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Extraction of pyridine derivatives from human urine using electromembrane extraction coupled to dispersive liquid–liquid microextraction followed by gas chromatography determination

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ABSTRACT

In the present work, some of pyridine derivatives were analyzed for the first time in complicated biological fluids by coupling electromembrane extraction with dispersive liquid–liquid microextraction (EME–DLLME). 3-Methylpyridine, 2,4-lutidine, quinoline and 4-dimethylaminopyridine (DMAP) were extracted from urine and water samples. Effective parameters on the efficiencies of EME and DLLME were optimized by one variable at a time method and face-centered central composite design (FCCCD), respectively. The supported liquid phase (SLM) employed for the extraction of the analytes was a mixture of 90% 2-nitrophenyl octyl ether (NPOE) and 10% di-(2-ethylhexyl) phosphate (DEHP) which was immobilized in the pores of a piece of hollow fiber. An electric field was applied to carry over the analytes into acceptor solution. The acceptor solution was transferred to 1 mL of an alkaline solution (pH=13) and then DLLME procedure was performed. Preconcentration factors in the range of 40–263 and satisfactory repeatabilities ($2.3 < RSD\% < 5.3$) were obtained in different matrices. The method offered a good linearity with coefficient of determination greater than 0.9948 and was utilized for determination and quantification of pyridine compounds in smokers' urine samples. The proposed technique can be introduced as a simple, fast and inexpensive method for diagnosis of smokers.

1. Introduction

Pyridine derivatives are such compounds which have relatively high toxicities and are particularly hazardous. Most of the pyridine derivatives exist in cigarette smoke. A single puff of a cigarette exposes the body to over 4000 chemical compounds and 600 additives. Pyridine derivatives cause increase in heart rate, heart disease and stroke, blood pressure and lung cancer. So, it is obvious that how much harmful these compounds can be for human health. The presence of these toxic compounds in cigarette smoke has been recognized as an important incentive for development of rapid, sensitive and accurate quantitative methods to analyze them. There are few available techniques for the determination and quantification of 3-methylpyridine (MPY), 2,4-lutidine (LU), quinoline (QUI) and 4-dimethylaminopyridine (DMAP); such as electrostatic precipitation [1], gas chromatography (GC) [2,3], on-line hollow fiber liquid-phase microextraction [4] and gas chromatography–mass spectrometry (GC–MS) [5–9] in various matrices. Most of these methods were conducted in water and not in complex matrices. Due to the high number of interferences occuring in complicated matrices, such as biological fluids, and to improve detection limits, sample preconcentration and cleanup must be carried out before determination of them. A large number of modern sample preparation techniques, including solvent-free extractions or extraction procedures with very high sample to solvent ratio, for such liquid-phase microextraction (LPME) methods have been introduced. Among the LPME methods, dispersive liquid–liquid microextraction (DLLME) and hollow fiber-based liquid-phase microextraction (HF-LPME) have been applied more frequently by analytical chemists. However, these techniques have some drawbacks; DLLME is efficient only for simple matrices, because it creates crowded chromatograms for extracts from complicated matrices, especially biological fluids. Membrane technology has overcome this problem and HF-LPME based on passive diffusion is the major application of this type of technology. This method provides high degree of clean-up, particularly for complex matrices like biological fluids, and also good selectivity by choosing proper organic solvents. But in the case of HF-LPME, the extraction time needed is usually long; so that extraction times of 30–50 min are commonly reported for it [10]. In 2006, Pedersen-Bjergaard et al. reported a novel microextraction technique called electromembrane extraction (EME) [11]. EME can be used to extract ionizable compounds from plasma samples and other complicated biological matrices without protein precipitation

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[12,13]. In this method, an electric voltage is applied which facilitates the extraction of analytes across a hollow fiber membrane. This voltage causes EME to be more efficient than passive HF-LPME and extracts the analytes in a short time relative to the long time required for HF-LPME [14]. In this process, the ionized target analytes are extracted from an aqueous sample into an organic solvent located in the pores of a porous hollow fiber, and then transported into an aqueous acceptor solution inside the lumen of hollow fiber by the force of the electric potential across the SLM. One of the main disadvantages of EME is its incompatibility with gas chromatography (GC) instrument. Moreover, GC instrument is simpler, faster, and less expensive compared to high performance liquid chromatography instrument. It can easily be conjugated with different kinds of sensitive detectors; for example, flame ionization detector (FID) and mass spectrometry (MS). Since the direct injection of aqueous acceptor phase in EME may cause some difficulties for GC instrument, some attempts have been made to transfer analytes into GC-compatible phases [15–18]. Recently, Yamini et al. combined EME with DLLME and in this way, benefited from the high cleanup ability of the EME method as well as the DLLME compatibility with GC instrument [17]. Therefore, extraction of analytes from complex matrices such as biological fluids became possible and the final solution could be analyzed by GC.

In this work, EME joined to DLLME (EME–DLLME) was exploited for extraction and determination of pyridine derivatives in urine samples. To this end, ionized forms of the analytes were first extracted into an aqueous acceptor phase, located inside the lumen of a hollow fiber, under an electrical field using EME technique. Subsequently, DLLME was employed to transfer the target analytes into a final organic phase which was GC compatible. This simple and cost-effective method can be utilized straightforwardly in clinical centers to recognize smokers which may be a useful test for some worried families or employers.

2. Experimental

2.1. Equipments for EME–DLLME technique

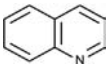
A 24-milliliter vial with an internal diameter of 2.5 cm and height of 5.5 cm was used as the sample compartment. Electrodes applied in this research were platinum wires with diameter of 0.25 mm, and were obtained from Pars Pelatine (Tehran, Iran). The electrodes were coupled to a power supply model 8760T3 with programmable voltages in the range of 0–600 V and current outputs in the range of 0–500 mA from Paya Pajoohesh Pars (Tehran, Iran). During the extraction, EME unit was stirred with speeds in the range of 0–1250 rpm via a heater-magnetic stirrer model 3001 from Heidolph (Kelheim, Germany) using a 1.5×0.3 cm^2 magnetic bar.

A 40-kHz, 0.138-kW (Tecno-Gaz SpA, Italy) ultrasonic water bath with temperature control was exploited to emulsify the organic solvent in the aqueous solution. A Selecta lab model Tl320 centrifuge (Barcelona, Spain) was employed for phase separation of the cloudy solution.

2.2. Chemicals and materials

MPY, LU, QUI, and DMAP were acquired from Sigma (St. Louis, MO, USA). Chemical structures and amounts of log P and pK_a of the analytes are presented in Table 1. Compounds 2-nitrophenyl octyl ether (NPOE), 2-nitrophenyl pentyl ether (NPPE), tris-(2-ethylhexyl) phosphate (TEHP) and di-(2-ethylhexyl) phosphate (DEHP) were purchased from Fluka (Buchs, Switzerland). Acetone, chloroform, methanol, acetonitrile, dichloromethane, trichlorobenzene, carbon tetrachloride and carbon disulfide were supplied by Merck.

Table 1
Chemical structures and amounts of pK_a and log P for the analytes.

Compound	Abbreviation	Chemical structure	pK_a	Log P
3-Methylpyridine	MPY		5.63	1.11
2,4-Lutidine	LU		6.46	1.65
Quinoline	QUI		4.81	2.03
4-Dimethylaminopyridine	DMAP		9.53	−0.9

All of the chemicals used were of analytical-reagent grade. The porous hollow fiber utilized for the SLM was a PPQ3/2 polypropylene hollow fiber from Membrana (Wuppertal, Germany) with inner diameter of 0.6 mm, wall thickness of 200 μm, and pore size of 0.2 μm. Ultrapure water was prepared by a Younglin aquaMAx purification system 370 series (Kyounggi-do, Korea).

2.3. Biological and standard solutions

Urine samples were collected from three smokers and one person who had not smoked at all (as a match matrix for plotting the calibration curves). All samples were stored at −4 °C, thawed and shaken before extraction. A stock solution, containing 1 mg mL^{-1} of the analyte in methanol, was prepared and kept at −4 °C protected from light. Working standard solutions were prepared by diluting the above stock solutions with methanol.

2.4. Gas chromatography apparatus

Separation and detection of MPY, LU, QUI, and DMAP were performed by an Agilent 7890A gas chromatograph (Palo Alto, CA, USA) equipped with a split-splitless injector and a flame ionization detector (FID). A 30-m HP-5 Agilent fused-silica capillary column (0.32 mm i.d. and 0.25 μm film thickness) was applied to the separation of the target compounds. Helium (purity 99.999%) was used as carrier gas at constant flow rate of 2.0 mL min^{-1}. Temperatures of the injector and the detector were set at 280 °C and 300 °C, respectively. Oven temperature program was as follows: 40 °C for 2 min, increasing to 130 °C with a ramp of 15 °C min^{-1}, increasing to 280 °C with a ramp of 100 °C min^{-1}, and holding at 280 °C for 3 min.

2.5. EME–DLLME procedure

The equipment exploited for EME–DLLME procedure is shown in Fig. 1. Twenty four milliliters of the sample solution, containing the target analytes, was transferred into the sample vial. To impregnate the pores of hollow fiber with the organic solvent, a piece of the hollow fiber was cut out (4 cm) and dipped in the solvent for 5 s and then the excess amount of solvent was gently wiped away by air blowing using a medical syringe. The upper end of the hollow fiber was connected to a medical needle tip as guiding tube which was inserted through the rubber cap of the vial. A 100 mmol L^{-1} HCl solution (as an acceptor phase) was introduced into the lumen of hollow fiber by a microsyringe and then the lower end of hollow fiber was mechanically sealed. One platinum cathode was introduced into the lumen of the fiber. The fiber, containing the cathode, the SLM and the acceptor solution,

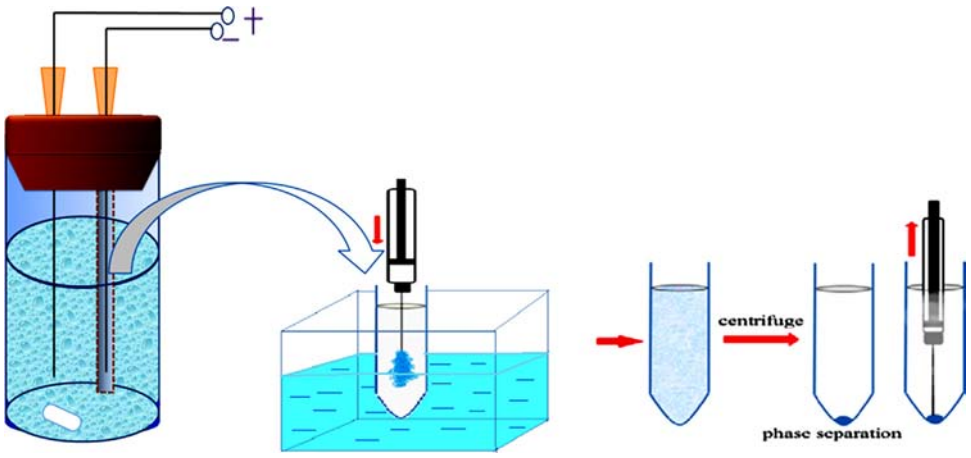


Fig. 1. A schematic presentation of EME–DLLME set-up for extractions of MPY, LU, QUI, and DMAP.

was afterwards moved into the sample solution. The anode was transferred directly into the sample solution. The electrodes were subsequently joined to a power supply and the extraction unit was placed on a stirrer with stirring speed of 700 rpm. The voltage was turned on and the extraction was executed for 20 min. When the extraction was completed, about 10 μL of the acceptor solution was collected by a microsyringe and injected into 1 mL of an alkaline solution (pH=13) in a 5-mL screw cap glass test tube with conical bottom for converting the extracted analytes to their neutral forms. After that, the DLLME procedure was carried out on the above solution according to Yamini et al.'s work with some modifications [17]. The glass centrifuge tube was immersed in an ultrasonic water bath. The ultrasonic water bath was switched on and a mixture of methanol (as a disperser solvent, 50 μL) and chloroform (as an extraction solvent, 20 μL) was slowly injected into the water sample via a 250-μL gas-tight syringe. After a few seconds of sonication (25 ± 3 °C), the emulsion formed was centrifuged at 2000 rpm for 10 min to separate the phases. One microliter of the sedimented phase was taken by a 5.0-μl Hamilton gas-tight syringe and injected into GC. The sedimented phase volume was approximately 3.0 ± 0.2 μL.

2.6. Calculation of preconcentration factor and relative recovery

Preconcentration factor (PF) was defined as the ratio of final analyte concentration in the acceptor phase ($C_{f,a}$) to initial concentration of analyte in the sample solution ($C_{i,s}$)

$$\mathrm{PF} = \frac{C_{f,a}}{C_{i,s}} \quad (1)$$

wherein $C_{f,a}$ was calculated from a calibration graph obtained via the direct injection of standard solutions (0.5–100 mg L^{-1}) in chloroform into GC.

Relative recovery (RR%) and accuracy (Error%) was calculated by the equations below

$$\mathrm{RR\%} = \frac{C_{found} - C_{real}}{C_{added}} \times 100$$

$$\mathrm{ER\%} = \mathrm{RR\%} - 100 \quad (2)$$

where C_{found}, C_{real}, and C_{added} are the concentration of analyte after the addition of a known amount of standard into the real sample, the concentration of analyte in the real sample and the concentration of a known amount of standard spiked into the real sample, respectively.

3. Results and discussion

In order to find out the best extraction conditions, two different types of variables had to be optimized which were related to EME and DLLME techniques. EME variables, such as composition of supported liquid membrane (SLM), applied voltage, extraction time, stirring rate and concentration of HCl in donor and acceptor phases, were initially optimized by one variable at a time method. Types of extraction and disperser solvents and their volumes, salt effect and pH of donor solution were effective parameters, which were considered for optimization of DLLME. All optimizations were done in ultra pure water.

3.1. Optimization of EME variables

3.1.1. Selection of organic liquid membrane composition

One of the most effective parameters on EME is the composition of SLM. A suitable liquid membrane can enhance the extraction recovery and selectivity. Composition of membrane affects the diffusion coefficient of analyte and also determines the range of applied voltage. To achieve the best SLM composition, some experiments were conducted. It was demonstrated that nitro-aromatic compounds, such as NPOE and NPPE, are the most favorable choices for extraction of cationic species [11,19–21]. Meanwhile, to extract relatively hydrophilic compounds, some carriers might be necessary. Firstly, NPOE and NPPE were examined as SLM. The reported results in Fig. 2A confirm that pure NPOE or NPPE are not able to effectively extract the analytes. Hence, the different percentages of TEHP and DEHP in the SLM were tested. A mixture of NPOE and DEHP was the best organic liquid membrane. As seen from Fig. 2A, increasing the amount of DEHP from 5% to 10% improves the extraction efficiency. DEHP could form ion-pair complexes with the analytes and facilitate their entrance into the organic phase. Presence of more than 10% of this carrier in the membrane diminished the extraction recovery owing to a decrease in the electrical resistance of SLM and consequently an increase in the current level that created bubbles around the fiber as a result of electrolysis reactions. As pH slightly increased in the acceptor solution (for extraction of basic analytes) due to electrolysis, analyte back-extraction into the donor phase led to the reduction of extraction efficiency. Eventually, NPOE containing 10% of DEHP was chosen as the optimum SLM.

3.1.2. Effect of applied voltage and extraction time

It has been proven that in EME, the electrokinetic migration of the analytes across the SLM into the acceptor solution is substantially

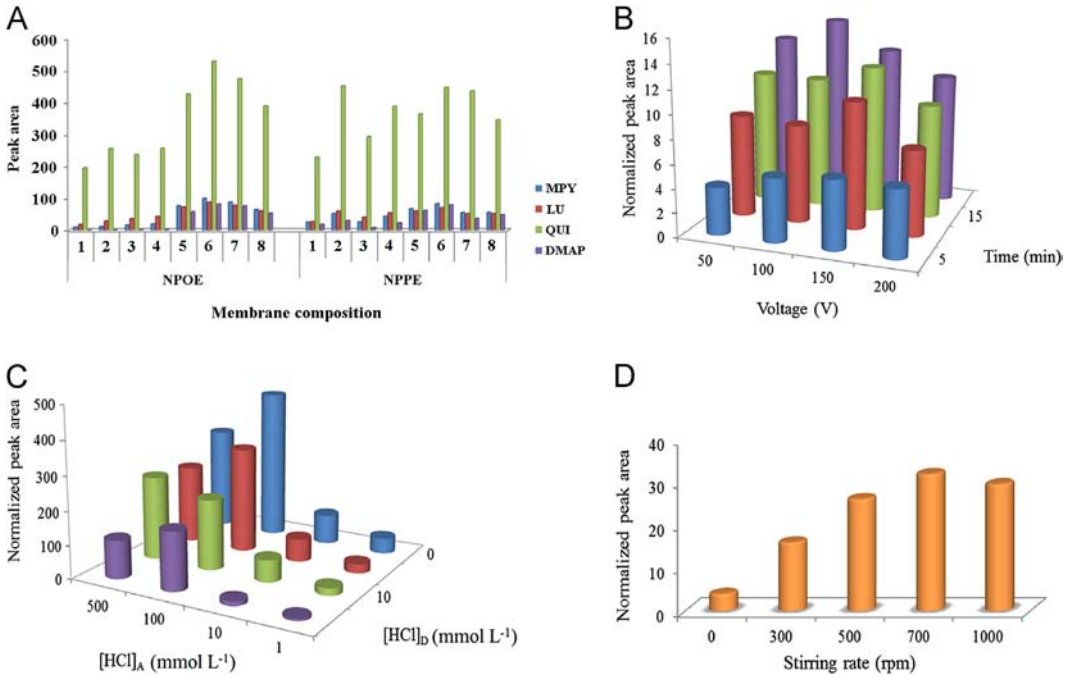


Fig. 2. Effects of SLM composition; 1: Pure solvent, 2: 5% TEHP, 3: 10% TEHP, 4: 15% TEHP, 5: 5% DEHP, 6: 10% DEHP, 7: 15% DEHP, 8: 5% TEHP + 5% DEHP (A), applied voltage and extraction time (B), HCl concentration in donor and acceptor phases (C) and stirring rate (D), on extraction efficiencies of MPY, LU, QUI, and DMAP by EME method. Spiked concentration of the analytes: 500 ng mL^{-1}; sample volume: 24 mL.

dependent on the applied voltage that provides the main driving force for the extraction procedure [22]. Thus, applying a voltage across a SLM is an important factor to be regarded for efficient extraction of basic compounds. In three-phase microextraction, mass transfer is a time-dependent process; so, time is another parameter which can affect the flux of analytes. Both time and voltage directly enhance the extraction recovery; but there is an antagonistic effect when they are simultaneously considered. Therefore, an increase in extraction time limits the voltage and vice versa. For attaining the optimum extraction voltage and time, these parameters were investigated at the same time. All experiments for optimization of applied voltage and extraction time were initially accomplished in ultrapure water. Extraction of the analytes was studied at different EME durations, ranging from 5 min to 20 min, and applied electric potentials, varying in the range of 50–200 V. The analytes were extracted from a neutral sample solution (analytes in pure water), which was agitated by stirring at a rate of 700 rpm, into a 100 mmol L^{-1} HCl solution. The normalized peak area for each run was selected as response objective for the study [23–26]. To normalize the peak areas of the analytes, the whole experiments were first performed and then the peak area of each analyte was divided by its smallest peak area that was gained in all of the experiments. The normalized peak areas for the pyridine compounds were subsequently added for each run and used in calculation of the total normalized peak area. The results are summarized in Fig. 2B. It was revealed that the chromatographic peak areas for the analytes increased by increasing the applied voltage and the extraction time up to 100 V and 20 min, respectively. Further enhancement of voltage leads to a drop in response owing to mass transfer resistance as a result of built-up of a boundary layer of ions at the interfaces at both sides of the SLM, increase of the current level, some instability and bubble formation. As a consequence, 100 V electrical potential was applied for 20 min for the rest of the experiments. Nevertheless, maximum amounts of time and voltage are restricted by composition of SLM and salt content of sample medium. Increasing the concentration of carrier in the SLM improves the conductivity of membrane as well as the ion transfer ability [20]. But in urine samples, there is a large number of ionic interferences. Hence, the number of ion transportations through the SLM noticeably increases. This causes an increase in the friction between the ions and the organic solvent, excessive heat production (Joule heating) and instability of SLM; so in some cases, puncture of SLM and arc generation between platinum electrodes have been reported [27]. Thus, time and voltage were again optimized in urine samples. Herein, extraction of the analytes was studied at various EME durations ranging from 5 to 20 min, while the electrical potentials were applied in the range of 20–100 V. Maximum peak areas were reached when 50 V was applied for 20 min.

3.1.3. *pH of donor and acceptor phases*

During the rest of optimization process, effect of pH of donor and acceptor solutions on the extraction efficiency was investigated. It was shown that the ratio of total ionic concentration of the donor phase to that of the acceptor phase, which is defined as ion balance (χ), affects the flux over the membrane [14,22]. The flux may decline with an increase in the above ratio, as described by theoretical models [22]. To scrutinize the impact of this parameter, HCl concentration in the donor phase was changed from 0 mmol L^{-1} (i.e., ultra-pure water) to 100 mmol L^{-1}, while it was altered in the range of 1–500 mmol L^{-1} in the acceptor phase (Fig. 2C). Maximum amounts of the analytes were extracted when the concentration of HCl in the acceptor phase was 100 mmol L^{-1} and ultra-pure water (pH 6.5) was employed as the sample solution. As it was expected, maximum extraction was acquired for a minimum value of χ. Nonetheless, a decrease in the concentration of HCl in the acceptor phase resulted in partial deprotonation of the analytes and accelerated their back-diffusion into the donor solution. Therefore, 100 mmol L^{-1} HCl was selected as a suitable acceptor solution for the subsequent experiments.

3.1.4. *Stirring rate*

As it is known, stirring speed plays an essential role in enhancing the kinetics and the efficiency of extraction by

increasing the mass transfer and reducing the thickness of double layer around SLM; so, stirring is absolutely vital during extraction, since it influences the migration of analytes. It was anticipated that the extraction efficiency would improve by increasing the stirring rate, and the obtained results in this study, for stirring rates up to 700 rpm, confirmed the prediction (Fig. 2D). On the other hand, a decline in extraction recovery was observed at higher speeds due to formation of intense vortex and bubbles formation in the sample solution. Consequently, a stirring rate of 700 rpm was chosen as the optimum value to achieve maximum extraction recoveries.

3.2. Optimization of DLLME variables using face-centered central composite design (FCCCD)

As it was mentioned, six different variables comprising types of extraction and disperser solvents and their volumes, salt effect and pH of donor solution had to be optimized in order to reach the best extraction conditions for DLLME. In this work, central composite design (CCD) was exploited to find the optimal point. CCD is a second-order model correlating the response function with the independent factors; so that the amount of response can be anticipated at any point within the factor domain, even though that point is not included in the design. The model takes the following general form for independent variables [28]:

$$y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} a_{ij} x_i x_j + \sum_{i=1}^{n} a_{ii} x_i^2 \quad (3)$$

where y is the dependent variable; x_i is the independent variable; a_i terms depict the regression coefficient of the model and a_0 is the difference between observed and predicted responses at the design point. CCD consists of factorial points, center points and star (axial) points. One kind of CCD is face-centered central composite design (FCCCD) which is considered unity in α (star point). The total number of design points needed (N) is determined by the equation below

$$N = 2^f + 2f + N_0$$

wherein f is the number of variables and N_0 is the number of center points. Hence, with six factors and 3 center points, totally 79 experiments had to be carried out for the FCCCD; whereas one of the principal aims of experimental methodologies is to attain the most desirable operating conditions with least runs. Thus, the number of variables had to be decreased logically to diminish the required experimental runs.

Investigation of the types of extraction and disperser solvents as a factor in experimental design increases the number of runs and complicates the design. Since study of this parameter separately could simplify the experimental design and reduce the number of runs, it was initially optimized. This design comprised 27 experiments with three center points performed in random order.

3.2.1. Selection of extraction solvent

Selection of an appropriate extraction solvent is very critical to achieve good recoveries and high PFs for target compounds. The extractant must have some properties such as greater density than water, high extraction capability of the analytes, low solubility in water, and good chromatographic behavior. Based on these facts, some extraction solvents including dichloromethane, chloroform, carbon tetrachloride, carbon disulfide and trichlorobenzene were tested as the extraction solvents. To choose the best extraction

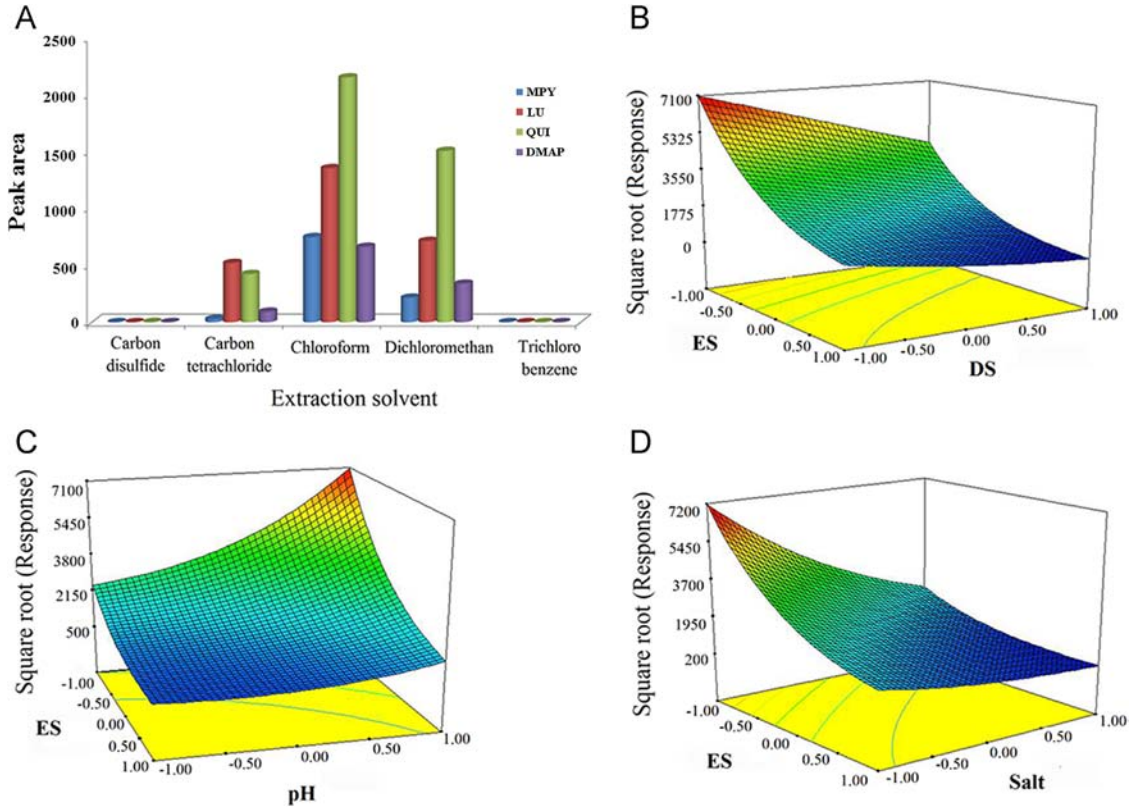


Fig. 3. Effect of type of extraction solvent on extraction efficiencies of MPY, LU, QUI, and DMAP by DLLME method (A). Three-dimensional response surfaces against: extraction and disperser solvent volumes (B), extraction solvent volume and pH of donor solution (C), extraction solvent volume and salt% in donor solution. Spiked concentration of the analytes: 500 ng mL^{-1}; Donor solution volume: 1 mL.

solvent, 100 μL of methanol solvents each containing proper amount of an extraction solvent, to obtain the same settled phase volumes (6.0 μL), were evaluated. The results are displayed in Fig. 3A. The results indicate that in the case of chloroform the peak areas of the analytes are maximized. So, chloroform was selected as extraction solvent.

Table 2
Experimental factors, levels and matrix of face-centered central composite design (FCCCD) for determination of pyridine derivatives.

Factor			Symbol	Level		
				Low (−1)	Center (0)	High (+1)
Volume of extraction solvent (μL)			ES	20	60	100
Volume of disperser solvent (μL)			DS	50	100	150
pH of donor solution			pH	7	10	13
Salt%			Salt	0	5	10
Run	*ES*	*DS*	*pH*	*Salt*		*Normalized peak area*
1	0	0	0	0		125.4193
2	1	−1	−1	1		281.9894
3	1	−1	1	1		170.5543
4	−1	1	1	1		2317.49
5	1	−1	1	−1		508.7305
6	−1	1	−1	−1		3373.394
7	−1	−1	1	−1		7080.861
8	−1	1	−1	1		1950.981
9	1	1	−1	1		1237.995
10	−1	−1	1	1		1999.866
11	1	−1	−1	−1		525.8606
12	1	1	1	1		4.088126
13	−1	1	1	−1		3078.545
14	1	1	−1	−1		361.8313
15	0	0	0	0		656.2145
16	1	1	1	−1		374.9147
17	−1	−1	−1	−1		3101.935
18	−1	−1	−1	1		12.49351
19	0	−1	0	0		499.3686
20	0	0	−1	0		594.1633
21	0	0	0	0		797.4958
22	0	0	0	−1		840.8565
23	0	1	0	0		268.6247
24	1	0	0	0		144.8081
25	0	0	1	0		747.1367
26	−1	0	0	0		1755.517
27	0	0	0	1		251.5966

3.2.2. Selection of disperser solvent

The key point for choosing the disperser solvent is its miscibility in both the extraction solvent and the aqueous sample [29] which enables the extraction solvent to be dispersed as fine droplets in aqueous phase to construct a cloudy solution (water/disperser solvent/extraction solvent). In such a case, the surface area between extraction solvent and aqueous phase (sample) is definitely large and leads to the enhancement of extraction efficiency.

Therefore, methanol, acetone and acetonitrile were selected as disperser solvents. A series of sample solutions were explored by using 100 μL of methanol, acetone and acetonitrile containing suitable amounts of chloroform (to gain identical settled phase volumes). According to the obtained results, methanol gave the highest peak areas compared to acetonitrile and acetone, and thus it was utilized as disperser solvent in the subsequent experiments.

3.2.3. Optimization of extraction and disperser solvent volumes, salt addition and pH of donor solution

Ultimately, the impacts of the volumes of extraction and disperser solvents, salt% and pH of donor phase were scrutinized

Table 4
Operating conditions for simultaneous extraction of pyridine derivatives from human urine.

Extraction method	Variable	Optimal point
EME	SLM	NPOE + 10% DEHP
	Voltage	50 V
	Extraction time	20 min
	Donor phase	Water
	Acceptor phase	100 mM HCl
DLLME	Type of extraction solvent	Chloroform
	Type of disperser solvent	Methanol
	Volume of extraction solvent	20 μL
	Volume of disperser solvent	50 μL
	pH of donor solution	13.0
	Salt%	0.0

Table 3
Analysis of variance (ANOVA) table for response surface quadratic model.

Source	Sum of squares	df	Mean square	*F* value	*p*-Value Prob > *F*	
Model	8674.12	14	619.58	10.5	0.0001	Significant
A-ES	3763.28	1	3763.28	63.76	< 0.0001	
B-DS	0.018	1	0.018	3.02E-04	0.9864	
C-pH	137.11	1	137.11	2.32	0.1534	
D-Salt	1342.17	1	1342.17	22.74	0.0005	
AB	56.88	1	56.88	0.96	0.3456	
AC	567.67	1	567.67	9.62	0.0092	
AD	434.63	1	434.63	7.36	0.0188	
BC	751	1	751	12.72	0.0039	
BD	593.3	1	593.3	10.05	0.0081	
CD	57.01	1	57.01	0.97	0.3451	
A\widehat2	120.53	1	120.53	2.04	0.1785	
B\widehat2	1.45	1	1.45	0.025	0.8779	
C\widehat2	84.57	1	84.57	1.43	0.2544	
D\widehat2	13.72	1	13.72	0.23	0.6384	
Residual	708.24	12	59.02			
Lack of fit	539.86	10	53.99	0.64	0.7427	Not significant
Pure error	168.38	2	84.19			
Cor total	9382.36	26				
R-squared	0.9245					
Adj *R*-squared	0.8364					

via FCCCD. For investigation of the four factors with 3 center points, totally 27 experiments had to be executed. The low (−1), central (0), and high (+1) levels of these variables are given in Table 2. The normalized peak area for each run was chosen as response objective for the study. Through multiple regression analysis, the experimental responses (shown in Table 2) were correlated with the four significant factors. As it is observed, the coefficient of determination, R^2, is more than 0.9 which denotes that the obtained equation has good adequacy for correlating the experimental results. The model was described as follows:

$$\begin{aligned}\text{Square root (Response)} = {} & 20.64 - 14.46(\text{ES}) - 0.031(\text{DS}) \\ & + 2.76(\text{pH}) - 8.64(\text{Salt}) - 1.89(\text{ES})(\text{DS}) \\ & - 5.96(\text{ES})(\text{pH}) + 5.21(\text{ES})(\text{Salt}) \\ & - 6.85(\text{DS})(\text{pH}) + 6.09(\text{DS})(\text{Salt}) \\ & - 1.89(\text{pH})(\text{Salt}) + 6.85(\text{ES})^2 \\ & - 0.75(\text{DS})^2 + 5.73(\text{pH})^2 + 2.31(\text{Salt})^2 \end{aligned} \quad (5)$$

ANOVA was performed and showed that the model was significant and the "lack of fit" was not significant ($P=0.05$) which implied that the model was fitted (Table 3). Response surface methodology was applied to analyze the effect of independent variables on the response. Fig. 3(B–D) illustrates the relationship between explanatory and response variables in a three-dimensional representation of the response surface. With this purpose, two variables were maintained at their central levels and the others were varied within the experimental range. Based on the analysis of variance and the response surface plot, it is noticed that the normalized peak areas of the pyridine derivatives increase in a quadratic manner with decreasing the volumes of extraction and disperser solvents and the salt% and enhancing the pH of donor solution. It is obvious that the sedimented phase volume declines by decreasing the volumes of extraction and disperser solvents. Diminishing the volume of final solution results is improving the total preconcentration factors. Therefore, as the results exhibit, the chromatographic signals of the analytes increase by decreasing the volumes of extraction and disperser solvents. Conversely, as can be seen in Fig. 3D, salt% may possess a negative effect on extractability by changing the physical characteristics of the Nernst diffusion film, thereby reducing the rate of diffusion of the analytes into the organic phase [26]. Also, it may cause the limitation of mass transfer owing to increasing the viscosity of solution. Thus, maximum response was yielded in the absence of salt. To acquire the most favorable extraction conditions, a pH of 13.0 was considered to be most appropriate for the donor solution. The direct extraction of ionized forms of analytes into hydrophobic media is difficult. So, the donor solution must be basic enough to prevent from the ionization of analytes and promote their distribution into organic solvent. Table 4 presents the attained optimum conditions used for the rest of the work.

Table 5
Figures of merit of EME–DLLME for extraction of pyridine derivatives from water and human urine samples.

Sample	Analyte	LOD (ng mL^{-1})	Linearity (ng mL^{-1})	R^2	PF[a]	RSD%[b]	
						Intra-day	Inter-day
Water	3-Methyl pyridine	0.25	0.5–500	0.9973	238	5.0	12.3
	2,4-Lutidine	0.5	1.0–500	0.9966	175	2.2	10.1
	Quinoline	0.1	0.5–500	0.9978	134	3.6	12.7
	2,4-Dimethyl amine pyridine	1.0	2.0–500	0.9989	109	2.3	9.6
Urine	3-Methyl pyridine	1.0	2.0–500	0.9988	42	4.2	12.3
	2,4-Lutidine	1.0	2.0–500	0.9948	43	2.4	12.7
	Quinoline	0.25	0.5–500	0.9982	202	3.0	13.1
	2,4-Dimethyl amine pyridine	2.0	5.0–500	0.9962	40	5.3	10.4

[a] At 10 ng mL^{-1}.
[b] Intra-day and inter-day RSDs% were obtained at 50 ng mL^{-1} by five and three replications, respectively.

Table 6
Comparison of the proposed method with other analytical techniques for determination of MPY, LU, QUI and DMAP.

Analytical method[a]	Analyte	Matrix	LOD (ng mL^{-1})	Donor phase volume (mL)	PF	RSD%	Ref.
SPE/LC–MS–MS	MPY	Cigarette	4.19 (ng/Cig)	50 mL	–	< 9	[9]
	QUI		3.79 (ng/Cig)		–	< 9	
Water trap/GC–MS	MPY	Cigarette	–	200 mL	–	< 8	[8]
	LU		–		–	< 8	
On-line-HF-LPME/HPLC-UV	MPY	Cigarette	1.0	20 mL	115	4.1	[4]
	LU		0.5		213	3.2	
	QUI		0.5		220	5.4	
DPV	QUI	Petroleum Fuels	5.05	–	–	< 5	[30]
EME–DLLME–GC	MPY	Water	0.25	24 mL	238	5.0	This work
	LU		0.5		175	2.2	
	QUI		0.1		134	3.6	
	DMAP		1.0		109	2.3	
	MPY	Urine	1.0	24 mL	42	4.2	This work
	LU		1.0		43	2.4	
	QUI		0.25		202	3.0	
	DMAP		2.0		40	5.3	

[a] Solid phase extraction (SPE), liquid chromatography (LC), mass spectrometry (MS), differential pulse voltammetry (DPV), dispersive liquid–liquid microextraction (DLLME).

3.3. Method validation

To verify the practical applicability of the proposed technique, calibration curves were plotted in ultra-pure water and analyte-free urine samples under the optimized extraction conditions, and figures of merit for the method were evaluated. The results are summarized in Table 5. To this end, the samples were spiked with the analytes and the extractions were accomplished (urine samples were diluted (1:4) with ultra-pure water prior to extraction). The pH values of all samples were adjusted by dropwise addition of NaOH and/or HCl solutions, so that the final pH of the samples was 6.5. As seen from Table 5, this method has the potential for extraction and determination of target analytes with admissible preconcentration factors in the ranges of 42–238, 43–175, 134–202 and 40–109 for MPY, LU, QUI, and DMAP, respectively, in different matrices. However, the lower PF values for urine sample in comparison with water sample may be attributed to high content of interference ions in urine as well as lower optimum extraction voltage. Satisfactory limits of detection were gained (LODs < 0.25 ng mL^{-1}) which confirmed the ability of this technique to analyze trace amounts of analytes in complicated matrices. The linearity of the method was studied up to 500 ng mL^{-1} and coefficients of determinations higher than 0.9948 with acceptable repeatabilities (intra-day RSDs $< 5\%$ and inter-day RSDs < 13.1) were achieved. Comparison of the suggested technique with other existing methods for extracting and determining these analytes is provided in Table 6. It is apparent that this technique possesses the capacity to effectively extract the analytes from complex samples. Relatively high preconcentration factors and small detection limits allow the presented method to

Table 7
Determination of MPY, LU, QUI and DMAP in different urine samples.

Sample	Analyte	C_{real} (n mL^{-1})	C_{added} (ng mL^{-1})	C_{found} (ng mL^{-1})	RSD% ($n=3$)	Accuracy (Error%) ($n=3$)	RR%
Urine 1	MPY	47	50	99	3.6	+4	104
	LU	nd[a]	50	48	4.3	−4	96
	QUI	nd	50	51	5.1	+2	102
	DMAP	nd	50	54	4.9	+8	108
Urine 2	MPY	< LOQ	50	53	2.7	+6	106
	LU	nd	50	48	4.4	−4	96
	QUI	nd	50	51	5.2	+2	102
	DMAP	nd	50	49	3.3	−2	98
Urine 3	MPY	30	50	83	3.9	+6	106
	LU	nd	50	52	5.0	+4	104
	QUI	nd	50	49	6.4	−2	98
	DMAP	nd	50	53	4.5	+6	106

[a] Not detected.

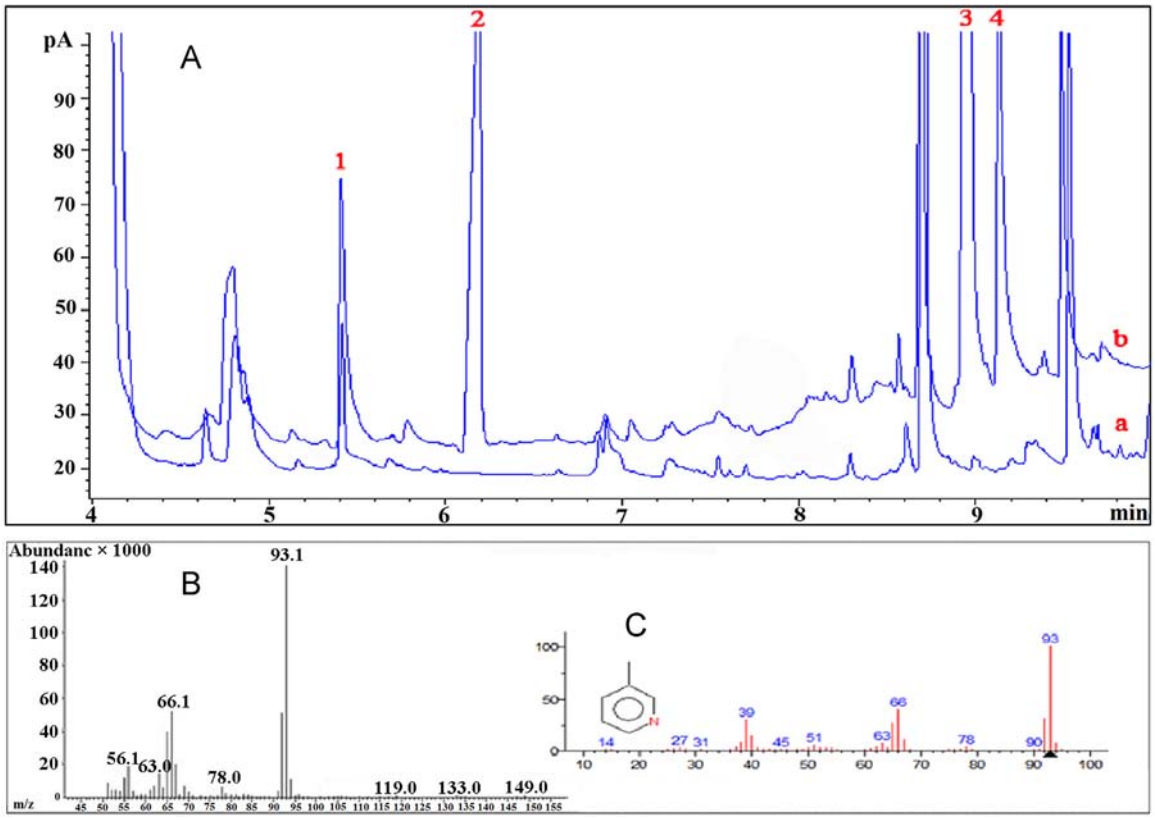


Fig. 4. Chromatograms obtained after performing (A) EME–DLLME process with (a) non-spiked urine sample (b) urine sample spiked at a concentration of 50 ng mL^{-1} of the analytes. (1) MPY, (2) LU, (3) QUI, (4) DMAP. (B) Mass spectra of detected MPY in urine sample, and (C) mass spectra of MPY from the database.

analyze trace amounts of the pyridine derivatives. Since EME–DLLME offers high sample cleanup, it can be exploited for determination and quantification of these analytes in complicated matrices.

3.4. Analysis of real samples

In order to investigate matrix effects and applicability of the proposed technique to extract the analytes from real samples, some final experiments were carried out on various urine samples taken from smokers. The urine samples were diluted 1:4 with ultra-pure water and their pHs were adjusted to 6.5 by dropwise addition of NaOH solution. Afterwards, 24 mL of each solution was transferred into the sample vial and the extraction process was conducted. Nearly 10 μL of the acceptor phase was transferred into 1 mL of an alkaline solution in a screw-cap glass test tube with conical bottom to perform the DLLME method. All of the procedures were repeated three times for each sample under the optimal conditions. The obtained results are illustrated in Table 7. Relative standard deviations (RSDs%) were within the range 2.7–6.4%. Besides, to examine the accuracy of the proposed method, 50 ng mL^{-1} of each analyte was spiked into the samples, EME–DLLME was executed and relative recoveries were calculated (Table 7). The relative recoveries for the spiked samples were in an acceptable range (96–108%) and there was no significant difference between the media, used to plot the calibration curves, and the real sample matrices. Therefore, the calibration curves could be employed directly to calculate the amounts of analytes in the samples. The chromatograms obtained from non-spiked and spiked urine samples with 50 ng mL^{-1} of the analytes are depicted in Fig. 4A. As observed in Fig. 4A, 3-methylpyridine can be detected in smokers' urine samples. For the real sample 1, the occurrence of MPY was justified by GC/MS analysis in the full scan mode (m/z in the range of 10–300) and comparing the obtained mass spectrum with MS database of instrument library. Fig. 4B shows GC/MS chromatogram of the urine sample after extraction. Fig. 4C indicates mass database of GC/MS library. As it is noted, the GC/MS results prove the presence of MPY in the urine sample.

4. Conclusions

The present work discloses the feasibility of extraction and determination of pyridine derivatives in human urine and water samples. To achieve this goal, EME–DLLME technique was used to benefit from high sample cleanup ability of the EME method as well as DLLME compatibility with GC instrument. The proposed technique was successfully developed for determination of pyridine derivatives in smokers' urine samples. Compared to the existing methods, this technique made evident the possibility of extraction of the analytes from complicated matrices. Furthermore, its application to real samples became possible in addition to affording EME advantages such as minimum consumption of organic solvents, short extraction time, efficient sample clean-up and very simple and inexpensive equipment. The suggested method may become a very powerful and innovative technique for diagnosis of smokers in clinical centers.

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