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Combination of electromembrane extraction with dispersive liquid–liquid microextraction followed by gas chromatographic analysis as a fast and sensitive technique for determination of tricyclic antidepressants

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ABSTRACT

For the first time, combination of electromembrane extraction (EME) and dispersive liquid–liquid microextraction (DLLME) followed by gas chromatography–flame ionization detection (GC/FID) was developed for determination of tricyclic antidepressants (TCAs) in untreated human plasma and urine samples. Response surface methodology (RSM) was used for optimization of experimental parameters, so that extraction time of 14 min, voltage of 240 V, donor phase of 64 mM HCl and acceptor phase of 100 mM HCl were obtained as optimal extraction conditions. Matrix effect and carry-over were investigated in this work. The results indicated matrix effect for urine and plasma samples in comparison with neat solutions, so match matrix method was used for drawing working calibration curves. However, no carry-over was appeared at the retention time of investigated TCAs (S/N < 3). With application of optimized values, good linearity in the range of 2–500 $\mu g\,L^{-1}$ was obtained for TCAs with the correlation of determination values (r^2) above 0.9968. The limits of detection (S/N = 3) for TCAs were found 0.25, 3.0, and 15 $\mu g\,L^{-1}$ in water, urine, and plasma, respectively. The preconcentration factors of TCAs in water, urine, and plasma were from 383 to 1065. The intra- and inter-assay precisions (%) were in the ranges 6.4–11.8% and 6.2–10.8%, respectively, and the intra- and inter-assay accuracies were >86.5%. The results showed that EME–DLLME–GC/FID is a promising combination for analysis of TCAs present at low concentrations in biological matrices.

1. Introduction

Treatment of depression includes various forms of psychotherapy as well as pharmacotherapy with medicines. The tricyclic antidepressants (TCAs), one of the largest groups of drugs for treatment of psychiatric disorders, are widely used for treatment of depression [1]. The measurement of concentration of these drugs in biological fluids is becoming increasingly important as an aid for effective control of therapy and forensic medical examinations. For this group of drugs, distinct ranges of optimum plasma concentration for therapy are required (100–300 $\mu g\,L^{-1}$ for most of the TCAs) [2]. Direct analysis of biological fluids would be best; however, in the majority of cases, it is not feasible due to the inherent complexity of these samples, which limits the selectivity and sensitivity of the determinations. Because of this, there exists a need for sample pre-treatment, which is intended to improve the sensitivity and specificity of the assay by removing the majority of the matrix interference while concentrating the analyte.

Liquid–liquid extraction (LLE) and solid phase extraction (SPE) have been usually applied as the useful sample preparation procedures for determination of drugs. However, both methods have certain drawbacks. LLE is a time consuming and tedious procedure and uses large amounts of high-purity and hazardous organic solvents. SPE techniques often introduce artifacts into the sample extracts, have limitations for pH ranges of sample solution, and may require lengthy processing such as washing, conditioning, eluting, and solvent evaporation [3].

Recently, modern trends in analytical chemistry are toward simplification, miniaturization, and minimization of organic solvent used in sample preparation. Stir-bar sorptive extraction (SBSE), solid-phase microextraction (SPME), and liquid phase microextraction methods (LPME) are miniaturized techniques, which have been developed for sample preparation. SPME and SBSE are simple and solventless methods. However, the major disadvantages of SPME are its high cost, sample carry-over, fiber fragility, and limited lifetime of the fiber [4]. SBSE needs relative long extraction (30–120 min) and desorption time, and also have carry-over problems [5]. According to the literature over different LPME methods, dispersive liquid–liquid microextraction (DLLME) and hollow fiber-based liquid phase microextraction (HF-LPME) have shown more

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interests among analytical chemists. DLLME is a simple and fast method and provides high preconcentration factors. HF-LPME provides high preconcentration factors and produces clean extracts without any need for solvent evaporation and re-constitution as required for LLE and SPE [6]. Also, sample carry-over can be avoided in HF-LPME because the hollow fibers are inexpensive enough to be disposed after each use. Because the LPME tolerates a wide pH range, it can be used in applications, which would not be suitable for SPE or SPME.

However, these techniques have some drawbacks; DLLME is only efficient for simple matrices, so that it creates crowded chromatograms for extracts from complex matrices, especially biological fluids. This intensifies distinguish among peaks of interferences and analytes. Therefore, sample pretreatment is an unavoidable step for this technique.

Lately, much more interests have been focused on the combination of DLLME with other extraction techniques such as SPE [7], supercritical fluid extraction (SFE) [8], SBSE [9] and molecular-imprinted polymer (MIP) extraction [10] as hyphenated techniques for the determination of a variety of compounds in complex matrices. These hyphenated techniques provide higher purification ability and selectivity.

In the case of HF-LPME, the extraction time needed is usually high and common extraction times of 30–50 min have been reported [11]. Recently, Pedersen-Bjergaard and Rasmussen introduced a novel microextraction technique called electromembrane extraction (EME) [12]. In EME, an electrical voltage is applied, which facilitates the extraction of analytes across hollow fiber membrane. This voltage causes EME to be more efficient than HF-LPME and analytes can be extracted in a short time in comparison with the long time needed for HF-LPME. Also, EME can extract analytes with no sample pretreatment, which removes the resulted problems due to this step [13]. EME was performed effectively for extraction of various compounds from different matrices so far [12,14–24].

One of the disadvantages of EME is its incompatibility with gas chromatographic (GC) instrument. Moreover, GC is simpler, faster, and less expensive than the high performance liquid chromatography instrument. It can easily be coupled with different types of sensitive detectors like flame ionization detector (FID) and mass spectrometry (MS). More recently, Guo et al., reported electro membrane extraction followed by low-density solvent based ultrasound-assisted emulsification microextraction (EME-LDS-USAEME) combined with derivatization for determining chlorophenols (CPs) in water samples and analysis by GC–MS [25]. However, this method has been applied for analysis of CPs in simple matrices and USAEME can solely create good results for this purpose, but it can be very efficient for complicated matrices.

The aim of this study was to present the first attempt at combining the advantages of EME with DLLME to develop a new pretreatment method for the extraction of TCAs from biological matrices. Regarding the mentioned points about SPE, SFE and SBSE, coupling of EME with DLLME have noticeable supremacies in comparison with other introduced hyphenated techniques. For example, the present method removes the need to relatively high cost SPE cartridge as well as tedious extraction steps especially solvent evaporation. In the case of SFE, the required instrument is expensive and this technique is more proper for solid samples such as soils and sediments.

EME–DLLME enables the easy application of DLLME toward complex matrices, removes the limitation of EME technique, increases the sensitivity of analysis due to collecting of analytes in very low microvolumes of the extraction solvent ($\leq$3 μL) as well as providing of high sample clean-up.

2. Experimental

2.1. Chemicals and materials

Amitriptyline (AMI), trimipramine (TRI), and doxepin (DOX) were purchased from Razi Pharmaceutical Company (Tehran, Iran). Selection of these drugs was done based on approved drug list by Food and Drug Administration (FDA) to treat depression as well as considering common antidepressant drugs which use in our country. 2-Nitrophenyl octyl ether (NPOE), tris-(2-ethylhexyl) phosphate (TEHP), and di-(2-ethylhexyl) phosphate (DEHP) were purchased from Fluka (Buchs, Switzerland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), methanol (MeOH) and carbon tetrachloride (CCl_4) were from Merck (Darmstandt, Germany). All the chemicals used were of analytical reagent grades. The porous hollow fiber used for the supported liquid membrane (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 obtained from a Younglin370 series aqua MAX purification instrument (Kyounggi-do, Korea).

2.2. Biological and standard solutions

Midstream urine samples were collected from a volunteer patient (25-year-old male) undergoing therapy with TCA drugs and one person who had not taken TCAs at all (as match matrix for drawing the calibration curves) with respect to human ethical guidelines. Also, the protocol was approved by an Internal Review Board.

Drug-free human plasma samples (blood group A^+) were obtained from Iranian Blood Transfusion Organization (Tehran, Iran). The samples were stored in sterilized bottles at −4 °C, thawed and shaken before extraction. A stock solution containing 1.0 mg mL^{-1} of AMI, TRI, and DOX were prepared in acetonitrile and stored at −4 °C. Working standard solutions were prepared by dilution of the stock solutions in acetonitrile.

2.3. Gas chromatography analysis

Separation and detection of AMI, TRI, and DOX were performed by an Agilent 7890A gas chromatography system (Palo Alto, CA, USA) equipped with a split–splitless injector and a flame ionization detector (FID). A 30 m HP-5 (5% phenyl–95% dimethyl polysiloxane) Agilent fused-silica capillary column (0.32 mm i.d. and 0.25 μm film thickness) was applied for separation of target compounds. Helium (purity 99.999%) was used as carrier gas at constant flow rate of 4 mL min^{-1}. The temperature of injector and detector were set at 280 and 300 °C, respectively. The injection port was operated at split mode (1:5). Oven temperature program was developed by our research group and was 185 °C for 12 min, increased to 280 °C with a ramp of 30 °C min^{-1}, and held for 3 min at 280 °C.

An Agilent (Wilmington, USA) 7890A GC coupled to an Agilent MSD 5975C quadrupole mass spectrometer was performed in the full scan mode (m/z in the range of 10–450) to confirm the presence of analytes after extraction in real samples. The GC was fitted with an Agilent HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium (99.999%) was used as the carrier gas at 1.0 mL min^{-1}. The MS quadrupole and the MS source temperatures were set at 150 and 230 °C, respectively. The same GC/FID temperature program was employed for GC/MS analysis. The filament delay time was set as 3 min.

2.4. Equipments for EME–DLLME technique

A twenty four-milliliter vial with internal diameter of 2.5 cm and height of 5.5 cm was used. The electrodes used in this work were

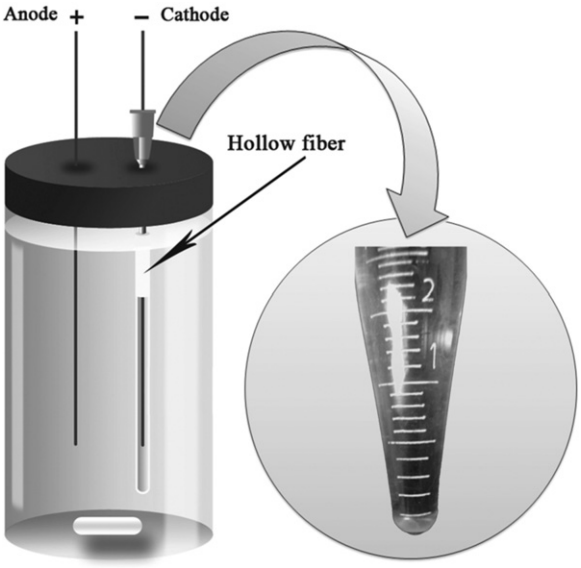

Fig. 1. A schematic of the EME–DLLME set-up for extraction of TCAs.

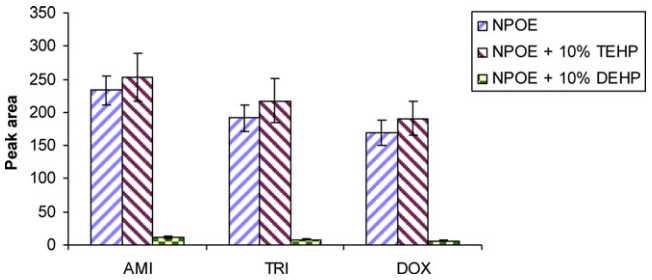

Fig. 2. Effect of SLM composition on extraction efficiencies of TCAs by EME–DLLME; spiked concentration: 100 μg L^{-1}, voltage: 150 V, sample volume: 24 mL, donor solution: 1 mM HCl, acceptor solution: 100 mM HCl, extraction time: 10 min, and stirring rate: 700 rpm. Standard deviations were calculated for $n = 3$. DLLME procedure was performed according to the literature [26] with some modifications (see Section 2.5).

platinum wires with diameters of 0.25 mm, and were obtained from Pars Pelatine (Tehran, Iran). The electrodes were coupled with a power supply model 8760T3 with a programmable voltage in the range of 0–600 V and with a current output in the range of 0–500 mA from Paya Pajoohesh Pars (Tehran, Iran). During the extraction, the EME unit was stirred with a stirring speed in the range of 0–1250 rpm by a heater-magnetic stirrer model 301 from Heidolph (Kelheim, Germany) using a 1.5 cm × 0.3 cm magnetic bar.

A 40 kHz and 0.138 kW (Tecno-GazSpA, Italy) ultrasonic water bath with temperature control was applied to emulsify the organic solvent in the aqueous solution. A Sepand Teb Azma centrifuge (Tehran, Iran) was used for phase separation from cloudy solution.

2.5. Procedure for EME–DLLME

A schematic of EME–DLLME procedure is shown in Fig. 1. Twenty-four milliliters of the sample solution containing target analytes in 64 mM HCl was transferred into the sample vial. To impregnate the organic solution in the pores of the hollow fiber wall, 3.8 cm piece of hollow fiber was cut out and dipped in the solution for 5 s and then the excess of organic solution was gently wiped away by air blowing using a 500 μL Hamilton syringe. The upper end of the hollow fiber was connected to a medical needle tip as a guiding tube, which was inserted through the rubber cap of the vial. Ten microliters of 100 mM HCl (the acceptor solution) was introduced into the lumen of the hollow fiber by a microsyringe and the lower end of the hollow fiber was sealed with a small piece of aluminum foil. One of the electrodes, the cathode, was introduced into the lumen of the fiber. The fiber containing the cathode, SLM, and the acceptor solution was afterward directed into the sample solution. The other electrode, the anode, was led directly into the sample solution. The electrodes were subsequently coupled to the power supply and the extraction unit was placed on a stirrer with stirring speed of 700 rpm. The predetermined voltage was turned on and extraction was performed for 14 min. Under the voltage applied, the target analytes migrated from aqueous sample to SLM, and then transported into the acceptor phase. When extraction was completed, the acceptor solution was collected by a microsyringe and injected into 1.0 mL of the alkaline solution (pH = 12) in a 5 mL screw cap glass test tube with conic bottom for converting extracted analytes to their neutral forms. Following that, the DLLME procedure was performed on this solution based on Ito et al.'s work with some modifications [26].

The centrifuge glass tube was immersed into an ultrasonic water bath. The ultrasonic water bath was switched on and a mixture of MeOH (as a disperser solvent, 150 μL) and CCl_4 (as an extraction solvent, 10 μL) was slowly injected into the water sample by a 250 μL syringe. After a few seconds of sonication (at 25 ± 3 °C), the emulsion formed was centrifuged at 5000 rpm for 3 min to separate the phases. Two microliter of the sedimented phase was removed by a 5.0 μL Hamilton gas-tight syringe and injected into GC. The sedimented phase volume was about 3.0 ± 0.2 μL.

2.6. Data handling and processing

To obtain the optimum conditions for simultaneous extraction of TCAs, a central composite design (CCD) was used. For this purpose, Design-Expert software trial version 8.0 (Stat-Ease Inc., MN, USA) was employed to generate the experimental matrix and evaluate the results.

3. Results and discussion

3.1. Selection of organic solvent

According to earlier works in EME, NPOE solely or in combination with alkylated phosphates (TEHP and DEHP) provides an efficient extraction for basic compounds [16]. As can be seen in Fig. 2, addition of TEHP increased the extraction efficiencies of TCAs whereas DEHP has noticeable negative effect. This can be attributed to the formation of strong ion-pair complexes among analytes and DEHP into SLM [16]. Significantly better reproducibility and membrane stability was observed for pure NPOE in comparison with its mixture with TEHP in urine and plasma samples. Electric current in EME system is at the μA level [14,16]. Addition of alkylated phosphates increases the polarity and transportation ability of membrane, and therefore the current level. Although addition of alkylated phosphates may noticeably improve extraction efficiencies, they increase the risk of Joule heating into SLM and also increase its instability due to the high content of other ions into urine and plasma media. Furthermore, this increases the bubble formation in both donor and acceptor phases, as a result of increasing the current level. Therefore, NPOE was selected for subsequent experiments.

3.2. Optimization of EME–DLLME

In order to reach the optimal values of EME–DLLME, face-centered central composite design (FCCCD) was conducted. Central composite design (CCD) consists of factorial points, center points,

and star (axial) points. One type of CCD is FCCD, in which α (star point) is considered unity. This design includes 17 experiments with three central points performed in random order.

Different variables can affect the extraction efficiency of EME procedure, including type of organic solvent (SLM), volume of sample solution, pH of the donor and acceptor phases, stirring rate, salt%, extraction time, and voltage.

Variables were chosen with the aim of reducing the extraction time and process cost. The proposal includes parameters such as extraction time, voltage, pH of the donor and acceptor phases. Separate study of membrane organic solvent can give optimal SLM as well as simplicity of experimental design method and reduction of number of runs. Therefore, this parameter was separately optimized at first. Furthermore, initial experiments showed that addition of salt has negative effect on extraction efficiencies of antidepressant drugs and increasing of stirring speed higher than 700 rpm is not experimentally possible due to formation of intense vortex and bubble formation into sample solution at higher speeds. Therefore, these factors were not taken into account in the experimental design. In the following, HCl concentration in the donor and acceptor phases was combined as the ion balance (χ), i.e., the ratio of the total ionic concentration in the sample solution to that in the acceptor solution [19], to further reduce the number of runs. For this purpose, in all experiments the acceptor solution was kept constant at 100 mM HCl and the donor phase was varied between 1 and 100 mM HCl for making χ in the range of 1–0.01. Increasing concentration of HCl in the acceptor phase increases releasing rate of analytes in the acceptor/SLM interface. However, such a high HCl concentration should be avoided to preserve risk of electrolysis reaction in the acceptor phase. Therefore, an acceptor concentration of 100 mM was chosen. Table 1 represents the design matrix of the variables in both coded and natural units. For each run, the sum of peak areas of analytes was used as experimental response.The data obtained were evaluated by analysis of variance (ANOVA). A p-value less than 0.05 in the ANOVA table indicates the statistical significance of an effect at 95% confidence level. For an experimental design with three factors, the model including linear, quadratic, and cross terms can be expressed as the following equation:

$$Response = a_0 + a_1F_1 + a_2F_2 + a_3F_3 + a_4F_1F_2 + a_5F_1F_3 + a_6F_2F_3 + a_7F_1^2 + a_8F_2^2 + a_9F_3^2 \quad (1)$$

Within Eq. (1), F_1–F_3 are the variable parameters, and a_0–a_9 are the coefficient values obtained through multiple linear regression using Design-Expert software. Data in Table 2 indicate that extraction time (F_2) is the most important parameter for the extraction efficiencies of TCAs by EME. In addition, there is no evidence of lack of fit at the 95% confidence level, meaning that the model is significant and explaining the observed differences in the response variable.

Table 1
Design matrix for the factors under study (F_i) and the corresponding response variable (sum of peak areas).

Factor	Description	Level		
		Low (−1)	Center (0)	High (+1)
F_1	Voltage (V)	100	200	300
F_2	Time (min)	5	10	15
F_3	Ion balance (χ)	0.01	0.051	1.0

Run	Voltage	Time	Ion balance (χ)	Sum of peak areas
1	−1	−1	−1	254.635
2	1	1	1	1209.969
3	0	0	0	1066.170
4	0	0	1	1027.432
5	0	0	−1	852.537
6	1	0	0	903.587
7	−1	1	−1	813.585
8	−1	1	1	799.823
9	0	−1	0	426.435
10	1	−1	−1	365.440
11	1	−1	1	268.563
12	−1	0	0	1101.533
13	1	1	−1	853.477
14	0	0	0	1168.324
15	0	0	0	1204.135
16	0	1	0	1279.032
17	−1	−1	1	276.812

Response surface methodologies (RSMs) were applied to analyze the effect of independent variables on the response. RSM graphically illustrate relationships between parameters and responses and are the way to obtain an exact optimum. Also, a two-dimensional contour plot on the basis of the model equations was shown below the response surfaces, which display the interaction between the independent variables and assist in determining the optimal operating condition for the desirable responses. The coefficients of the obtained models are illustrated in Table 3. The model has the coefficient of determination (adjusted-R^2) of 0.8764 that indicates a high degree of correlation between the response and model.

Fig. 3 shows the overall response surfaces. As can be seen in this figure, increasing of voltage and time enhanced extraction for all target analytes, reaching a maximum and thereafter gradually declined. The positive effect of time and voltage upon extraction in EME has been discussed by Pedersen-Bjergaard et al. [19]. Increasing the voltage pushes the system further from equilibrium and

Table 2
Analysis of variance (ANOVA) of the quadratic model to predict the increase in extraction efficiencies.

Factor	SS	df	MSS	F	p
Model	19,900,000	9	221,200	13.61	0.0012 (*significant*)
F_1	12,577.52	1	12,577.52	0.77	0.4082
F_1^2	28,564.98	1	28,564.98	1.76	0.2266
F_2	1,132,000	1	1,132,000	69.63	<0.0001
F_2^2	171,600	1	171,600	10.56	0.0141
F_3	19,618.26	1	19,618.26	1.21	0.3083
F_3^2	73,678.51	1	73,678.51	4.53	0.0708
F_1F_2	15,092.97	1	15,092.97	0.93	0.3673
F_1F_3	7887.68	1	7887.68	0.49	0.5085
F_2F_3	21,780.98	1	21,780.98	1.34	0.2850
Residual	113,800	7	16,252.71		
Lack of fit	103,500	5	20,703.65	4.04	0.2103 (*not significant*)
Pure error	10,250.74	2	5125.37		
Corrected total	2,104,000	16			

SS: sum of square; df:degree of freedom; MSS: mean sum of squares; F: Fisher value; p values <0.05 were considered to be significant, where F_1: voltage, F_2: time, and F_3: ion balance.

Table 3
Regression coefficients and standard errors (SE) of components of the quadratic model.

Coded term	Coefficients of the regression (a)	SE
Intercept (a_0)	1123.13	54.55
F_1	35.46	40.31
F_1^2	−103.25	77.89
F_2	336.40	40.31
F_2^2	−253.08	77.89
F_3	44.29	40.31
F_3^2	−165.83	77.89
F_1F_2	43.44	45.07
F_1F_3	31.40	45.07
F_2F_3	52.18	45.07
R^2-adjusted: 0.8764		

SE: standard error; F_1: voltage, F_2: time, and F_3: ion balance.

thus creates a stronger force driving transfer from the donor to the acceptor phase. It should be noted that EME is a non-exhaustive process. At the beginning of the process, recoveries increased rapidly by increasing the extraction time and voltage, but declined thereafter. The decreased peak area after these time and voltage values may be attributed to mass transfer resistance and built-up of a boundary layer of ions (from hydrochloric acid) at the interfaces at both sides of SLM or saturation of the analyte in the acceptor phase and analyte back-extraction into the donor phase as pH increased slightly into acceptor solution due to electrolysis [12]. In addition, the gradual suppression of analyte net transfer resulted from heat generation at higher time and voltages can also decrease extraction efficiency [17]. Furthermore, increasing the acidity of the donor phase enhances the protonation of the analytes. However, increase of proton ion concentration into donor phase increases competition among them with analyte ions for migration into the acceptor phase. This decreases the extraction efficiency and risk of heat generation into SLM and electrolysis reactions in both donor and acceptor phases.

According to the overall results of the optimization study, extraction time of 14 min, voltage of 240 V, and 64 mM HCl concentration of the donor phase were chosen for analysis of TCAs into real samples.

3.3. Analytical performance

The validation of the method was performed under optimal conditions by establishing linearity, preconcentration factor (PF), limits of detection (LOD) and quantification (LOQ), matrix effect (ME%), intra- and inter-assay precision (RSD%) and accuracy (Error%), relative recovery (RR%), carry-over and stability in drug-free water, urine, and plasma samples, according to recommendations of Food and Drug Administration (FDA).

The urine and plasma samples were diluted at the ratios of 1:4 and 1:9 with 64 mM HCl, respectively. Calibration curves were found to be linear in the range of 2–500 $\mu g\,L^{-1}$, 10–500 $\mu g\,L^{-1}$, and 40–500 $\mu g\,L^{-1}$ for water, urine, and plasma, respectively. Values for the coefficient of determination, r^2, were >0.9968 for the target analytes. The PF was defined as the ratio of the final analyte concentration in the sedimented phase to the initial concentration of analyte in the sample solution. The obtained PF values for TCAs in water, urine, and plasma were in the range of 383–1065. The obtained LODs (S/N = 3) and LOQs for TCAs were in the range of 0.25–15 $\mu g\,L^{-1}$ and 2–40 $\mu g\,L^{-1}$ in water, urine, and plasma, respectively, indicating good sensitivity for the presented method. The results are summarized in Table 4. An improvement in detection limits of the methodology could be achieved using GC/MS analysis due to compatibility of this technique with gas chromatography instrument. Fig. 4 depicts the GC/FID chromatograms of the TCAs related to spiked water, urine, and plasma samples at the concentration of 20 $\mu g\,L^{-1}$, respectively.

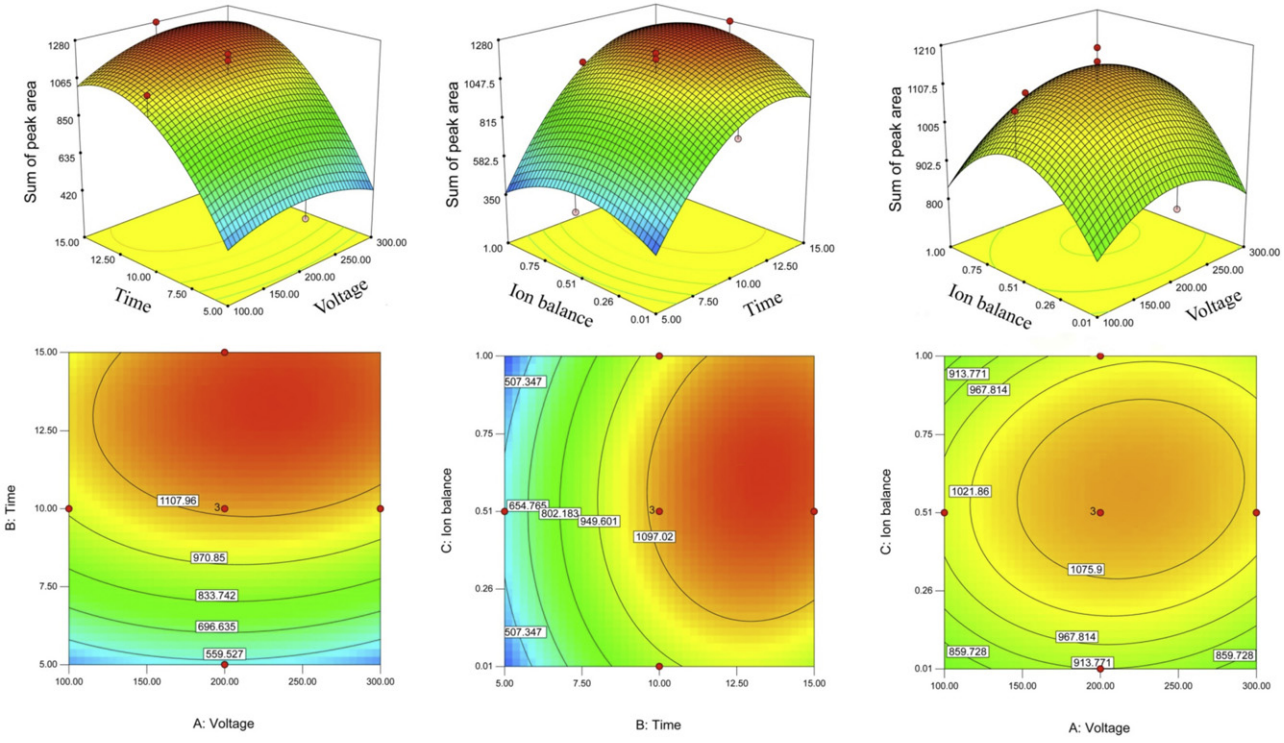

Fig. 3. Three dimensional-response surfaces with contour plots of sum of peak area against different operating variables for extraction of TCAs by EME–DLLME. Variables that are not shown in any plot were held constant at middle levels.

Table 4
Method validation of EME–DLLME–GC/FID for analysis of TCAs in water, urine, and plasma samples.

Matrix	Analyte	LOD (μg L^{-1})	Linearity (μg L^{-1})	r^2	LOQ (μg L^{-1})	PF	Matrix effect (ME%)[b]		
							10	100	400
Water	AMI	0.25	2–500	0.9995	2.0	1065	95.5	97.7	96.3
	TRI	0.25	2–500	0.9994	2.0	990	96.4	97.4	96.9
	DOX	0.25	2–500	0.9991	2.0	1009	96.0	97.3	96.2
Urine[a]	AMI	3.0 (0.6)	10–500	0.9987	10 (2.0)	775	72.9	76.4	75.9
	TRI	3.0 (0.6)	10–500	0.9990	10 (2.0)	753	75.9	79.1	80.2
	DOX	3.0 (0.6)	10–500	0.9988	10 (2.0)	781	77.3	80.3	82.6
Plasma[a]	AMI	15 (1.5)	40–500	0.9968	40 (4.0)	388	36.4	47.1	46.7
	TRI	15 (1.5)	40–500	0.9977	40 (4.0)	397	40.1	50.1	52.4
	DOX	15 (1.5)	40–500	0.9973	40 (4.0)	383	37.8	48.8	50.5

[a] These data are based on the initial volumes of urine and plasma samples and dilution effect was considered for calculation of them. The reported data into parentheses are according to diluted urine (1:4) and plasma (1:9) samples.
[b] All concentrations are in μg L^{-1}.

By FDA's definition, a matrix effect is the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample. There are many sources of matrix effects in bio-analysis, including endogenous substances from the sample matrix, components in the mobile phase, as well as molecules deriving from contaminations during sample preparation [27]. Of the endogenous substances, the phospholipids are the greatest source of matrix effects.

No significant interfering peaks were appeared at the retention times of target analytes (AMI: 12.9 min, TRI: 13.3 min and DOX: 13.4 min) after extraction from water, urine and plasma samples, demonstrating a good selectivity of the proposed method as well as a correct separation of target analytes during the chromatographic process.

One procedure for evaluating matrix effects is described by Matuszewski et al. [28]. The matrix effect was calculated by comparing the absolute peak areas in the neat solutions with those that obtained for the standards spiked (10, 100 and 400 μg L^{-1}). The obtained values were in the ranges of 95.5–97.7% in water, 72.9–82.6% in urine and 36.4–52.4% in plasma (Table 4). Thus, the results indicate that the matrix effect exists in urine and plasma and the quantitative determinations should be carried out by the working curve procedure such as match matrix or standard addition methods. Due to simplicity of match matrix method in comparison with standard addition method, at first this procedure was investigated in the study. The application possibility of match matrix method, were calculated by comparing the peak area ratios of TCAs from the spiked urine and plasma samples to those obtained in working curve solutions (drug-free water, urine and plasma samples) at the concentrations of 10, 100 and 400 μg L^{-1}. The obtained RR% values in Table 5 indicate good matching peak areas and applicability of match matrix method.

Diluting of the sample or reduce the volume of sample being injected, reduces the amount of interfering compounds. However, the best alternative to reduce the remaining matrix effects is probably to improve the sample clean up.

EME–DLLME creates high efficient sample clean-up during analysis of biological fluids. This can be attributed to use of hollow fiber membrane which acts as a filter so that phospholipids and other interferences can be removed to a higher degree in the preparation step; application of electrical driving force that only transfers cationic or anionic analytes into the acceptor phase depending on the charge of platinum electrode which is located into the lumen of the fiber; and DLLME step with non-polar extraction solvent (CCl_4) that only extracts neutralized compounds with low polarity. As can be seen from GC/FID chromatograms, considerable sample clean-up has been obtained due to dilution of urine and plasma samples as well as application of EME–DLLME method.

Precision, defined as the relative standard deviation (RSD%) and accuracy (Error%) were determined by intra- and inter-assays using three determinations in each of the three levels in the range of expected concentrations. The results are shown in Table 5.

One of the other common analytical problems is carry-over and it can compromise the accuracy of an assay. It was investigated

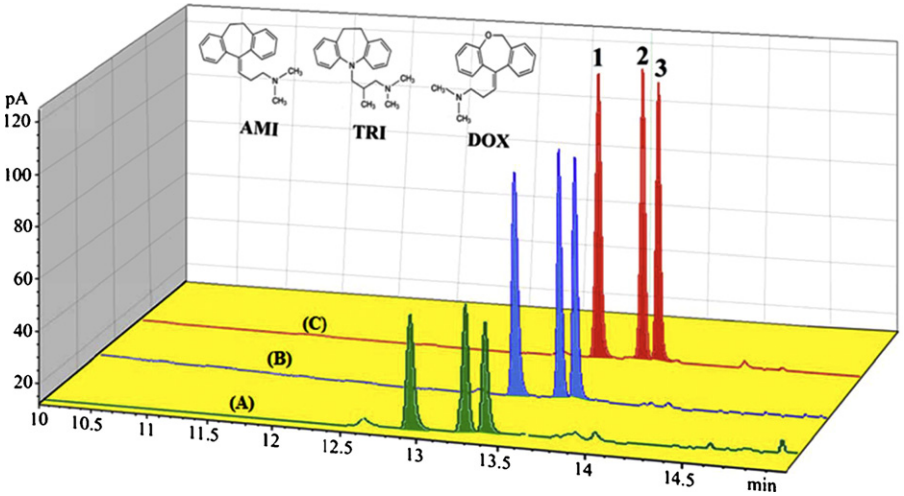

Fig. 4. Chromatograms of TCAs after extraction from the diluted plasma (A), diluted urine (B), and water (C) samples using EME–DLLME–GC/FID. (1) AMI, (2) TRI, (3) DOX at concentration level of 20.0 μg L^{-1} (100 and 200 μg L^{-1} proper to undiluted urine and plasma samples, respectively).

Table 5
Accuracy, precision, and relative recovery of the proposed method for determination of AMI, TRI and DOX in a drug-free urine sample.

Analyte	Conc. ($\mu g\,L^{-1}$)[a]	Accuracy (Error%)						Precision (RSD%)						RR%		
		Intra-assay ($n=3$)			Inter-assay ($n=3$)			Intra-assay ($n=3$)			Inter-assay ($n=3$)			W	U	P
		W[b]	U[c]	P[d]	W	U	P	W	U	P	W	U	P			
AMI	10	−4.5	−7.7	−10.8	−5.5	−7.6	−9.7	8.9	11.3	11.8	7.4	8.8	10.2	95.5	92.3	89.2
	100	−2.3	−6.8	−8.9	−5.2	−8.4	−7.7	7.4	9.8	10.3	6.6	7.7	10.1	97.7	93.2	91.2
	400	−3.7	−5.8	−6.6	−3.8	−6.9	−8.1	6.0	8.4	8.7	6.9	8.3	9.3	96.3	94.2	93.4
TRI	10	−3.6	−7.6	−10.4	−4.8	−7.1	−10.0	9.2	11.7	11.0	6.8	9.3	10.8	96.4	92.4	89.6
	100	−2.6	−6.1	−8.5	−4.9	−8.9	−8.4	7.7	9.3	10.1	6.5	9.4	9.8	97.4	93.9	91.5
	400	−3.1	−6.3	−7.1	−2.8	−6.4	−9.1	6.7	7.8	8.8	6.2	7.9	9.0	96.9	93.7	90.9
DOX	10	−4.0	−8.1	−11.2	−5.3	−8.0	−9.5	8.5	10.5	11.5	7.5	8.5	10.5	96.0	92.4	88.8
	100	−2.7	−6.5	−8.7	−5.8	−8.7	−7.3	8.2	9.6	10.5	6.8	8.0	9.5	97.3	93.5	91.3
	400	−3.8	−6.2	−7.4	−3.2	−6.6	−8.5	6.4	8.1	8.5	6.7	7.8	8.4	96.2	93.8	92.6

[a] The added concentrations for urine and plasma samples are based on diluted samples.
[b] Water.
[c] Urine.
[d] Plasma.

according to Haeckel's proposals for the description and measurement of carry-over effects in clinical chemistry [29]. Investigation of carry-over for EME–DLLME–GC/FID method by injecting two repeated extraction containing 500 $\mu g\,L^{-1}$ of each AMI, TRI and DOX followed by three repeated extraction of target analytes at concentration level of 10 $\mu g\,L^{-1}$ in each water, urine and plasma samples show no significant peaks or signals (S/N < 3) at the retention time for AMI, TRI and DOX.

Table 6
Comparison of the proposed method with other microextraction techniques for determination of AMI, TRI, and DOX in different samples.

Extraction technique[a]	Analytes	Sample	Linear range ($\mu g\,L^{-1}$)	r^2	LOD ($\mu g\,L^{-1}$)	PF	RSD%	Analysis time (min)[b]	Ref.
HF-LPME–HPLC/UV[c]	AMI	Water, urine, plasma	5–500[c]	0.9978[c]	0.5[c]	313[c]	<12[c]	>30	[34]
In-tube SPME–LC/MS	AMI	Urine	1–50 (DOX) 1–500 (AMI)	>0.9986	0.06–2.84	5.6–6.4[c]	<16.5	17	[35]
	DOX	Plasma	1–500	>0.9933	0.07–2.95	5.6–6.4[c]		>32	
HF-DDSME–GC/MS	AMI	Blood	100–1000	>0.997	25	–	2.5	10	[36]
HF-LPME–ESI/IMS[d]	TRI	Urine	–	–	5.0 (LOQ)	–	<6.0	20	[37]
		Plasma	–	–	5.0 (LOQ)	–	<6.0	>27	
DSDME–GC/FID	AMI	Water, urine	50–20,000[c]	>0.9992[c]	40	167	7.5	20	[38]
HF-LPME–LC/MS	AMI DOX	Water	0.025–0.5 (AMI) 0.037–0.5(DOX)	>0.982	<0.011	>18,000	<20.6	120	[39]
SPME–HPLC/UV	AMI	Plasma	75–500	0.995	75 (LOQ)	–	<15	>60	[40]
SPME–micro-HPLC/UV	AMI	Urine	5–500	0.991	3.0	–	–	>210	[41]
Wire-in-tube SPME–micro-HPLC/UV	AMI	Urine	–	–	–	58.8–110	–	10	[42]
DLLME–GC/MS	AMI	Urine	2–100	0.999	0.5	–	<7.9	>3	[26]
DLLME–GC/FID	AMI	Water	5–16,000	0.9960	5.0	740.04	5.6	>10	[43]
		Plasma	7–21,000	–	7.0	–	6.1	>25	
HF-LLLME–HPLC/UV[c]	AMI TRI	Water, urine, plasma	0.2–200[c] (AMI) 0.5–200[c] (TRI)	>0.997[c]	0.08–0.1[c]	>680[c]	<6.3[c]	>40[c]	[44]
EME–DLLME–GC/FID	AMI	Water	2–500	>0.9991	0.25	>990			
	TRI	Urine	10–500	>0.9987	3.0	>753	<11.7	17	This work
	DOX	Plasma	40–500	>0.9968	15	>383			

[a] Drop-to-drop solvent microextraction (DDSME), directly suspended droplet microextraction (DSDME), electro spray ionization–ion mobility mass spectrometry (ESI/IMS), and liquid–liquid–liquid microextraction (LLLME).
[b] The mean of this time is the total time needed before injection of extracts into analysis instrument such as sample pretreatment and extraction time.
[c] The data reported are related to water samples.
[d] No data has been reported for extraction procedure. However, linearity of 5–1000 $\mu g\,L^{-1}$, $r^2 > 0.997$, and LOD = 1.0 $\mu g\,L^{-1}$ have been reported for direct calibration of standard solutions in this article.

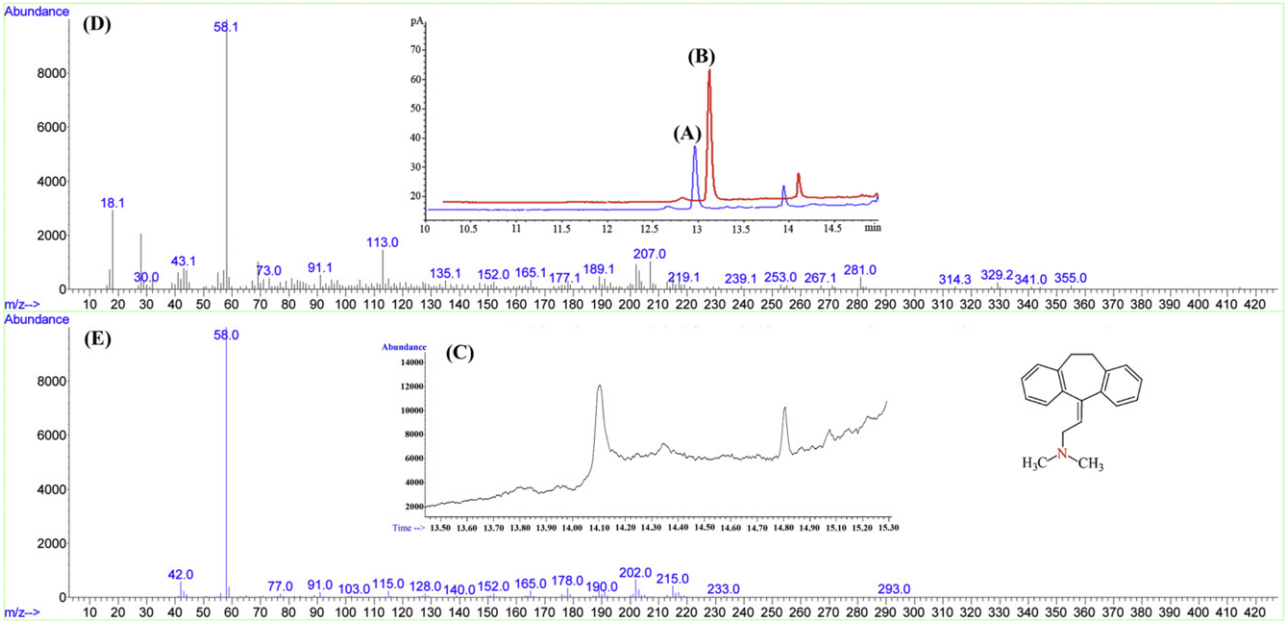

Fig. 5. GC/FID chromatogram of a real urine sample before (A) and after (B) spiking at concentration level of 5.0 μg L^{-1} (25 μg L^{-1} proper to undiluted urine); GC/MS chromatogram of the urine sample after extraction (C); mass spectra of detected AMI in the urine sample (D) and MS database of GC/MS library (E).

The stability of AMI, TRI and DOX including analyses stability (in processed), short-term and long-term storage stability as well as freeze–thaw cycles were established in different literatures and were therefore not evaluated during this validation [30–33]. As can be seen in the literature, TCAs show similar stability behavior and it can be attributed to their chemical structure similarity. TCAs in plasma are stable for at least five days at room temperature and six months frozen at −20 °C. Their stability in urine has been reported 7 days at room temperature and fourteen days at −20 °C [31].

3.4. Comparison of the proposed method with other existing techniques

A comparison of the proposed method with different methods for extraction and determination of AMI, TRI, and DOX is shown in Table 6. The results showed an excellent applicability of the new proposed method for determination of TCAs from the investigated samples.

Despite of several advantages of DLLME such as simplicity and rapidity, this technique is not well compatible for extraction of TCAs from these types of samples due to the interaction of matrix components in biological samples like urine and plasma with organic solvent [42]. It is not possible to produce sedimented phase without high dilution ratios of real samples [42]. Also, due to the direct contact between the extracting phase and sample matrix, crowded chromatograms are created after extraction, which leads to several problems such as sensitivity of determination and separation. As mentioned before, SPME suffers from high cost, sample carry-over, fiber fragility, and limited lifetime of the fiber [4]. The main disadvantages of SBSE are relative long extraction and desorption time as well as carry-over problems [5].

Recently, EME–GC/FID analysis of two antidepressant drugs including imipramine and clomipramine have been reported [45]. Although, it has been claimed that direct injection of water does not create critical problem but in fact the life time of GC columns decrease noticeably by successive injection of water. On the other hand, a lot of limitations are created for direct injection of water in GC. The large expansion volume of water can cause backflash and excess water can extinguish the FID flame [46].

Water analyzed on a nonpolar stationary phase or on a moderately polar stationary phase could cause the flame on the FID to be extinguished. This is because the water will not partition properly and will “bead up” on the phase, producing a large plug of water that passes through the detector and extinguishes the flame [46]. To minimize the possibility of extinguishing the flame and reduce the effect of vapor expansion, selection of a polar stationary phase and application of special liners are necessary for more compatibility with water [46]. Also, existing of nonvolatile compounds in aqueous solution can damage GC columns or created carryover problems. This causes need to multiple replacement of injection port glass wool during analysis [45]. In addition, injection of low volumes of water (≤1 μL) and using of high split ratios should be used in direct injection of aqueous solution in GC for preventing of mentioned problems [45,47] and this may decrease analysis sensitivity.

Apart from all these, direct injection of aqueous solution in GC can be proper for compounds which do not need to derivatization before analysis whereas wide group of chemical compounds need to derivatization for analysis with GC. In these cases, water acts as a strong nucleophile and destroys derivatization reagents. Combination of EME with DLLME which is presented in this work is a simple and fast trick that can remove all of these problems.

As shown in comparison with other techniques, EME–DLLME along with its simplicity demonstrated high sensitivity and an acceptable reproducibility with an important emphasis on the high sample clean-up and short total analysis time. The results obtained indicate that combination of EME with DLLME is a promising technique for analysis of antidepressant drugs present in low concentrations from biological matrices and can be applied for analysis of a broad range of different ionizable compounds.

3.5. Analysis of real samples

The Error%, and RSDs% for analysis of AMI, TRI, and DOX in plasma and urine samples based on three replicate extractions and determinations are shown in Table 7.

Table 7
Analytical results for extraction and determination of TCAs in urine and plasma samples.[a]

Matrix	AMI	TRI	DOX
Urine 1			
$C_{initial}$	30.1	Not detected	Not detected
C_{added}[b]	20.0	20.0	20.0
C_{found}	49.5	19.4	19.7
RSD% ($n=3$)	9.4	9.7	10.1
Error%	−3.0	−3.0	−1.5
Urine 2			
$C_{initial}$	Not detected	Not detected	Not detected
C_{added}	20.0	20.0	20.0
C_{found}	18.8	18.5	19.1
RSD% ($n=3$)	10.6	11.7	10.8
Error%	−6.0	−7.5	−4.5
Plasma 1			
$C_{initial}$	Not detected	Not detected	Not detected
C_{added}	20.0	20.0	20.0
C_{found}	18.1	18.5	18.3
RSD% ($n=3$)	10.2	11.3	11.6
Error%	−9.5	−7.5	−8.5
Plasma 2			
$C_{initial}$	Not detected	Not detected	Not detected
C_{added}	20.0	20.0	20.0
C_{found}	17.5	18.0	17.3
RSD% ($n=3$)	10.1	10.6	9.5
Error%	−12.5	−10.0	−13.5

[a] All concentrations in this table are in $\mu g\,L^{-1}$.
[b] The added concentrations for urine and plasma samples are based on diluted samples (100 and 200 $\mu g\,L^{-1}$ for undiluted urine and plasma samples, respectively).

The Error% values of the method were in the range of 1.5–7.5% for urine and 7.5–13.5% for plasma samples, indicating the good performance of the presented method for determination of the TCAs in complex matrices. The relative standard deviations for determination of target analytes in the real samples examined were located less than 11.7%. Results demonstrated a good performance and accuracy of the presented method for determination of the TCAs in complex matrices.

Fig. 5 illustrates the GC/FID chromatograms of a real urine sample before (A) and after (B) spiking with AMI standard solution at concentration level of 5.0 $\mu g\,L^{-1}$ (25 $\mu g\,L^{-1}$ proper to undiluted urine) which shows the presence of AMI. For all the real samples analyzed, the presence of TCAs was confirmed by GC/MS analysis in the full scan mode (m/z in the range of 10–450) and comparison of the obtained mass spectrum with MS database of instrument library. Fig. 5C shows GC/MS chromatogram of the urine sample after extraction. Fig. 5D and E indicates mass spectra of detected AMI in the urine sample and database of GC/MS library, respectively. As can be seen, the GC/MS results confirm the presence of AMI in the urine sample.

4. Conclusions

In the present work, for the first time a simple, rapid, and sensitive method was used for determination of TCAs in untreated human plasma and urine samples using combination of two interesting microextraction techniques; EME and DLLME followed by GC/FID. This technique not only removes the incompatibility problem of EME with gas chromatographic systems, but also provides high sample clean-up and sensitivities. Also, high preconcentration factors were obtained in EME–DLLME due to collection of extracted analytes in very small volume of the acceptor phase. In comparison with other extraction techniques, the present work has the supremacies such as short total analysis time, low cost, noticeable extraction clean-up and decreasing the risk of working with biological samples. The results showed an excellent applicability of the proposed new method for the determination of TCAs in biological fluids. In overall, EME–DLLME in combination with GC/FID enabled a rapid simple and sensitive determination of TCAs in urine and plasma samples.

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