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Preconcentration and analysis of cannabinoid compounds (THC-9, CBN, CBD) in urine samples by IL-ISFME/D-µ-SPE/HPLC-DAD

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ABSTRACT

In this work we applied a recently developed ionic liquid assisted in-situ solvent formation microextraction coupled with magnetic nano-particle based dispersive micro-solid phase extraction following by HPLC diode array detector (IL-ISFME/D-µ-SPE/HPLC-DAD) for preconcentration and measurment of cannabinoid compounds (THC-9, CBN, CBD) in urine samples. The extraction involved a primary extraction based on IL-ISFME technique followed by a D-µ-SPE step. In the final, the extracted analytes introduced to HPLC-DAD by means of methanol. The condition of the technique were optimized regarding to six effective factors including IL amount, ion pairing salt amount, magnetic nano-particle amount, organic solvent volume, ionic strength and organic solvent type. The whole optimization procedure was done within 159 experimental runs. Optimization procedure was performed by a full factorial central composite design (CCD). This new technique provided up to 98-113 fold preconcentration of the analytes under the optimized conditions. Good repeatabilities (with RSDs 1.8-2.1%) were obtained. Detection limits were in the range of 0.2–1.4 ng/ml.

Keywords: cannabinoids, THC-9, CBN, CBD, ISFME, D-µ-SPE

INTRODUCTION

Determination and analysis of trace amounts of cannabis group psychoactive compounds in biological samples such as urine, saliva, blood plasma, sweat, hair and nails is becoming increasingly important due to the need to understand more about the psychedelic and toxic effects of these drugs in the case of forensic investigation or legislation procedures. The abuse of marijuana products can be detect by analyzing of urine samples for findings cannabinol (CBN), cannabidiol (CBD) and tetrahydrocannabinol (THC) compounds (figure 1). In the case of light cannabis use, THC has been confirmed as a marker of recent consumption [1]. Also, CBN and CBD are proposed as markers of recent cannabis intake [2-4].

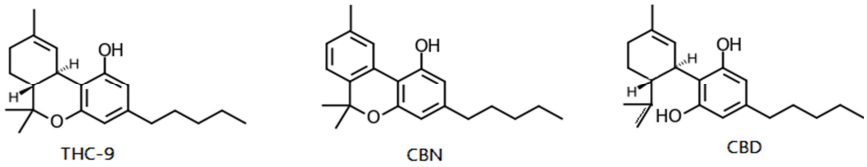

Fig. 1: The chemical structure of studied analyses

Marijuana intake can be detected up to 2–5 days after exposure for infrequent users; for heavy users: 1–15 days; for chronic users and/or users with high body fat: 1–30 days. Many authors have investigated the concentrations of cannabinoids in urine samples. Reports have shown that the urine concentration of THC decreases slowly, after

smoking cigarettes containing 16 and 34 mg cannabis. This caused average concentration of 20 ng/mL (range 5-35 ng/mL) for THC in urine samples after 7 days [5, 6].

Urine is the specimen of choice for monitoring cannabinoids use, because its collection is physically noninvasive, and large volumes of sample are often available [3]. By today several methods have been introduced to detection and measurement of these compounds in urine samples such as gas chromatography – mass spectrometry (GC-MS) [7], two dimensional gas chromatography coupled with mass spectrometry (GC-GC-MS) [8], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [9, 10], solid phase extraction coupled with liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS) [11], and hollow fiber-based liquid phase microextraction liquid chromatography-tandem mass spectrometry (HF-LPME-LC-MS/MS) [12].

In this work we apply and optimize a new version of ionic liquid-linked dual magnetic microextraction (IL-DMME) coupled with HPLS-DAD to determination THC and its major metabolites, as well as CBN and CBD in urine samples. In this technique, satisfactory enrichment factor and recovery can be readily achieved through the two-step microextraction linked by MNPs. The first step is includes an in situ solvent formation microextraction (ISFME) and thereafter the procedure followed by MNPs in the dispersive micro-solid-phase extraction (D-µ-SPE) step. ISFME is a sub division of homogenous liquid-liquid microextraction (HLLME). This method was proposed by Shemirani et al in 2009 [13]. An important advantage of ISFME over other procedures is the high capability to solutions with complex matrices and high content of salt [14]. It is a very good characteristic in the case of handling biological fluids, especially urine, which has a complex matrix and high level of contaminate salt [15]. A possibility for the recovery of ILs after DLLME is the combination in sequence of ISFME with dispersive micro-SPE (D- µ -SPE). The D- µ-SPE can be considered a miniaturized version of conventional SPE, using dispersion of hydrophobic magnetic NPs (MNPs). Most MNPs contain Fe, Ni, Co and their oxides as the magnetic core [16]. MNPs are employed as sorbents for retrieving the extractant containing the analytes, which are finally desorbed (e.g., by sonication). MNPs, employed in nanoscale magnetic separations, display a large surface area and high sorption capacity and can be isolated from the sample solution by an external magnetic field [16]. Recently several reports about the application of the mentioned dual extraction technique have been appeared in literature [16-20]. The optimization of all including numerical and categorical factors has been taken out by response surface methodology.

MATERIALS AND METHODS

Materials

The standard materials of THC-9, CBN and CBD with 99% purity were kindly donated by the Research Center of Antinarcotic Police (Tehran, Iran). 1-propanol, acetone, ethanol, acetic acid, 1-methyl-3-octylimidazolium tetrafluoroborate ($omimBF_4$) and all salts used were purchased from Merck (Darmstadt, Germany). Sodium hexafluorophosphate ($NaPF_6$) ACROS (Geel, Belgium). The viscosity of ILs is high and their handling is difficult, so working solution ($omimBF_4$), was diluted to the arbitrary concentration by acetone. Similar to ($omimBF_4$) story, ($NaPF_6$) was prepared by dissolving appropriate amount this salt in doubly distilled water.

Stock solution of magnetic nanoparticles Fe_3O_4 (20 nm) was purchased from Aladdin (Milwaukee, USA). Working stock solutions of NOPs were prepared in acetonitrile at a final concentration of 100 mg L^{-1}.

Instrumentation

Chromatographic separations were performed with a HPLC system from Waters (Milford, USA) consisted of a 1525 binary pump, a 717 plus automatic injector, a 1500 series column heater, and a 2998 photodiode-array detector. The separations were carried out on ODS-3 column (250 mm × 4.0 mm, with 5 m particle size) from Waters. It was thermostated at 27.0 ± 0.5 ◦C. Chromatographic data were recorded and analyzed using Empower^{TM} software. An isocratic elution was performed at a flow rate of 1.0 mL min^{-1}. Eluent A was 1% (v/v) orthophosphoric acid in water containing 4 mL n-hexyl amine whose pH was adjusted at 6.0 by dropwise addition of 4 M NaOH and/or orthophosphoric acid 1 M and eluent B was acetonitrile (87:13). Total analysis time was 15 min. Quantification of all amphetamines was accomplished by measuring peak areas at 220 nm. Calibration was run by injecting 10 µL of standards and samples were injected into the chromatographic system for analysis.

Extraction procedure

ISFME procedure: Five milliliters of the sample or standard solution containing 0.5 mM, acetate/acetic acid buffer (pH 6.5) and predetermined amount of ($omimBF_4$) was transferred to 15 mL screw-cap conical-bottom glass

centrifuge tube. After a sonication step, a determined amount of (NaPF6) was added to the solution by a pipette and a cloudy solution was formed.

D-µ-SPE step: The extraction procedure was followed by adding a known amount of MNPs to the conical tube. In the following, the vial was sealed and placed on a vortex agitator. After that, the Fe_3O_4 magnetic nanoparticles were deposited at the bottom of the vial by applying an ordinary magnet and subsequently the aqueous part was withdrawn by a syringe. In the case of desorption of IL as well as analyte compounds, a known volume of organic solvent was added into the vial and sonicated for minutes. Finally, 5 mL of organic solvent was collected and injected into the HPLC system for analysis (Figure 2).

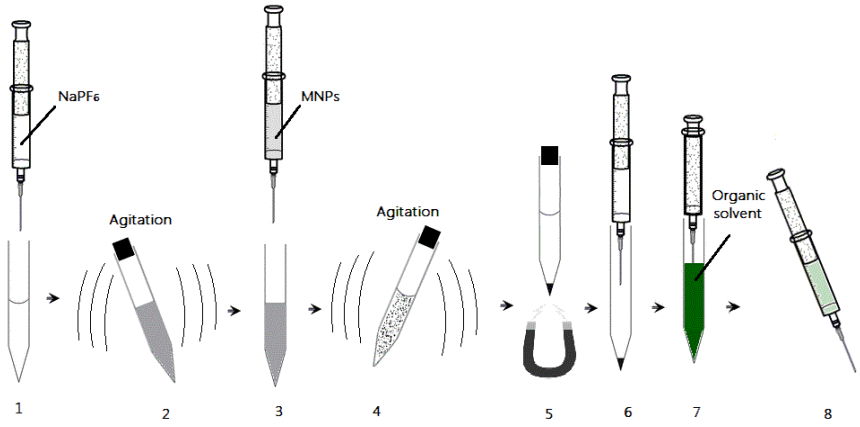

Fig. 2: IL-ISFME/D-µ-SPE/HPLC-DAD procedure

1) The injection of NaPF6 to the solution containing analyte and omimBF_4, 2) agitation, 3) Injection of MNPs to the analyte solution, 4) agitation, 5) deposition of the MNPs by applying an ordinary magnet, 6) withdrawing the remained aqueous solution, 7) desorption of IL as well as analyte compounds by injecting an organic solvent and 8) injection the final solution to the HPLC.

Blank sample and real sample preparation

Blank sample: Drug-free samples were obtained from five healthy laboratory members with no records of using any kind of medicine and drugs of abuse lately and used for the preparation of calibration curves and for the repeatability and matrix effect studies. The samples were stored at −4 ◦C, thawed and shaken prior to extraction.

Real sample preparation: A total volume of 90 ml urine samples were collected from 3 young males who were not consumed cannabis products at all and one female person who was suspicious to consumption hashish recently. The samples were stored at −4 ◦C, thawed and shaken prior to extraction.

Optimization strategy and data handling: The optimization procedure was done by application of a central composite full factorial design. The experimental design generally includes various combinations of different factor levels, which enables it to depict the interactions among different factors and to be more efficient to deal with a large number of factors, compared with one factor at a time design [21]. This method decreases the number of experiments, time and material resources.

In a full factorial design, every possible combination of each factor level is tested. An appropriate polynomial model can be used to describe the effects of the factors studied on a response and then optimize the response when necessary [21]. The number of experiments is geometrically relative to the number of factor that is chosen. Central composite design (CCD) is widely used as an experimental design method to estimate a second-order polynomial approximation to a response in that region. In this work we chose five numerical factors and one categorical factor (Table 1) as effective factors on the ISFME/D-µ-SPE/HPLC-DAD procedure. The average peak area of all three analyte compounds for experimental runs assumed as a numeric response in the case of data handling.

Experimental design matrices were constructed and the response surfaces were carried out using the Design Expert® (Version 7.1.5) statistical software (State- Ease Inc., USA).

RESULTS AND DISCUSSION

Optimization and the analytical characteristic of IL-ISFME/D-µ-SPM/HPLC-DAD: Based on similar reported studies, six factors that may affect the analytes extraction yields were evaluated by CCD methodology. According to the selected factors (Table 1) the software proposed a matrix of experimental runs including 159 experiments. All the experimental runs were performed randomized within 5 days (5 blocks) in order to protect against the effects of lurking variables.

The obtained responses for all runs were transferred to a mathematical model (Equation 1) by using the analysis of variance (ANOVA) and the analysis of the residues generated between predicted values and observed values (Table 2).

Equation 1:

$$Area = -5183227+196296.3*OmimBF_4+227580*NaPF_6+43300.79*MNP\ amount-2617.5*Ionic\ strength+521812.5*Organic\ Solvent\ Volume+20423.55*\ OmimBF_4*\ NaPF_6+19934.6*\ OmimBF_4*MNP\ amount-3399.76*\ OmimBF_4*Ionic\ strength+21964.38*\ OmimBF_4*Organic\ Solvent\ Volume+15849.52*\ NaPF_6*MNP\ amount-2703.06*\ NaPF_6*Ionic\ strength+17463.35*\ NaPF_6*Organic\ Solvent\ Volume-3437.36*MNP\ amount*Ionic\ strength+43932.62*MNP\ amount*Organic\ Solvent\ Volume-3787.36*Ionic\ strength*Organic\ Solvent\ Volume-35575.1*\ OmimBF_4^2-34855.8*\ NaPF_6^2-61123.7*MNP\ amount^2-9314.41*Ionic\ strenght^2-45786.7*Organic\ Solvent\ Volume^2$$

Table 1. Factors and levels used in the central composite design

			Coded factor levels				
Variable	**Unit**	**Symbol**	**−1.5 (low)**	**-1**	**0**	**1**	**+1.5 (High)**
OmimBF4	mg/ml	A	3	5.6	7.5	9.4	12
NaPF6	mg/ml	B	0	400	700	1000	1400
MNP amount	mg/ml	C	1:00	13:30	23:00	32:15	45:00
Ionic strenght	(w/w)%	D	0:00	3:00	5:00	7:00	10:00
Organic Solvent Volume	ml	E	6	8	9.5	11	13
Organic solvent type		F	**Solvent 1**		**Solvent 2**		**Solvent 3**
			Methanol		Acetonitrile		Acetone

The evaluation of the proposed model fitting quality can be representing by the coefficient of determination (R^2 and adjusted-R^2). R^2 of 0.94 and adjusted-R^2 of 0.90 showed a good relationship between experimental data and fitted model, also high potential of model in prediction of response. From the ANOVA summary, the models were found to be significant with a p-value less than 0.0001.

As it represented in equation one, factors A and B have a strong positive linear effect on the response. Also there were significant negative quadratic coefficient of A and B, but these coefficients are way smaller than the linear ones. This indicates that the response value increases with the increase of these parameters, then it reaches a maximum, and finally it starts to decrease at very higher values which are out of the range of studied levels. For factor A and B this phenomenon can be explained from two aspects. First by increasing the amount of ($omim^+$) and (PF_6^-) ions, the total volume of insoluble IL will increases, which has a positive effect on the extraction of analytes into it. But in another aspect, which involves the negative quadratic effect, increasing the amounts of (omim BF_4) and (Na PF_6) affects the density and viscosity of the solution which has a dramatic effect on the mass transfer to the IL droplets [13]. The behavior of these factors in the studied intervals is represented in figure three. As it seen in figure 3 the interaction of factors A and B with each other has a positive effect on the response value. The high and positive coefficient of the AB component in the proposed model proves this statement. According to the molar mass of $omim^+$ (Mw=172.33 g/mole) and PF_6^- (Mw=174.92 g/mole), the best responses belongs to the equal molar concentration of $omim^+$ and PF_6^-, which represents the 1:1 stochiometriy ratio of cation and anion.

Table 2. ANOVA for the proposed model

Source	Sum of Squares	df	Mean Square	F-Value	p-value (Prob > F)	
Block	2.16E+12	4	5.41E+11			
Model	7.6E+13	32	2.38E+12	24.14804	< 0.0001	significant
A-OmimBF4	4.38E+12	1	4.38E+12	44.48138	< 0.0001	
B-NaPF6	2.75E+12	1	2.75E+12	27.99408	< 0.0001	
C-MNP amount	2.64E+12	1	2.64E+12	26.80067	< 0.0001	
D-Ionic strenght	1.18E+12	1	1.18E+12	12.00115	0.0007	
E-Organic Solvent Volume	4.48E+12	1	4.48E+12	45.49666	< 0.0001	
F-Organic solvent type	3.21E+12	2	1.61E+12	16.31951	< 0.0001	
AB	3E+12	1	3E+12	30.542	< 0.0001	
AC	1.32E+12	1	1.32E+12	13.39804	0.0004	
AD	1.7E+11	1	1.7E+11	1.727168	0.1912	
AE	1.77E+12	1	1.77E+12	18.02259	< 0.0001	
AF	2.28E+11	2	1.14E+11	1.157109	0.3178	
BC	8.33E+11	1	8.33E+11	8.469512	0.0043	
BD	1.07E+11	1	1.07E+11	1.091822	0.2981	
BE	1.12E+12	1	1.12E+12	11.3929	0.0010	
BF	1.43E+11	2	7.16E+10	0.728212	0.4849	
CD	8E+10	1	8E+10	0.812981	0.3690	
CE	3.27E+12	1	3.27E+12	33.20048	< 0.0001	
CF	1.39E+11	2	6.95E+10	0.706034	0.4956	
DE	1.08E+11	1	1.08E+11	1.093594	0.2977	
DF	6.16E+10	2	3.08E+10	0.312937	0.7319	
EF	2.29E+11	2	1.14E+11	1.163329	0.3159	
A^2	1.7E+13	1	1.7E+13	172.6161	< 0.0001	
B^2	1.63E+13	1	1.63E+13	165.7063	< 0.0001	
C^2	1.06E+13	1	1.06E+13	108.0414	< 0.0001	
D^2	4.85E+12	1	4.85E+12	49.28414	< 0.0001	
E^2	7.32E+12	1	7.32E+12	74.43117	< 0.0001	
Residual	1.2E+13	122	9.84E+10			
Lack of Fit	1.2E+13	104	1.15E+11			
Pure Error	0	18	0			
Cor Total	9.02E+13	158				

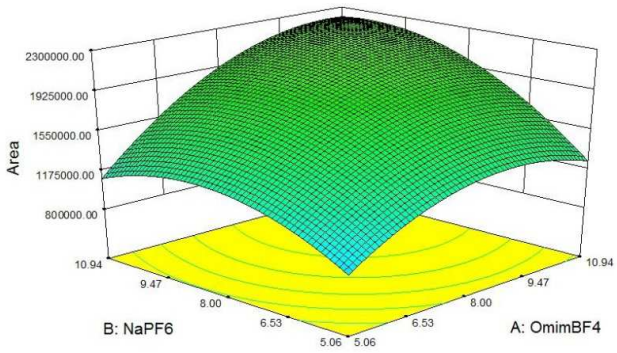

Fig. 3: The 3D central composite design plots for the effects of variables A and D on response

As it shown in figure 4, factors C and E have positive effect on the response values. The significant positive coefficients of these factors in the mathematical models are consonant with this phenomenon, also according to the ANOVA table there is a good significant interaction between C and E factors. Increasing the MNPs amount leads to better sedimentation of the IL droplets on the surface of the sorbents which results in higher yield of extraction. In the other hand increasing the organic solvent volume results in better desorption of the sedimented IL droplets from the MNTs surface. Similar to the A and B factors, C and E factors have negative quadratic coefficient but smaller than the linear components. As it could be seen in figure 4, the negative quadratic effect of C and D in the next of the lowest intervals of the counter factor is obvious. In the case of C factor, the low responses near the lowest level of solvent volume shows ineffective IL desorption from the MNPs surface, caused by aggregation of the MNPs. Surely according to the negative quadratic coefficient of C, this observations could be seen in very higher amounts

of the MNPs for the studied domain of factor E. Similarly, the decrease of the response value for higher volume of solvent in the lowest level of C factor is caused by dilution of the incompletely extracted analytes.

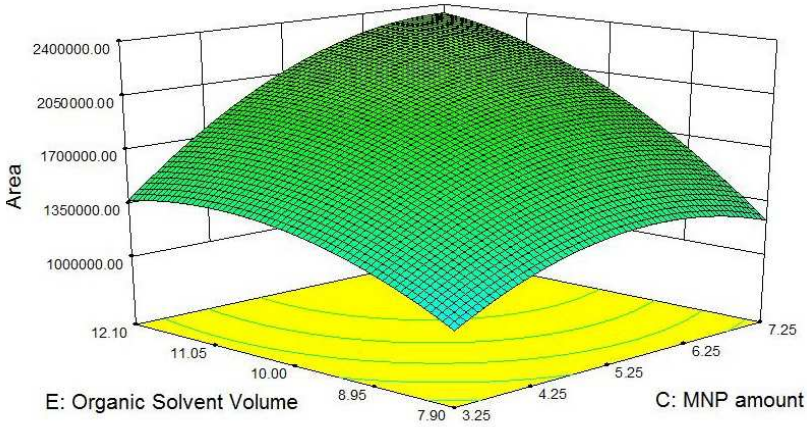

Figure 4: The 3D central composite design plots for the effects of variables C and E on response

According to the ANOVA table, unlike other studied numerical factors, ionic strength has no significant interaction with the rest factors. In the proposed model factor D has a relatively weaker negative linear effect on the response value. In the other hand, the D^2 component has a significant negative coefficient which explains the decrease of response in higher levels of D. The ionic strength has a direct effect on the solubility of ILs in the water based solvents. By increasing the ionic strength of the solubility of the IL will increase and in result of this, phase separation cannot occur completely. Also At higher salt content, the density of solution increases. This would be affecting the mass transfer negatively [13, 14]. As it shown in figure 5, by increase of the ionic strength the response value decreases polynomial which is totally consonant with the proposed statements.

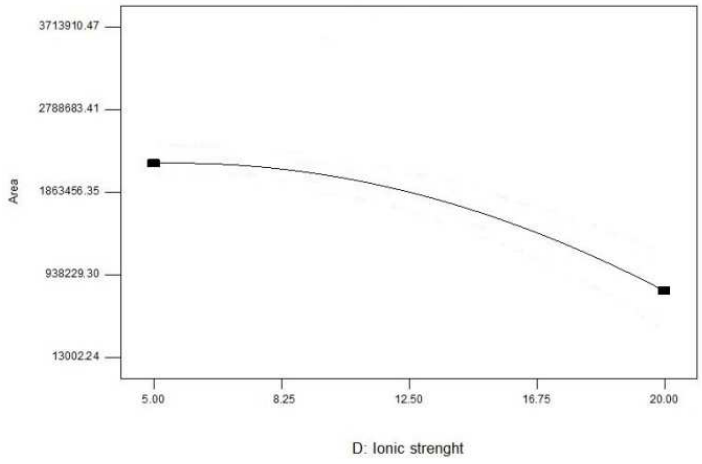

Fig. 5: The 2D central composite design plots for the effects of variable D on response

The only categorical studied factor was the effect of desorption solvent type. In this work we studied three types of organic solvents including: Methanol, Acetonitrile and Acetone. Based on the reported p-value in the ANOVA table changing the organic solvent had a significant effect on the extraction performance. As it represented in the figure 6, desorption of IL droplets as well as analyte compound from sorbent particles were done significantly better by mean of methanol in comparison with two others.

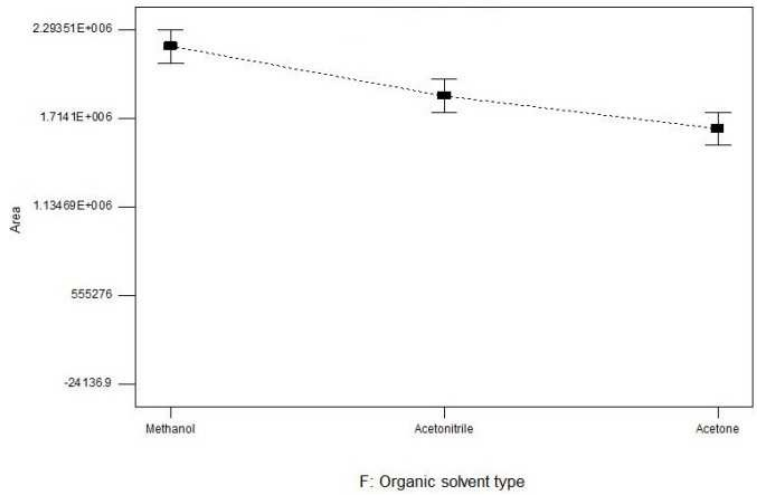

Figure 6: The 2D central composite design plots for the effects of variable F on response

The best proposed optimum points for each factor are listed in table 3. To evaluate the practical applicability of the proposed IL-ISFME/D-µ-SPE/HPLC-DAD technique, under optimized extraction conditions, the figures of merit of the method were investigated in cannabinoid-free human urine sample (Table 6). Under optimum conditions, the calibration curves were observed to be linear in the concentration range of 1-40 ng/mL for all three analyte compounds. Also the correlation coefficients of the calibration curve equations were 0.99 for THC, CBN and CBD, which indicates that a good linear regression between the response values and the concentrations were established. The limit of detections (LODs) for studied analytes were in the range 1.3-2.4 ng/mL which are lower than the natural concentration of cannabinoid compounds in the urine samples of a user person [5, 6]. The limits of quantitation (LOQ) for the target analytes were 0.25– 5 ng/mL. The precision is expressed as percent relative standard deviation (%RSD), was carried out using 7 experiments at the concentration of 25 ng/mL for each analytes. The RSD amounts are reported in table 4. The reported figure of merits under the achieved optimum points shows that the proposed method has high sensitivity and precision for analyzing and determination of THC, CBN and CBD compounds in urine real samples.

Table 3: Optimum level of all 6 factors for the best response

Factor	**A (mg/ml)**	**B (mg/ml)**	**C (mg/ml)**	**D (w/w%)**	**E(ml)**	**F**
Optimum points	9.5	9.5	6.5	<10	11.5	Methanol

Table 4. Performance of the IL-ISFME/D-µ-SPM/HPLC-DAD for extraction and determination of canabinoides

Analyte	**Equation**	**LOD (ngmL^{-1})**	**R^2**	**Recoveries (%)**	**RSD % (n = 7)**
THC-9	y = 51062x - 68025	0.8	0.99	113	1.8
CBN	y = 44312x + 92581	1.1	0.99	110	2.2
CBD	y = 32942x + 45735	1.4	0.99	98	2.1

Analysis of real samples: The proposed method was applied to the determination of THC-9, CBN, and CBD in urine samples. The real samples were collected from four young males who were not consumed cannabis products at all and one female who was suspicious to consumption hashish recently. The urine samples were spiked to assess the effect of different urine matrixes. The reported recovery in table 5 shows no significant matrix effect arising from different urine samples. The typical chromatograms of the studied analytes after a full extraction procedure for a spiked and blank urine samples are illustrated in figure 4.

Table 5. Results obtained from analysis of the amphetamines in the real samples

Analyte	THC-9		CBN		CBD	
Samples	Funded ($ngmL^{-1}$)	Recovery (%)±SD	Funded ($ngmL^{-1}$)	Recovery (%)±SD	Funded ($ngmL^{-1}$)	Recovery (%)±SD
Urine1 (3 $ngmL^{-1}$ added)	2.9	98±0.04	2.9	98±0.05	3.1	102±0.06
Urine 2	1.2	-	0.7	-	1.2	-
Urine3 (12 $ngmL^{-1}$ added)	20	153±0.04	12	100±0.02	16	106±0.04
Urine 4(5 $ngmL^{-1}$ added)	5.2	104±0.07	5.1	102±0.06	5.0	100±0.03
Urine 5 (15 $ngmL^{-1}$ added)	16.2	107±0.04	15.3	102±0.06	15.2	101±0.05

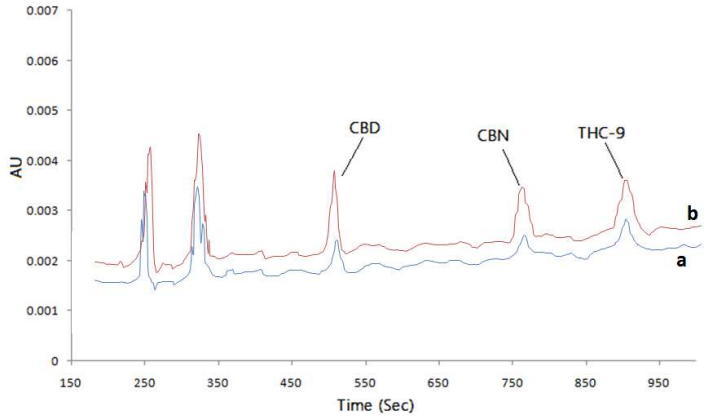

Fig. 7: (a) Chromatograms of the actual urine sample related to a person (24-year-old female) suspicious of amphetamines consumption and (b) the corresponding spiked ones at concentration level of 5ng/ml for all three analytes.

Table 6. Comparison of the proposed method with other methods applied for Preconcentration and analysis of cannabinoid compounds (THC-9, CBN, CBD).

Analyte	Sample	method	LOD (ng mg^{-1})	LDR (ng mg^{-1})	RSD%	Extraction time (min)	References
THC-CBD-CBN	Hair	HS-SPDE-GC/MS	0.9-0.14 ng mg^{-1}	1.8-20	3.2-4.0	5	[22]
THC-CBD-CBN	Hair	HF-LPME-GC/MSMS	0.5-15	1-500	2.0-3.6	20	[23]
THC-CBD-CBN	Plasma, Urine	LC/MSMS	0.2-1.0	0.5-100	1.3-2.2	-	[2]
THC	Oral fluid	SPE-LC/MS	1	2.0-100	1.7-2.9	10	[24]
THC-9, CBN, CBD	Urine	IL-ISFME/D-µ-SPE/HPLC-DAD	0.2-1.4	0.5-100	1.8-2.3	3	This work

Comparison of the proposed method with other methods applied for Preconcentration and analysis of cannabinoid compounds (THC-9, CBN, CBD): Comparison of the resulting data of the proposed method with the result of other methods with reference to the limit of detection, relative standard deviation, linear range and extraction time for extracting and determining the amphetamines in urine samples is provided in Table 6. As can be seen the limits of detection, linear ranges and analysis time of the proposed method are superior to those reported before. However, the relative standard deviations (RSDs) of the proposed method are about the same with those reported for the other methods and sometimes are better. All these results indicate that the proposed IL-ISFME/D-µ-SPE/HPLC-DAD is a sensitive, fast, reproducible and simple technique that can successfully be used for the preconcentration and determination of cannabinoid in human urine samples.

CONCLUSION

In the present study, we used ionic liquid assisted in-situ solvent formation microextraction coupled with magnetic nano-particle based micro-solid phase extraction following by HPLC detection (IL-ISFME/D-µ-SPM/HPLC-DAD)

for quantitative measurements of THC-9, CBN and CBD in urine samples. For the ISFME step we applied (omimBF_4) as insoluble ionic liquid. In D-µ-SPM step we used Fe_3O_4 magnetic nano-particles as solid sorbent in the case of capturing ILs containing analyte compounds. The separation of phases was done by using a conventional magnet. For the optimization of the effective parameters we applied central composite design methodology, by selecting 5 numerical and 1 categorical factors including IL amount, ion pairing salt, magnetic nano-particle, organic solvent volume, ionic strength and organic solvent type. The whole optimization procedure was done within 159 experimental runs. All of the results demonstrated that this method is sensitive and effective procedure for detection and measurement some of the common marker of cannabinoide uses in urine samples. For conclusion, the proposed method has a good potential to get use in both researches-based and/or routine-based analytical procedures.

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