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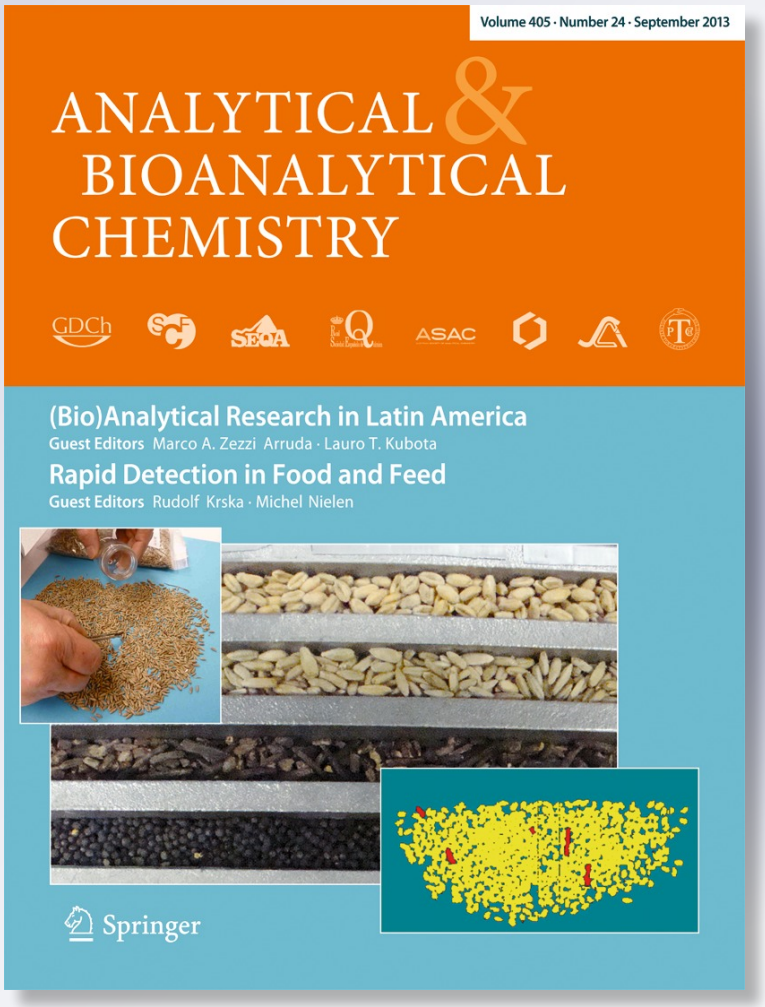


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REVIEW

# Bioanalytical separation and preconcentration using ionic liquids

**Leticia B. Escudero · Alexander Castro Grijalba · Estefanía M. Martinis · Rodolfo G. Wuilloud**

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**Abstract** Ionic liquids (ILs) are novel solvents that display a number of unique properties, such as negligible vapor pressure, thermal stability (even at high temperatures), favorable viscosity, and miscibility with water and organic solvents. These properties make them attractive alternatives to environmentally unfriendly solvents that produce volatile organic compounds. In this article, a critical review of state-of-the-art developments in the use of ILs for the separation and preconcentration of bioanalytes in biological samples is presented. Special attention is paid to the determination of various organic and inorganic analytes—including contaminants (e.g., pesticides, nicotine, opioids, gold, arsenic, lead, etc.) and functional biomolecules (e.g., testosterone, vitamin $B_{12}$, hemoglobin)—in urine, blood, saliva, hair, and nail samples. A brief introduction to modern microextraction techniques based on ILs, such as dispersive liquid–liquid microextraction (DLLME) and single-drop microextraction (SDME), is provided. A comparison of IL-based methods in terms of their limits of detection and environmental compatibilities is also made. Finally, critical issues and challenges that have arisen from the use of ILs in separation and preconcentration techniques are also discussed.

**Keywords** Ionic liquids · Microextraction · Separation · Preconcentration · Biological samples

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## Abbreviations

### Extraction techniques

| | |
|---|---|
| ATPS | Aqueous two-phase system |
| CV-ILAHS-SDME | Cold vapor ionic-liquid-assisted headspace single-drop microextraction |
| CVG | Cold vapor generation |
| cycle-flow SDME | Cycle-flow single-drop microextraction |
| DI-SDME | Directly immersed in a stirred solution single drop microextraction |
| DLLME | Dispersive liquid–liquid microextraction |
| dLPME | Dynamic liquid-phase microextraction |
| FI-AFS | Flow-injection atomic fluorescence spectrometry |
| HF-LPME | Hollow-fiber liquid-phase microextraction |
| HS-SDME | Headspace single-drop microextraction |
| LLE | Liquid–liquid extraction |
| LLME | Liquid–liquid microextraction |
| LPME | Liquid-phase microextraction |
| MA-DLLME | Microwave-assisted dispersive liquid–liquid microextraction |
| SBSE | Stir-bar sorptive extraction |
| SDME | Single-drop microextraction |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SI-DLLME | Sequential injection dispersive liquid–liquid microextraction |
| SPE | Solid-phase extraction |
| SPME | Solid-phase microextraction |

| | |
|---|---|
| TA-DLLME | Temperature-assisted dispersive liquid–liquid microextraction |
| UA-DLLME | Ultrasound-assisted dispersive liquid–liquid microextraction |

**Detection technique**

| | |
|---|---|
| CE-DAD | Capillary electrophoresis–diode array detector |
| CV-AAS | Cold-vapor atomic absorption spectrometry |
| ETAAS | Electrothermal atomic absorption spectrometry |
| ETV-ICP-MS | Electrothermal vaporization–inductively coupled plasma–mass spectrometry |
| FAAS | Flame atomic absorption spectrometry |
| GC | Gas chromatography |
| GC-MS | Gas chromatography–mass spectrometry |
| HPLC | High-performance liquid chromatography |
| MALDI | Matrix-assisted laser desorption/ionization |

**Ionic liquids**

| | |
|---|---|
| $[C_4mim][BF_4]$ | 1-Butyl-3-methylimidazolium tetrafluoroborate |
| $[C_6mim][BF_4]$ | 1-Hexyl-3-methylimidazolium tetrafluoroborate |
| $[C_4mim]$ $[CF_3SO_3]^-$ | 1-Butyl-3-methylimidazolium trifluoromethanesulfonate |
| $[C_2mim]$ $[(CF_3SO_3)_2\ N]$ | 1-Ethyl-3-methylimidazolium bis[(trifluoromethylsulfonyl)]imide |
| $[C_8mim]$ $[(CF_3SO_3)_2\ N]$ | 1-Octyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide |
| $[C_4mim][Cl]$ | 1-Butyl-3-methylimidazolium chloride |
| $[C_6mim][Cl]$ | 1-Hexyl-3-methylimidazolium chloride |
| $[C_4mim][OH]$ | 1-Butyl-3-methyl imidazolium hydroxide |
| $[C_4mim][PF_6]$ | 1-Butyl-3-methylimidazolium hexafluorophosphate |
| $[C_6mim][PF_6]$ | 1-Hexyl-3-methylimidazolium hexafluorophosphate |
| $[C_8mim][PF_6]$ | 1-Octyl-3-methylimidazolium hexafluorophosphate |
| $[C_4mpd][Br]$ | 1-Butyl-3-methylpyridinium bromide |
| $[C_4mprd][Br]$ | 1-Butyl-3-methylpyrrolidinium bromide |
| $[C_4tmsim][PF_6]$ | 1-Butyl-3-trimethylsilylimidazolium hexafluorophosphate |
| CYPHOS® IL 101 | Trihexyl(tetradecyl)phosphonium chloride |
| $[EC_2mim]$ $[(CF_3SO_2)_2\ N]$ | 1-Ethoxyethyl-3-methylimidazolium bis[(trifluoromethylsulfonyl)]imide |
| [Nmim][Cl] | *N*-methylimidazolium chloride |
| $[C_1oim][BF_4]$ | 1-Methyl-3-octylimidazolium tetrafluoroborate |
| $[PPmim][PF_6]$ | *N*,*N*-bis[2-methylbutyl]imidazolium hexafluorophosphate |
| $(PY\ BS)_3$ $PW_{12}O_{40}$ | Keggin-based ionic liquid |

## Introduction

Ionic liquids (ILs) are liquid salts with melting points close to or below room temperature [1]. They generally consist of a nitrogen- or phosphorus-based organic cation that is counterbalanced by an organic or inorganic anion [2]. It has been reported that the first IL was observed in the nineteenth century [3]. However, it took a very long time for these solvents to begin to attract the attention of researchers; for instance, 1-alkyl-3-methylimidazolium chloroaluminate was first reported in 1982 by Wilkes et al. [4].

Recently, there has been considerable interest in the use of ILs as an alternative to regular solvents that produce volatile organic compounds (VOCs). Besides their low melting points, ILs displays many other useful physicochemical properties, including air and moisture stability, good thermal stability (even at high temperatures), wide electrochemical windows, relatively favorable viscosity, and good abilities to extract metal ions and organic compounds [5].

Due to the drive for "green chemistry," the use of state-of-the-art solvents such as ILs has been increasing in the field of analytical chemistry. Thus, ILs have been proposed as innovative stationary phases in gas chromatography (GC) [6–9], electroosmotic flow modifiers in capillary electrophoresis (CE) [10, 11], additives to mobile phases for high-performance liquid chromatography (HPLC) [12], matrices for matrix-assisted laser desorption/ionization (MALDI) [13, 14], as well as solvents for electroanalytical applications [15, 16]. In the last few years, ILs have been widely used as novel media for the extraction and separation of different analytes [17–20]. These procedures, when combined with suitable analytical instrumentation, have been applied for the determination of metal ions and organic and biological compounds. However, due to the very low concentrations of some analytes in the samples studied, preconcentration steps have usually been incorporated. Moreover, preconcentration is important because it can minimize or even eliminate matrix effects and concomitants, improving detection limits and enhancing the sensitivity of detection techniques towards several analytes.

Some reviews referring to separation and preconcentration techniques that use ILs have been published in recent years [21, 22]. However, to the best of our knowledge, there are no reviews that have specifically covered applications of ILs in the field of bioanalysis. Therefore, a timely review of the recent applications of ILs in bioanalytical separation and preconcentration procedures is presented here. This work focuses on the determination of several organic and

inorganic analytes, including contaminants (e.g., pesticides, nicotine, arsenic, lead, etc.), as well as functional biomolecules (e.g., testosterone, vitamin B12, hemoglobin) in biological samples such as urine, blood, saliva, hair, and nail samples. It is divided into a number of sections. The ILs that are most commonly used in analytical chemistry for bioanalysis are described, as well as classes of ILs and some of their properties. A brief introduction to modern extraction techniques based on ILs, such as liquid-phase microextraction (LPME), solid-phase extraction (SPE), single-drop microextraction (SDME), solid-phase microextraction (SPME), and stir-bar sorptive extraction (SBSE), is given. Furthermore, instrumental techniques for IL-based methods, including electrothermal atomic absorption spectrometry (ETAAS), flame atomic absorption spectrometry (FAAS), cold-vapor atomic absorption spectrometry (CV-AAS), high-performance liquid chromatography (HPLC), GC, CE, as well as a comparison of IL-based methods in terms of their analytical performance and environmental compatibility are presented. Critical problems and challenges are also discussed. Finally, a perspective on the future application of ILs to organic compound and metal determination is included.

## Classes and properties of ionic liquids used in bioanalysis

Over the last two decades, the number of publications concerning ILs has increased substantially, highlighting the continually growing interest in them in many different research fields. As can be observed in Fig. 1, the number of publications on ILs barely increased from 1992 to 2000. However, since then, significant growth in this number has occurred, which indicates the great potential of ILs for chemical analysis. Indeed, this rise in IL-related publications also shows that researchers have begun to explore chemistry that is more eco-friendly, innovative, and sustainable. Moreover, it is estimated that there could be as many as $10^{18}$ ILs that are potentially available for different applications [23].

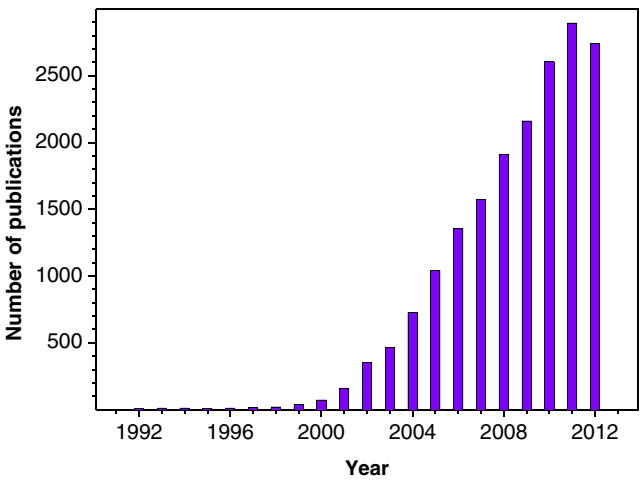



**Fig. 1** Number of articles on ILs published worldwide according to year of publication. Data obtained from a search of the Scopus database (http://www.scopus.com) using the term "ionic liquids" as a single search filter

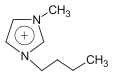
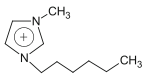
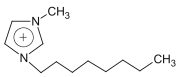
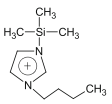
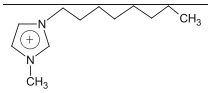
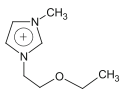
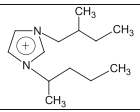
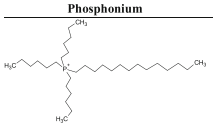
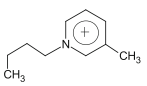
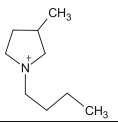
Typically, ILs used in analytical chemistry consist of organic cations (including imidazolium, phosphonium, pyrrolidinium, pyridinium, or quaternary ammonium) and anions such as hexafluorophosphates, tetrafluoroborates, alkylsulfates, alkylsulfonates, trifluoromethanesulfonate, bis(trifluoromethylsulfonyl)imide, nitrate, acetate, hydroxide, chloride, or bromide [21, 24, 25]. However, only a few types of ILs have been used in bioanalytical separation and preconcentration procedures. Table 1 summarizes the ILs used in this field and their main properties.

Imidazolium-type ILs have been widely utilized in analytical chemistry. This could be due to their relatively low cost and straightforward synthesis. Furthermore, they offer a variety of properties that depend on the length of the alkyl chain of the imidazolium ring and the counteranion, such as low melting points, reusability, tunable viscosity, and solubility. In previous contributions, it was observed that viscosity increases in proportion with the length of the alkyl chain, while solubility in water decreases [24]. Therefore, both parameters must be considered when selecting an appropriate extraction phase, since low solubility allows minimal IL consumption, while high viscosity could cause practical drawbacks during microextraction procedures. Furthermore, it should be considered that an increase in the length of the alkyl chain is often followed by the formation of aggregates of ILs in water above a certain concentration (IL-based surfactants) [26, 27].

As shown in Table 2, 1-Hexyl-3-methylimidazolium hexafluorophosphate ($[C_6mim][PF_6]$) and 1-Butyl-3-methylimidazolium hexafluorophosphate ($[C_4mim][PF_6]$) have been the ILs most commonly used in bioanalytical preconcentration and separation techniques. This could be expected, considering the high chemical affinity that imidazolium ILs show for different metal ions and biological and organic compounds. Although 1-Octyl-3-methylimidazolium hexafluorophosphate ($[C_8mim][PF_6]$) has a very low solubility in water, is highly capable of extracting analytes, and shows good chromatographic behavior [28], it has not been widely used for bioanalytical applications. The high viscosity of $[C_8mim][PF_6]$ is probably the main disadvantage of this IL.

On the other hand, phosphonium-type ILs have been barely used in this field. It is well known that tetraalkylphosphonium-type ILs are thermally and chemically stable [22]. Unlike imidazolium ILs, phosphonium ILs have lower densities than water, which is a significant drawback in classical liquid–liquid microextraction (LLME) techniques, as the IL phase remains in the upper part of the extraction vessel.

**Table 1** Main properties of ionic liquids used for bioanalytical separation and preconcentration

| Cation | Anion | Ionic liquid | Molecular weight (g $mol^{-1}$) | Melting point (K) | Density (g $ml^{-1}$) | Viscosity (cP, 25°C) | Ref. |
|---|---|---|---|---|---|---|---|
| **Imidazolium** | | | | | | | |
|  | $[Cl]^-$ | 1-Butyl-3-methylimidazolium chloride | 174.67 | 314.15 | 1.08 | 3950 | [92,93] |
| | $[PF_6]^-$ | 1-Butyl-3-methylimidazolium hexafluorophosphate | 284.18 | 283.15 | 1.35 | 397 | [92] |
| | $[OH]^-$ | 1-Butyl-3-methylimidazolium hydroxide | 156.22 | n.a. | n.a | n.a. | - |
| | $[BF_4]^-$ | 1-Butyl-3-methylimidazolium tetrafluoroborate | 226.03 | 198.15 | 1.19 | 219 | [94] |
| | $[CF_3SO_3]^-$ | 1-Butyl-3-methylimidazolium trifluoromethanesulfonate | 288.29 | 289.55 | 1.29 | 90 | [95] |
|  | $[Cl]^-$ | 1-Hexyl-3-methylimidazolium chloride | 202.73 | 198.15 | 1.03 | 716 | [96] |
| | $[PF_6]^-$ | 1-Hexyl-3-methylimidazolium hexafluorophosphate | 312.24 | 212.15 | 1.30 | 560 | [24] |
| | $[BF_4]^-$ | 1-Hexyl-3-methylimidazolium tetrafluoroborate | 254.08 | 190.75 | 1.16 | 310 | [97,98] |
|  | $[PF_6]^-$ | 1-Octyl-3-methylimidazolium hexafluorophosphate | 340.29 | 233.15 | 1.22 | 810 | [92,98] |
| | $[BF_4]^-$ | 1-Octyl-3-methylimidazolium tetrafluoroborate | 282.13 | 194.15 | 1.11 | 440 | [98] |
| | $[(CF_3SO_3)_2N]^-$ | 1-Octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide | 475.47 | n.a. | 1.32 | 119.3 | [99,100] |
|  | $[PF_6]^-$ | 1-Butyl-3-trimethylsilylimidazolium hexafluorophosphate | 300.26 | n.a. | n.a. | n.a. | - |
|  | $[BF_4]^-$ | 1-Methyl-3-octylimidazolium tetrafluoroborate | 282.13 | n.a. | 1.12 | n.a. | - |
|  | $[(CF_3SO_2)_2N]^-$ | 1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide | 391.32 | 256.15 | 1.52 | 18 | [98] |
| | $[PF_6]^-$ | 1-Ethyl-3-methylimidazolium hexafluorophosphate | 256.13 | 331.15 | 1.40 | - | [98,101,102] |
| | $[BF_4]^-$ | 1-Ethyl-3-methylimidazolium tetrafluoroborate | 197.97 | 279.15 | 1.248 | 66 | [98] |
|  | $[(CF_3SO_2)_2N]^-$ | 1-Ethoxyethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide | 435.36 | n.a. | n.a. | n.a. | - |
|  | $[PF_6]^-$ | N,N-bis[2-methylbutyl]imidazolium hexafluorophosphate | 356.96 | n.a. | n.a. | n.a. | - |
|  | $[Cl]^-$ | N-methylimidazolium chloride | 118.56 | 345.15 | 1.03 | n.a. | [103] |
| **Phosphonium** | | | | | | | |
|  | $[Cl]^-$ | Trihexyl(tetradecyl)phosphonium chloride Cyphos®IL 101 | 519.30 | 203 | 0.895 | 2077 | [104] |
| **Pyridinium** | | | | | | | |
|  | $[Br]^-$ | 1-Butyl-3-methylpyridinium bromide | 230.14 | 230 | n.a. | n.a. | [105] |
| | $[PW_{12}O_{40}]^{3-}$ | Keggin based ionic liquid | n.a. | n.a. | n.a. | n.a. | [90] |
| **Pyrrolidinium** | | | | | | | |
|  | $[Br]^-$ | 1-Butyl-3-methylpyrrolidinium bromide | 222.17 | n.a. | n.a. | n.a. | - |

n.a., not available

**Table 2** Analytical performances of IL-based methods involving bioanalytical separation and preconcentration

| Analyte | Sample | Sample preparation | IL | IL amount | Purpose of IL | Detection limit ($\mu g\ L^{-1}$) | Detection technique | Reference |
|---|---|---|---|---|---|---|---|---|
| Testosterone | Urine | ATPS | $[C_4mim][Cl]$ | 200 mg | Extraction solvent | 1 | RP-HPLC-UV | [62] |
| Thallium | Urine | SI-DLLME | $[C_6mim][PF_6]$ | 37 μL | Extraction solvent | 0.86 | FAAS | [65] |
| Cobalt | Urine and saliva | DLLME | $[C_6mim][PF_6]$ | 60 mg | Extraction solvent | $3.8\times10^{-3}$ | ETAAS | [64] |
| Arsenic | Urine | DLLME | $[C_6mim][PF_6]$ | 60 mg | Extraction solvent | $1\times10^{-2}$ | ETAAS | [37] |
| Arsenic | Urine and blood | DLLME | $[C_4mim][PF_6]$ | 200 mg | Extraction solvent | $5\times10^{-3}$ | FI-HGAAS | [36] |
| Vitamin B12 | Urine | ATPS | $[C_6mim][Cl]$ | 200 mg | Extraction solvent | 90 | HPLC-UV–vis | [63] |
| Celastrol | Urine | DLLME | $[C_6mim][PF_6]$ | 45 μL | Extraction solvent | 1.6 | HPLC-DAD | [68] |
| Antiinflammatory drugs | Urine | DLLME | $[C_4mim][PF_6]$ | 280 μL | Extraction solvent | 8.3–32 | LC-UV | [34] |
| Antiinflammatory drugs | Urine | dLPME | $[C_4mim][PF_6]$ | 50 μL | Extraction solvent | 38–70 | LC-UV | [18] |
| Phenothiazine | Urine | dLPME | $[C_4mim][PF_6]$ | 50 μL | Extraction solvent | 21–60 | LC-UV | [69] |
| Clozapine | Urine and serum | LLME | $[C_2mim][(CF_3SO_2)_2N]^-$ | 100 μL | Extraction solvent | 3–11 | CE-DAD | [35] |
| Emodin | Urine | DLLME | $[C_6mim][PF_6]$ | 50 μL | Extraction solvent | 0.1 | HPLC-UV | [38] |
| Nicotine | Urine and plasma | HF-LPME | $[C_4mim][PF_6]$ | 5 μL | Extraction solvent | 50 | HPLC-UV | [70] |
| Proteins | Urine | ATPS | $[C_4mim][Cl]$ | 300 μL | Extraction solvent | 800 | UV–Vis and FTIR | [19] |
| PCBs and PBDEs | Urine | DLLME | $[C_8mim][PF_6]$ | 40 μL | Extraction solvent | 0.1 | HPLC-DAD | [66] |
| Antihypertensive drugs | Urine | DLLME | $[C_8mim][PF_6]$ | 50 mg | Extraction solvent | 1.5–3.3 | HPLC-DAD | [67] |
| Benzophenone | Urine | SDME | $[C_6mim][PF_6]$ | 5 μL | Extraction solvent | 1.3 | LC-UV | [71] |
| Amphetamine | Urine | SPME | $[EC_2mim][(CF_3SO_2)_2N]$ | n.a. | Extraction solvent | 0.5 | GC-MS | [72] |
| Sarcosine | Urine | SPME | n.a. | n.a. | Extraction solvent | n.a. | GC-MS | [6] |
| Antidepressant | Urine | SPE | $[C_4mim][CF_3SO_3]$ | n.a. | Additive | 12.3–90.1 | HPLC-UV | [75] |
| Co, Hg, and Pb | Serum | SDME | $[C_4mim][PF_6]$ | 3 μL | Extraction solvent | $1.5\times10^{-3}$ to $9.8\times10^{-3}$ | ETV-ICP-MS | [85] |
| Pesticides | Hair | HF-SPME | $[C_4mim][OH]$ | 75 mg | Aid incorporation of nanoparticles into extractant phase | $3.0\times10^{-3}$ to $8.0\times10^{-3}$ | HPLC-DAD | [89] |
| Au(III) and Ag(I) | Hair | DLLME | $[C_6mim][PF_6]$ | 250 μL | Extraction solvent | Au(III)=$4.8\times10^{-3}$ Ag(I)=$2.6\times10^{-3}$ | ETAAS | [50] |
| As(III) | Hair and nails | LLE | $[C_6mim][PF_6]$ | 30 mg | Extraction solvent | $6\times10^{-3}$ | ETAAS | [49] |
| Hemoglobin | Blood | LLE | $[C_4tmsim][PF_6]$ | 50 μL | Extraction solvent | 0.1 | UV–vis | [77] |
| Pd | Blood | TA-DLLME | $[C_6mim][BF_4]$ | 60 μL | Extraction solvent | 0.2 | UV–Vis spectrometry | [82] |

**Table 2** (continued)

| Analyte | Sample | Sample preparation | IL | IL amount | Purpose of IL | Detection limit ($\mu g L^{-1}$) | Detection technique | Reference |
|---|---|---|---|---|---|---|---|---|
| Sulfonamides | Pig plasma | MADLLME | $[C_6mim][PF_6]$ | 100 μL | Extraction solvent | 0.018–0.033 | HPLC–fluorescence spectrophotometry | [83] |
| Pb | Blood | TA-DLLME | $[C_4mim][PF_6]$ | 45 μL | Extraction solvent | 0.13 | FAAS | [48] |
| Δ6-Monounsaturated fatty acids | Hair and nails | - | n.a. | n.a. | Stationary phase modifier | n.a. | GC-MS | [88] |
| Organophosphorus pesticides | Hair | HF-SPME | (PY BS)3PW12O40 (pyridinium IL) | 30 mg | Facilitates particle incorporation into the extractant phase | 0.0074–1.3000 $\mu g\ g^{-1}$ | HPLC–PDA | [90] |
| Hg species | Hair | CV-ILAHS-SDME | CYPHOS® IL 101 | 0.1 mg | Extraction solvent | 0.01 | ETAAS | [91] |
| $Ag^+$ | Hair | TA-DLLME | $[C_6mim][PF_6]$ | | Extraction solvent | $5.2\times10^{-3}$ | ETAAS | [106] |
| Isoquinoline alkaloids | Blood | TA-DLLME | $[C_8mim][PF_6]$ | 200 μL | Extraction solvent | 0.089–0.124 | AFS | [81] |
| Carvedilol | Serum | SBSE | $[C_1mim][BF_4]$ | n.a. | Desorption solvent | 0.3 | HPLC-UV | [78] |
| Cytochrome c | Blood | SPE | $[PPmim][PF_6]$ | 9.5 g | Template for the preparation of porous nano-$TiO_2$ particles | n.a. | SDS-PAGE | [79] |
| Hemoglobin | Blood | DLLME | $[C_4mim][PF_6]$ | 2 mL | Water-in-ionic liquid reverse microemulsion | n.a. | SDS-PAGE | [107] |
| Basic proteins | Blood | SPE | [Nmim][Cl] | n.a. | Inmobilization on PVC to adsorb proteins | n.a. | UV–vis | [80] |
| Hemoglobin | Blood | SPE | $[PPmim][PF_6]$ | 1 g | Adsorption improvment on nanoparticles | n.a. | UV–vis | [108] |
| Proteins | Serum | No extraction | $[C_x mim][BF_4]$ *x*: 2,4,6,8<br>$[C_4mim]Cl$<br>$[C_4mim]Br$<br>$[C_4mpd]Br$<br>$[C_4mprd]Br$ | 20 μL | Polyacrylamide gel modifier | n.a. | SDS-PAGE | [84] |
| Sildenafil | Blood | CE | 1-Methylimidazolium chloride | n.a. | Capillary modifier | n.a. | CE-DAD | [109] |
| Vanadium | Saliva | On-line TA-DLLME | $[C_4mim][PF_6]$ | 40 μL | Extraction solvent | $4.8\times10^{-3}$ | ETAAS | [87] |
| Nitrite | Saliva | DLLME | $[C_8mim][(CF_3SO_2)_2\ N]^-$ | 22 μL | Extraction solvent | $5\times10^{-2}$ | HPLC-UV | [86] |

*n.a.* not available

Pyridinium ILs has not been frequently used for bioanalytical separation and preconcentration techniques. Although pyrrolidinium ILs can be used instead of imidazolium or pyridinium ILs, they have also been rarely applied in the field of bioanalysis.

## Sample preparation using ionic liquids

Sample preparation is a very important step in most analytical methods. It remains a challenging part of chemical studies, especially when biological samples are involved [29]. Instrumental techniques are either not often sensitive enough to allow analyte determination in biological samples, or the results are distorted by interfering species. Therefore, the isolation of toxicologically relevant compounds or functional biomolecules from biological matrices is essential for their successful detection and identification. Different methods have been applied to isolate analytes from biological specimens; the most frequently used procedures are LLE and SPE [30]. LLE is recommended as it is a simple procedure and works especially well with biological fluids. Moreover, it is based on well-defined thermodynamic relationships and has a wide dynamic range. However, one major current research trend is the miniaturization of traditional LLE approaches, with the aim being to reduce costs, analysis time, reagents, and sample consumption while increasing separation efficiency and enabling automation [31]. Therefore, the implementation of ILs in modern microextraction techniques has attracted considerable attention in recent years in relation to the analysis of biological samples [32, 33]. The main advantages of this recently devised analytical strategy are very low consumption of solvent and high extraction performance. Moreover, the utilization of ILs in microextraction techniques offers important advantages when developing environmentally friendly analytical methods. The equipment needed for IL-based LPME is generally simple and inexpensive. Several reports have been published on the successful application of ILs in the extraction of organic and inorganic analytes from biological samples using LPME systems [34–36]. Thus, there are a wide range of novel LPME techniques, including SDME in different modes (direct SDME, cycle-flow SDME), hollow-fiber liquid-phase microextraction (HF-LPME), and dispersive liquid–liquid microextraction (DLLME) in its various forms (Fig. 2).

SDME is an interesting and promising approach in which the extraction phase is a drop of solvent, usually suspended in the needle of a syringe, which is directly immersed in a stirred solution (DI-SDME) or placed in close contact with its headspace (HS-SDME). This technique is characterized by its simplicity and affordability, as a particular commercial source is not required. Moreover, the unique properties of hydrophobic ILs allow their direct implementation as extraction solvents in SDME techniques.

Despite the practical advantages of ILs in SDME and cycle-flow SDME, such as the high stability of a microdrop, the hanging drop can still suffer from limited stability and it may be knocked into the sample during extraction. Furthermore, the volume of IL used as the extraction solvent is limited to a few microliters. In order to overcome this problem, a novel alternative to LPME, termed HF-LPME, was proposed. HF-LPME is a solvent-minimized technique in which a hollow fiber containing the extraction solvent is affixed to the tip of a syringe needle and used to extract analytes from the sample. The sample can be stirred or shaken vigorously without any loss of extraction liquid as it is protected mechanically.

In order to reduce the analysis time, a novel version of the LPME technique termed DLLME, based on a ternary-component solvent system or the application of ultrasound or heat, has been recently proposed [34, 36–38]. In this method, an appropriate mixture of extraction and dispersive solvents is rapidly injected into the sample by a syringe, and a cloudy solution is formed. The analyte in the sample is extracted into the fine droplets of extraction solvent that were initially formed. After extraction, phase separation is performed by centrifugation, and analyte enriched in the sedimented phase is determined by the detection technique. DLLME is the method most commonly applied to treat biological samples due to its simplicity, rapidity, and low cost. Additionally, high recovery and enrichment factors can be obtained due to the infinitely large surface area formed between the IL phase and the sample. However, this technique has some drawbacks related to the high viscosity shown by hydrophobic ILs and the need for a third component to obtain a dispersion. The use of a third component (a surfactant, a hydrophilic IL, or an organic solvent) as a disperser in DLLME could result in a decrease in the partition coefficients for the partitioning of analytes into the extraction solvent [39]. Temperature-assisted DLLME (TA-DLLME) can solve this problem, as dispersion occurs upon heating due to the resulting decrease in the solubility of the IL. Furthermore, this technique is much safer than DLLME, which requires the use of volatile organic solvent. Ultrasound irradiation can also be applied to avoid the use of volatile organic solvent as the disperser in DLLME-based IL methods, in a mode known as ultrasound-assisted DLLME (UA-DLLME) [40–42]. Furthermore, since ILs absorb microwave irradiation extremely well and transfer energy quickly by ionic conduction [43], IL-based microwave-assisted dispersive liquid–liquid microextraction (MA-DLLME) has been developed. This strategy could improve extraction efficiency and speed up analysis.

An IL-based aqueous two-phase system (ATPS) was recently developed. This technique represents an alternative

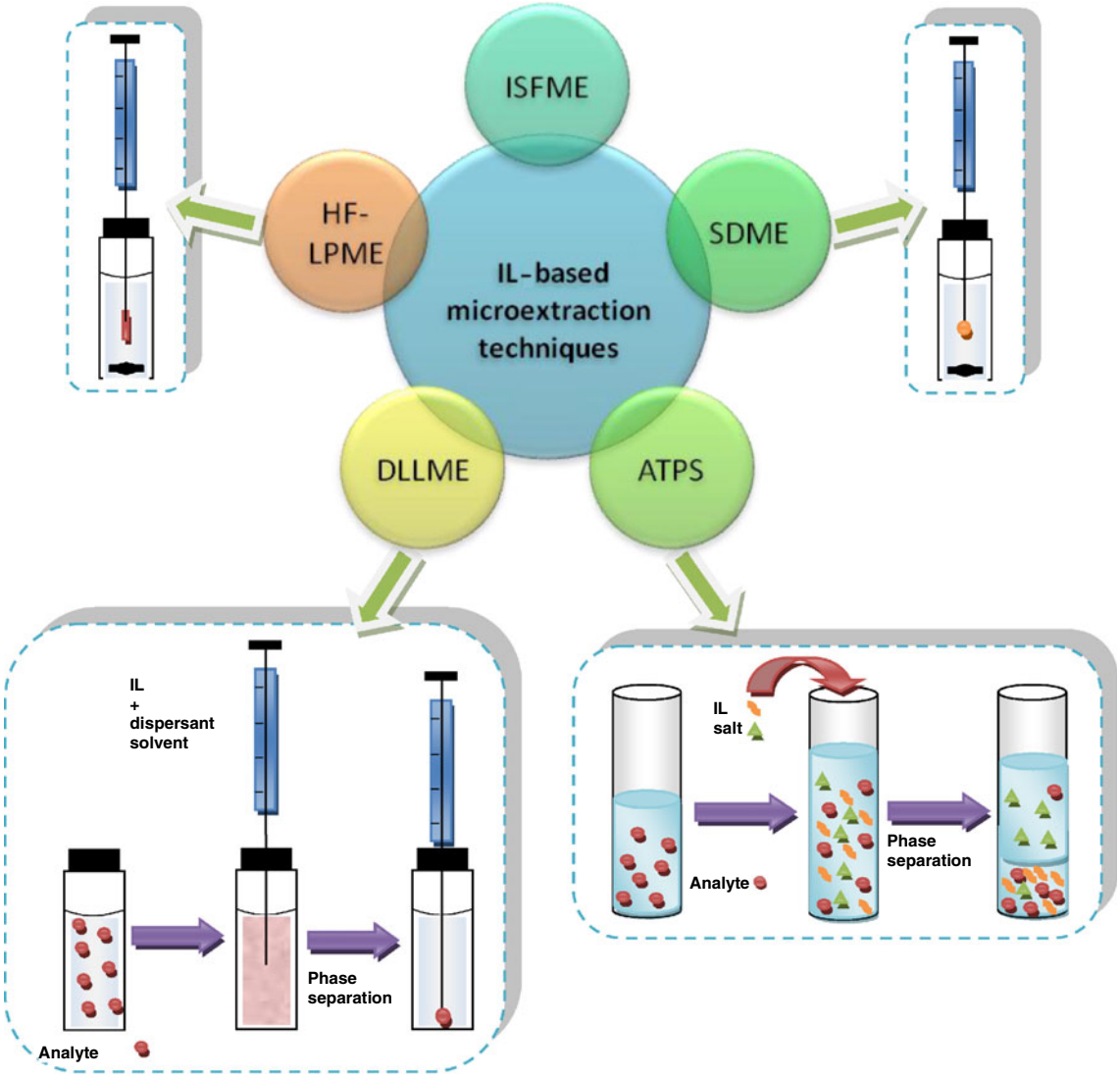



**Fig. 2** IL-based microextraction techniques applied for the analysis of biological samples

to analyte extraction that uses less viscous ILs. ATPS methods involve the application of hydrophilic ILs and a salt. During ATPS, a dispersion forms, thus generating a large interfacial contact area between the IL phase and the sample.

Another microextraction technique based on ILs is called in situ solvent formation microextraction (ISFME) [44, 45]. In this method, sodium hexafluorophosphate ($NaPF_6$) is used as an ion-pairing agent. A small amount of the salt is added to a sample solution containing very small amounts of 1-alkyl-3-methylimidazolium tetrafluoroborate ILs. A cloudy solution is then observed as a result of the formation of fine droplets of hydrophobic ILs. The IL-enriched phase is separated by means of centrifugation. ISFME is a fast, simple, and suitable method for the extraction and preconcentration of analytes from sample solutions with high salt contents. However, it should be mentioned that additional reagents are needed to synthesize the extractant phase in situ.

Most works related to the application of ILs to metal determination in biological samples have dealt with their use as extractant solvents. However, other uses of ILs have been reported. Figure 3 shows a schematic diagram of the main applications of ILs in bioanalytical separation and preconcentration techniques.

## Instrumental techniques in IL-based methods

Several instrumental techniques have been applied to bioanalytical separation and preconcentration methods, including ETAAS, flame absorption spectroscopy (FAAS), cold-vapor generation atomic absorption spectroscopy (CV-AAS), electrothermal vaporization–inductively coupled plasma–mass spectrometry (ETV-ICP-MS), UV–Vis spectrophotometry, CE, GC, and HPLC.

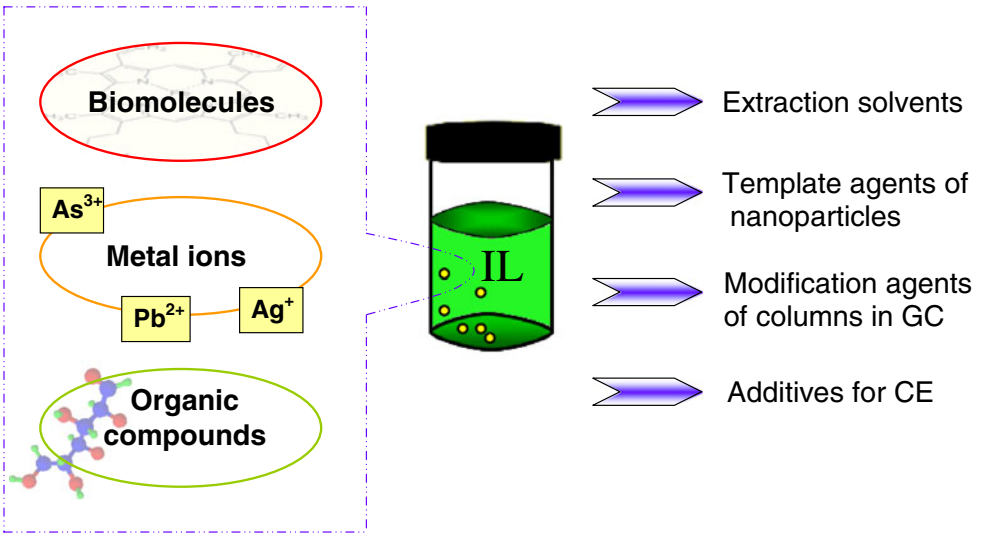



**Fig. 3** Schematic diagram of the main applications of ILs in bioanalysis

The high viscosities of most ILs need to be carefully considered during the development of a method. Thus, previous works have proposed conditioning the IL phase before introducing it into the nebulizer of the FAAS technique through the addition of a suitable diluting agent [46, 47]. For instance, Shah et al. added an aliquot of acidic methanol to the IL phase prior to its analysis by FAAS to determine trace levels of Pb in blood samples from children with different respiratory disorders [48]. In the case of the ETAAS technique, a simple alternative involves the direct and manual injection of the IL phase into the graphite furnace [49, 50]. The IL phase is usually dissolved in a minimal volume of suitable solvent to diminish the viscosity of the extractant and achieve a reproducible injection.

Ionic liquids have been successfully used for the enhanced cold-vapor generation (CVG) of transition and noble metals in aqueous solution with $KBH_4$ by flow-injection atomic fluorescence spectrometry (FI-AFS) [51, 52]. In fact, it has been shown that ILs improve the CVG of $Au^0$, which is hypothesized to be due to electrostatic interactions between the metal species and the ILs. These interactions could stabilize the unstable volatile Au species and help to rapidly isolate the analyte from the reaction mixture [52].

Regarding ICP-based detectors, the main difficulty is to directly introduce a complex matrix with a high organic load, such as an IL phase, into the instrument. This problem makes it tricky to use these instrumental techniques. It can, however, be overcome by utilizing methods involving an acid back-extraction step with a nitric acid solution. On the other hand, ETV-ICP-MS could be a suitable alternative that can easily solve this problem.

When UV–Vis detection is involved, sample introduction does not cause further drawbacks. The IL-rich phase is usually dissolved in organic solvents such us ethanol and transferred directly to the quartz cell. Nevertheless, spectral interferences and low sensitivity are potential problems with this detector.

In GC, ILs have been employed to modify stationary phases of columns [53]. Two basic approaches are used to obtain such a modified column. The first consists of coating the wall of a capillary column with the IL; the second involves coating the IL onto a support to produce a packed column. These modified columns show remarkable properties that derive from the typical properties of ILs, including high viscosity, low surface tension, high thermal stability, and low vapor pressure at elevated temperatures. Although their selectivities for different analytes are dominated by the solvation interactions of the cations and anions, all of the ILs exhibit a clear, unique, dual-natured selectivity that is quite unlike other popular nonionic stationary phases. This dual-natured selectivity provides the stationary phases with the ability to separate nonpolar molecules (like a nonpolar stationary phase) as well as polar molecules (like a polar stationary phase) [54]. However, these IL stationary phases have not been widely applied in the field of bioanalysis.

The viscosity of ILs and their spectral properties make them appropriate for use as mobile phases in HPLC. One trend has been to apply these compounds as mobile-phase additives at low concentrations (e.g., 1–10 mmol $L^{-1}$) [55]. Ionic liquids have been used as additives in mobile phases due to their role as masking agents for the residual silanols that are used in columns for reversed-phase chromatography, thus improving analyte separation and peak resolution [56]. The use of ILs as mobile-phase additives in HPLC has not yet been widely explored in bioanalysis. In fact, ILs have been used mainly as extraction agents in clean-up treatments prior to HPLC analysis.

In CE, ILs have been used as background electrolytes, bulk-solution pseudo-stationary phases, and capillary wall modifiers [57]. It has been found that the nature of the

anionic part of the IL has a slight effect on the electrophoretic mobility, but that the IL concentration influences the general electrophoretic mobility of the separation medium. Neutral analytes cannot be separated by conventional CE, but they can be separated using ILs. The analytes can be charged in the presence of the ILs or can form complexes with IL anions. This last process is known as heteroconjugation [58]. However, a disadvantage of using ILs in this technique is the extensive time required to characterize the ILs and to develop the method. Thus, it is necessary to gather information about the specific properties of the ILs that could influence the electrophoretic separation, such as their viscosity, conductivity, polarity, solubility, etc.

## Ionic-liquid-based methods for bioanalysis

The analysis of biological samples for diagnosis and monitoring can be very useful as a means to detect environmental pollutants (atmospheric or occupational), drug abuse, local and systemic diseases, and it can also provide valuable information for diagnostics, treatment, and forensic investigations [59, 60]. Bioanalytical separation and preconcentration techniques utilizing ILs have been developed for the determination of organic and inorganic compounds. However, organic analytes have received more study in this context (Fig. 4a). Urine is the most commonly analyzed type of biological sample (Fig. 4b). The application of ILs to the analysis of typical biological samples will be discussed in detail in the following sections. Applications of ILs in bioanalysis are summarized in Table 2.

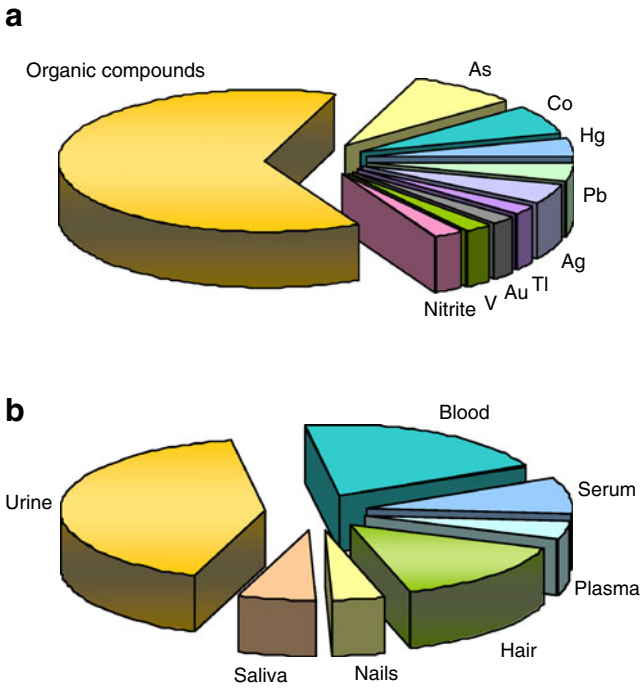



**Fig. 4 a–b** Applications of IL-based separation and preconcentration techniques. *Exploded wedges* represent the elements included in bioanalyses (**a**) and the types of biological samples analyzed (**b**)

### Urine

Urine is the sample type that is most commonly employed to test for opioids and illicit drugs, such us cocaine, opiates, and amphetamines. However, urine analysis is a great challenge due to the high complexity of the matrix. It has been previously reported that the use of ILs during sample preparation can alleviate this problem to some extent [61].

One concern in the sporting world is the abuse of drugs such as anabolic androgenic steroids by atheletes in order to improve their performances. He et al. analyzed testosterone and epitestosterone in human urine samples using an aqueous two-phase system (ATPS) consisting of the ionic liquid 1-Butyl-3-methylimidazolium chloride [$C_4$mim][Cl] and salt ($K_2HPO_4$) [62]. This novel extraction technique was coupled to reversed-phase high-performance liquid chromatography (RP-HPLC). Good extraction efficiencies were obtained for both analytes (80–90 %) in a one-step extraction. Moreover, [$C_4$mim][Cl] has been applied for the direct extraction of proteins from urine samples [19]. Hydrophilic ILs have been also used in ATPS for the separation and enrichment of vitamin $B_{12}$ in human urine samples. The composition of the system used in this case was similar to that described above, but 1-Hexyl-3-methylimidazolium chloride [$C_6$mim][Cl] IL was used instead of [$C_4$mim][Cl]. After the extraction procedure, the IL phase was directly injected into the (HPLC) system for analysis [63]. Good extraction efficiency was also obtained under optimum conditions (97 %).

IL-DLLME has been widely applied to extract a variety of analytes. A DLLME has been developed to allow cobalt (Co) determination in human urine samples using ETAAS [64]. Initially, Co was complexed with the reagent 1-nitroso-2-naphthol (1N2N). [$C_6$mim][$PF_6$] IL was used as the extractant and methanol as the dispersant solvent. Finally, the IL-rich phase was solubilized in methanol and directly injected into the graphite furnace of the ETAAS instrument. Moreover, the speciation and preconcentration of arsenic (As) were successfully studied using IL-DLLME-ETAAS and flow injection coupled with hydride-generation atomic absorption spectrometry (FI-HG-AAS) in urine and whole-blood samples [36, 37]. The best detection limits were obtained with FI-HGAAS. However, this method led to higher sample and IL consumption and a lower enrichment factor than the IL-DLLME-ETAAS technique. Anthemidis et al. developed a novel on-line sequential-injection dispersive liquid–liquid microextraction (SI-DLLME) method based on [$C_6$mim][$PF_6$] IL as an extractant solvent for thallium (Tl) determination in urine samples [65]. Dispersion of [$C_6$mim][$PF_6$] IL into the

aqueous phase containing the analyte ($[TlBr_4]^-$) was developed in a continuous mode. Since the surface area between the IL and the aqueous phase was very large, the complex was observed to easily migrate into the fine droplets of the IL. The extractant phase was retained in a packed microcolumn and finally eluted with methylisobutyl ketone (MIBK) into the FAAS nebulizer. Such methods could potentially use any IL, since they do not depend on the density of the extractant as compared to that of the aqueous phase. Moreover, manual operation and contamination risk are reduced.

As mentioned previously, ILs have been widely used to extract organic compounds. Zhao et al. developed a method based on TA-DLLME-HPLC for the determination of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in urine samples [66]. This method provides good enrichment factors (278–343) under optimized conditions. IL-DLLME assisted by organic solvents has also been developed to study the pharmacokinetics and metabolism of antihypertensive drugs [67]. The IL-DLLME technique exhibits notable advantages, such as rapidity and accuracy. Sun et al. developed an ultrasound-assisted IL-DLLME combined with HPLC for the determination of celastrol, which is a natural compound derived from a traditional Chinese medicine with anti-inflammatory properties [68]. An enrichment factor of 110 and low consumption of organic solvent was observed. Other nonsteroidal anti-inflammatory drugs (NSAIDs), including ketoprofen, naproxen, flurbiprofen, and indomethacin, have been determined by LC following in-syringe IL-DLLME assisted by an organic solvent [34]. The proposed method consisted of the use of a syringe to inject the extractant and disperser mixture and subsequent IL-phase recovery. The implementation of the syringe avoided the need for centrifugation, leading to a significant reduction in the extraction time. Acceptable enrichment factors were obtained, ranging between 73.7 (for ketoprofen) and 84.6 (for indomethacin). However, the extraction efficiency values were very low (40 %). The same NSAIDs, and others such as tolmetin and fenbufen, have been determined via a similar sample preparation step called dynamic liquid-phase microextraction (dLPME) using an IL [18]. This work applied a flow configuration to improve the reproducibility by controlling the volumes and flow rates of the different solutions. Recoveries of between 72.2 and 90.3 were obtained. Nevertheless, low preconcentration factors were also obtained (10.69 to 13.93). Phenothiazine derivatives—a group of basic substances that are widely used as antipsychotic, antiparkinsonian, and antihistaminic drugs—have also been determined by dLPME coupled with LC [69]. Active compounds of medicinal Chinese plants, including emodin, have been determined by DLLME [38]. Also, HF-LPME has been applied for the determination of natural alkaloids (such as nicotine) and drugs (including clozapine) [35, 70].

A method involving IL-based SDME coupled to HPLC analysis has been developed for the determination of benzophenone-3 , a compound normally used as a UV filter in cosmetic products [71]. A 5-μL drop of $[C_6mim][PF_6]$ was used as the extractant phase. After the microextraction process, the extractant was injected into a liquid chromatography system. The proposed method is fast, inexpensive, and useful for both preconcentration and cleanup steps. Moreover, the method offers the advantages of robustness and enhanced surface-to-volume ratios. The limit of detection of the method was on the order of 1.3 ng $mL^{-1}$. An IL-modified SPME fiber was fabricated for the forensic determination of methamphetamine and amphetamine [72]. This modified SPME fiber has shown some advantages over commercially available SPME fibers, including good thermal stability and hence greater compatibility with GC-MS. ILs have been recently used as novel GC stationary phases due to their physicochemical properties, such as negligible vapor pressure, high thermal stability, low viscosity, good wettability on the inner walls of fused silica capillaries, and selectivity towards a specific class of compounds [9, 73]. Moreover, the use of ILs as GC stationary phases can enable enhanced resolution of a specific class of compounds, allowing more reliable qualitative and quantitative determinations [25]. For example, Bianchi et al. developed an SPME procedure coupled to GC-MS that utilizes a new IL column for the determination of sarcosine and *N*-ethylglycine in human urine [6]. Sarcosine is a differential metabolite that can be found at high levels during prostate cancer progression to metastasis [74]. For this reason, its determination is very important. Finally, drugs used for the treatment of depression and obsessive compulsive disorders have been analyzed by SPE coupled to HPLC with UV detection [75]. The use of silica-based columns can be a real drawback in reversed-phase chromatography of basic compounds because of the underivatized free silanol groups present, which can cause severe tailing of the chromatographic peaks [76]. In this work, successful chromatographic separation was achieved in a reversed-phase $C_8$ column using an IL as an alternative to traditional additives. The IL was applied as a silanol activity suppressor that prevented peak tailing and improved chromatographic resolution.

## Blood

For this type of biological sample, whole blood or some of its fractions (e.g., plasma or serum in animals and humans) is/are analyzed. Cheng et al. directly extracted hemoglobin from blood using ILs but no chelating reagent [77]. The special interaction between the IL 1-Butyl-3-trimethylsilylimidazolium hexafluorophosphate ($[Btmsim][PF_6]$) and the Fe atom present in the protein heme group was studied. It was found that the electronic configuration of Fe allows the

formation of a coordinated covalent bond between this atom and the free electronic pair of the nitrogen atom of the imidazole group, facilitating the extraction of the hemoglobin [77]. An advantage of this methodology was that the whole blood did not need any complex pretreatment to extract the analyte; just a simple dilution was required, thus avoiding sample manipulation and improving sensitivity. Moreover, the IL was the only reagent required for the efficient extraction of the analyte.

Lin et al. developed a method based on HF-LPME coupled to HPLC for the determination of nicotine in plasma [70]. The extraction efficiency attained using the IL $[C_4mim][PF_6]$ was markedly higher than those obtained using conventional organic solvents (toluene, 1-octanol, and dimethylbenzene). The recoveries obtained were between 91.1 % and 99.4 %, indicating that the effect of the sample matrix was minor. It should be mentioned that the lower vapor pressure of the IL than those of volatile organic solvents led to better extraction results, as the reduced volatility of the IL makes extraction and retention in the liquid phase more efficient.

The IL 1-Methyl-3-octylimidazolium tetrafluoroborate ($[C_1oim][BF_4]$) has been used as a desorption solvent (methanol) in stir-bar sorptive extraction (SBSE) for the analysis of carvedilol [78]. This analyte is a nonselective β-adrenergic antagonist that is widely used to treat hypertension, angina, and congestive heart failure. An advantage of using this IL is the elimination of the memory effect (carryover) that occurs during the desorption step in SBSE when organic solvents are used. Two polymeric phases were used as coating materials in this method: poly(methyl methacrylate/ethyleneglycol dimethacrylate) (PA-EG) and polydimethylsiloxane (PDMS). The analyte showed a stronger interaction with the coating in the extraction with PA-EG desorbed with the IL.

Another interesting property of ILs relates to the fact that they are able to form self-assembly templates with particles with high surface areas. Thus, Meng et al. have used the hydrophobic IL *N*,*N*-bis[2-methylbutyl]imidazolium hexafluorophosphate ($[PPmim][PF_6]$) as a template for the preparation of porous nano-$TiO_2$ particles with tetrabutyl titanate (TBOT) as the precursor, which were used to adsorb several analytes from blood [79]. These particles made it possible to isolate cytochrome c from blood. The separation was dependent on the pH of the sample solution. The use of the IL in this methodology led to a 30 % improvement in analyte adsorption efficiency compared to that achieved with the particles without ILs.

ILs have been shown to play an interesting role as agents facilitating immobilization onto a solid support. Shu et al. immobilized *N*-methilimidazole onto a polyvinyl chloride material for the selective separation of basic proteins [80]. The main isolated analyte was hemoglobin. The adsorption was thought to be due to electrostatic interactions between the protein species and the surface of the [NmimCl]-PVC hybrid. However, a disadvantage of this method was that the adsorption efficiency decreased at pH values higher than the isoelectric point of the analyte. Therefore, it is very important to completely characterize the protein or analyte in terms of its isoelectric point before the isolation.

In a different application, four alkaloids were extracted from plasma samples by the IL 1-Octyl-3-methylimidazolium hexafluorophosphate ($[C_8mim][PF_6]$) using a TA-DLLME method [81]. These alkaloids could not be detected by fluorescence since the resulting intensity of the signal was negligible. However, their fluorescence signal increased considerably when these analytes were extracted into the IL. It was supposed that this effect originated from an interaction between the anions of the IL and the alkaloid cations. Nevertheless, more studies must be done to fully understand this phenomenon. The proposed methodology could replace standard methodologies that are used to extract and determine these analytes by HPLC, and would avoid the need to use dangerous solvents that would be volatilized into the environment. Vaezzadeh et al. have also applied TA-DLLME for the preconcentration and determination of palladium in blood [82]. The proposed method was demonstrated to be robust against high to medium salt contents (up to 40 %), inexpensive, easy, and safe for the preconcentration and separation of this analyte in biological samples.

IL-based microwave-assisted dispersive liquid–liquid microextraction (MA-DLLME) has been developed for the extraction and preconcentration of six sulfonamides (SAs) in animal plasma prior to determination by HPLC [83]. Sulfonamides (SAs) are a class of antimicrobial agents that are commonly used in animal husbandry to promote growth. The proposed method showed recoveries of between 95 % and 110 % and enrichment factors of around 30, and also offered advantages such as rapidity and practical convenience.

An interesting publication on the separation of proteins in human serum described the use of the IL 1-Butyl-3-methylpyrrolidinium bromide ($[C_4mprd][Br]$) [84]. In this work, imidazolium-type, pyridinium-type, and pyrrolidium-type ILs with a $C_2$–$C_8$ alkyl chain were employed during the preparation of polyacrylamide gel, and the modified gel was then used for human serum protein separation. The authors concluded that IL-SDS-PAGE provided higher resolution and separation efficiency than ordinary SDS-PAGE for proteins with low and moderate relative molecular masses in human serum.

Furthermore, Xia et al. developed a modified IL-based SDME technique for Co, Hg, and Pb preconcentration from biological and environmental samples [85]. In this case, the analytes were extracted by exposing an IL droplet to a flowing sample stream; in other words, mechanical stirring was replaced with a continuous flow of sample in this

approach. This technique was named cycle-flow SDME. The use of the IL [$C_4$mim][$PF_6$] led to higher extraction efficiency than attained with conventional organic solvents such as carbon tetrachloride. Moreover, the proposed method is inexpensive due to the small amount of IL needed for each determination.

### Saliva

Saliva can be obtained by noninvasive techniques, and this is helpful when a series of samples are needed [59]. This makes saliva an easy-to-collect, low-cost matrix that is very useful for screening large populations [60]. As mentioned before, ILs have shown certain benefits when employed as extraction solvents for toxicologically relevant compounds or functional biomolecules from biological matrices such as urine or blood. However, in contrast to the growing number of applications of ILs in biological samples, the potential of these solvents for separation, preconcentration, and speciation in saliva samples has still not been fully explored. In fact, to date, IL-based methods have only been applied by Berton et al. and He et al. for the analysis of saliva samples [64, 86, 87]. In pioneering work, Berton et al. developed a simple and fast microextraction procedure based on IL-DLLME for the selective determination of cobalt (Co) via ETAAS detection. This IL-DLLME procedure was performed using 60 mg of the IL [$C_6$mim][$PF_6$] as extractant and methanol as the dispersant. An enrichment factor of 120 was obtained with only 6 mL of sample solution and under optimal experimental conditions. The resulting limit of detection (LOD) was 3.8 ng $L^{-1}$. Furthermore, Berton et al. have developed an original flow-injection (FI) system for the on-line microextraction of V [87]. Vanadium was complexed with the chelating reagent 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP) at pH 4.0. A 40-μL volume of ([$C_4$mim][$PF_6$]) IL was mixed with 5 mL of sample solution containing the V–5-Br-PADAP complex. Then, a fully on-line TA-DLLME procedure involving analyte microextraction and final on-line separation of the IL phase with a Florisil-packed microcolumn was developed. Vanadium was removed from the microcolumn with a 10 % (v/v) nitric acid (in acetone) solution and finally measured by ETAAS. The detection limit achieved after preconcentrating only 5 mL of sample solution was 4.8 ng $L^{-1}$. The on-line retention of the dispersed IL phase in a Florisil-packed microcolumn significantly simplified the microextraction technique by reducing manual operation and contamination risk.

He et al. have, for the first time, employed 1-Octyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide ([$C_8$mim][$(CF_3SO_3)_2$ N]) as the extraction solvent in DLLME combined with HPLC for the determination of nitrite ion in saliva samples [86]. The method involved the color reaction of nitrite with *p*-nitroaniline in the presence of diphenylamine in acid medium. Subsequently, the product of the insoluble diazo coupling reaction in the water phase was extracted into the dispersed fine droplets of [$C_8$mim][$(CF_3SO_3)_2$ N]. The concentration of nitrite ion was indirectly determined from the azo product. Various factors that influence extraction performance, including reaction and extraction conditions, were investigated. Under optimal conditions, this [$C_8$mim][$(CF_3SO_3)_2$ N] based IL-DLLME procedure provided a high enrichment factor (430-fold) and a good extraction recovery (91.7 %) for nitrite ion. The limit of detection of the method (S/N=3) was 0.05 μg $L^{-1}$. An interesting comparison of [$C_8$mim][$(CF_3SO_3)_2$ N] with other extraction solvents used in the DLLME technique, such as conventional organic solvents ($CCl_4$, $C_2H_4Cl_2$, $C_6H_5Cl$) and [$C_8$mim][$PF_6$], was performed. The authors concluded that [$C_8$mim][$(CF_3SO_3)_2$ N] was superior to [$C_8$mim][$PF_6$] and conventional organic solvents for the extraction and enrichment of nitrite ion in DLLME, due to the low solubility of [$C_8$mim][$(CF_3SO_3)_2$ N] in the aqueous sample. However, the extraction mechanism and analyte interaction involved in this process should be investigated.

### Hair and nails

There are very few studies on the application of ILs for separation, preconcentration, and speciation in hair and nail samples. In turn, nails are a relatively rarely studied type of sample. An interesting application is related to the modification of a stationary phase with ILs for the GC separation and determination of Δ6-monounsaturated fatty acids in hair and nail samples [88]. A commercially available column was used in that work. The column modified with ILs was able to separate two *cis* isomers that usually are very difficult to separate, even on a biscyanopropyl siloxane phase, the phase most commonly used for such an application. The highlight of this work was the identification of petroselinic acid in human skin, hair, and nails—a compound that had never previously been identified by GC. Unfortunately, the IL that was used to coat the column was not reported, as a commercial column was used. These IL-modified GC columns show a series of advantages over conventional columns, including their very low volatility (the columns have longer lives), their ability to remain in the liquid state over a wide temperature range, their lack of active hydroxyl groups (the columns are more resistant to moisture and oxygen), and their high polarity.

SPME fibers based on ILs have also been fabricated. Thus, six pesticides (diazinon, fenitrothion, malathion, fenvalerate, phosalone, and tridemorph) were analyzed with a fiber modified with the IL 1-Butyl-3-methylimidazolium hydroxide [$C_4$mim][OH] [89]. The amount of [$C_4$mim][OH] used in this preconcentration procedure was found to be a

critical factor in obtaining a high extraction efficiency; 75 mg of IL were observed to be enough to obtain the highest preconcentration factor for each analyte. These values were in the range between 264 (for tridemorph) and 1,290 (for the pesticide fenvalerate). This approach offers several advantages, such as simplicity, good precision and accuracy, short extraction time, low cost, and minimal organic solvent consumption. However, the main disadvantage of it was that the modified fiber could only be used once, so it was necessary to repeat the modification each time an extraction had to be performed. That said, using a new hollow fiber each time could help to reduce cross-contamination and carryover effects. The analytes were not found in hair samples. For this reason, recovery studies were performed to assay possible matrix effects, and the results were found to be acceptable (86.2–98.8 %), implying that this method is a useful tool for this kind of biological sample.

Ebrahimi et al. synthesized a new IL to use in SPME coupled to HPLC aimed at the determination of organophosphorus pesticides in hair [90]. The IL was a mix of a Keggin heteropolyacid (HPW12O40), pyridine, and 1,4-butane sultone, so the resulting IL was (PY BS)3PW12O40. In this case, 30 mg of IL were enough to achieve the best extraction efficiency. Utilization of the new IL helped the authors to obtain a hollow fiber that showed strong interactions between the analytes and the sorbent. The preconcentration factors obtained with this device were very high, with values of between 360 and 1,500. The analytes were not found in hair samples. However, the recovery study yielded very good values of between 83 % and 92 %, showing only minor matrix effects. Therefore, this method is a reliable alternative for the determination of organophosphorus pesticides in hair.

Additionally, Majidi et al. developed an IL-based extraction technique named ISFME for arsenic speciation in hair and nails [49]. This extraction method was proposed for the analysis of samples with high salt contents, such as foods and drugs, and it was also applied to hair and nails. Interestingly, an enrichment factor of 200 was obtained with this extraction method, whereas similar works have shown values between 0.01 and 50.

Finally, trihexyl(tetradecyl)phosphonium chloride (CYPHOS® IL 101) has been applied as an extraction solvent in a new technique named cold-vapor ionic-liquid-assisted headspace single-drop microextraction (CV-ILAHS-SDME) for mercury species determination in human hair [91]. The proposed method demonstrated wide applicability to different complex matrix samples, including tuna fish, hair, and wine, and reached an enhancement factor of 75. Both organic and inorganic mercury species were found in human hair samples; the concentration of organic mercury species was observed to be higher than that of inorganic mercury species.

## Conclusions

The application of ILs for separation and preconcentration is an increasing trend in the field of bioanalysis. In this review, ILs have been presented as efficient tools for improving limits of detection, selectivity, and sensitivity through their implementation in liquid–liquid microextraction techniques during the sample preparation step. This approach is highly advantageous from a bioanalytical perspective, as the amount of sample is a critical consideration during the analysis of biological samples. Furthermore, ILs are better choices than volatile organic solvents due to their high preconcentration factors and analytical recoveries, which allow more sensitive and accurate determinations.

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