Pre-treatment strategies based on aqueous two-phase systems comprising ionic liquids to improve the adrenal cancer diagnosis

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Abstract
Cancer remains the cause of millions of deaths every year worldwide, and reliable early-stage diagnosis methods are essential to prevent them. Cancer biomarkers quantification in human fluids has gained relevance in detecting cancer in an early-stage. Among these, the levels of the biomarkers epinephrine, norepinephrine and vanillylmandelic acid in urine have been used for the diagnosis of adrenal cancer. However, due to their low levels in human fluids, expensive and multistep pretreatment methods are necessary to increase their concentration to meet the detection limits of the analytical equipments. In this work, we propose the use of aqueous two-phase systems (ATPS) composed of ionic liquids (ILs), K3PO4 and synthetic/natural urine as an alternative pretreatment strategy to improve the concentration of adrenal cancer biomarkers from human fluids. The ATPS ternary phase diagrams, as well as the respective tie-lines and tie-line lengths, were determined at 25 °C. The performance of these IL-based ATPS for the extraction and concentration of epinephrine, norepinephrine and vanillylmandelic acid from human urine was then evaluated. The obtained results show that in the studied systems, with the exception of IL-based ATPS constituted by ILs with longer alkyl side chains at the cation, all the studied cancer biomarkers preferably migrate to the IL-rich phase. The best ATPS investigated is constituted by 1-ethyl-3-methylimidazolium methylsulfate ([C2mim][CH3SO4]) that allows the complete extraction of all cancer biomarkers to the IL-rich phase in a single-step and a concentration factor up to 500-fold. All biomarkers were accurately quantified in the IL-rich phase after the extraction from urine and the concentration step by UHPLC. According to the obtained results, IL-based ATPS could be used as a novel and effective method for the pretreatment and concentration of cancer biomarkers from human fluids to improve cancer diagnosis.

1. Introduction

Nowadays, one in seven deaths is associated to cancer, and 19.3 million new cancer cases and 10.0 million cancer deaths occurred in 2020 [1]. These numbers will increase from the expected population increase; however, the disease development factors are derived from other aspects, such as social and economic factors [2]. Cancer affects all ages, with survival expectations different in children and adults for several types of cancer [3]. Although the cases of child or adolescent diagnosed with cancer have been low compared to adults, the disease is still the second leading cause of death in children [4]. Among the several types of cancer, childhood cancers differ from those that occur in adults, with brain and lymphoma system carcinomas correspond to 38% of pediatric cancers [3].

Adrenocortical carcinoma (ACC) is an aggressive neoplasm that occurs in children, being correlated to malignant tumors [5]. Mortality rate associated with endocrine system tumors is directly related to the ability to detect the disease at an early-stage. Patients who have access to early detection have higher chances of disease progression containment [5]. ACC diagnosis is different according to the patient age, with children having most effective diagnosis compared to adults at the same cancer stage [5]. Due to the necessity of early cancer detection high attention is now given to cancer biomarkers.

Cancer biomarkers are molecules with biological activity or expression that are present in human fluids (saliva [6], urine [7], tears [8] and serum [9]), which are at altered levels due to the
impact of pathologic conditions. Along with the advancement of genomics, proteomics and molecular biology, several biomarkers arose as potential tools for early cancer detection [10]. Biochemical cancer biomarkers include DNA [11], mRNA [12], enzymes [13], metabolites [14] and proteins [15]. However, these are at very low concentrations in human fluids, representing a major challenge in cancer biomarkers quantification by available analytical equipment [16]. Several techniques have been applied for cancer biomarkers detection and quantification, such as polymerase chain reaction (PCR) [17], enzyme-linked immunosorbent assay (ELISA) [18], colorimetric assays [19], electrochemical assays [20] and fluorescence methods [21]. However, most of these methods are multistep and expensive [16]. Furthermore, human fluids are a complex matrix comprising several metabolites at higher concentrations than cancer biomarkers, representing an additional challenge to perform the accurate cancer biomarkers detection and quantification. Currently, blood samples are the most employed type of biological fluids to biomarkers detection for several cancers [22]. However, urine samples have been attracting additional attention as a non-invasive alternative to blood samples [23], with urine catecholamine levels been used in the diagnosis of ACC in pediatric patients (<19 years) [24]. Due to low levels of epinephrine (EPI), norepinephrine (NOR) and vanillylmandelic acid (VMA) in urine (μg/day⁻¹), which correspond to other well-known biomarkers representative of ACC and whose chemical structures are illustrated in Fig. 1, samples need to be collected during 24 h to achieve minimum values for analytical equipment detection, leading to an uncomfortable experience and further requiring several pre-treatment steps.

In order to overcome some disadvantages of the used pretreatment methods of biological fluids, aqueous two-phase system (ATPS) could be used as an alternative further allowing the cancer biomarkers concentration. ATPS is a liquid–liquid extraction method usually applied to biomolecules. It is mainly composed of water thus promoting a stable environment for active biomolecules [25]. ATPS are also an environmentally-friendly technology since they avoid the use volatile organic solvents. Composed of different pairs of water-soluble compounds, ATPS are usually constituted by two polymers, a polymer and a salt or two salts that above given concentrations undergo separation in two coexisting phases. In order to overcome some limitations of traditional polymer-based ATPS, Gutowski et al. [26] proposed ionic-liquid-based ATPS. Ionic liquids (ILs) are molten salts constituted by a large organic cation and an inorganic/organic anion, with a structure that allows a variety of different interactions. Due to their ionic nature, most ILs present unique characteristics, namely a negligible volatility, non-flammability, high thermal and chemical stabilities, and a strong solvation capability for several compounds [27]. Besides these properties, ILs are “designer solvents” and can be tailored to a specific task, which is a property that assist the high performance of IL-based ATPS [28]. IL-based ATPS have been explored as a versatile platform for the extraction and purification of proteins [29], alkaloids [30], antioxidant [31] and drugs [32]. Furthermore, remarkable results concerning the concentration of endocrine disruptors from human fluids were achieved using IL-ATPS [33]. The authors reported concentration factors of up to 100-fold on the extraction and concentration of bisphenol A from synthetic urine.

Due to low levels of EPI, NOR and VMA in urine and the problems that must be addressed in cancer diagnosis, the main goal of this work is the development of a new pre-treatment strategy of human urine to improve their concentration and detection/quantification. In this way, novel phase diagrams constituted by IL, inorganic salt (K₃PO₄) and synthetic/natural urine were determined and employed for the extraction and further concentration of ACC biomarkers from urine. The effects of the IL chemical structure were investigated aiming at reaching the complete extraction and maximum concentration of ACC biomarkers in the IL-rich phase in a single-step, which can be accurately quantified by UHPLC.

2. Materials and methods

2.1. Materials

The ATPS studied in this work are composed of potassium phosphate tribasic 97% pure from Sigma Aldrich. The ionic liquids...
studied were the following: 1-Hexyl-3-methylimidazolium chloride, [C₆mim]Cl; 1-Butyl-3-methylimidazolium chloride, [C₄mim]Cl; 1-Ethyl-3-methylimidazolium tosylate, [C₂mim][Tos]; 1-Ethyl-3-methylimidazolium thiocyanate, [C₂mim][SCN]; 1-Ethyl-3-Methylimidazolium Bromide, [C₂mim]Br; 1-Ethyl-3-methylimidazolium dimethylphosphate, [C₂mim][DMP]; 1-Ethyl-3-methylimidazolium Methylsulfate; [C₂mim][CH₃SO₄]; and 1-ethyl-3-methylimidazolium dicyanamide, [C₂mim][N(CN)₂]. All ILs were purchased from Iolitec. The choice of these ionic liquids was based on the systematic study of the effect of these compounds in aqueous solutions in the presence of urine, using the expertise already widely publicized by our research group. The molecular structures of the investigated ILs are displayed in Fig. 1. K₃PO₄ (>98 wt% pure) acquired by Sigma-Aldrich was chosen because of its high salting-out capacity, facilitating phase separation, which could be impaired by the presence of salting-in compounds in the urine. The Epinephrine, Norepinephrine and Vanillylmandelic acid were purchased from Sigma Aldrich, with the respective chemical structures given in Fig. 1.

**Fig. 2.** Phase diagrams for the systems composed of IL + K₃PO₄ + synthetic urine at 25 °C: (A) [C₂mim]Cl, [C₄mim]Cl and [C₆mim]Cl; (B) [C₂mim]Cl, [C₂mim][DMP], [C₂mim][CH₃SO₄] and [C₂mim][N(CN)₂]; (C) Phase diagrams for the systems composed of [C₂mim][CH₃SO₄] + K₃PO₄ at 25 °C: H₂O, synthetic urine and real urine.
2.2. Phase diagrams and tie-lines

Aqueous solutions of each IL (between 40 and 80 wt%) and synthetic urine solutions containing K₃PO₄ at 35 wt% were prepared and used for the determination of the binodal curves. This salt was chosen due to its strong salting-out ability, thus allowing long tie-line lengths and high concentration factors as demonstrated. The phase diagrams were determined through the cloud point titration method at 25 °C and atmospheric pressure. The system compositions were determined by the weight quantification of all components added within ± 10⁻³ g.

The tie-lines (TLs) were determined by a gravimetric method originally proposed by Merchuk et al. [34]. Different mixture points at the biphasic region were prepared in small glass ampoules (ca. 5 mL) especially designed for the purpose, vigorously stirred and allowed to reach equilibrium by the separation of the phases for at least 12 h at 25 °C. After separation of the two phases, both the top and bottom phases were weighted. Each individual TL was determined by application of the lever-arm rule to the relationship between the top weight phase composition and the overall system composition. The experimental binodal curves were correlated using Eq. (1):

\[
[I]_L = A \exp \left( B [\text{salt}]^{0.5} \right) \left( C [\text{salt}]^3 \right)
\]

The determination of the TLs was accomplished through the solution of the following system of four equations (Eqs. (2) to (5)) to determine four unknown values ([IL]₀, [IL]salt, [salt]₀ and [salt]salt):

\[
[I]_L = A \exp \left( B [\text{salt}]^{0.5} \right) \left( C [\text{salt}]^3 \right) \quad (2)
\]

\[
[I]_{\text{salt}} = A \exp \left( B [\text{salt}]^{0.5} \right) \left( C [\text{salt}]^3 \right) \quad (3)
\]

\[
[I]_{\text{IL}} = \left[ \frac{[I]}{[\text{M}] - \left( 1 - \frac{\alpha}{\alpha} \right) [\text{salt}]} \right] [I]_{\text{salt}} \quad (4)
\]

\[
[\text{salt}]_{\text{IL}} = \left[ \frac{[\text{salt}]}{[\text{M}] - \left( 1 - \frac{\alpha}{\alpha} \right) [\text{salt}]} \right] [\text{salt}]_{\text{salt}} \quad (5)
\]

where the subscripts “IL”, “salt” and “M” represent the top and bottom phases and the mixture composition, respectively. The parameter \( \alpha \) is the ratio between the weight of the top phase and the weight of the overall mixture. For the calculation of each tie-line length (TLL), Eq. (6) was employed:

\[
TLL = \sqrt{([\text{salt}])^2 + ([I]_{\text{IL}} - [I]_{\text{salt}})^2} \quad (6)
\]

Additionally, the impact of using real urine was also studied in systems based on [C₂mim][CH₃SO₄] and K₃PO₄ at 25 °C and atmospheric pressure. The real urine sample was kindly provided by a healthy male donor after signing an informed consent according to the Declaration of Helsinki. The collection of urine samples was approved by the Ethics Committee of Cova da Beira University Hospital Center (CE 41/2015). The collection of data complied with the General Data Protection Regulation (Reg. (EU) 2016/679) and all procedures were carried out in accordance with relevant guidelines and regulations.

2.3. Extraction of cancer biomarkers

The ternary mixture compositions used in the partitioning experiments were chosen based on the phase diagrams determined in this work for each IL-salt-synthetic urine system. Different mixture compositions were studied to evaluate the effect of the concentration of the phase-forming components through the extraction of EPI, NOR and VMA at initial concentrations of 1.0 g L⁻¹. Each mixture was vigorously stirred and left to equilibrate for 12 h at 25 °C, allowing the EPI, NOR and VMA partitioning between the coexisting phases. After, a careful separation of the phases was performed and the amount of EPI, NOR and VMA in each phase was quantified by Varian Cary 50Bio UV–Vis Spectrophotometer at 285 nm using a calibration curve previously established. Blank controls were employed whenever necessary.

The percentage extraction efficiency of EPI, NOR and VMA (EE_{EPI}, EE_{NOR} and EE_{VMA}), is the percentage ratio between the amount of biomarkers in the IL-rich aqueous phase to that in the IL-rich aqueous phase, being defined according to Eqs. (7) to (9):

\[
EE_{EPI} = \frac{w_{\text{EPI}}}{w_{\text{EPI}} + w_{\text{EPI}}^0} \times 100
\]

\[
EE_{NOR} = \frac{w_{\text{NOR}}}{w_{\text{NOR}} + w_{\text{NOR}}^0} \times 100
\]

\[
EE_{VMA} = \frac{w_{\text{VMA}}}{w_{\text{VMA}} + w_{\text{VMA}}^0} \times 100
\]

Table 1

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<th>Weight fraction composition (wt %)</th>
<th>IL + K₃PO₄ + synthetic urine</th>
<th>IL</th>
<th>[IL]₀</th>
<th>[IL]salt</th>
<th>[IL]₀</th>
<th>[IL]salt</th>
<th>[IL]₀</th>
<th>[IL]salt</th>
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<tr>
<td>[C₆mim][CH₃SO₄]</td>
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<td>24.70</td>
<td>0.01</td>
<td>55.42</td>
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</table>
where $w_{\text{EPI}}^\text{IL}$, $w_{\text{NOR}}^\text{IL}$, and $w_{\text{VMA}}^\text{IL}$ are the total weight of each biomarker in the IL-rich and $w_{\text{EPI}}^\text{Salt}$, $w_{\text{NOR}}^\text{Salt}$, and $w_{\text{VMA}}^\text{Salt}$ are the total weight of each biomarker in the salt-rich aqueous phases. In all systems the top phase corresponds to the IL-rich phase whereas the bottom phase is mainly constituted by the salt and synthetic urine.

2.4. Concentration of cancer biomarkers in the [C$_2$ mim][CH$_3$SO$_4$]-based aqueous two-phase system

After identifying the best IL and ATPS to carry out the cancer biomarkers extraction, a fixed and long TL was selected to guarantee the possibility of achieving high concentration factors. Along the same TL, the composition of the phases is maintained while varying the weight or volume ratio between them. For each mixture, the salt and ionic liquid percentages were varied to obtain the desired concentration factor (CF) according to equation (10):

$$\text{CF} = \frac{w_{\text{Synthetic urine}}}{w_{\text{IL}}}$$

where $w_{\text{Synthetic urine}}$ and $w_{\text{IL}}$ correspond to the weight of the synthetic urine containing EPI, NOR and VMA (at 1 ng.L$^{-1}$ individually) and the weight of the IL-rich phase in the biphasic mixture obtained.

The concentration factor of ACC biomarkers along the characterized TL was evaluated by the preparation of ternary systems at different compositions and at different weight ratios (weight of synthetic urine added to the system per weight of the IL-rich

![Fig. 3. Extraction efficiency (EE%) of epinephrine (A), norepinephrine (B) and vanillylmandelic acid (C) in ATPS composed of 30 wt% of IL + 15 wt% K$_3$PO$_4$ at 25 ºC.](image_url)
phase): 5 to 500. The effect of real urine from male was then addressed. The mixtures were carefully separated and weighted to determine the CF according to equation (10). EPI, NOR and VMA were quantified using an UHPLC system equipped with an auto sampler (Shimadzu SIL20AOCR), detector SPD/TOF and a column Supelco® Silica and C18 Saturator Columns. Chromatographic separation was carried out with a mobile phase consisting of 0.1 molL⁻¹ phosphate buffer (pH 7.4) at a flow rate of 0.5 mL min⁻¹. The total chromatographic run time was 10 min, and the injection volume was 50 μL. The wavelength was set at 280 nm whereas the retention time of EPI, NOR and VMA were found to be ca. 2 min. MS spectra at 2 min were searched according the database using the compounds molecular weight as criteria.

3. Results and discussion

3.1. Phase diagrams

Novel phase diagrams were determined for the several systems consisting of IL + K₃PO₄ + synthetic urine at 25 °C and atmospheric pressure. These phase diagrams are displayed in Fig. 2a, b and c, allowing the evaluation of the IL cation and anion effect in forming ATPS. Detailed data are provided in the Supporting Information (Table S1-S4). In all phase diagrams, the monophasic region is located below the solubility curve while the biphasic region is above. Thus, phase diagrams with a higher area above the binodal curve present a higher ability to form biphasic systems, i.e., they are more easily salted-out by K₃PO₄. Fig. 2a displays the effect of the IL alkyl side chain length in ATPS formation, following the rank: [C₄mim]Cl > [C₆mim]Cl > [C₈mim]Cl. The longer the alkyl chain length, the more hydrophobic the IL is, thus being more easily salted-out by the salt. This trend, although achieved with synthetic urine, is in agreement with the trend previously reported for ATPS with a larger biphasic region. The K₃PO₄ also allows better extraction of real urine instead of water was analyzed. Due to the presence of different metabolites (salt, urea, proteins, ADN and RNA) [40] in urine, in Fig. 2c it is provided a comparison between ATPS composed of water, synthetic urine and real human urine. The use of human urine allows an increase in the biphasic area when compared to synthetic urine and to water, which turns into an advantage to achieve higher concentration factors. The evaluation of real male urine induces the same behavior on the phase diagrams.

The experimental data corresponding to the binodal curves were fitted using Eq. (1) as shown in Fig. 2a, b and c. The regression parameters estimated by least-squares regression, standard deviations (σ) and correlation coefficients (R²) are displayed in Supporting Information (Table S5). Eq. (1) adequately describes the experimental binodal curves as confirmed by the correlation coefficients obtained. The experimental TLs in each ATPS, along with their respective length, and at the compositions for which extraction and concentration studies were conducted are reported in Table 1.

3.2. Extraction of biomarkers

The effect of the IL in the extraction of EPI, NOR and VMA with ATPS was evaluated using a common mixture composition (30 wt% of IL, 15 wt% of K₃PO₄ and 55 wt% of synthetic urine containing EPI, NOR and VMA at 1 g.L⁻¹). The respective extraction efficiencies are depicted in Fig. 3 (Detailed data are provided in the Supporting Information Table S6). The top and bottom phase of all systems correspond to the IL-rich phase and salt-rich phase, respectively. In general, all ATPS show a high ability to extract EPI, NOR and VMA. With the exception of the ATPS constituted by [C₈mim]Cl, for which EPI, NOR and VMA preferentially partitions to the salt-rich phase, in all other ATPS the cancer biomarkers extraction occurs to the IL-phase and is substantially high, reaching extraction efficiencies of 99.10% in a single-step.

The effect of the IL cation alkyl chain length on the cancer biomarkers extraction can be appraised with the series of 1-alkyl-3-methylimidazolium cations with the same anion (Cl⁻). The ILs extraction ability for the 3 cancer biomarkers follows the

**Fig. 3.** Graphical representation of concentration factors determined by the lever-arm rule in ATPS composed of [C₈mim][CH₃SO₄] + K₃PO₄ + synthetic urine: (A) Different compositions along the same TL obtained by applying the lever-arm rule which allow obtaining different concentration factors. (B) Comparison between experimental and predicted concentration factors studied.
rank: \([\text{C}_2\text{mim}]\text{Cl} > [\text{C}_4\text{mim}]\text{Cl} > [\text{C}_6\text{mim}]\text{Cl}\). EPI, NOR and VMA are relatively hydrophilic molecules, i.e. small organic molecules with aromatic rings and several hydroxyl groups that favor their interaction with water. Therefore, increasing the cation alkyl chain length leads to a less polar character of the IL-rich phase and lower affinity of biomarkers to this phase.

Since the higher extraction efficiencies were achieved using the IL with the shorter cation alkyl chain length, further systems containing \([\text{C}_2\text{mim}]\)-based ILs with different anions were studied. The extraction efficiencies of EPI, NOR and VMA decrease in the order: \([\text{CH}_3\text{SO}_4]^- > \text{Cl}^- > [\text{N(CN)}_2]^- > [\text{DMP}]^- > \text{Br}^- > [\text{Tos}]^- > [\text{SCN}]^-\).

The highest extraction efficiency of all biomarkers was achieved using an aqueous two-phase system composed of \([\text{C}_2\text{mim}]\text{][CH}_3\text{SO}_4]\) and \(K_3\text{PO}_4\), with 99.1%. Since 100% of extraction efficiency was not obtained, a different mixture composition (30 wt% of \([\text{C}_2\text{mim}]\text{][CH}_3\text{SO}_4]\) + 25 wt% of \(K_3\text{PO}_4\) + 45 wt% of an aqueous solution containing EPI, NOR and VMA at 1 g.L\(^{-1}\)) was used, increasing the salt concentration to promote the biomarkers salting-out to the opposite phase. With this composition, the complete extraction of EPI, NOR and VMA was achieved in the IL-rich phase in a single step, being this the mixture point with the tie-line that will be used in the concentration factor studies (Table 1).

Overall, the extraction efficiencies obtained in this work are higher than those observed in traditional solid-phase extraction methods commonly applied for the extraction of EPI, NOR and VMA [41,42]. Daneshfar and Khezeli (2015) [41] developed a micro-solid-phase extraction procedure with \(\text{Fe}_3\text{O}_4@\text{MIL-100(Fe)}\) core–shell nanoparticles grafted with pyrocatechol to extract dopamine, epinephrine and norepinephrine from human fluids (human urine and serum) using deep eutectic solvents (DES) as solvents. While achieving a high recovery yield (>91.4%) this procedure is expensive and requires extra steps, such as the target analytes desorption/elution before analysis. In order to increase the selectivity in solid-phase extraction, aminophenylboronic acid-functionalized magnetic nanoparticles were applied to extract the same metabolites from human serum and urine [42]. The recovery yields obtained varied between 96.8 and 97.5% from human urine samples, but facing the same disadvantages associated to solid-phase extraction. Remarkable extraction efficiencies for EPI, NOR and VMA were obtained in this work applying IL-based ATPS, allowing the simultaneous extraction, concentration and sampling for analysis.

### 3.3. Concentration of biomarkers in \([\text{C}_2\text{mim}]\text{][CH}_3\text{SO}_4]\)-based aqueous two-phase systems

Aiming at concentrate the cancer biomarkers required to promote an ACC early diagnosis, the ATPS with the mixture...
composition of 30 wt% of [C2mim][CH3SO4] + 25 wt% of K3PO4 + real urine (containing EPI, NOR and VMA at 0.1 ng.L−1) was studied. This mixture composition allows the cancer biomarkers complete extraction in one step and provides a long TL that affords high concentration factors. Accordingly, several ATPS were prepared at different compositions along the same TL (Fig. 4a), while reducing the IL-rich phase volume and increasing the concentration factor. In the same TL, concentration factors up to 500-fold were achieved. The results obtained for the IL concentration effect and a comparison between theoretical (by the application of the lever-arm rule) and experimental concentration factors are displayed in Fig. 4b. Each IL-rich phase was then analyzed by UHPLC, guaranteeing that the concentration factor achieved is key and still allows the complete extraction of EPI, NOR and VMA. The extraction efficiency results corresponding to each system are depicted in Fig. 5, all corresponding to the complete extraction of all biomarkers to the IL-rich phase in one step. These results also demonstrate that there is no saturation of the IL-rich phase with the biomarkers, further confirming the high solvation ability afforded by ILs.

The UHPLC chromatograms obtained from EPI, NOR and VMA are shown in Fig. 6, demonstrating that after their extraction from human urine cancer biomarkers are only present in the IL-rich phase. Furthermore, biomarkers can be quantified individually without interference of the IL present in the phase. Overall, the major novelty of this work consists on the development of an easy, fast and low-cost pre-treatment method that allows the concentration of cancer biomarkers in a single-step. An easy handling procedure can thus be used to pretreat human fluids and increase the concentration of biomarkers, further contributing to the early stage diagnosis of ACC.

4. Conclusions

In order to overcome the challenges in cancer early-stage detection, in this work IL-based ATPS were evaluated as pre-treatment strategies of human fluids, i.e. to extract and concentrate adrenal cancer biomarkers, namely epinephrine, norepinephrine and vanillylmandelic acid, from human urine. For that purpose, phase diagrams composed of IL + K3PO4 + urine, and respective tie-lines and tie-line lengths, were determined. In general, the studied cancer biomarkers preferentially migrate to the IL-rich phase, with the exception of the ATPS constituted by the IL with a longer alkyl chain ([C2mim][Cl]). The tailoring of the amount of phase-forming components (IL and salt) allows the complete extraction of cancer biomarkers to the IL-rich phase in one step, while resulting in a long tie-line length that allows high concentration factors. Along the same TL, the IL-rich phase volume was tuned to achieve a concentration factor up to 500-fold. In this mixture composition and concentration factor, all cancer biomarkers are completely extracted from human urine in a single-step, further allowing their identification and quantification by UHPLC. According to the reported results, IL-based ATPS can be foreseen as a novel, fast and low-cost pretreatment strategy of human fluids to improve cancer detection and monitoring.

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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Appendix A. Supplementary data

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