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An insight into the determination of trace levels of benzodiazepines in biometric systems: Use of crab shell powder as an environmentally friendly biosorbent

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ABSTRACT

A vortex assisted dispersive solid phase extraction approach (VADSPE) based on crab shell powder as biodegradable and biocompatible μ-sorbent was developed for simultaneous analysis of three benzodiazepines (BZPs): Oxazepam, Flurazepamand Diazepam, in biological matrixes included blood, nail, hair and urine samples. The effective parameters in VADSPE process, including the volume of uptake solvent, the dosage of sorbent, extraction time and back extraction time, were optimized using response surface methodology(RSM) based on central composite design(CCD). The suggested technique allows successful trapping of BZPs in a single-step extraction. Under the optimized extraction conditions, the proposed approach was exhibited low limits of detection (0.003–1.2 μg·mL^{-1}), an acceptable linearity (0.04–20 μg·mL^{-1}). Method performance was assessed by recovery experiments at spiking levels of 10 μg·mL^{-1}(n = 5) for BZPs in blood, nail, hair and urine samples. Relative recoveries were determined by HPLC, which were between 36%and 95.6%.

1. Introduction

Benzodiazepines are a large class of drugs with a wide spectrum of therapeutic result, involving soothing-hypnotics, anticonvulsants, muscle-relaxants and anxiolytics [1]. Benzodiazepines are now in the thick of the greatest customary prescribed drugs, which sharply intensify their possibility for the sexual attack, suicide, abuse in connection with crime and addiction driving under the impact of the considered drugs [2]. Hence, on the authority of the forensic and clinical toxicology significance of BZPs, predictable, rapid and sensitive analytical techniques are necessitated for the accurate parallel determination of BZPs in complicated matrices. Fig. S1 was supplied with Electronic Supplementary Material (ESM), which shows the chemical structure of target analytes. From the physicochemical perspective, BZDs have somewhat high octanol-water partition coefficient (e. g. for diazepam log$P_{o/w}$ = 2.8) which is related to the lipophilic composition [3].

Quite a few quantitative approaches have been well-expressed in the literature for the parallel determination of BZPs as well as: capillary electrophoresis (CE), liquid chromatography mass spectrometry, or tandem mass spectrometry, gas chromatography–mass spectrometry (GC–MS) and electrochemical approaches [4]. Generally, chromatographic methods, required isolation procedure of BZPs from complicated biological matrices. For this purpose, solid phase extraction, Liquid–liquid extraction (LLE), liquid phase microextraction (LPME) and solid-phase microextraction (SPME), have been progressed. SPME has benefited from the more advantages such as excellent enrichment factor, cost saving and less exhaustion of organic solvents [5,6].

Despite of the SPE is definitely the most widely employed technique for clean-up, it suffers from some drawbacks such as subordinate wastes, a time-consuming process, solvent loss and a requisite for the complicated apparatus. Dispersive micro- solid phase extraction (D-μ-SPE) classified as an SPE method. The D-μ-SPE indicates some benefits of conventional SPE, such as moderated solvent exhaustion, an excellent amenity for the efficiency of recovery, straightforward, cost-effective and easy to use. Various sorbents can be exerted with D-μ-SPE. Equate to long-established SPE sorbents, nanomaterials acquire short diffusion path and considerable surface area, which may result in great extraction efficiency and fast extraction dynamics. To overcome the serious drawbacks of utilizing nano-materials packed into a cartridge, such as lengthy sample loading time and higher back pressure; vortex assisted dispersive solid-phase extraction (VADSPE), as an efficient SPE mode, has been introduced based on a wide range of adsorbents

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including crab shell powder [7].

Crab shells powder contained chitin, calcium carbonate and proteins. Chitin, poly-β-(1, 4)-*N*-acetyl-D- glucosamine, is one of the structural ingredients in the external body skeleton of crabs. It is cellulose-like biopolymer, with a number of different functional groups, such as hydroxyls and amines, which increase the uptake efficacy of many drugs and chemicals and maximizes chemical loading. Chitosan, is the product of Chitin's de-acetylation and is the most important derivative of it. The mechanism of chitosan formation from chitin exhibited graphically in Fig. S2 [8]. It has captivated special attentiveness on account of its unique aspects, including adsorption capacity, large surface area, biodegradability, good biocompatibility, renewability non-toxic, film forming ability and hydrophobicity [9].

Due to the presence of a multitude of free hydroxyl and amino groups in its molecular structure and because of protonation of the–NH2 functional groups introduce into the C-2 position of the D-glucosamine reiterating division, it is capable of forming hydrogen bonding interactions, ion-exchange and electrostatic attraction. Further, in the acidic aqueous medium chitosan back bone suffers solubilization. This generates chitosan well afford as a unique pseudo-natural polymer which can be implemented as film, fibers and the hydrogel form [10]. In addition, the chitosan is an environmentally friendly, readily available, effective, low-cost material. Additionally it has capacity for the coupling easily, modification and has an excellent adsorption ability for the oil, grease, metals and other matters [11]. Accordingly chitosan has been extensively applied for miscellaneous fields including drug delivery, food sector, agriculture, separation, pharmaceutical, sewage treatment and medicine [12–14]. For these reason, this bio-available sorbent has been used.

In this research, a response surface methodology approach was applied to identify the optimum conditions for analysis during method development. The traditional method of optimization is the one-factor-at-a-time approach, which has been extensively employed via the past investigations. Even if, it is well approved that this technique persistently was unsuccessful to predict optimal separation conditions and demand a proportionate massive number of trials. It is predominately long-lasting, labor exhaustive, and can tend towards misconstruction of the results due to an inadequacy to contemplate feasible interactions between factors. Quite the opposite, multivariate optimization based on the statistical experimental model attitude affords superiorities including enhanced statistical construction of the results, cutback on the number of trials significantly and that refrain from ambiguous outcomes. Additionally, the impression of a particular criterion can be determined at various stages of the further characteristics, so the outcomes are logical over a wide series of experimental conditions [15].

The RSM is a capable model for optimizing an assortment of proceedings where several variables and interactions influence in the expected response. It can use the quantitative figures of the well-chosen experimental design to estimate manifold variables along with their interactions by set up a short-lived and less arduous mathematical style [16].

In some previous works [11,12], the considerable enhancement of analyte extraction which was accomplished in SPE by supplying an integrated method of vortex assisted solid phase extraction had been projected. Following to those works, we have tried to progress this method in order to develop method precision with lower detection limits. In addition, central composite design was applied to optimize the extraction parameters.

So, in the present work, crab shell has drawn a lot of our attention for pre-concentration and determination of three BZPs; oxazepam, flurazepma and diazepam in various biological matrix as a result of its unique aspect such as: green, low cost and biodegradability.

Indeed, our intent was to examine an eco-friendly adsorbent to isolate the target drugs from bio-matrixes. Cellulose-like backbone of the crab shells also played an important role in the benzodiazepine isolation. The results show that crab shells, a marine waste, could be used as a promising adsorbent in the bio-matrix treatment process. Therefore, a novel process of benzodiazepines isolation, which is environmentally friendly, practical and economical for actual sewage and bio-matrix treatment and easy to operate, should be developed.

Further, in this research for the first time, crab shell as an environmental-friendly adsorbent has been provided for the extraction of benzodiazepines from complicated matrix, due to its adhesives aspect for isolation of biomolecules.

Though in similar researches, the chitosan as an adsorbent mainly in simple environments such as aqueous solutions for extraction or removal of species had been reported. It is noteworthy that the porosity of crab shell is much more than the chitosan. So, in addition to chemical absorption, physical adsorption is involved in the extraction of target analytes because of its entrapment and adsorption properties. Furthermore, crab shell consisting of great reactive groups for chemical activation and cross-linking. Another highlighted feature of the considered biopolymer is high surface charge density, which may play the important role in the extraction procedure due to the electrostatic interactions. However, it suffers some drawbacks including the narrow pH stability range of crab shell back bone and poly-dispersion of the crab shell micro particles [17].

2. Experimental

2.1. Instrumentation

The HPLC system is composed of a Waters 600 E (Millipore, Milford, MA, USA), LC-600pump, C1Cheminert injector valve equipped with a 20 μL sample loop (Switzerland), a Waters 486 tunable UV–Vis detector and a Waters 746 integrator. A C18 column (125 mm length, 4.0 mm diameter, and 5 μm particle size) was applied for separation.

This column was packed in our laboratory with a Knauer packing system involving a Knauer pneumatic HPLC pump (Berlin, Germany), utilizing packing material (Eurospher 100, C18). The degassed mobile phase was a mixture of acetonitrile- methanol–pure water optimized on (80:10:10, v/v/v). The UV detection wavelength was set at 238 nm and the mobile phase flow-rate was 1 mL/min. The column was utilized at room temperature (22 ± 0.5 °C) [18].

2.2. Reagents and solutions

The HPLC-grade solvents including: methanol, pure water, acetonitrile and other analytical grade were purchased from Merck (Darmstadt, Germany). Crab shells were obtained from the supermarket, which was powdered by the ball mill (planetary mill, Iran) with the resolution scale of 2 μm.

Subsequently, the powdered shells soaked in an acidic solution (5% HCl) to remove calcium salt and other minerals for one hour at room temperature. After a washing step with deionized water to remove residual HCl, the powder was soaked in a hot alkaline solution (50% NaOH, 80 °C) for at least an hour, until it has been deacetylated. At this stage, the protein is also removed. After that, the powder was rinsed in deionized water, the pH was adjusted to neutral by HCl treatment and then the bio-sorbent was air-dried. The sorbent was stored in a suitable container until use [19].

Purified free BZPs (Fig. S1) with a purity of > 99% were purchased from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide was purchased from Farabi Co. (Tehran, Iran). Stock solutions of discussed analytes (0.1 mg/mL) were prepared in methanol and kept at 4 °C. Standard sample solutions were supplied diurnal at various concentrations by diluting the stock standard solution with distilled water, which was purified on a Milli-Q ultra-pure water-purification system (Millipore, Bedford, MA, USA).

2.3. Preparation of real sample

All biological samples, mandatory for technique validation and elaboration, were collected from a healthy individual volunteer. The deprivation of target compounds was checked utilizing HPLC analysis on genuine samples.

2.3.1. Hair samples treatment

Hair samples of patients and one of the researchers who was fascinated by this investigation were gathered as the biological matrixes which were conjectured to contamination with the benzodiazepines. Fat and further surface contaminations on collected hair samples with a length of 2–4 cm should be detached. As follows, the hair was rinsed by successive solvents on a hierarchy arrangement as follow:

Cleaning was accompanied by 20 mL dichloromethane, 20 mL acetone and 15 mL methanol, respectively at ambient temperature and then they were desiccated. To facilitate the digestion procedure, hair samples was ultimately cut into relatively 1 mm divisions and digested by the succeeding process (For adjusting the pH value at 7.4 a phosphate buffer solution was chosen). 50 mg of hair samples were introduced into a 10 mL screw-cap vessel which contained a volume of 2.0 mL methanol as an extracting solvent. Then hair samples were heated at 55 °C for 5 h. Finally, residual solid hair matrix was sieved and washed with 0.5 mL ethanol and added to the extracted solution. The remaining alcoholic was diluted with appropriate deionized water [20].

2.3.2. Nail samples treatment

50 mg of nail samples was introduced into a digestion vessel with 3.1 mL of nitric acid and 1 mL of 30% high purity hydrogen peroxide. At the first step, the container was heated up to 140°C at 400 W for 10 min, after that the temperature was risen to 190°C at 600 W for 25 min applying a Milestone Ethos Plus microwave digestion system. Subsequent to ta king the containers away from the microwave, samples were chilled and shifted to acid extracted 50 mL polypropylene vessels and watery to 8 mL with high purity water. Succeeding digestion the solutions were clear designating perfect oxidation of organic substance [21].

2.3.3. Blood samples treatment

Blood samples were collected from the volunteers at the hospital clinic of Ebne-Sin (Mashhad, Iran) with permission that was obtained from the patients due to the regulation of the local ethics committee. Due to acid digestion in a microwave, blood samples were decomposed. The digestion was conducted in PTFE containers are as follows: 1 mL of nitric acid and 0.3 mL of hydrogen peroxide were added straight into the container which is included 1 mL of blood matrix. The container was then covered and warmed up 6 min at 294 W in the microwave. Subsequent to heat abating in an ice bath, the container was unfastened and the admixture was watery by expanding upon 5 mL. A fraction of 200 μL was introduced into an auto-sampler cup jointly with the well-chosen quantity of matrix modifiers and was watery to 400 μL [22].

2.3.4. Urine samples treatment

Blank human urine samples were gathered from volunteers, which were not affected by mentioned drugs. Urine samples did not have any particular requirements for pretreatment for SPE procedure. However, diluting with water or a buffer to obtain the proper pH prior to sample addition is necessary. In some cases, to make sure that the analytes of interest is solvated simplistic in the urine matrix, acid hydrolysis (for basic compounds) or base hydrolysis (for acidic compounds) are required. The following process is included heating the urine samples (25 mL of urine samples were diluted 4-fold) for 15–20 min, cool and diluting with an appropriate buffer solution to make adjusted pH for SPE process. For frees bound compounds or drugs, enzymatic hydrolysis could be suggested. At the end of the mentioned pretreatment procedure, for extraction via VADSPE technique, the pH of urine samples were adjusted to pH 7 with sodium hydroxide [23].

2.4. VADSPE procedure

VADSPE procedure was fulfilled in accordance with the succeeding steps: 10 mL of aqueous solution consisting of the three benzodiazepines was put in a centrifuge tube. Afterwards, 35 mg of treated crab shell powder was introduced into the feed solution, which was vortexed 5 min to certify the particles were dispersed extensively the samples. The stirrer was then switched on. The extraction was carried out for a period of time at room temperature and then the solution was transferred into the centrifuge tube. Following the mixture was centrifuged at 5000 rpm for 10 min and the upper aqueous phase cautiously was withdrawn. The analytes were desorbed from the subjacent crab shell powder applying 1.5 mL of methanol with 6 min of vortex treatment. After 10 min of centrifugation, 10 μL of the uptake solvent including the mentioned analytes was introduced into the HPLC apparatus for further analysis.

2.5. Optimization strategy

Various variables may influence the efficiency of benzodiazepines extraction applying VADSPE such as extraction solvent volume, extraction time, back extraction time and dosage of sorbent. In order to progress the vortex assisted dispersive solid phase extraction approach for these benzodiazepines analysis, it is essential to contemplate and optimize specified parameters. RSM depends on CCD, a multivariate statistical approach was implemented to optimize the level of profitable parameters for enhancing the effectiveness of three discussed benzodiazepines extraction. The variety of variables altering the responses to target compounds was represented in Electronic Supplementary material (Table S1); these factors consist of extraction solvent volume, extraction time, back extraction time and dosage of sorbent. The model involved six replicate of the central point. Hence, the applied model composed of 31 accomplished experiments at random. Three-replicate extractions and quantitative analysis were carried out for each experiment. The model matrix involved the responses (experimental values), which was presented in Table S2. The whole experiments were carried out applying working solutions that were included 1 mg L^{-1} of each mentioned benzodiazepines. For estimating the extraction efficiency of benzodiazepines, the sum of the peak areas was assumed as the HPLC response. The response surface schemes describing the design and the modeled CCD information were achieved via statistical determination. The model creation and statistical evaluations were fulfilled by taking advantage of the software mini-tab version 17 for windows [24,25].

3. Results and discussion

The proper separation and detection of three benzodiazepines was obtained by the optimization of four parameters which could alter the extraction efficiency in the VADSPE process: the volumes of extraction solvent, extraction time, back extraction time and dosage of sorbent. The response surface model was applied for response optimization of the four parameters.

3.1. Characterization of crab shell powder as adsorbent and its modified forms

The main sorption crab shell sites obviously are hydroxyl- or amino groups. In other words, crab shell may be readily interacted with mentioned BZDs by utilizing the reactivity of the primary amino group (predominantly) and primary and secondary hydroxyl groups. Further, another possibility for extraction mechanism of the analytes by crab shell may consist of two types of the intermolecular interactions: The CH/π interaction is a kind of hydrogen bond operating between a soft acid CH and a soft base π-system (aromatic rings) in other words interaction of aromatic parts of analytes with C—H bonds of crab shell. And also, formation of hydrogen bond between carbonyl group of

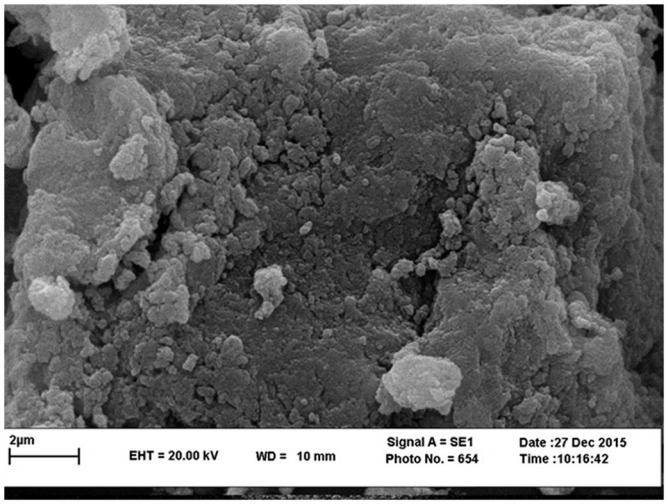

Fig. 1. SEM image of crab shell powder.

analytes and hydroxyl- and amino groups of crab shell [26].

Characterizations of the crab shell powder were performed including SEM and FT-IR. The morphology of mentioned green adsorbent was certified by SEM (Fig. 1). According to the particle size analysis of the crab shell powder, it was exhibited microspheres owning an average diameter of about 51.429 μm.

According to Fig. 2.a, in the FT-IR spectrum of crab shell powder, the presence of the broadened bond at 3200–3600 cm^{-1} was providing evidence of overlapping \OH and \NH stretching vibration to some extent [27]. Further, absorption bands typical of polysaccharides appeared in the spectral expanse of 3500 $cm^{-1}$and 2800 cm^{-1}. Ether linkage of chitosan backbone was proved by the presence of the C—O stretching vibration and carbonyl stretching vibration (amide I) at 1661 cm^{-1} and 1155 cm^{-1}.

As mentioned in the Fig. 2.b, due to the interaction of the amino and carboxyl groups, the main changes were occurred in the range of 1800–1600 cm–1. The vibration of the carbonyl group of pectin could be observed at 1723 cm–1. The asymmetric stretching vibration of the carbonyl group of the carboxylate (COO–) groups in pectin (~1631 cm–1) and the amide I (~1658 cm–1) and amide II (~1600 cm–1) vibrations of the amide groups of chitosan providing evidence of the formation of inter-chain or inter-molecular ionic salt bonds i.e. PEC between amino groups of chitosan and carboxyl groups of pectin [27,28].

In the FT-IR spectra of CHI-PP coated crab shell (Fig. 2.c), the appearance of the peak at 3444 cm-1 was attributed to the O—H stretching vibrations of chitosan and N—H stretching vibrations of polypyrrole. The peak was observed at 1318 cm-1 is for O—H bending vibrations. In addition, the observed peak at 1561 cm-1 was assigned to NH3 group [29].

FTIR spectra of CHI-PEG (Fig. 2.d), the PEGylation of the hydroxyl group was discerned by the presence of the band corresponding to the hydroxyl group of chitosan had been moderately shifted. Notwithstanding, the reaction on some of the hydroxyl groups of chitosan was proved by the appearance of new-found peaks at 1032 (C\O), 1280, 1313, 1433, and 3200–3600 (\CH2\O\CH2) which coincided with the diminution in the peak dimension. [30].

The infrared spectra of CHI- PANI -functionalized crab shell (Fig. 2.e) demonstrated the effective coating of polyaniline on the chitosan back bone. Further, the presence of band comparable with the N—H stretching vibration (secondary amine) was observed at 3426 cm^{-1}. The appearance of the peaks at 1567 cm-1 and 1492 cm-1 were assigned to quinoid ring and benzene ring, respectively. Approximate to the infrared spectrogram of the native chitosan, the N—H bending vibration peak at 1661 cm-1 (primary amine) moderately

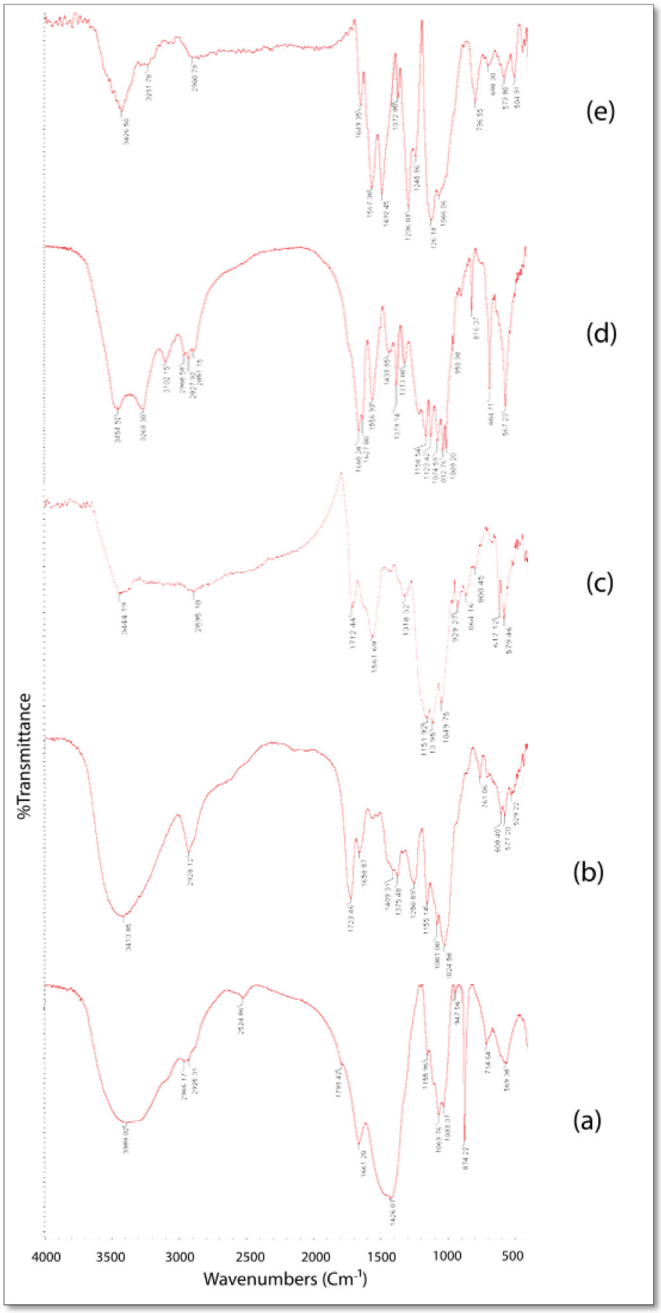

Fig. 2. FT-IR spectrum of (a) crab shell powder, (b) polyelectrolyte complex (PEC) modified crab shell, (c) CHI-PP coated crab shell, (d) CHI–PEG coated crab shell, (e) PANI-CHI functionalized crab shell.

shifted to that of 1649 cm-1 (secondary amine). In addition, the N—H bending vibration peak proved the coating of PANI on the crab shell powder [31].

In addition, the particle size analysis of crab shell powder indicated that the average size of the 90% of the particles is about 51.429 μm (See Fig. S3).

3.2. Type of adsorbents

Desorption capacity of some various adsorbents based on crab shell powder, for the extraction of mentioned analytes at the optimum situations was compared. To sum up from these investigations, the foremost adsorbents preference was different depending on the considered compounds, so the final choice should be hang on the purpose

Table 1
Comparison of the results of various adsorbents in the extraction.

Type of sorbent	Peak area of oxazepam	Peak area of florazepam	Peak area of diazepam	Microextraction mode
Crab shell micro powder	0.16	0.19	0.18	VADSPE
Crab shell micro powder	0.18	0.18	0.06	HF-SLPME
Synthetic Chitosan	0.12	0.06	0.1	VADSPE
Sol-Gel [16]	0.53	0.11	0.2	HF-SLPME
Crab shell micro powder-Pectin Poly Electrolyte Complex*	0.13	0.33	0.23	VADSPE
Crab shell micro powder-Pectin Poly Electrolyte Complex added to sol-gel	0.59	0.13	0.36	HF-SLPME
Polyaniline coated crab Shell micro powder**	2.37	0	0.75	VADSPE
Polyaniline coated crab Shell micro powder added to sol-gel	0.18	0.13	0.06	HF-SLPME
Polypyrrole coated crab shell micro powder**	0.34	0.17	0.03	VADSPE
Polypyrrole coated crab shell micro powder added to sol-gel	0.26	0.19	0.02	HF-SLPME
Polyethylene glycol coated crab shell micro powder**	0.42	0	0.17	VADSPE
Polyethylene glycol coated crab shell micro powder added to sol-gel	0.01	0.02	0.03	HF-SLPME

* Synthesized according to the reference # [17].
** Synthesized according to the reference # [18].

Table 2
Analytical features of the proposed approach.

Matrix	Analyte	Correlation Coefficient	Equation	L.O.D(μg·mL^{-1})	L.O.Q(μg·mL^{-1})	D.L.R(μg·mL^{-1})
water	Oxazepam	0.9968	y = 0.3896x − 3E-06	0.01	0.042	0.042–15
	Flurazepam	0.9934	y = 1.0635x + 2E-05	0.003	0.01	0.01–15
	Diazepam	0.9995	y = 0.0492x − 0.0053	0.052	0.18	0.18–15
Hair	Oxazepam	0.9919	y = 0.1049x + 3E-06	0.23	0.79	0.79–20
	Flurazepam	0.9997	y = 1.5661x + 1E-07	0.011	0.04	0.04–10
	Diazepam	0.992	y = 0.9302x + 3E-05	0.012	0.04	0.04–10
Nail	Oxazepam	0.9911	y = 0.0038x + 3E-06	0.94	3.15	3.15–20
	Flurazepam	0.9942	y = 0.0042x − 4E-05	0.61	2.11	2.11–20
	Diazepam	0.996	y = 0.0041x − 7E-06	0.31	1.07	1.07–20
Blood	Oxazepam	0.9935	y = 0.2392x − 4E-05	0.1	0.3	0.3–15
	Flurazepam	0.9922	y = 0.0059x − 2E-05	1.1	3.38	3.38–15
	Diazepam	0.9977	y = 0.0052x + 1E-05	1.2	3.8	3.8–15

Table 3
Comparison of RSD% and L.O·Ds in the sequential HPLC-VADSPE method and recent approaches for the analysis of Benzodiazepines.

Instrument	Extraction approach	Matrix	number of target analytes	L.O.D	R.S.D.%	Year	Ref.
HPLC/UV	VSDSPE	Urine, Nail, Hair, Blood	3	0.003–1.2 μg·mL^{-1}	2.63–6.19	Present Work	
LC–MS/MS	LLE	Urine	23	5*10^-4–3*10^-4 μg·mL^{-1}	5–11.8	2011	[31]
HPLC/UV	HF-SLPME	Water, Urine, Hair	4	8*10^-5-500 μg·mL^{-1}	1.1–4.6	2014	[32]
LC–MS/MS	μ.Elution solid-phase extraction	Blood	6	5*10^-5–25*10^-3 μg·mL^{-1}	0.1	2013	[33]
UPLC-PDA	DLLME	Human Plasma	7	1.7–10.6 μg·mL^{-1}	0.1–8.6	2013	[34]
HPLC-DAD	HF-LPME	Water, Urine, Plasma	5	0.5–0.7 μg·mL^{-1}	4.3–6.1	2012	[35]
GC-TOF-MS	SPE	Urine	35	4*10^-4–15.3*10^-3 μg·mL^{-1}	2.49–9.68	2011	[36]
HPLC/UV	L.L.E and S.P.E	Human Plasma	7	2*10^-5 μg·mL^{-1}	8.9–14.8	2009	[37]

of the research. The adsorbents that were compared include: crab shell micro powder, synthetic chitosan, polyethylene glycol (PEG) modified crab shell, polyaniline-coated chitosan (CHI- PANI) functionalized crab shell, chitosan-polypyrrol (CHI-PP) coated crab shell, chitosan– polyethylene glycol(CHI-PEG) functionalized crab shell.

According to the result, (See Table 1) the most helpful and manageable status was crab shell powder by VADSPE approach. Hence, this green adsorbent was applied for the rest of experiments in this investigation. The surface density of functional groups may affect the outcome [7,27,32,33].

3.3. Optimization of VADSPE method

The aim at this procedure is to gain the optimal values of the noteworthy parameters to achieve the foremost response and enhance the sensitivity of three mentioned benzodiazepines extraction efficiencies by dispersive-solid phase extraction [34]. Some preliminary experiments for optimizing the extraction conditions by CCD approach must be performed. The sum of total design point necessitated (N) is ascertained by the following equation:

$$N = 2^f + 2f + C_p \quad (1)$$

where f is the number of parameters and Cp is the number of centre point [35]. Accordingly, thoroughly 31 experiments in one block and at random manner to minimize the effect of uncontrolled variables on the response were executed for the CCD (C_p = 6) [36–40]. The peak area of discussed benzodiazepines was considered as the experimental response. A quadratic design was qualified as the achieved information. This design was applied to predict the response to any point, despite those was not involved in the model. Interpretation of regression analysis was demonstrated in the ESM file (See Tables S4.a–c, S5 and S6). To estimate the importance of each variable and interaction terms, analysis of variance (ANOVA) was applied. The data was shown in Table S7.a–c. The graphical interpretation of the interactions, was demonstrated in the usage of three-dimensional (3D) plots of the design (See ESM,file). Scatter plots of predicted response of three analytes vs actual response of them from RSM design were illustrated in Fig. S8.a–c.

Table 4
Relative recovery and founded concentration of target analytes of proposed method for target BZPs in the biological matrixes.

Nail				Blood			
		RR%	Founded			RR%	Founded (n = 3)
Oxazepam							
	46 years old man	-	not qualified				
	110 years old woman	-	not qualified	Flurazepam			
Flurazepam					55 years old woman after 24 h	95.60%	0.74 ± 0.04 μg·mL^{-1}
	46 years old man	-	not qualified		55 years old woman after 48 h	-	not qualified
	110 years old woman	-	not qualified				
Diazepam							
	110 years old woman	-	not qualified				
	46 years old man	-	not qualified				
Hair				**Urine**			
		RR%	Founded			RR%	Founded
Oxazepam				Oxazepam			
	110 years old woman	50%	2.95 ± 0.06 μg·mL^{-1}		55 years old woman after 24 h	55%	0.051 ± 0.005 μg·mL^{-1}
	46 years old man	50%	2.19 ± 0.05 μg·mL^{-1}		55 years old woman after 48 h	-	not qualified
Flurazepam				Flurazepam			
	110 years old woman	50%	0.17 ± 0.05 μg·mL^{-1}		55 years old woman after 24 h	36%	0.51 ± 0.05 μg·mL^{-1}
	46 years old man	55%	0.14 ± 0.03 μg·mL^{-1}		55 years old woman after 48 h	-	not qualified
Diazepam					46 years old man	66%	0.38 ± 0.03 μg·mL^{-1}
	110 years old woman	60%	0.1 ± 0.05 μg·mL^{-1}				

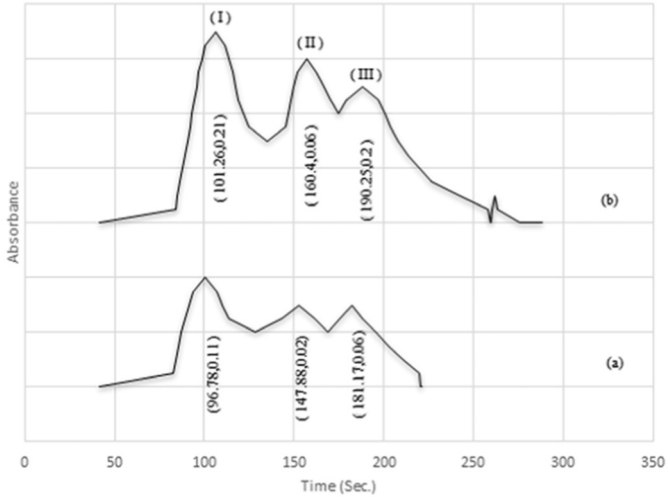

Fig. 3. HPLC chromatogram of hair matrix (a) before spike and (b) after spike: (I) Oxazepam, (II) Flurazepam, (III) Diazepam.

4. Quantitative analysis of benzodiazepines

The sequential VADSPE –HPLC method was estimated in accordance with LODs, linearity, recoveries and precision under the mentioned-optimized conditions. The proved consequences were provided in Table 2. The RSDs of the considered analytes ranged from 2.9 to 5.4%. The limits of detection (LODs) were calculated experimentally at a signal-to-noise ratio of 3, which were analyzed at a spiked level of 1 μg·mL^{-1} of target analytes and extended over 1.50 to 6.12 μg·mL^{-1}. The limit of quantifications (LOQs) (S/N ratio of 10:1) was between 0.01 and 3. 8 μg·mL^{-1}. The obtained calibration graph for target benzodiazepines was linear and in the concentration range of 0.01–20 μg·mL^{-1}. Satisfactory correlation coefficients of all calibration graphs were obtained ($R^2 \geq 0.9910$).

The VADSPE – HPLC results for extraction and determination of discussed compounds compete with the reciprocal literature data utilizing other techniques. It indicated that the suggested approach has an excellent recovery, comparable detection limit, acceptable linear attitude with correlation coefficients higher than0.99 and satisfying repeatability in biological matrixes. The extraction and determination of the discussed benzodiazepines applying the suggested technique approximated to other approaches [4,11–13,41–42], and the results were provided in Table 3 which proved that sequential VADSPE embody green chemistry principle in analytical chemistry. In addition, the technique of effortless procedure could accomplish performances about to alternative manners without exerting progressive gadgets. Thus, in time to come, the proposed VADSPE method was predicted to be broadly applied to the determination of target compounds in complicated matrix. So, for demonstrating the clinical applicability of the assay, the method was used successfully to quantitative BZPs in human hair, nail, urine and blood samples that were obtained from patients who were treated by BZPs. The obtained extraction relative recoveries were ranged from 36 to 95.6%. The corresponding equation for calculating the relative recovery was given in Eq. (1). Supporting Information of real sample analysis was provided in Table 4. HPLC Chromatograms of considered BZPs in hair matrices before the spike and after spiked with 1.0 μg·mL^{-1}of BZPs were exhibited in Fig. 3a and b respectively. Some of the real sample chromatograms were added to the supplementary file (Figs. S4–S6). Relative Recovery% is calculated based on the following formula.

$$\text{Relative Recovery\%} = \frac{C_{added} - C_{real}}{C_{added}} \times 100$$

In this equation, C_{real} and C_{added} are the concentrations of an analyte in the real sample and the spiked one, respectively.

5. Conclusion

This investigation offered a fast analytical methodology (VADSPE-HPLC-UV) which was progressed for the extraction and analysis of three BZD at trace levels in complicated biological matrixes. Chemometric approach, RSM based on CCD was applied to optimize effective variables on the performance of extraction procedure which identified the interaction and quadratic effects of variables. Hence, the mathematical design and 3D response surfaces assisted us to select the most accomplished experimental situations for the effective variables more accurately with minimum trial experiments (See Figs. S7 and S8). The Excellent precision, linearity, detection limits and accuracy for the suggested technique were achieved. The suggested approach has

evidenced various practical utilities, as well as; straight forwardness, promptness of extraction and analysis, a rebuff from matrix component, minimal usage of toxic organic solvent and short extraction time which begets that the performance applicable for the clinical and forensic investigations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2018.05.046.

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