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Review

Liquid-phase and dispersive liquid–liquid microextraction techniques with derivatization: Recent applications in bioanalysis☆

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ABSTRACT

Liquid phase microextraction (LPME), especially hollow fiber liquid-phase microextraction (HF-LPME), and dispersive liquid–liquid microextraction (DLLME) offer high enrichments of target analytes in a single step. The analytical usefulness of these techniques is significantly enhanced by coupling them with suitable derivatization methods. Due to their simplicity, diverse bioanalytical applications have recently been reported. This review focuses on the recent developments of the combined LPME (mainly HF-LPME and single drop microextraction (SDME)) and DLLME techniques with derivatization for the analysis of biological samples. A broad range of sample matrices such as urine, blood, plasma and human hair samples with various derivatization methods for polar or ionizable organic compounds will be considered. These techniques can also be extended to the determination of trace metal ions, such as the heavy metal ions (Hg, Pb, and Co) and Se. Future trends of the techniques will also be discussed.

Contents

1. Introduction

The determination of drugs and related substances in biological samples such as whole blood, plasma, serum, tissues and cells is referred to as bioanalysis. This term was coined in the 1970s in relation to various techniques designed for the study of pharmacokinetics of drugs [1,2]. Bioanalysis is routinely carried out in forensic medicine and for toxicological studies. However, prior to the analytical determination, the analytes of interest are first subjected to a suitable sample preparation procedure [3]. These include the traditional liquid–liquid extraction (LLE), protein precipitation and solid-phase extraction (SPE).

To improve productivity, coupled with the increasing demands for green chemistry approaches in analytical determinations, the liquid-phase microextraction (LPME) method was introduced in the mid-to-late 1990s. This is a modified form of the LLE that utilizes microliters of solvents for the extraction process. The LPME technique is able to overcome some of the problems that are often encountered in solid phase microextraction (SPME). These problems include, but are not restricted to, the bending of syringe, leaching of fiber coating materials and the fragility of the fiber itself. The microextraction techniques received favorable responses and various modifications have been introduced, e.g., single-drop microextraction (SDME), continuous-flow microextraction (CFME)

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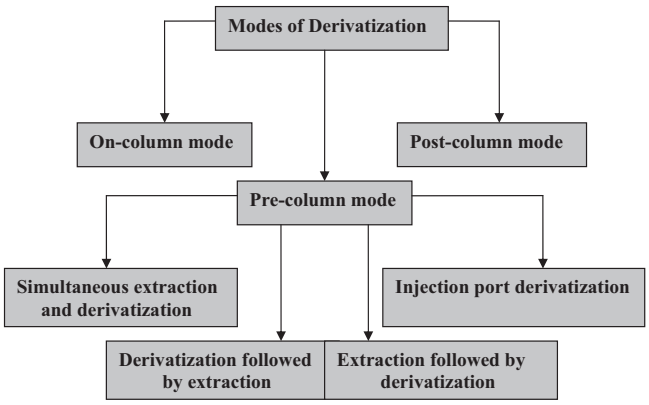

Fig. 1. Different modes of derivatization.

and hollow-fiber liquid-phase microextraction (HF-LPME). Details of these techniques are described elsewhere [4–7]. A more recent technique which does not involve the use of either fiber or syringe has been termed dispersive liquid–liquid microextraction (DLLME). As the name suggests, it involves an interaction between microliter amounts of disperser solvent with sample matrix containing the analytes [8]. Originally, only solvents with higher density than aqueous samples were used in order to ease their collection as they settle below the aqueous phase by centrifuging. However, a new method adopted by a group of researchers [9] in which organic solvents with densities less than 1 g/mL were used has reversed this trend.

To date, numerous LPME [10–13] and DLLME [14] methods have been applied for bioanalysis in various matrices. One of the main areas of considerable research interest is the possible integration of different steps (e.g., preconcentration, derivatization and sample cleanup) into a single one. In particular, the HF-LPME technique is able to meet these tasks.

The main reasons for derivatization in chromatographic analysis are: to improve the separation characteristics of the analyte, its thermal stability and to improve sensitivity of the determination. Usually, analytes are converted by derivatization either into volatile compounds able to be analyzed by GC with sensitive detectors (FID, ECD, and MS), or into fluorescent compounds to be analyzed by HPLC–fluorescence detector. Polar analyte needs to be derivatized in gas chromatographic (GC) analysis to decrease its polarity and/or to increase the detector sensitivity. In the case of HPLC analysis, some of the analytes (e.g., aliphatic biogenic amines) do not have chromophore. Therefore, derivatization is required to ensure that the analyte is amenable to sensitive detection.

Because of polarity, many substances of interest from environmental and biological samples are, unfortunately, unable to be determined. This problem can be overcome by introducing derivatization reactions on the HF-LPME method which can improve their recovery, separation, selectivity and sensitivity [15,16]. The focus of most of derivatization methods, therefore, has been mainly on the treatment of polar compounds to convert them into more easily extractable, thermally stable, more volatile analytes, with better chromatographic behavior. For GC determinations, in particular, derivatization is employed to prevent the decomposition of analyte by improving its thermal stability. Thus sensitivity is enhanced, peaks become sharper and tailing is reduced.

Different modes of derivatization could be envisaged (Fig. 1). In simultaneous mode, both extraction and derivatization take place in one-step. Hydroxycarbonyls, such as hydroxyacetone and 3-hydroxy-2-butanone can be derivatized simultaneously during their extraction using a headspace microextraction as in the method of Chen and Huang [17]. Here, a microdrop of the extracting solvent was suspended at the end of a needle which was pushed through the septum of a 1.5 mL vial into the headspace and contact established with the derivatization reagent just below it. In this way, extraction of the analytes and their derivatization were concurrently achieved. In sequential mode, derivatization of analytes can only occur before or after extraction is achieved. This type was used in the in situ derivatization of biogenic amines in food samples [18]. A 10 mL of prepared sample solution was spiked with 0.5 μg/mL of a mixture of different biogenic amine standards, poured into 16 mL sample vial and saturated with sodium hydrogen carbonate to give a mixture with pH 9.5. This was followed up by the addition of 200 μL of dansyl chloride as the derivatization reagent. With this contact of sample with dansyl chloride, in situ derivatization of the analyte occurred giving rise to a stable dansylation product with UV–Vis absorbing property prior to the actual extraction of the analyte into the lumen of the hollow fiber membrane. Cumbersomeness is one of the demerits usually put forward as possible disadvantages of derivatization, in addition to the use of toxic and environmentally non-friendly chemicals [19]. Fortunately, the in situ approach for derivatization is simple and does not involve many steps. However, some problems with this approach may impede its effective utilization in many instances – it is prone to side reactions, higher possibilities of interference from the sample matrix and cannot be performed with moisture-sensitive derivatization reagents (e.g., N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide). To overcome these shortcomings, an in-fiber mode is employed: as a micro liquid drop (solvent phase) extracts the analyte from the donor phase (sample solution), the drop is retracted back into an acceptor phase within a hollow fiber where derivatization reaction takes place. Thus, interference from the sample matrix is avoided since derivatization only occurs when the extracted analyte comes into contact with the acceptor phase.

Other terminologies commonly encountered are the pre- and post-column derivatization. When derivatization of analytes is performed prior to loading the sample onto GC or LC column, this mode is called pre-column derivatization. This is especially suitable for thermally unstable samples and polar/ionic pesticides. The aforementioned example of in situ derivatization for biogenic amines falls within this category. A second example can be found in the method of Tong et al. [20], where nitroaniline and dinitroaniline were derivatized with fluorescamine before their determination using HPLC–UVD. For post-column mode, derivatization takes place only after the analytes are separated in GC or LC columns. They are then converted to a form more amenable to detection. Example of this is found in the analysis of phenylurea and propanil herbicides. After the analytes were separated on LC column, they were converted photochemically into strongly fluorescent photoproducts using UV irradiation [21]. This is a typical photochemically induced fluorimetry (PIF) in which the analytes can be detected using a fluorescence detector. A derivatization mode that is in-between these two is the so-called on-column derivatization, in which both the analyte and derivatization chemical are loaded onto separation columns where the derivatization is performed. This was applied for the analysis of carbamate pesticides in tap water and waste water [22]. After extracting the analytes, 1 μL was withdrawn into a microsyringe and injected directly into GC column together with same volume of the derivatization reagent. In this way, degradation of the carbamates into their corresponding phenols and amines would be avoided or drastically reduced. An on-column reaction of amino acids with fluorenylmethyl chloroformate (FMOC-Cl) may be used for a high resolution GC-based determination of chiral amino acids.

A comprehensive review on the different types of derivatization reactions in LPME has been written by Xu et al. [23]. While there are several important reviews [24–26] that consider various applica-

tions in different solvent minimization extraction techniques, we report here progress that has been made regarding applications of LPME and DLLME methods, with specific reference to derivatization for bioanalysis in different matrices. A summary of these techniques is presented in Table 1.

2. Human urine matrix with LPME

In competition sports, there is always the problem of some athletes trying various means of cheating in order to outdo other competitors. One of these is the abuse of drugs with the ability to enhance performance and subdue fatigue, giving the athletes undue advantage over their rivals. To keep this under control, the Medical Commission of the International Olympic Committee has stipulated a limit of urinary concentration for two classes of drugs known as the beta blockers and beta agonists which dilate airways and help to relieve symptoms of dyspnea. Numerous chromatographic techniques for their determination can be found in the literature. Efficient and sensitive methods for extraction and pre-concentration are necessary for the detection of these drugs or their metabolites which are usually present at very low concentrations in biological fluids. Liu et al. [27] have employed a fiber-protected LPME with in situ derivatization coupled with GC–MS for the analysis of these compounds. Test urine samples from a healthy male volunteer that was treated with about 50 mg of metoprolol tablet were collected at various intervals. A blank was also collected prior to the volunteer taking the tablet. To optimize the conditions for the extraction, various extraction solvents (benzene, cyclohexane, n-hexane, chloroform and methylbenzol) were tested. Following comprehensive evaluation of factors affecting extraction and derivatization, including water immiscibility, polarity matching with the derivatized analyte and its stability throughout the extraction process, methylbenzol was finally selected. This solvent and the derivatization reagent, N-methyl-N-(-trimethylsilyl)trifluoroacetamide (MSTFA), were mixed in equal proportions (v/v, 1:1). A hollow fiber was fixed on the tip of a microsyringe containing 5 μL of this mixture and then immersed in the extraction solvent for 20 s to engorge the pores of the membrane. Thereafter, the syringe plunger was depressed to release its content into the hollow fiber which was then immersed in the sample solution for a 20 min optimized extraction time, during which derivatization of the analyte simultaneously took place along with the extraction process. Only 1 μL of the derivatized analyte was injected into GC system for analysis. The method showed good extraction efficacy (recoveries of 93.73–109.04%) and is very sensitive for the analysis of β-agonists and β-blockers in human urine samples since the extracted ion current chromatograms of derivatized metoprolol (EIC, $m/z = 72$) were markedly distinguished from the blank urine as detected by GC/MS in SCAN mode.

Another important class of drugs commonly abused in competition sports is the anabolic steroid. A simple and efficient method for extracting this class of drugs from body fluids is important for its quick and accurate determination. LLE may give better recoveries than fiber-supported LPME due to the stronger adsorption of steroids to the polypropylene fiber. However, 25% increase in extraction recoveries was reported for drained fibers that were flushed and extracted with hexane after the LPME step [28]. In this experiment, MSTFA was also used as the derivatization agent and 20 μL dihexylether (DHE) was used as the extracting solvent for four different anabolic steroids from human urine samples. The LC–MS technique yields limit of detection (LOD) of 2 ng/mL. In addition, the effect of salting-out on the recovery was examined by the addition of different concentrations of sodium chloride to the aqueous phase. It was found that with 30%(w/v) of the salt, recoveries of the steroids were 28–50% lower than when no salt was added.

Amphetamine (AM) and methylenedioxyamphetamine (MDA) are two important central nervous system stimulants that are often abused both by drug addicts and recreational users. Methods for the determination of these drugs and their metabolites in biological samples have been reported. However, for simplicity, lower cost and low organic solvent consumption, Chiang and Huang [29] developed a new method for the simultaneous extraction and derivatization of AM and MDA. This method utilizes a headspace HF-LPME and GC–MS for the analytical determination. Pentafluorobenzaldehyde (PFBAY), the derivatizing reagent, was added to only 3 μL of the extraction solvent, facilitating the release of the volatile and basic analytes from the sample matrix into the headspace, followed by the extraction and derivatization within the solvent. Good precision (%RSD less than or equal to 4%) and low LODs were obtained. The low detection limits were most probably the result of derivatization and dynamic extraction mode. This result sharply contradicts the claim by Xiong et al. [30] that derivatization might bring about poor precision and high background of quantitation since the precision of less or equal to 4% obtained with derivatization is better than what the authors achieved in their method for the quantitation of AM, caffeine and ketamine in urine samples, utilizing HF-LPME with GC–FID in the absence of derivatization (% RSD, 6.9–14.1%). The result is also better than the 4.9% and 4.7% for AM and methamphetamine (MA), respectively, previously determined without derivatization, in the single drop liquid–liquid–liquid microextraction (SDLLLME) method of He and Kang [31] coupled with HPLC–UV.

Another drug of interest to addicts and recreational users is marijuana. Its urinary metabolite, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) is preferentially used for its determination. Due to some limitations with using microextraction drop for complex matrices such as urine, the use of polymer membrane tubing by Kramer and Andrews [32] for LPME had improved the extraction efficiency and enabled more rigorous stirring. A combination of N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA, and octane (20 μL) was used both as extraction solvent as well as derivatization reagent. After 8-min of extraction (without prior filtration of the urine sample), an aliquot was injected into the GC unit for separation and analysis. The estimated LOD was 1.0 ng/mL.

Cyanide exposure, accidental, suicidal or homicidal, has attracted considerable attention in forensic science for some time. In the environment, species of cyanide are generated, among others, by industries involved with electroplating and petroleum refining. Exposures via inhalation of tobacco smoke and motor vehicle exhaust fumes are also encountered. Due to its easy accessibility and acute toxicity, cyanide is a potential weapon of terrorism. Therefore, the importance of a fast and simple method for its extraction and determination in biological samples cannot be overstated. Meng et al. [33] used a home-made fiber to develop a HF-LPME method coupled with capillary electrophoresis (CE). In this study, the effect of temperature on the extraction solvent was examined using human urine matrix. It was observed that the extraction efficiency decreased after 45 °C as a result of the depletion of the extraction solvent. Overall extraction recoveries of 92–103.4% were obtained which indicate good accuracy of the method. The inherently low sensitivity of the CE technique has been adequately compensated by the high enrichments of the HF-LPME technique which employs the formation of stable $Ni(CN)_4^{2-}$ complex in the presence of Ni^{2+}–NH_3 as derivatization reagent for the quantitative determination of free cyanide.

Among the group of anti-homeostatic compounds collectively named as endocrine disrupting chemicals (EDCs), 10 phenolic types in the urine have been successfully determined by ion-pairing assisted in-drop LPME [34]. The analytical procedure was performed in a 5-mL vial fitted with screw cap having a polytrifluoroethylene (PTFE)-lined silicon septum. Three milliliters of the

Table 1
LPME with derivatization for bioanalysis.

Analyte	Matrix	Extraction solvent/volume	Derivatization agent	Detection	LOD	Reference
Co, Hg and Pb	Human serum, hair	1–3 μL 1-butyl-3-methylimidazolium hexafluorophosphate	1-(2-Pyridylazo)-2-naphthol (PAN)	ICP-MS	1.5–9.8 pg/mL	[40]
β-Agonists and β-blockers	Human urine	5 μL methylbenzol/N-methyl-N(-trimethylsilyl) trifluoroacetamide (MSTFA)	Methylbenzol/MSTFA	GC–MS	0.08–0.10 ng/mL	[27]
Hexanal and heptanal	Human blood	2 μL decane	o-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride	GC–MS	0.12–0.16 nM	[42]
Anabolic steroid	Human urine	20 μL dihexylether (DHE)	MSTFA	LC–MS	2 ng/mL	[28]
THC-COOH	Human urine	20 μL N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + octane	BSTFA + octane	GC–MS	1.0 ng/mL	[32]
Se(IV)	Human urine, plasma	20 μL 1-octanol	o-Phenylenediamine	UV	0.02–1.00 μg/L	[38]
Bisphenol A	Human urine	Toluene	Acetic anhydride	GC–MS	0.02 ng/mL	[36]
Amphetamine and methylene dioxyamphetamine	Human urine	3 μL 1,2,4-trichlorobenzene, n-octanol, n-nonanol	Pentafluoro benzaldehyde	GC–MS	0.25–1.00 ng/mL	[29]
NO	PC12 cells	20 μL carbon tetrachloride (CCl_4)	1,3,5,7-Tetramethyl-2,6-dicarbetoxyl-8-(3,4-diaminephenyl)-difluorobora diaza-s-indacene (DAMBO-CO_2-Et)	UV–Vis/fluorescence	2.5×10^{-13} mol/L	[57]
Acetone	Human blood	2 μL decane	o-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine (PFBHA)	GC–MS	2.0 nM	[44]
Hexanal and heptanal	Human blood	10 μL methylcyanide	2,4-Dinitrophenylhydrazine (DNPH)	LC–UV	0.79–0.80 nmol/L	[41]
Cyanide	Human urine, saliva	7 μL acceptor phase; Ni(II), NH_3 pyromellitic acid, sodium carbonate	Ni(II)/NH_3	CE-UV	0.01 μmol/L	[33]
Chlorophenols	Human urine	3 μL toluene	Acetic anhydride	GC–MS	0.1–1.0 ng/mL	[37]
Endocrine disruptors	Human urine	2 μL chloroform/n-octanol (1:1)	Ethylchloroformate	GC/MS/FID	0.2–26.5 ng/mL	[34]
Bodipy-FL C_5	Human urine	1 μL 1-octanol	BODIPY	CE/LIF	–	[35]
Pb	Human urine	40 μL CCl_4	1-Phenyl-3-methyl-4-benzoyl-5-pyrazolone (PMBP)	GFAAS	39 ng/mL	[39]
Cr	Cerebrospinal fluid	5 μL N^1-hydroxy-N^1,N^2-diphenylbenzamidine (HOA) in dichloromethane	HOA	DRS-FTIR	0.01 μg/g	[58]
Methylmercury	Human hair	4 μL toluene	Thiourea	GFAAS	0.1 μg/L	[53]
As	Human hair	4 μL toluene	Ammonium pyrrolidine dithiocarbamate (APDC)	ETAAS	0.12 ng/L	[54]

sample, 0.5 mL buffer (0.5 M disodium hydrogen phosphate–NaOH) and 0.5 mL ion-pairing reagent were introduced into this vial and the content agitated at 250 rpm with a stir bar. From an organic solvent consisting of n-pentadecane as an internal standard and an ion-pairing reagent, 3 μL was drawn into a microsyringe previously rinsed with the organic solvent several times to remove air bubbles from the barrel of the microsyringe. Two microliter drop of this was depressed onto the tip of the microsyringe and made to touch the surface of the aqueous donor phase containing the sample in order to accomplish both extraction and derivatization at the same time. After a set time run, the drop was retracted back into the syringe, and the content injected into GC system for analysis. Many extraction solvents were tested, but the combination of chloroform and n-octanol (v/v, 1:1) provided the best reproducible results and hence chosen for subsequent analysis. Two types of detectors were used for the quantitative determination of the compounds under consideration. However, GC with MS proved to be more sensitive compared to FID. This is demonstrated by their limit

of detection; 0.2–1.3 ng/mL and 8.5–26.5 ng/mL for MS and FID, respectively. Derivatization may sometimes encounter some hindrance, especially in a two-phase system. In this method, tetrabutyl ammonium bromide (TBAB) was employed as an ion-paring agent to bring about rapid interaction between the aqueous phase and the organic phase, in order to obtain more derivatized extract within a short possible period. Under vigorous stirring, contact between the analytes and the organic acceptor phase was enhanced in the presence of the ion-pairing reagent.

Probably the reason for the low extraction recovery in the recent method of Sikanen et al. [35] was the absence of ion-pairing reagent and the stagnant condition under which LPME was performed. Only 6% of Bodipy-FL C_5 was recovered from the urine. Extraction was carried out in about 10 μL well pressed into a 1 cm × 5 cm piece of aluminum foil. This well was then filled with 5 mM $Na_2B_4O_7$ (10 μL) acceptor phase. 1 cm × 1 cm piece of Celgard 2500 microporous membrane with 55% porosity and 0.2 μm × 0.05 μm pores was used for the immobilization of supported liquid membrane (SLM). A micropipette was used to transfer 1 μL of 1-octanol to the polypropylene membrane for rapid immobilization. Excess solvent was removed with the aid of a medical wipe. This membrane was subsequently placed on top of the acceptor phase. As the membrane squeezed the acceptor, a liquid–liquid contact was established. 15 μL sample droplet containing 10 mM hydrochloric acid (HCl) to suppress ionization of the acidic compound was placed on top of the membrane in direct contact. Extraction was then initiated and continued for about 5 min. The analyte was determined using CE with laser-induced fluorescence detection. Since this is the first report of LPME under stagnant conditions, there is still a lot of room for improvement regarding the low recovery of acidic extracts.

In a different application, Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, with estrogenic activity has been reported to be released from polycarbonate plastics. Its determination in biological fluids becomes imperative for the assessment of environmental exposure. In this regard, Kawaguchi et al. [36] have developed and applied a miniaturized HF assisted LPME method with in situ derivatization and GC–MS for the analysis of Bisphenol A in urine samples of human subjects (22–27-years old). Extraction time is one of the factors that could impact on the efficacy of the extraction procedure. To optimize this, therefore, the investigators used 5 ng/mL standard BPA to follow the extraction time profile of the acyl derivative. To obtain this derivative, human urine sample (1 mL) was taken in 2 mL vial. This was spiked with the surrogate standard of BPA, and 100 μL of 1.0 M ammonium acetate was added in addition to 10 μL each of β-glucuronidase (10,000 U/mL) and sulfatase (3540 U/mL). This mixture was incubated at 37 °C for 3 h to achieve the hydrolytic de-conjugation of the BPA. After this, 100 μL (1.0 M) sodium hydroxide solution was added to adjust the pH. The derivatization reagent, 20 μL of acetic anhydride was then added. On contact with the released BPA, an in situ derivatization of the BPA to its acyl derivative was established. This was extracted and 2 μL of the extract injected into GC–MS system for analysis. An optimum extraction/derivatization was achieved at 15 min, and the method was able to detect trace amounts of BPA with good linearity in the range of 0.1–50 ng/mL.

The same method was applied by Ito et al. [37] for the determination of chlorophenols (CPs) in urine with minor alterations. The human urine sample in this case was spiked with 50 and 200 ng/mL of the surrogate standards containing 2,4-dichlorophenol (DCP), 2,4,6-trichlorophenol (TrCP), 2,3,4,6-tetrachlorophenol (TCP) and pentachlorophenol (PCP). While the volume of toluene used for extraction was missing in the application for BPA, 3 μL was used in this instance, which was aspirated into a microsyringe previously rinsed 10 times with acetone and toluene to avoid carryover effect and formation of bubbles. Other factors such as type of derivatization reagent, de-conjugating enzymes, and optimum time for the extraction and derivatization remain the same. It was found that after the 15 min optimum time, the relative peak areas of the analytes decreased, probably due to evaporation of the toluene to insufficient levels. The LOD was between 0.1 and 1 ng/mL at S/N of 3 to greater than 10.

Selenium is an important co-factor necessary for the normal activity of some enzymes in the human systems. However, at abnormal concentrations, it could lead to deleterious health effects. A simple and highly sensitive method has been developed for the determination of Se(IV) in human urine based on the reaction between Se(IV) and o-phenylenediamine to form piazselenol [38]. This derivatized form of selenium was extracted into the lumen of a HF-LPME support and the resulting solution subsequently injected into HPLC with an ultraviolet detector (HPLC–UV). 20 μL of the extraction solvent (1-octanol) was used along with the other optimized parameters such as volume of organic phase, pH of solution, rate of stirring and ionic strength to give a low LOD (0.02–0.1 μg/L), and linearity of at least $R^2 = 0.995$.

3. Human urine matrix with DLLME

Pb in the urine was determined via a new method that employs a dispersive liquid–liquid microextraction (DLLME) for sample preconcentration [39]. Five microliter portions of sample were placed in a screw-capped, conic-bottom glass test tube (10 mL) followed by the addition of 0.5 mL ethanol dispersant solvent containing 1 mg/mL of 1-phenyl-3-methyl-4-benzoyl-5-pyrazolone (PMBP). Following the addition of 40 μL of carbon tetrachloride, CCl_4, a cloudy solution was formed in which Pb was chelated by PMBP and extracted with CCl_4. This was centrifuged at 3000 rpm for 5 min and 20 μL of the sediment phase injected into graphite furnace atomic absorption spectrometric (GFAAS) set-up for analysis. The procedure being fast and time independent is a clear advantage over other techniques, as evident from the enrichment factor of 78 obtained in just 5 min. This was better than the value of 60 obtained when ionic liquids (ILs) were used with SDME in serum matrix [40].

4. Blood, plasma and serum matrices with LPME

Aldehydes (e.g., hexanal and heptanal) are products of free-radical mediated degradation of lipids and cellular components. Elevated blood levels are indicative of pathological conditions. Even though a variety of analytical procedures could be garnered for their identification, accurate quantification poses some challenges which would necessitate derivatization to improve detectability. Recently, a sensitive method was developed which utilizes the acid-catalyzed reaction between 2,4-dinitrophenyl hydrazine (2,4-DNPH) and aldehydes for their indirect determination via the product hydrazone [41]. This was achieved with the aid of a polychloroprene rubber as the carrier of organic solvent that contained 2,4-DNPH reagent. Blood samples, obtained from 5 healthy volunteers and 12 lung cancer patients, were extracted in a thermostated hot water bath under ultrasonication. Separation and detection were done using HPLC–UV. A low LOD of 0.79–0.80 nmol/L was obtained with minimal consumption of the extraction solvent, methylcyanide. Previously, a head-space single drop microextraction (HS-SDME) method for the determination of hexanal and heptanal in human blood matrix was reported [42]. In this method, instead of 2,4-DNPH and 10 μL of methylcyanide, o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA-HCl) and 2 μL decane were used as the derivatization reagent and extraction solvent, respectively. The extraction was performed at 40 °C without ultrasonication, and a better LOD (0.12–0.16 nmol/L) in GC–MS was obtained which is lower than that obtained earlier in the HS-SDME–GC/MS method

espoused by Li et al. [43] for the extraction of different compounds including heptanal and hexanal in complex biofluid of blood using 1-octanol as organic solvent and PFBHA as derivatization reagent.

Acetone is an important component of cellular metabolism which is utilized as a biomarker in tracking cellular anomaly. Its level in the blood may increase sharply during diabetes mellitus where insulin deficiency results in the accumulation of acetoacetate. Decarboxylation of the acetoacetate generates acetone, which can be determined by 2,4-DNPH derivatization. A simple and fast GC method which employs a HS-SDME and simultaneous derivatization using o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) was recently developed for the extraction, separation and quantification of acetone in human blood [44]. About 100 μM of acetone-spiked blood sample was introduced into the headspace sample vials and the extraction performed at 4 °C. A stirring rate of 500 rpm and 2 μL decane as the extraction solvent were used. An impressive LOD of 2 nmol/L was claimed.

Trace amounts of Co, Hg and Pb were recently determined in human serum using a SDME combined with electrothermal vaporization inductively coupled plasma mass spectrometry (ETV-ICP-MS) [40]. This method is based on IL cycle flow. ILs are important candidates with the potential of replacing toxic, flammable and volatile solvents in separation and other traditional chemical operations in the near future. Trace amounts of the three metals were determined, with LOD of 1.5, 9.8 and 6.7 pg/mL, respectively, underlining the sensitivity of the method. After only 10 min into the extraction procedure, enrichment factors calculated for Co, Hg and Pb were 350, 50 and 60, respectively. A method with better enrichment factor for the trace metals Cd and Pb has been demonstrated by Li et al. [45]. This is also based on SDME coupled with ETV-ICP-MS. The metals were analyzed based on the high-intense signal-giving 8-hydroxyquinoline (8-HQ)-metal complex. The 8-HQ chelating compound was prepared as a 0.1 mol/L solution by dissolving 0.363 g of 8-HQ in 25 mL of chloroform. SDME was performed according to the procedure explained elsewhere [46] which depends on a continuous flow system. Briefly, the extraction system was filled with the analyte matrix, in this regard serum. To this was added 5 μL of extraction solution (8-HQ in chloroform) with the aid of 10-μL syringe. 4 μL of this solution was used to form a drop on the Perfluoroalkoxy (PFA) tube outlet in the extraction chamber. The trace metals were chelated and extracted from the sample solution as it flowed around the microdrop. After 15 min, the microdrop was withdrawn by the syringe and injected into ICP-MS for analysis. The two metals, Cd and Pb, were enriched to the factor of 140 and 190-fold, respectively. Impressive LOD of 2.9 pg/mL for Pb and 4.6 pg/mL for Cd were also recorded in this method. It was further noted that various concentrations of the commonly found metal ions in the environment did not show any significant suppression in the signals of the two analytes, which indicates good selectivity.

The HF-LPME method by Saleh et al. for the determination of Se(IV) was validated using human plasma samples [38]. These samples were obtained from the Iranian Blood Transfusion Organization (IBTO) in Tehran and stored at −20 °C before use. The samples were diluted 1:10 using ultra-pure water before the addition of the derivatization reagent, o-phenylenediamine, in order to reduce matrix effect. Extractions were performed using HF-LPME and concentration of Se(IV), determined in the plasma using standard addition method, was 46 ± 4 μg/L.

GBP or Gabapentin (1-(amino methyl)cyclohexaneacetic acid) is an analogue of γ-Aminobutyric acid (GABA) that can be extracted from complex biological matrices using HF-LPME. This drug, commonly used as a treatment for neuropathic pain [47], lacks any chromophore for UV detection. Derivatization coupled with HPLC–UV determination has been suggested for its monitoring in biological systems [48]. Recently, a procedure of three-phase HF-LPME together with derivatization using 1-fluoro-2,4-dinitrobenzene (FDNB) was adopted by Ebrahimzadeh et al. [49] for this purpose. Plasma sample previously diluted to 1:3 ratio with ultra pure water was placed in a test tube. Then 1 mL GBP solution (30 μg/L), 1 mL of 0.25 mol/L borate buffer (pH 8.2), 3.6 mL acetonitrile and 30 μL FNDB were added to the test tube. This was then heated at 60 °C for 10 min and cooled at ambient temperature. The mixture was afterwards diluted to 8.5 mL with 2.0 mol/L HCl and used as the source or donor phase in the traditional three phase HF-LPME in which the product of GBP and FNDB reaction in the source phase was separated using 25 μL DHE as the organic phase and borate buffer (pH 9.1) as the acceptor or receiving phase. LOD for this method is 0.2 μg/L (0.2 ppb), which is lower than the 0.01 μg/mL (10 ppb) obtained in a previous non-LPME method by Jalalizadeh et al. [50] in which HPLC–UV was also used together with FNDB pre-column derivatization for the determination of GBP in human plasma.

5. Blood, plasma and serum matrices with DLLME

Recently, a method for the determination of volatile aldehydes (hexanal and heptanal) from human blood samples collected from healthy people and from lung cancer patients was explained [51]. This is another dispersive liquid–liquid microextraction technique that is based on solidification of floating organic droplet (DLLME-SFO). 50 μL of methanol was used as the dispersive solvent, DNPH as derivatization reagent and 1-dodecanol as extractant. Blood serum was obtained for analysis by addition of 750 μL methanol to the blood sample followed by centrifugation to remove protein and other substances. 500 μL of the resultant supernatant was then diluted with ultrapure water. For the DLLME-SFO procedure, 5 mL sample solution containing the analytes at concentrations of 1 μmol/L was placed in a 6 mL screw-capped glass test tube. This was followed by the addition of 0.75 g salt (NaCl), 20 mmol/L DNPH (30 μL) and 40 μL formic acid. The tube was then conditioned in water bath at 40 °C for 10 min. After that, a combination of 50 μL 1-dodecanol and 50 μL methanol was rapidly injected into the tube and the screw cap replaced. This tube was thereafter shaken, leading to the formation of a cloudy solution which was subjected to centrifugation at 4000 rpm for 2 min. This yielded a floating organic solvent droplet over a denser aqueous phase below. The tube was subsequently chilled in an ice-bath to solidify the organic solvent. A small medicine spoon was used to scoop out the floated portion into a 1.5 mL conical vial. Under room temperature, this solid quickly melted due to the low melting point (24 °C) of 1-dodecanol. About 50 μL of methanol was added to reduce the viscidity of the 1-dodecanol before analysis. Only 5 μL of the mixture was injected into HPLC–UV system. The mobile phase used was 87:13 (v/v) mixture of methanol:water flowing at 1.0 mL/min. Analytes were detected at $\lambda_{360\,nm}$. The method has achieved 67.84–70.28% recovery. Compared to the method of Xu et al. [23], this method has higher solvent consumption but offers better reproducibility of measurements (% RSD, <5%).

In another study, Pb was separately determined in human serum matrix using an ingenious technique that combines a laser-induced thermal lens spectrometry (LI-TLS) with DLLME [52]. This method primes itself with high preconcentration factor and good reproducibility (% RSD, <4%). The serum sample was prepared for analysis by removing lipid and protein contents. For this end, serum:acetonitrile (1:1) was centrifuged for 5 min and the colorless supernatant was further diluted to a 1:10 ratio with high purity water. At its tail, DLLME was performed by taking 10 mL of this sample in a glass test tube having a screw cap. This was spiked with Pb standard at concentration of 25 μg/L, quickly followed by the addition of 12 μL dithizone chelating agent (1×10^{-3} mol/L 1,5-

diphenylthiocarbazone). An insulin syringe with a capacity of 1 mL was used to introduce 0.4 mL ethanol (disperser solvent) containing 40 μL extractant solvent (CCl_4). A cloudy mixture of water, ethanol and CCl_4 was formed. Pb–dithizone complex was extracted into the CCl_4 fine droplets. Centrifuged at 3500 rpm for 5 min, contents of the tube were segregated, with the dispersed droplets of CCl_4 settling at the bottom of the tube as sediment. 25 μL of this sediment phase was recovered with a micropipette and manually injected into the 20 μL microcell of a locally designed single laser thermal lens spectrometer having a 1 mm band pass and operated at 532 nm wavelength and laser power of 50 mW. Another important merit of this method is its large enhancement factor, 1000 (calculated by taking the ratio of slopes of calibration curves before and after ethanol was used as disperser solvent). To eliminate interference, mixture of potassium cyanide (0.2×10^{-3} M) and sodium citrate (0.1×10^{-4} M) was used as modifier. This led to high recovery (>94%) and improved detectability. The detection limit registered for this method is in sub-parts per billion (0.01 μg/L).

6. Hair matrix with LPME

In order to validate the method of Xia et al. [40] for the trace determination of Hg, Pb and Co, certified reference material of human hair (GBW07601) was used to determine if the values were in agreement with the certified results. To achieve this goal, 25 mg of the human hair samples in 5 mL PTFE beaker to which 0.5 mL concentrated HNO_3 was added together with 0.2 mL $HClO_4$ were used. These samples were then digested to release the metal contents by warming at low temperature while adding drops of H_2O_2. The resulting solution was then heated to near dryness, dissolved with buffer solution and diluted to 5 mL with deionized water. Then 1.5 mL of this was extracted with 1-(2-pyridylazo)-2-naphthol (PAN) and analyzed for the presence of the metals. Quantitative determination was performed by ETV-ICP-MS. Results obtained showed the presence of these metals at different concentrations in μg/g (Co, 0.02 ± 0.004; Hg, 0.34 ± 0.07; Pb, 8.9 ± 0.8), which agreed with the certified reference concentrations provided. These results indicate that Pb has preponderance for accumulation in biological systems.

Methylmercury (MeHg) and arsenate have also being determined in human hair samples via similar methodologies. For methylmercury [53], hair samples finely cut from the nape of the neck near the scalp section were collected from inhabitants of Wuhan (Wuhan, China), washed with non-ionic detergent solution and rinsed thoroughly with large amount of ultra pure deionized water and acetone. It was then air dried and 0.1 g of the dried material plus 4 mL of 5 mol/L HCl were transferred to a 10 mL centrifuge tube and sonicated for 30 min. The suspension was centrifuged at 4000 rpm for 10 min and its supernatant transferred to a 10 mL flask. Sonication and centrifuging were repeated for the residue and the two supernatants combined in high purity deionized water. This sample solution was then divided into two portions. One portion was directly analyzed for total mercury by ICP-MS while the other portion was subjected to a three-phase liquid–liquid–liquid microextraction (LLLME) procedure: 4 μL thiourea (4% thiourea in 1 M HCl) was used as acceptor solution/chelating agent. Methylmercury being hydrophobic was able to cross the organic phase into the acceptor phase by forming stable complex with thiourea. Following extraction for 10 min, the acceptor phase was drawn back into the microsyringe and its content was used for analysis with GFAAS. Aside this, two-phase microextraction (HF-LPME) was also performed. Though, this provided for better precision, the former displayed higher enrichment factor (204 against 55) and better limit of detection (HF-LLLME, 0.1 μg/L; HF-LPME, 0.4 μg/L). This method is very good for separating inorganic mercury from the organic ones, since the former would remain in the aqueous sample during the LLLME process. Similar mechanism was applied for the separation of As(III) from its +5 oxidation state species, where the former in the form of ammonium pyrrolidine dithiocarbamate (APDC)–As(III) complex was extracted during HF-LPME while the latter, As(V), was left behind in the aqueous phase [54]. After obtaining and washing the hair samples as explained above for MeHg determination, the hair strands were mixed with water (0.5 g hair/10 mL water) and heated in polypropylene tube for 6 h at 90 °C. The content of the tube was filtered and the filtrate stored overnight at 4 °C. Next day, 2.7 mL of this sample was subjected to HF-LPME for extracting the As(III) species using APDC as complexing compound. For total mercury, As(III) in the aqueous sample was converted to As(V) with 0.5% (w/v) L-cysteine before determination. Quantification was done at $\lambda_{193.7\,nm}$ with electrothermal atomic absorption spectrometry (ETAAS) that uses Pd as modifier and a hollow cathode lamp (HCL) running at 15 mA current. The method recorded good recoveries for the spiked samples (86–109%). However, the extraction efficiency (65 ± 9%) is far less than reported using cloud point extraction (CPE) with ETAAS [55]. This may well be attributed to effect of the surfactant used in the CPE which maximizes extraction efficiency through minimization of phase volume ratio.

7. Miscellaneous matrices with LPME

Various other matrixes have been considered under this review. As an intracellular messenger, the level of nitric oxide (NO) is considered a physiological marker in the assessment of pathological states of both Alzheimer's and Parkinson's diseases. A model cell line commonly employed in monitoring these degenerative conditions of the neurons is the PC12 cell derived from the Pheochromocytoma of rat *adrenal medulla*. Direct measurement of NO is very difficult due to its extreme lack of stability [56]; it is easily converted to NO_2^- and NO_3^- in oxygenated aqueous environments. Therefore, a trapping molecule with good detectable property was needed. High fluorescence quantum efficiency and large molar extinction coefficient, two important properties of the derivatization reagent used, difluoroboradiaza-S-indacene (BODIPY), could be harnessed for the determination of NO. Huang et al. [57] have reported the use of a derivative of BODIPY, 1,3,5,7-tetramethyl-2,6-dicarbethoxy-8-(3,4-diaminophenyl)difluoroboradiaza-S-indacene (DAMBO-CO_2Et), for the derivatization and quantification of NO in the PC12 tumor cells using ultrasound assisted LPME–HPLC. DAMBO-CO_2Et has an excellent ability for cell membrane penetration, and its reaction to form the corresponding triazole, DAMBO-CO_2Et-T, produced an enrichment factor of 150 in just 2.5 min. To obtain this, PC12 cells were first cultured in a Dulbrecco's Modified Eagle's Medium (DMEM) which was supplemented with 5% heat-deactivated fetal bovine serum, 10% heat-deactivated horse serum, penicillin G sodium (100 U/mL) and streptomycin sulfate (0.1 mg/mL). This culture was maintained at 37 °C under 5% CO_2 and 95% humidity. The rate of NO production in the model cells (200 ± 5) was found to be about 1.76 fmol/30 min, determined with UV–Vis detector ($\lambda_{500\,nm}$) and fluorescence detector ($\lambda_{ex/em}$ = 500 nm/510 nm) having an LOD of 2.5×10^{-13} mol/L at 3σ.

Chromium in the form of dichromate ion was determined in many biological fluids including cerebrospinal fluid (CSF) using SDME and KBr press diffuse reflectance Fourier transform infrared spectrometry (DRS-FTIR) [58]. CSF (0.5–1 mL) was obtained from the spines of hospitalized patients using propene intravenous cannula. After separating the proteins by centrifugation, the fluid

was diluted with 1% nitric acid (1:4), and spiked with chromium standard at 10 μg/L. Extraction was performed in an 8-mL vial which was sealed with a silicon septum having a PTFE coating. A 10 μL gas-tight Hamilton manual injection microsyringe was filled with 5 μL chelating agent (0.01 mol/L N^1-hyroxy-N^1,N^2-diphenylbenzamidine (HOA) in dichloromethane). The barrel of this microsyringe was pushed into the sample vial septum and its tip maintained at about 1 cm below the surface of the sample. The 5 μL microdrop was squeezed out of the syringe and dangled on the tip of the needle. This sample was continuously agitated at 300 rpm with a stir bar for 5 min. Thereafter, the drop was retracted back into the microsyringe and the syringe removed from the vial. This microdrop containing Cr(IV)–HOA complex was carefully delivered over a 0.1 g finely ground KBr powder and dried over 60 °C water bath for 2 min. After this step, the KBr carrying the analyte matrix was mixed adequately with a spatula, and filled into a sample holder for analysis with DRS-FTIR using 902 cm^{-1} vibrational peak for the identification of chromium. This determination is about the first application of LPME to CSF matrix. Chromium was determined at 1.3–3.1 μg/L (n = 8, % RSD, 3.1–4.5) in the CSF samples, a far cry from the 14.6 ± 6.3 ng/mL obtained from 43 healthy human volunteers using electrothermal atomization atomic absorption spectrometry (ETA-AAS) with deuterium arc background correction [59].

HF-HS-SDME method by Meng et al. [33] was also applied to the extraction of cyanide in salivary fluids. Both smokers' and non-smokers' saliva were collected. Extraction was performed using 1.5 cm long hollow fiber membranes that were previously cleansed in acetone aqueous solution (v/v, 2:3) under sonication and then air dried before use. The segments of these fibers were then immersed in an acceptor solution composed of 0.5 mmol/L Ni^{2+}, 300 mmol/L NH_3, 6 mg/L pyromellitic acid and 1 mmol/L sodium carbonate and ultrasonicated for 20 min in order to impregnate the porous wall. Extraction was subsequently performed with 7 μL of the acceptor solution for 10 min optimized time under stirring speed of 900 rpm. Only 5 μL of the extract was introduced to CE for analysis. This method displayed good linearity (R^2 = 0.9987) between 0.1 and 20 μmol/L of CN^-. The amount of CN^- determined in smokers was 0.81 μmol/L compared to only 0.28 μmol/L in non-smokers. This result further shows the higher danger to which smokers are exposed via inhalation of different toxic materials present in cigarette.

Recently, a new method of small volume liquid extraction (SVLE) was employed for the extraction of amphetamines (AMs) in salivary fluid [60]. Since this method also involves two liquid phases and the use of microliter amount of extraction solvent (50–100 μL), SVLE can be considered a new form of LPME devoid of syringe and fiber membranes. AMs (AM, MA, MDA and 3,4-methylenedeoxymethamphetamine, MDMA) were determined in salivary fluid matrix using post-extraction derivatization followed by GC–MS quantification in SIM mode. To extract and derivatize the AMs, 1 mL of saliva from drug abusers was introduced into 2 mL polypropylene plastic centrifuge vial. This was spiked with 2-methyl-benzylamine internal standard followed by the addition of 0.5 mL of 1 M NaOH and chloroform (50–100 μL). The mixture was separated into two distinct phases following ultrasonication for 5 min. This was then centrifuged and 50 μL of the bottom layer containing the extracted analytes was transferred into a 2 mL glass vial and 20 μL N-methyl-bis-trifluoroacetamine (MBTFA) derivatization reagent was added. 1 μL of this was then injected into GC for analysis. Only AM and MA were detected in the salivary fluid using MS in SIM mode. The method is interestingly simple. Relative percent recoveries for the four analytes are 87–114% and their LODs are between 1 ng/mL and 5 ng/mL. The method is, however, very sensitive to sample volume variation which can affect reproducibility of results immensely.

8. Conclusions and future directions

The present paper has reviewed the current literature on LPME and DLLME coupled with derivatization approaches for bioanalysis. The techniques have been found to be attractive because they are inexpensive and are easy to handle. More applications are expected in the future to cover other matrices such as lachrymal fluid, synovial fluid and milk. As interests in this area continue to grow, we expect to see more modifications to instruments and parameters that would enable the achievement of higher pre-concentration factors that allow for even lower LODs to be attained.

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