of DNA. After the protein precipitation, all ABS were centrifuged at 1000 rpm for 2 min, and left at (25 ± 1) °C for 2 h. After equilibrium and precipitation of the protein, the phases were carefully separated and quantified in terms of DNA and HSA content by UV-Vis spectroscopy (Shimadzu UV-1800 UV-Vis spectrometer, USA), allowing to determine the extraction efficiencies and yields. The purification level of DNA was confirmed by the UV-Vis absorbance ratio of the IL-rich phase at 260 nm and 280 nm (A_{260}/A_{280} ratio).

After the purification step, the DNA stability in each ABS at different pH values was confirmed by UV-Vis (Shimadzu UV-1800 UV-Vis spectrometer, USA) and circular dichroism (CD, Jasco J-1500 CD spectrometer, Japan) spectroscopies. To appraise the long-term stability of DNA in the IL-rich phase, samples were kept (25 ± 1) °C for several months, in which UV-Vis and CD spectra were periodically acquired.

2.4. Cultivation of *E. coli* DH5α and extraction of gDNA

Growth of *E. coli* DH5α was carried out in shake-flasks at 37 °C and 250 rpm with 0.25 L of Terrific Broth medium (12 $g \cdot L^{-1}$ tryptone, 24 $g \cdot L^{-1}$ yeast extract, 4 $mL \cdot L^{-1}$ glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4). After 7 h of fermentation, the bacterial growth was suspended, the cells recovered by centrifugation, and the gDNA was extracted using the Wizard SV Genomic DNA purification kit (Promega, Madison, USA), according to the manufacturer's instructions. Quality control of gDNA (integrity, purity and concentration) was assessed by agarose gel electrophoresis and UV-spectroscopy. Finally, the extracted bacterial DNA was used to supplement human serum, foreseeing to evaluate the performance of [Ch][Gly]-based ABS to extract gDNA from complex human samples for clinical diagnosis.

2.5. Conventional PCR using *E. coli* gDNA dissolved in [Ch][Gly] ABS at pH 5

Preliminary experiments by conventional PCR were performed to ascertain the effect on the amplification efficiency of the concentration of the top and bottom phases of the [Ch][Gly] ABS at pH 5. A defined amount of *E. coli* gDNA was dissolved in the top and the bottom phases of the selected ABS, and conventional PCR was performed using the DreamTaq Green DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) in a T100™ Thermal Cycler from Bio-Rad (Hercules, CA, USA). The specific primers used in these experiments were 5'-ACACGGTCCAGAACTCCTACG-3' (forward) and 5'- CCGGTGCTTCTTCTGCGGGTAACGTCA -3' (reverse) [25] for the amplification of a 181-bp fragment of the 16S ribosomal RNA gene, and according to the following program: 95 °C for 10 min for initial denaturation, 30 cycles consisting of denaturation at 95 °C for 10s, annealing at 60 °C for 30 s, and extension at 72 °C for 10s, and a final elongation step at 72 °C for 5 min. These experiments were performed using top and bottom phases with different dilution ratio (undiluted, 1:5, 1:10, 1:100 and 1:1000), while keeping constant the amount of gDNA. The amplicons were then analyzed by horizontal electrophoresis using 1% agarose gels (Hoefer, San Francisco, CA, USA). Electrophoresis was carried out at 110 V, for 30 min, with TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8). The bands corresponding to gDNA were visualized in the agarose gel using UVItec FireReader system (UVItec, Cambridge, UK) after staining with greenSafe Premium (Nzytech, lisboa, Portugal).

2.6. Real-time qPCR quantification of extracted *E. coli* gDNA from human serum

The quantification of gDNA was performed in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the Maxima® SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific Inc.), by real-time qPCR. Selective amplification of a 181 bp DNA-fragment was performed with 16S ribosomal RNA primers, and according the following program: denaturation at 95 °C for 3 min, 40 cycles consisting of an initial denaturation at 95 °C for 30 s, annealing at 60 °C during 30 s, and final elongation at 72 °C for 30s. The concentration of template DNA can be related to the threshold cycle (Cq), defined as the cycle in which the fluorescent

signal surpasses a defined fluorescent threshold (higher DNA concentrations correspond to lower Cq values and vice versa) [26]. The Cq value is linearly related to the log of the initial concentration of template DNA undergoing amplification, and by generating a standard curve the efficiency of the reaction can be determined [26]. The initial step consisted in the determination of qPCR efficiency 16S rRNA primers using serial dilutions of gDNA to infer the influence of the concentration of top and bottom ABS phases in the amplification efficiency. Real-time qPCR efficiencies were calculated from the given slopes with MyIQ 2.0 software (Bio-Rad, Hercules, CA, USA). qPCR efficiencies within the range of 90-110 % were achieved using dilution ratios of 1:50 and 1:200, respectively, for top and bottom ABS phases, and these dilutions were also performed to quantify gDNA from spiked human serum. Then, standard curves in the concentration range of 0.075-75 and 0.75-75 ng/µL gDNA (in 1:50 top phase or 1:200 bottom phase, respectively) were generated. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95 °C with 10 s holds at each temperature (0.05 °C·s^{-1}). In the context of clinical diagnosis, this method was applied for the quantification of gDNA from a complex human matrix composed of human serum supplemented with *E. coli* gDNA, after extraction with pH 5 [Ch][Gly]-based ABS. ABS preparation and phase separation were performed as described above. The relative quantification of the gDNA was calculated by applying the comparative threshold cycle (Ct) method. Each sample was run in triplicate, and Ct values were averaged from the triplicate. The final data were averaged from three independent experiments.

3. Results and discussion

3.1. Separation of DNA and HSA

The liquid-liquid phase diagrams of ABS composed of water, PPG 400, and several hydrophilic ILs, namely cholinium [Ch][C_1CO_2], [Ch][C_2CO_2] and [Ch][Gly], with no pH control and at 25 °C were previously reported [27]. [Ch]Cl was also investigated as an ABS phase-forming component for comparison purposes. PPG 400 was chosen due to its lower toxicity profile compared to PEG [28,29], and due to its higher hydrophobic nature required to induce proteins precipitation. Additionally, it was previously shown that cholinium-based ILs require more hydrophobic polymers, like PPG, to undergo phase separation since some of them do not form ABS with PEG [30]. In the studied ABS, the top phase corresponds to the PPG-rich aqueous phase, while the bottom phase is mainly composed of IL and water. These ABS were evaluated in what concerns their ability to selectively separate mixtures of DNA and HSA. However, since electrostatic interactions play a relevant role in proteins partitioning in ABS [31], we additionally investigated ABS at different pH values (from pH 1 to 9). The ABS's pH was adjusted by adding the acid corresponding to each IL anion as part of the aqueous solutions (*cf.* Experimental section), thus not introducing new compounds in the medium.

The extraction efficiencies and recovery yields of the different IL-based ABS at the various pH values (pH 1-9) were firstly determined for each individual biomolecule, *i.e.*, for pure DNA (0.5 g·L^{-1}) and pure HSA (1.0 g·L^{-1}). The following mixture composition was used: 30 wt% of PPG 400, 20 wt% of IL/Acid and 50 wt% of water. Only for three systems composed of [Ch][C_1CO_2] at pH 5 and [Ch][C_2CO_2] at pH 5 (50 wt% of PPG, 20 wt% of IL and 30 wt% of water at pH 5), and [Ch][Gly] at pH 4 (50 wt% of PPG, 20

wt% of IL and 30 wt% of water at pH 4), a different mixture was used because it was not possible to obtain a biphasic region with these ILs at lower pH values.

Figure 2 depicts the extraction efficiencies (*EE*%) and recovery yields (*Y*%) of the studied ABS for both DNA and HSA. The extraction efficiencies are defined as the percentage ratio between the total weight of DNA or HSA in the IL-rich phase to that in both phases, whereas the recovery yield is defined as the total percentage ratio between the total weight of DNA or HSA quantified in the IL-rich phase to that initially added. Detailed equations and results are given in the Supplementary Material (Eqs. S1 to S4, Tables S1 and S2). Remarkable extraction efficiencies of DNA to the IL-rich phase were obtained with all investigated systems, ranging between 90 and 100% in a single-step. Furthermore, no significant losses of DNA were observed, as appraised by the respective yields at the IL-rich phase (100%). This preferential partition may result from hydrogen-bonding and electrostatic interactions between ILs and DNA, as previously demonstrated by MacFarlane and co-workers [20] and Athira and Gardas [31]. The unique exception to this trend was observed for the ABS formed at pH 1 with [Ch]Cl, in which DNA is denatured.

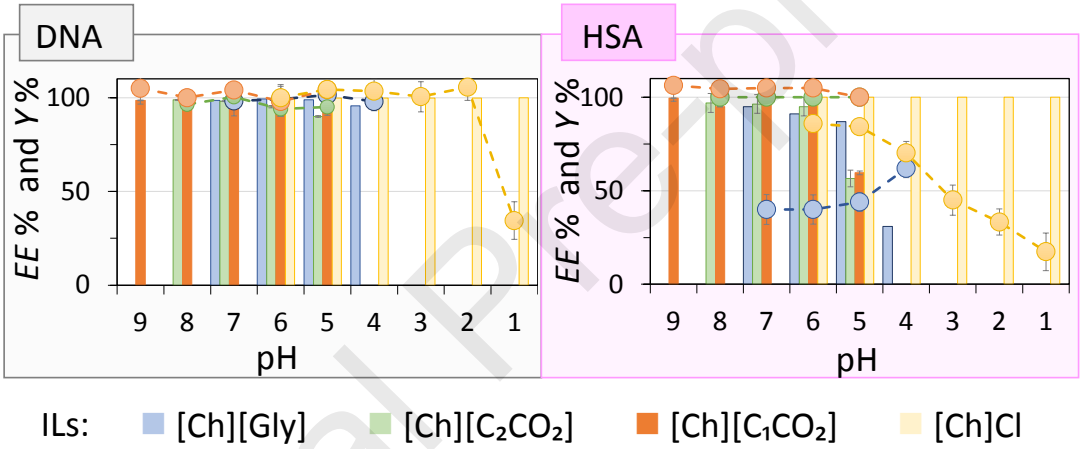

Figure 2. Extraction efficiency (*EE*%, bars) of DNA and HSA to the IL-rich phase, and recovery yield (*Y*%, symbols) of DNA and HSA in ABS formed by the different ILs.

Anderson and co-workers [32] applied water-miscible ILs to DNA aqueous solutions, and then added $LiNTf_2$ to promote the *in situ* formation of a water immiscible IL, allowing the phase separation and the enrichment of DNA at the IL-rich phase (with an extraction efficiency of *ca.* 49%, calculated according to the definition given above). Solutions of DNA at low concentrations (< 0.01 $mg \cdot mL^{-1}$) were used by the authors to achieve the highest extraction efficiencies. The authors demonstrated that when using DNA solutions at higher concentrations (> 0.1 $mg \cdot mL^{-1}$), the IL-rich phase reaches saturation and, consequently, the extraction efficiency for DNA decreases to 35% (estimated using the definition given above) [32]. In this work, we show that ABS may be used to extract DNA at higher concentrations, namely ≈ 0.5 $mg \cdot mL^{-1}$, without reaching the IL-rich phase saturation, and that the use of fluorinated species can be avoided. Furthermore, the phase separation is achieved using a biocompatible polymer – PPG 400, further allowing extraction efficiencies of 100% in a single-step.

Given the excellent performance of the studied ABS to extract DNA to the IL-rich phase, they were then optimized to separate DNA from HSA. For that, they were then evaluated in terms of HSA extraction performance. The extraction efficiency and yield of HSA are presented in Figure 2. With the exception of the system formed by [Ch]Cl, HSA preferentially partitions to the IL-rich phase at higher pH values and gradually shifts to the polymer-rich phase as the pH decreases. This protein has an isoelectric point (pI) of 4.9 [33], meaning that when positively charged (as in acidic media), it prefers the polymer-rich phase, whereas when negatively charged it prefers the IL-rich medium. This trend indicates preferential interactions between the IL cation and the negatively charged protein at higher pH values. Interestingly, significant precipitation of HSA in the [Ch][Gly]- and [Ch]Cl-based ABS was observed, particularly in acidic media, confirmed by the low recovery yields shown in Figure 2. These are the most hydrophilic ILs investigated, meaning that the respective ABS have a lower water content in the PPG-rich phase, being thus unsuitable for the enrichment of the protein at this phase. In particular, the system formed by [Ch]Cl, despite allowing a complete extraction of HSA to the IL-rich phase, has a low recovery yield due to a significant precipitation of the protein, corresponding to a protein-rich interphase [24,34,35].

Based on the described results, the selective separation of DNA and HSA using the studied ABS depends on two parameters: IL hydrophilicity and pH. Therefore, if these parameters are properly designed, DNA can be enriched at the IL-rich phase and the protein can be separated by precipitation at the interphase. The best ABS to proceed with this strategy is the one composed of [Ch][Gly].

3.2. Purification and preservation of DNA using [Ch][Gly]-based ABS

ABS formed by [Ch][Gly] at different pH values (4-7) were investigated to separate DNA from HSA prepared mixtures (employing also different DNA to HSA weight proportions, namely 1:1 to 1:5). With the exception of the ABS at pH 7, the remaining systems allow effective separation of DNA from HSA, as shown in Figure 3. Detailed results are given in the Supplementary Material in Tables S3 and S4. After the extraction step, DNA was isolated from the IL-bottom phase using ice cold ethanol and re-dissolved in a phosphate buffer aqueous solution (0.01 M, pH 7.2). The ethanol precipitation method is a highly effective technique for simultaneously precipitating DNA and removing IL from the DNA sample, thus avoiding interference in UV-Vis spectroscopy and circular dichroism measurements. It is worth noting that ethanol can be derived from renewable biomass sources such as corn and sugarcane, making it a bio-based solvent with potential environmental benefits. In addition, ethanol is approved as a food additive [36]. Therefore, the use of ethanol in DNA isolation does not affect the overall sustainability of the developed process.

A decrease in the DNA:HSA ratio in water leads to a decrease in the DNA purity, as shown in Table S5 in the Supplementary Material. The UV-Vis spectrum of DNA is characterized by a single characteristic absorbance band at 260 nm, and thus the purity of DNA may be appraised by the absorbance ratio at 260 nm and 280 nm (protein absorbance). For pure DNA in water this value corresponds to 1.8 [37]. According to the data given in Table S5 in the Supplementary Material, the best ABS to isolate DNA is the one formed by [Ch][Gly], at pH 5. Furthermore, for the DNA:HSA ratio of 1:1, 1:2 and 1:3, it is possible to obtain DNA with a purity > 98%. To the best of our knowledge, only

one work reports the use of IL-based ABS to purify DNA [38]; however, DNA was recovered with lower purity (absorbance ratio at 260 nm and 280 nm = 1.7). In addition to the higher DNA purity achieved by us, the [Ch][Gly]-PPG-based ABS investigated avoids the use of imidazolium-based ILs with fluorinated anions and the use of high concentrations of inorganic salts.

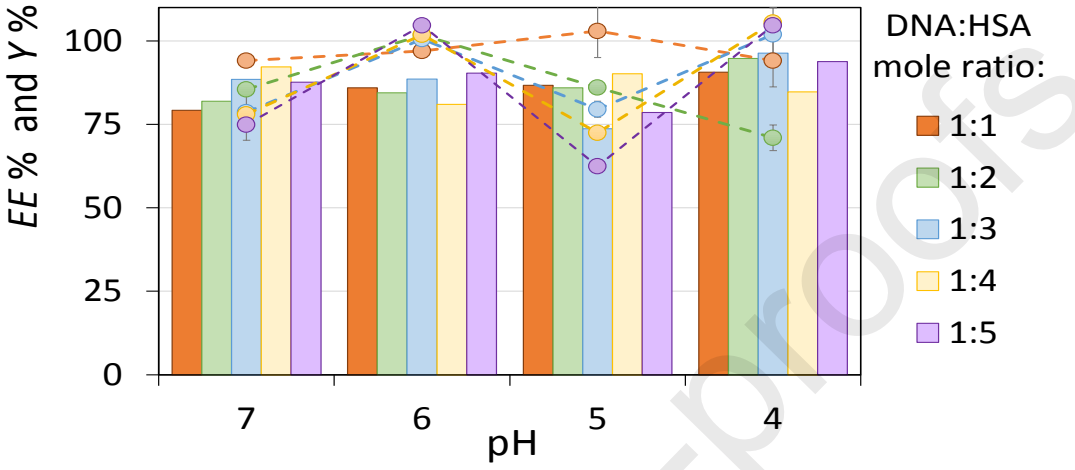

Figure 3. DNA extraction efficiency to the IL-rich phase (*EE*%, bars) and HSA yield (*Y*%, symbols) in ABS composed of [Ch][Gly] at different pH values.

The structural and conformational stability of the recovered DNA was appraised by Circular Dichroism (CD) – Figures 4A and 4B. In terms of DNA stability, the best ABS is also the one formed by [Ch][Gly] at pH 5, particularly at a DNA:HSA weight ratio of 1:1. The CD spectrum of stable β-form of DNA is characterized by a positive band at 277 nm, corresponding to π-π base interactions, and a less intense negative band at 246 nm, corresponding to the DNA helicity (Supplementary Material, Figure S1A). Thus, DNA with the highest purity (Supplementary Material, Figure 4B showing that HSA is not present) and stability (Supplementary Material, Figure S1A showing that the DNA β-form was not changed) is obtained with the ABS formed by [Ch][Gly] at pH 5. Overall, the ABS formed by [Ch][Gly] and PPG 400, at pH 5, leads to the recovery of DNA with high purity, as HSA precipitates at the interface of the system. It is worth noting that precipitation of HSA occurs only at low pH, while no such precipitation is observed at neutral pH. As for the recovery of HSA and DNA, HSA can be recovered by resuspension in a suitable buffer, while DNA with high purity can be successfully recovered from the IL-rich phase by ethanol precipitation, as mentioned above.


A


B

Figure 4. (A) Circular dichroism (CD) spectra of samples of DNA:HSA at different weight ratio after recovery from the IL-rich phase at pH 5. **(B)** CD spectra of the DNA:HSA sample at a 1:1 weight ratio after recovery from the IL-rich phase at different pH values.

The long-term stability of DNA (up to six months) at room temperature (25 °C) in the IL-rich phase was also appraised (detailed data shown in the Supplementary Material, Figure S1B). According to the respective CD spectra, there are no DNA conformational changes, confirming the structural and chemical stability of DNA for at least six months in the IL-rich phase. It should be noted, however, that DNA stored in PBS aqueous solutions (0.01 M, pH 7.2) was found to be degraded at the end of the six months of storage. These results support the potential of aqueous solutions of [Ch][Gly] to act as preservation media of DNA, in agreement with the results by Sharma *et al.* [22] for other cholinium-based ILs. This set of results reveals that the isolation of DNA can be effectively done using appropriate IL-based ABS, in which the IL-rich phase can be further used as storage medium up to the DNA analysis.

3.3. Real-time qPCR quantification of extracted *E. coli* gDNA from human serum

Based on the ability of ABS constituted by [Ch][Gly] at pH 5 to isolate DNA from DNA-HSA mixtures, and the improved stability shown by DNA in the IL-rich phase, this system was finally applied to isolate DNA from a real human serum sample supplemented with *E. coli* gDNA. Conventional PCR was initially performed in both top and bottom ABS phases at different dilution ratio, while keeping constant the concentration of gDNA. The respective results are depicted in Figure 5. It is demonstrated that the amplification of 16S rRNA gene is strongly inhibited at a dilution ratio below 1:100 and 1:10, respectively, for the top and bottom phases. These results provided the basis for selecting the most appropriate dilution ratio of the ABS top and bottom phases for further qPCR quantification. The amplification efficiency was determined by qPCR using gDNA at different dilution ratio of the top and bottom ABS phases. Efficiencies (%) of 103.8 and 90.3 were achieved (*cf.* Supplementary Material, Figure S2) using gDNA dissolved at dilution ratio of 1:50 and 1:200 for the top and bottom phases, respectively. The obtained values fit within 90 and 100 %, efficiencies that are acceptable in the validation of

quantitative qPCR methods and are indicative of real amplification (rather than signal drift) [39].

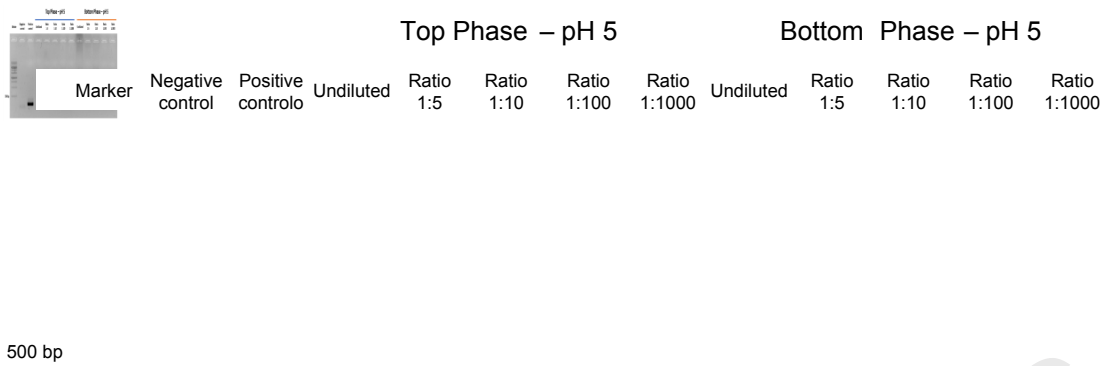

Figure 5. Agarose gel electrophoresis analysis of qPCR products using oligonucleotide primers specific for 16S RNA gene. *E. coli* DH5α gDNA was used as the template DNA and dissolved in the top or bottom phases from ABS at pH 5 at different dilution ratios (in 10 mM Tris, pH 8).

Based on the results obtained, the ABS formed by [Ch][Gly] at pH 5 was used with human serum spiked with *E. coli* gDNA, and after phase separation, the top and bottom phases at dilution ratio of 1:50 and 1:200 were subjected to qPCR analysis. The respective results are given in Table S6 in the Supplementary Material. The DNA recovery yield from this complex mixture in the bottom phase was (92 ± 4)%, being in close agreement with the results shown before for the aqueous mixtures of DNA and HSA (Figure 3).

Some studies on DNA extraction by ILs and their use for the signal amplification in PCR have been reported. It has been shown that only some ILs are efficient (mainly imidazolium-based) and under specific conditions [40]. Furthermore, Anderson and co-workers [5] showed that hydrophobic magnetic ILs are able to extract DNA from model systems or from human serum [26], but when they were used with conventional and qPCR, respectively, they had to create a special buffer to be able to read the sample due to the inhibition caused by the IL. In the current work with ABS there is no need to add an extra buffer, while using cholinium-based ILs, but it still requires a dilution of 200 times. Recently, a new generation of hydrophobic magnetic ILs with metal-containing cation was applied for the *in situ* dispersive liquid-liquid microextraction (DLLME) of long and short double-strand DNA, showing that this DNA extraction method presents advantages over existing ones due to its speed (3 min) and simplicity [41]. These magnetic ILs are easier and cheaper to prepare, making them a more affordable alternative for DNA extraction than commercially available kits; however, studies are necessary to design magnetic ILs compatible with qPCR [41].

In summary, the IL-based ABS here studied can be used to separate DNA from real matrices, the respective IL-rich phase can be further used to preserve the nucleic acid up to use, and the DNA can be directly quantified by real-time qPCR after an appropriate dilution. Regarding the reusability of IL, after DNA precipitation with cold ethanol, IL can be recovered and reused after the evaporating of ethanol and water. As for the polymer, there are already studies showing that the polymers can be efficiently recovered and reused in polymer-based ABS [42–44].

4. Conclusions

It was here demonstrated that ABS composed of [Ch][Gly] and PPG 400 at pH 5 are an efficient platform for the *E. coli* gDNA isolation from human serum samples, while leading to the precipitation/separation of HSA, and thus improving the diagnosis of bacterial infections. The selective precipitation of the HSA protein was observed at low pH, whereas no such precipitation was observed at neutral pH. After precipitation of HSA, > 98% pure DNA was isolated with 90% recovery yield. The DNA was found to retain its structural integrity during purification at different pH values using IL-based ABS and after storage at room temperature in the IL-rich phase for at least six months. The DNA at the IL-rich phase can be quantified by qPCR, paving the way for the molecular diagnosis of bacterial infections using ABS as an efficient pretreatment strategy of human serum.

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Supplementary Material

Towards the improved monitoring of bacterial infections by the isolation of DNA from human serum using ionic-liquid-based aqueous biphasic systems

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Equations

The percentage extraction efficiency of DNA (EE_{DNA}) and HSA (EE_{HSA}) corresponds to the percentage ratio between the total weight of DNA or HSA in the IL-rich phase to that in the two aqueous phases, and is defined according to Eq. S1 and Eq. S2, respectively:

$$EE_{DNA}\% = \frac{w_{DNA}^{IL}}{w_{DNA}^{IL} + w_{DNA}^{PPG}} \times 100 \qquad (Eq.S1)$$

$$EE_{HSA}\% = \frac{w_{HSA}^{IL}}{w_{HSA}^{IL} + w_{HSA}^{PPG}} \times 100 \qquad (Eq.S2)$$

where $w^{IL}$and $w^{PPG}$are the total weight of DNA or HSA in the IL-rich phase and in the PPG-rich phase, respectively

The recovery yield of DNA (Y_{DNA}) and HSA (Y_{HSA}) corresponds to the percentage ratio between the total weight of DNA or HSA in the IL-rich phase to that added in the initial mixture, and is defined according to Eq. S3 and Eq. S4, respectively:

$$Y_{DNA}\% = \frac{w_{DNA}^{IL}}{w_{DNA}^{Initial}} \times 100 \qquad (Eq.S3)$$

$$Y_{HSA}\% = \frac{w_{HSA}^{IL}}{w_{HSA}^{Initial}} \times 100 \qquad (Eq.S4)$$

where $w^{Initial}$is the weight of DNA or HSA added to the initial mixture.

Figures

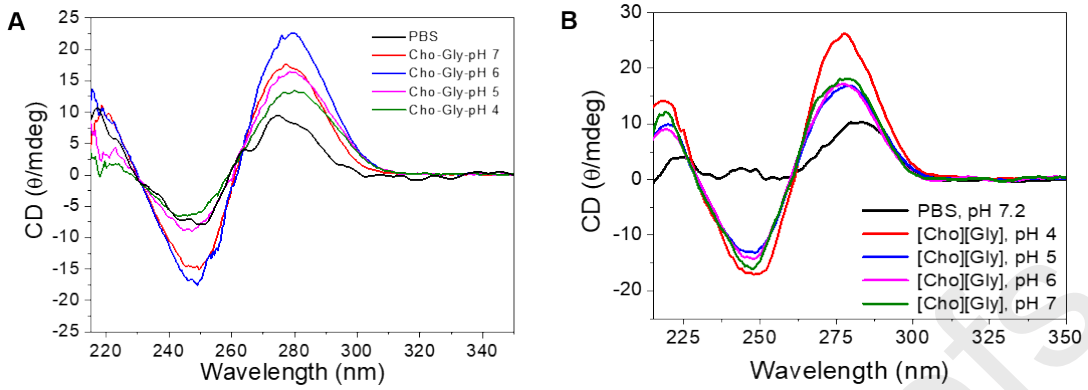

Figure S1. Circular dichroism (CD) spectra of standard DNA and DNA recovered from the bottom phase of [Ch][Gly]-based ABS at different pH values: **(A)** after extraction and immediate recovery; **(B)** after storage for six months at room temperature (*ca.* 25 °C) and recovery. All CD spectra were recorded in PBS (0.01 M, pH 7.2).

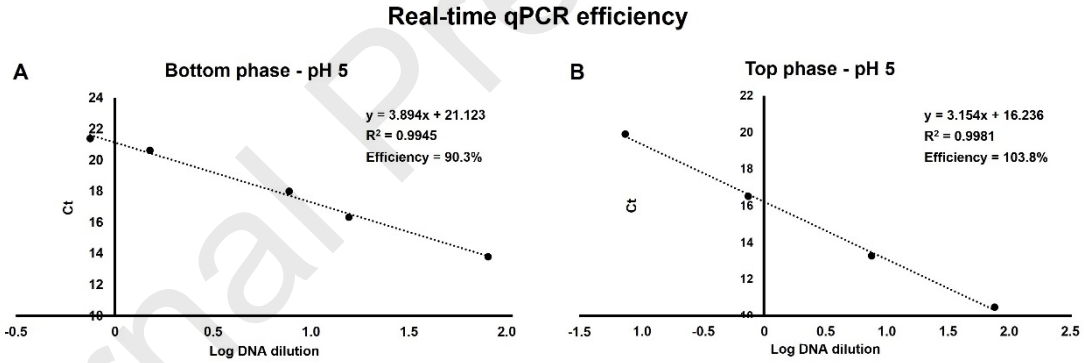

Figure S2. Standard curves to determine the amplification efficiency of 16S ribosomal RNA gene and subsequent quantification by real-time qPCR. **(A)** DNA in 1:200 bottom phase of [Ch][Gly] ABS pH 5 (0.75 – 75.0 ng·µL^{-1}); **(B)** DNA in 1:50 top phase of [Ch][Gly] ABS pH 5 (0.075-75.0 ng·µL^{-1}).

Tables

Table S1. Extraction efficiency to the IL-rich phase and yield of DNA and HSA. These experiments were carrying out with individual biomolecule solutions (DNA at 0.5 $g \cdot L^{-1}$ and HSA at 1.0 $g \cdot L^{-1}$).

		DNA		HSA	
IL	**pH.**	***EE*%**	***Y*%**	***EE*%**	***Y*%**
[Ch][Gly]	**7**	98.56 ± 0.08	98 ± 1	95.0 ± 0.5	40 ± 8
	6	99.3 ± 0.1	99 ± 1	91 ± 1	40 ± 8
	5	98.9 ± 0.2	101 ± 1	87 ± 2	44 ± 5
	4	95.6 ± 0.8	98 ± 4	31 ± 4	62 ± 5
[Ch][C_2CO_2]	**8**	98.8 ± 0.2	96 ± 6	97 ± 5	100 ± 2
	7	98.1 ± 0.2	100 ± 5	96 ± 5	100 ± 2
	6	95.2 ± 0.6	94 ± 1	95 ± 5	100 ± 4
	5	90.0 ± 0.5	95 ± 4	57 ± 4	100 ± 4
[Ch][C_4CO_2]	**9**	98.6 ± 0.1	105 ± 8	99.6 ± 0.1	106 ± 8
	8	98 ± 1	100 ± 7	95.0 ± 0.1	104 ± 4
	7	98.78 ± 0.06	104 ± 3	97.93 ± 0.05	105 ± 2
	6	98.67 ± 0.01	98 ± 2	96.8 ± 0.5	105 ± 8

	5	93.32 ± 0.09	104 ± 7	60 ± 1	100 ± 2
	6	100.00 ± 0.09	100 ± 2	100 ± 1	86 ± 2
	5	99.81 ± 0.02	104.3 ± 0.3	100 ± 1	84 ± 2
[Ch][Cl]	**4**	99.80 ± 0.02	103 ± 1	100.0 ± 0.9	70 ± 6
	3	99.83 ± 0.02	100.5 ± 0.5	100 ± 1	45 ± 8
	2	99.81 ± 0.01	105.5 ± 0.7	100 ± 1	33 ± 7
	1	99.95 ± 0.05	34 ± 5	100.0 ± 0.9	17 ± 7

Table S2. Identification of the ABS with precipitation (✓) or not (×) of HSA and DNA at different pH values.

pH	DNA				HSA			
	[Ch][Gly]	[Ch][C_2CO_2]	[Ch][C_1CO_2]	[Ch][Cl]	[Ch][Gly]	[Ch][C_2CO_2]	[Ch][C_1CO_2]	[Ch][Cl]
1	---	---	---	✓	---	---	---	✓
2	---	---	---	×	---	---	---	✓
3	---	---	---	×	---	---	---	✓
4	×	---	---	×	✓	---	---	✓
5	×	×	×	×	✓	×	×	✓
6	×	×	×	×	✓	×	×	---
7	×	×	×	---	✓	×	×	---
8	---	×	×	---	---	×	×	---
9	---	---	×	---	---	---	×	---

Table S3. Extraction efficiency of DNA to the IL-rich phase and respective yield in the ABS formed by [Ch][Gly] at different pH values.

pH	*EE* %					*Y* %				
	DNA:HSA ratio (w/w)					DNA:HSA ratio (w/w)				
	(1:1)	(1:2)	(1:3)	(1:4)	(1:5)	(1:1)	(1:2)	(1:3)	(1:4)	(1:5)
4	89.20	91.90	98.50	102.20	97.60	98.10	99.20	98.90	95.50	99.89
5	95.90	94.40	98.60	91.00	100.30	97.00	84.20	83.30	82.40	85.50

6	96.70	95.90	83.70	100.10	88.50	89.20	86.00	79.50	72.50	62.50
7	100.60	104.70	106.30	94.70	103.80	92.30	71.00	90.40	86.20	85.60

Table S4. Identification of the systems with precipitation (✓) or not (×) of HSA in ABS formed by [Ch][Gly] at different pH values.

pH	**DNA: HSA weight ratio**				
	(1:1)	**(1:2)**	**(1:3)**	**(1:4)**	**(1:5)**
7	×	×	×	×	×
6	×	✓	✓	✓	✓
5	✓	✓	✓	✓	✓
4	✓	✓	✓	✓	✓

Table S5. A_{260} to A_{280} ratio for DNA samples after recovery from the IL-rich phase.

Water/pH of the IL-rich phase	$A_{260/280}$ ratio of recovered DNA from the IL-rich phase					
	Std. DNA solution (1 g·L^{-1})	DNA:HSA (*mol:mol*)				
		(1:1)	(1:2)	(1:3)	(1:4)	(1:5)
Water	**1.85**	1.75	1.58	1.54	1.51	1.48
pH 7		1.77	1.70	1.66	1.64	1.59
pH 6		1.79	1.74	1.75	1.72	1.68
pH 5		**1.83**	**1.81**	**1.79**	1.77	1.70
pH 4		1.79	1.78	1.77	1.71	1.69

Table S6. Quantification of *E. coli* DH5α gDNA isolated from human serum using real-time PCR in the bottom phase of [Ch][Gly] ABS pH5 (samples were diluted by 1:200).

Sample	Ct	Ct average	Log_{10} ([DNA])^{A}	[DNA] (ng/µL)	DNA Mass (ng)^{B}	Real DNA Mass (µg)^{C}	DNA Recovery (%)
Initial Sample	16.95	17.04	1.049	11.18	2124.7	424.93	------
	17.13						
	16.13						
Sample	17.49	17.69	0.882	7.613	2017.7	403.54	94.97

1	17.89						
	16.60						
Sample 2	17.69	17.79	0.857	7.198	1907.5	381.50	89.78
	17.88						
	18.50						

[A]Calculated according to the standard curve from Figure S2: $Log_{10}([DNA]) = (Ct-21.123)/-3.894$.

[B]DNA mass in the total volume of initial sample/ABS bottom phase (respectively, 190.0 µL and 265.0 µL).

[C]DNA mass considering the dilution performed before qPCR analyses (1:200). Values in red were not considered in calculations.

- DNA purification by ionic-liquid-based aqueous biphasic systems (ABS).
- DNA is enriched in the ionic-liquid-rich phase.
- Proteins precipitate in the interphase of the ABS.
- The ABS comprising cholinium glycolate (pH 5) is the most effective.
- The stability of DNA is preserved after the purification step.

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: