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ARTICLE

Analysis of Benzodiazepines in Urine Samples by Solvent Bar Microextraction using HPLC-UV

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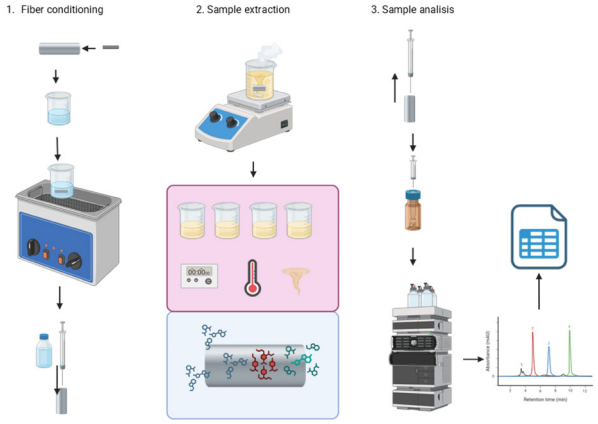

The identification of benzodiazepines (BZDs) in forensic samples has become a significant focus globally due to their increasing abuse in recent years. These compounds are readily assimilated by the body, leading to their increased use not only as over-the-counter medication but also in drug-facilitated sexual assaults. Consequently, this study investigated the behavior of selected BZDs in aqueous and spiked urine samples. Following method validation, real forensic urine samples were analyzed. To achieve this, a hollow fiber solvent bar microextraction (SBME) method was developed and optimized for the analysis of carbamazepine, nitrazepam, temazepam, and diazepam. The methodology involved the evaluation of factors such as microextraction time, temperature, agitation speed, and salt concentration. The optimization of the microextraction method was performed using a one-variable-at-a-time (OVAT) approach with an N + (N-1) design, considering each treatment with its respective replicates and the concentrations of the four standards. The optimal conditions for SBME were determined to be a temperature of 30 °C, a stirring speed of 400 rpm, a microextraction time of 30 min, and a salt concentration of 10% w/v. The analytical method was evaluated for sensitivity, linearity, precision, and accuracy, yielding RSD values below to 5%, in accordance with the guidelines for the validation of analytical methodologies. Finally, the developed method for the analysis of BZDs in urine by HPLC-UV following SBME was validated against more robust methods, including liquid chromatography-mass spectrometry, for final confirmation, demonstrating high performance in tests with forensic samples. The hollow fiber solvent bar microextraction technique allows for rapid analysis with small sample quantities, low costs, high recovery rates (86-96%), low detection (3.9-5.4 ng mL^{-1}) and quantification limits (in the range of 13.0 to 18.0 ng mL^{-1}), more efficient results, and greater eco-efficiency. This provides clear data for consumer protection and aids in clarifying criminal cases.

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INTRODUCTION

In the 60s, benzodiazepines (BZDs) began to be used gradually and displaced barbiturates due to their greater safety, therapeutic range, and pharmacological efficiency. Since then, more than 2000 different benzodiazepine-type drugs have been synthesized.[1] They are an essential class of drugs commonly used as minor tranquilizers, hypnotics, muscle relaxants, and anticonvulsants. The usefulness of BZD varies considerably because there are significant differences in the selectivity between these drugs.[2] They are among the drugs most frequently prescribed for the treatment of anxiety, sleep disorders, and status epilepticus.[3]

In addition, BZDs are used in the treatment of alcohol withdrawal, to relieve tension in the preoperative phase, for amphetamine-type drug overdose, and to induce anesthesia in surgical procedures. The over-the-counter use of these drugs is linked to different drug-facilitated sexual assaults (DFSA). It can potentially lead to dependence or adverse events like sudden death, respiratory depression, and coma.[4] Clinical and forensic toxicology laboratories, therefore, frequently receive requests for the determination of BZD in plasma, urine, or gastric fluids.[5] The analysis of BZD in biological fluids is laborious due to the diversity of these commercially available drugs and the fact that each product has a particular therapeutic and toxic range.[6] The concentration can also change due to post-mortem phenomena of redistribution or degradation.[1,7]

Their presence in forensic samples is usually associated with scenarios such as kidnappings, drug-facilitated crimes, recreational use, unintentional abuse associated with over-the-counter or self-administration, and replacement therapy in opioid withdrawal or to clinically treat the adverse effects of psychostimulant drug intoxication (e.g., cocaine overdose).[8] It has been proposed that criminal and violent acts produced under the effects of BZD may be related to low levels of serotonin through GABAergic effects.[9] Due to the partial transformation of BZD in the organism, different chemical adducts or metabolites appear, which leads to the need for updating information for the development of sufficiently sensitive and specific laboratory techniques for the reliable detection of BZDs in biological matrices. Clinical and forensic laboratory methodologies for BZD confirmation include a preliminary confirmation using liquid chromatography with ultraviolet detection (LC-UV), based on the comparison of the retention time and absorbance between a standard and real samples at a given wavelength. Immunoassay techniques such as fluorescent polarization immunoassay (FPIA), enzyme immunoassay (EIA), or multiplied enzyme immunoassay (EMIT) are also used; they are based on a competitive link between enzymatically labeled drugs (antigen) and the drugs in the analyzed matrix by the specific antibodies. The analytical principle is that a negative result indicates that when the drug is not present in the matrix, the antigen binds to the antibody and the substrate cannot bind to the enzyme; in this case, there are no changes in absorbance over time. However, when the drug is present in the analyzed matrix, the antigen is free to interact with the substrate and generates NADH, which absorbs at 340 nm; therefore, the concentration of the drug has a relatively proportional relationship to the absorbance.[10,11] Confirmatory analysis uses two focuses: 1-untargeted and qualitative assays based on structural identification, which regularly use gas chromatography coupled to mass spectrometry GC-MS, and liquid chromatography coupled to tandem mass spectrometry LC-MS/MS in full scan mode. 2-targeted (qualitative or quantitative) assays, which regularly use GC-MS or LC-MS in tandem mass spectrometry setup that uses a triple quadrupole (QqQ) or ion traps as mass filters, and the acquisition mode in selection reaction monitoring (SIM), multiple reaction monitoring (MRM), or target mode. The main goal of confirmatory assays is the univocal structural characterization, based on the match between the retention time, isotope ratio, and mass fragmentation patterns between the sample, drug certified reference standards, and spectral libraries.[8]

The solvent bar microextraction (SBME) was first introduced in 2004 by Jiang and Lee as an improvement on hollow fiber liquid-phase microextraction.[12] In this configuration, the fiber ends are sealed prior to use, allowing the fiber to move freely in contact with the sample during extraction. Its advantages include a simplified procedure, which enhances portability, and improved mass transfer from the sample to the fiber lumen, which reduces extraction times and allows for simultaneous extractions. Since its introduction, it has rapidly gained interest as a promising alternative in sample preparation for both inorganic and organic analysis, with applications in environmental, medical, and food analytical chemistry.[13]

SBME is a sample preparation technique that enables a high rate of mass transfer between phases, resulting in high recovery percentages. It is a rapid enrichment method with low interference, as the hollow fiber acts as a barrier. It can be easily integrated with various chromatographic systems, particularly HPLC, GC, and Capillary Electrophoresis (CE).[14,15]

Due to the different uses of BZD, their commitment to criminal acts in some cases, and in order to contribute to the implementation of modern methods of forensic analysis, it is necessary to develop a rapid, reliable, economical and environmentally friendly extraction method, as the membrane microextraction in solvent bar SBME in doped and forensic samples for the determination of four BZD. Its corresponding comparison with the usual method of analysis in the Legal Medicine laboratories, solid-phase extraction (SPE), will support whether there are significant differences in the recovery percentages between the two methodologies. The hollow membrane microextraction in solvent bar combines sampling, cleaning and enrichment of the sample in a single step, uses small amounts of solvent with a simple, easy, potentially automated sample preparation assembly and sequentially coupled to a separation procedure and detection for the determination of emergent pollutants in raw plastic industrial materials and forensic samples.[13,14]

Given the diverse applications of BZDs, their involvement in criminal activities, and the need to optimize forensic analysis methods, it is crucial to develop extraction techniques that are faster, more reliable, cost-effective, and environmentally friendly. This study aims to evaluate hollow fiber-supported liquid-phase microextraction for the detection of four BZDs in forensic samples, with the potential for use in legal medicine laboratories as a routine practice. The implementation of this methodology will help determine if significant differences exist in recovery rates compared to current techniques and will contribute to the modernization of forensic toxicology analytical processes.[15]

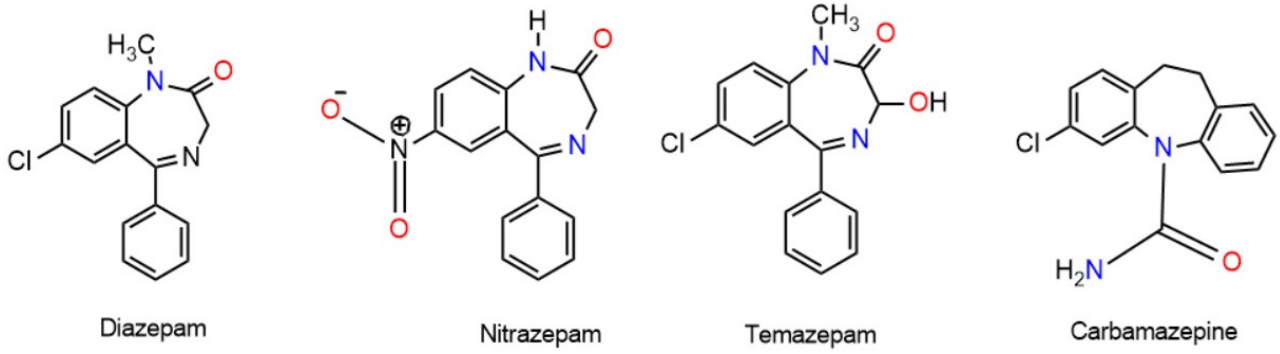

Figure 1. Molecular structure of target BZD.

MATERIALS AND METHODS

To implement the method, BZD standards were used in spiked samples, forensic samples, and the protocol applied for the technique. The method used for SBME-are also presented.

Reagents and solutions

All reagents and standards were acquired from Sigma-Aldrich Company through authorized distributors in Colombia (Quimirel Ltda and Commercial Outsourcing). Standard stock solutions of nitrazepam, carbamazepine, temazepam, and diazepam were prepared separately at a concentration of 1000 ng mL^{-1} in 10 mL of ultrapure Type I water (obtained from a Milli-Q purifier system). From these stock solutions, a 5 mL diluted mixture of the four analytes was prepared at a concentration of 30 ng mL^{-1} for proof-of-concept tests of the SBME procedure. All stock and working solutions were stored at 4 °C.

Sample collection and preparation

This research was approved by the Bioethics Committee of the Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Manizales, Colombia, where the study was conducted, with approval issued on June 14, 2022. Blank urine samples were obtained from healthy individuals with no prior exposure to medications or BZDs, as confirmed by a survey. The real forensic sample was voluntarily donated by a patient undergoing cancer treatment who had been administered morphine and diazepam intravenously 8 hours before sampling. The samples were hydrolyzed as follows: 500 µL of the forensic sample was mixed with 4450 µL of Type I water and 50 µL of β-glucuronidase. The mixture was incubated at 56 °C for 2.5 hours with gentle shaking. After hydrolysis, the urine pH was adjusted to 9 by adding a tetraborate buffer. The SBME was then performed under the optimized conditions, and the sample was analyzed by HPLC-UV.[4]

Instrumentation

HPLC-UV system

Chromatographic analysis was performed on a Thermo U-Dionex HPLC system equipped with an ultraviolet (UV) detector. An Agilent SB-C18 column (50 mm × 2.1 mm ID, 1.8 µm particle size) was used at 40 °C under isocratic conditions. The mobile phase consisted of a 5 mM KH_2PO_4/K_2HPO_4 buffer solution (pH 6.0), methanol, and diethyl ether (55:40:5 v/v/v) at a flow rate of 0.5 mL min^{-1} (the optimal flow was determined using the Van Deemter curve procedure). UV detection was performed at 245 nm. The analysis time was 5 min, and the injection volume was 20 µL. For corroboration of all results, two robust systems were used.

GC-MS system

An Agilent Technologies 7890A gas chromatograph equipped with a 5975C mass detector was used. The detector temperature was set at 250 °C. The column was an Rtx®-5 (30 m × 250 µm × 0.25 µm ID). The oven temperature program was as follows: initial temperature of 80 °C held for 3 min, then increased by 15 °C per minute to 200 °C, held for 5.33 min, then increased by 10 °C per minute to 300 °C, and maintained for 12 min. The total run time was 38.33 min. The injector temperature was 270 °C, operating in pulsed splitless mode at 20 PSI. The injection volume was 1 µL. The carrier gas was helium (99.999% purity).[16,17]

LC-MS system

Corroborative analysis of forensic samples was performed by HPLC-MS/MS on an Agilent Technologies 1120 liquid chromatograph coupled in tandem with a quadrupole filter and a 6200 series time-of-flight mass spectrometer. Analyte separation was carried out on a Zorbax Eclipse Plus C18 column (2.1 mm ID × 100 mm, 1.8 µm particle size), using 5 mM ammonium formate in 0.01% formic acid (A) and chromatography-grade acetonitrile (B) as mobile phases. The general source settings for positive electrospray ionization (ESI) were: capillary voltage, 3500 V; ion transfer tube temperature, 320 °C; and vaporizer temperature, 350 °C. The flow rate was 0.4 mL min^{-1}, the injection volume was 20 µL, and the scanned mass range was 80–1000 AMU. Data was analyzed using Agilent MassHunter Qualitative software with a lab-developed method for deconvolution and identification using dynamic combinatorial libraries (PDCL).

Calibration curve

To quantify the amount of BZD, a calibration curve was made with an external standard, in which the concentration versus the peak area of each of the standards worked was related. For nine concentration levels, three replications were made, for a total of 27 data points to estimate the relationship between concentration and area. A simple linear regression was performed by the least squares method, and the assumptions of normality, independence and homoscedasticity were verified for the four analytes studied. After verifying the assumptions of the model, the variance analysis of the regression was performed. This analysis was made taking into account all the replicates by concentration level and not by the average of each level, in order to a better adjustment of the model to the real situation according to the data shown in Figure 2.

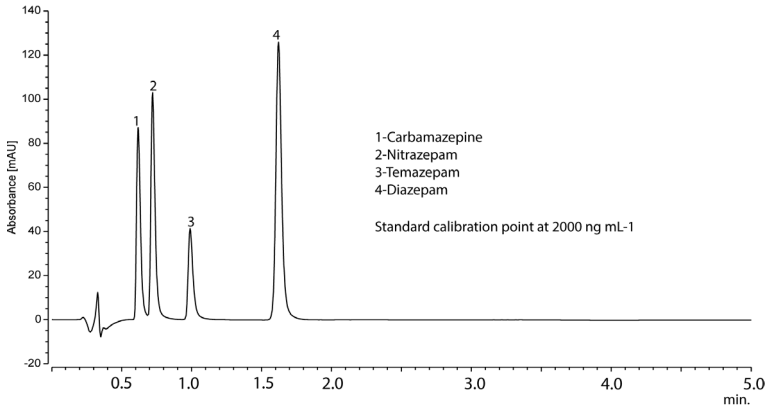

Figure 2. Chromatogram of the calibration curve. 1: carbamazepine (0.610 min), 2: nitrazepam (0.708 min), 3: temazepam (0.973) and 4: diazepam(1.598 min).

Solvent bar microextraction (SBME) procedure

Polypropylene Accurel PP S6/2 hollow fibers (Membrana, Wuppertal, Germany) were used as the support for the SBME experiments. The fibers had an inner diameter of 1,800 µm, a wall thickness of 450 µm, an average pore size of 0.2 µm, and a porosity of 72%. For each experiment, a 25 mm segment of the fiber was used. A metal pin was inserted into the fiber to allow it to be used as a magnetic stirrer. The SBME device was prepared by adding 30 µL of 1-octanol into the fiber lumen. The fiber was then immersed in 1-octanol to fill the pores and improve analyte transfer (two-phase mode). Both ends of the fiber were mechanically sealed to ensure that mass transfer occurred only through the membrane pores. The prepared SBME device was then introduced into a 5 mL vial containing the urine sample for extraction. Filtration of the matrix was not necessary, as this is facilitated by the membrane.

Optimization of SBME

The optimization of the SBME system was performed using a one-variable-at-a-time method[18] to study the effects of extraction temperature, stirring speed, extraction time, and the salting-out effect. An experimental design based on N + (N-1) was used, with four control variables, each at three levels. This design resulted in a maximum of nine experiments, which were conducted with a minimum of three repetitions for the four BZDs to establish the optimal operating conditions for the SBME system, as shown in Table I. The study was conducted using a One-Variable-at-a-Time (OVAT) approach to optimize the main factors affecting the response. For each factor investigated (e.g., temperature, time, stirring speed), a series of experiments was conducted where the level of that single factor was varied while all other factors were held constant at a baseline level. Each specific experimental condition (i.e., each level) was performed in triplicate (n=3) to ensure the reproducibility of the results and to calculate the mean and standard deviation for that point (see the errors bar in the Figures 4A-D).

Table I. Desing of Experiments N+(N-1)

Assay	Extraction temperature (°C)			Stirring speed (rpm)			Extraction time (min)			Salting out effect (w/v)		
Number	**10**	**20**	**30**	**400**	**700**	**1000**	**10**	**20**	**30**	**10**	**15**	**20**
1	+				+				+	+		
2		+			+				+	+		
3			+		+				+	+		

(continued on next page)

Table I. Desing of Experiments N+(N-1) (continued)

Assay	Extraction temperature (ºC)			Stirring speed (rpm)			Extraction time (min)			Salting out effect (w/v)		
Number	10	20	30	400	700	1000	10	20	30	10	15	20
4			+	+					+	+		
5			+			+			+	+		
6			+		+		+			+		
7			+		+			+		+		
8			+		+				+		+	
9			+		+				+			+

The levels for each factor were selected based on a combination of preliminary screening experiments and established findings in the relevant literatura.[19] The goal was to select a range that was known to be effective and relevant for this type of analysis.

While the levels were not always perfectly equidistant, they were chosen to adequately explore the operational range and identify the optimal working conditions. For example, if preliminary tests showed a sharp change between two points, we selected levels closer together in that region to better define the peak of the response curve. The term “9 experiments” referred to the total number of conditions tested for four factors (N-1) less 1 why one of them always keep constant with three levels each one ($3 \times (4 - 1) = 9$ conditions), with each condition being replicated three times.

SBME procedure

Unlike other modalities, both ends of the fiber are mechanically or thermally sealed after introducing the metal pin and the extraction solvent into the lumen, immobilizing the latter within the pores of the hollow membrane. Finally, the solvent bar is inserted into the sample, and once the extraction is complete, one end is opened to recover the acceptor phase.[6]

The complete procedure consists of cutting the membrane and the metal pin, then conditioning the membrane with acetone. After this, the lumen (hollow space) is filled with the appropriate solvent, and both ends are sealed using mechanical pressure. Next, the hollow fiber is functionalized as a magnetic stirrer by inserting a metal pin inside. The SBME system is then placed inside the doped sample container. After extraction, the SBME system is removed by cutting one end of the membrane, recovering the extract using a 30 µL Hamilton syringe. Finally, 30 µL of the extract is injected into the liquid chromatograph with the mobile phase,[7] as shown in Figure 3.

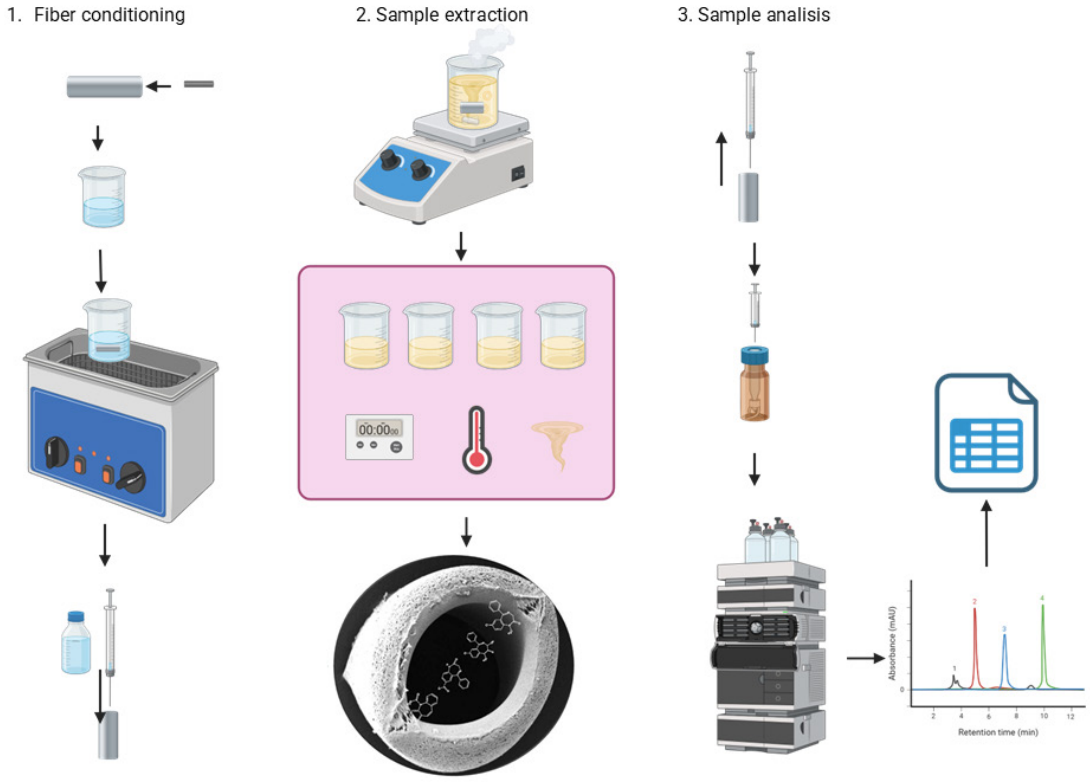

Figure 3. Flow diagram of the developed Solvent Bar Microextraction (SBME) system.

Calibration and statistical analysis

To quantify the BZDs, an external standard calibration curve was constructed by plotting concentration versus peak area for each of the four analytes. Nine concentration levels were prepared, with three replications for each, for a total of 27 data points. A simple linear regression was performed using the least squares method. The assumptions of normality (Shapiro-Wilks test), independence (Durbin-Watson test), and homoscedasticity (Bartlett's test) were verified for the four analytes under study. After verifying the model's assumptions, an analysis of variance (ANOVA) of the regression was performed. This analysis was conducted using all replicates for each concentration level, rather than the average of each level, to ensure a better fit of the model to the real data, as shown in Figure 2.

The SBME calibration curve was constructed using the four standards: carbamazepine, temazepam, nitrazepam, and diazepam. Spiked samples were prepared at six concentration levels: 100, 300, 500, 700, 900, and 1100 ng mL^{-1}, corresponding to typical concentrations found in the working matrix. Each sample was treated using the SBME procedure described above. Data analysis was conducted using SPSS and Statgraphics Centurion XI in demo mode.

RESULTS AND DISCUSSION

In this section, the setup and validation of the chromatographic methods used to quantify the analytes under study are presented, as well as the optimization of sample preparation methods with experiments, showing critical variables, as well as the best conditions for SPE with their recovery percentages. Also, the conditions for SBME are described, including the effect of microextraction speed, temperature, time, and the addition of NaCl, with their respective recovery percentages for both spiked and forensic samples. An analysis is performed using GC-MS and a corroboration of the case study by LC-MS/MS, demonstrating the reliability of the developed method.

Chromatographic method validation and analyte identification

Reversed-phase HPLC was chosen for the identification of the standards and analytes under study. The validation method involved the analysis of two blanks, two points from the calibration curve (200 ng mL^{-1} and 1100 ng mL^{-1}), a urine sample spiked with 1000 ng mL^{-1}, and an unspiked sample. Finally, the validation curve was analyzed. All analyses were performed in duplicate over five days to ensure thorough verification of the validation method.

The optimal separation conditions were established by alternating injections (20 µL) of standard, spiked, and unspiked samples, using an Agilent ZORBAX RRHD SB-C18 column (50 mm × 2.1 mm ID, 1.8 µm particle size). To determine the optimal mobile phase, tests were conducted based on previous work that performed chromatographic runs in isocratic mode at a flow rate of 0.5 mL min^{-1}. The optimal wavelength for analyte detection was determined by performing a spectral scan of the standard from 190 nm to 400 nm, which revealed the highest signal intensity at 254 nm. This is consistent with bibliographic references for BZD analysis. The optimal flow rate for chromatographic separation was determined using the Van Deemter curve, which shows the relationship between the linear velocity (u) of the mobile phase and the height equivalent to a theoretical plate (HETP). This curve was generated by injecting the four standards at flow rates ranging from 0.2 mL min^{-1} to 1.0 mL min^{-1}, using the optimal mobile phase described above. The lowest HETP (0.0025 mm) was obtained at a flow velocity of 0.05 cm s^{-1}, which corresponds to a flow rate of 0.5 mL min^{-1}. Therefore, this flow rate was determined to be optimal for the analysis of standards and samples.

Optimization of SBME conditions

To apply the SBME technique to the analysis of real samples, several variables were optimized. The pH of the samples was adjusted to and maintained at 7.0 prior to extraction. This pH was strategically chosen because the target analytes (carbamazepine, diazepam, temazepam, and nitrazepam) are all weak bases with p*K*a values significantly different from this pH. At a neutral pH, these compounds exist predominantly in their un-ionized, most hydrophobic form, which is essential for achieving maximum and reproducible extraction efficiency. These variables are discussed below.

Effect of extraction temperature

Temperature is a critical parameter in microextraction as it influences both the transfer rate and the partition coefficients of the analytes by affecting the kinetics and thermodynamics of the analyte adsorption process. The effect of temperature was evaluated at 10 °C, 20 °C, and 30 °C (see Table I and Figure 4A).

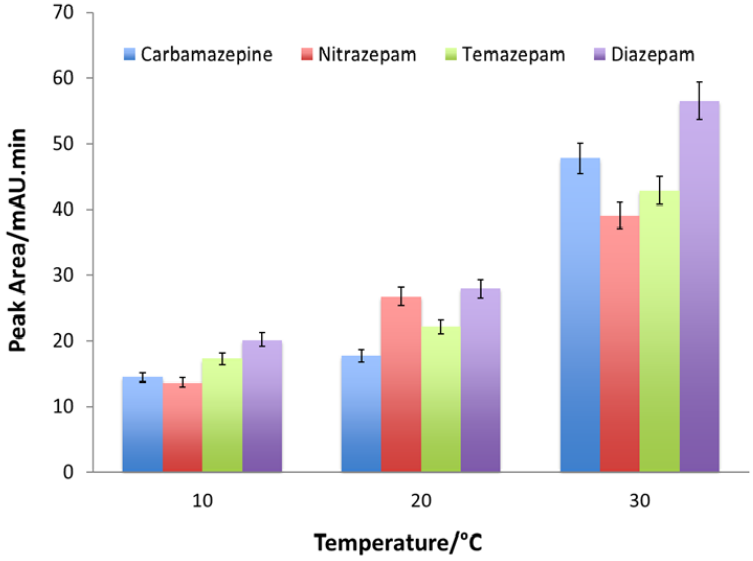

Figure 4A. Efect of extraction temperature.

It was observed that an increase in temperature enhances the microextraction of analytes, although some compounds are extracted at a higher rate. In general, the partition coefficient of some analytes can be improved with increasing temperature, and no significant increases were observed at 10 °C. Therefore, the optimal temperature was determined to be 30 °C.[14]

Effect of stirring speed

In the SBME system, stirring increases the transfer of analytes from the donor phase to the acceptor phase by reducing the thickness of the Nernst diffusion film. However, a very high stirring speed can cause significant loss of the extraction solvent or the formation of air bubbles in the pores of the SBME, which would prevent phase exchange. This is a limiting factor for the extraction. Stirring speeds of 400, 700, and 1000 rpm were evaluated (see Table I and Figure 4B). The best extraction results were obtained at 400 rpm, as significant losses of the organic solvent occurred at 700 and 1000 rpm.[19,20]

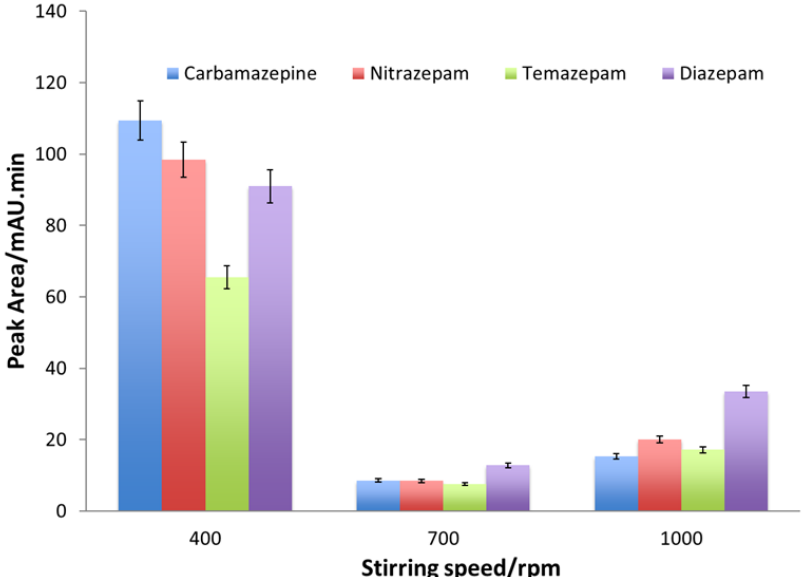

Figure 4B. Effect of stirring speed.

Effect of extraction time

The goal is to achieve system distribution equilibrium in the shortest possible time to extract the maximum amount of analyte. Extending the extraction time beyond equilibrium is futile, as the microextraction rate becomes constant and may even lead to loss of the organic solvent. The extraction time was evaluated at 10, 20, and 30 min (see Table I and Figure 4C). At an extraction temperature of 30 °C and a stirring speed of 400 rpm, the extraction rate increased up to approximately 30 minutes. Longer extraction times did not result in substantial improvements and could cause solvent loss, especially with this new type of hollow fiber arrangement.[21]

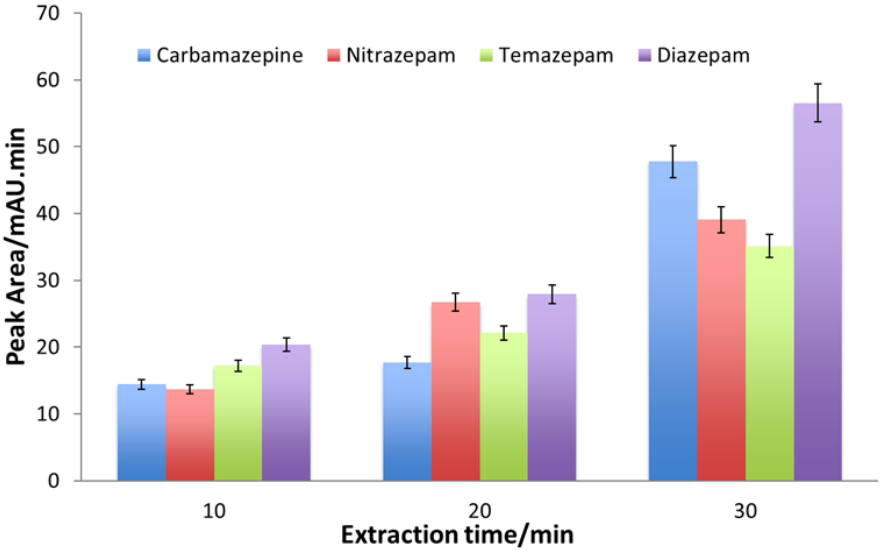

Figure 4C. Effect of extraction time.

Effect of NaCl addition

The ionic strength of the sample is a crucial parameter in any microextraction system, as it can significantly enhance the concentration of analytes. The effect of salt addition was evaluated at concentrations of 1%, 5%, and 10% NaCl (see Table I and Figure 4D). It was observed that increasing the ionic strength improves the solubility of the analyte in the organic phase. However, higher salt concentrations increase the viscosity of the solution and reduce mass transfer. Therefore, 10% NaCl was selected as the optimal concentration.[22]

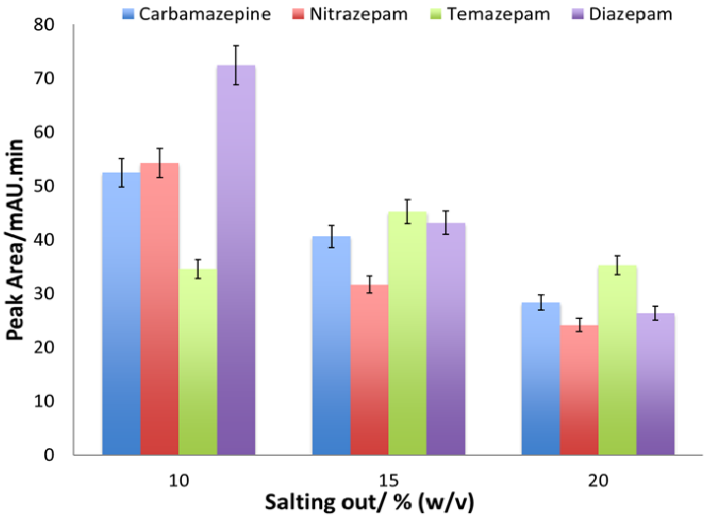

Figure 4D. Effect of salting out.

As shown, nine experiments were conducted with three levels and four control variables. Each experiment was replicated three times to determine the highest recovery percentage. Recovery percentages above 80% were obtained in experiment number 4 (see Table II), which had the following optimal conditions: 30 °C, 400 rpm, 30 minutes of stirring, and a 10% salt concentration.[23]

Table II summarizes the equations applied to calculate the validation parameters, namely the limit of detection (LOD), limit of quantification (LOQ), calibration features (slope, intercept and determination coefficient), accuracy, precision and enrichment factor (EF). The calculated values for carbamazepine, nitrazepam, temazepam, and diazepam were derived from the regression model parameters (intercept, slope, and their standard deviations). These results demonstrate the accuracy and sensitivity of the calibration model for each analyte.[24]

Table II. Calibration, sensitivity, accuracy, precision and preconcentration features

Analyte	Intercept	Slope	R^2	LOD ng mL^{-1}	LOQ ng mL^{-1}	Recovery %	RSD %	EF
Carbamazepine	0.0336	0.0013	0.9997	5.4	17.8	93.3	9.9	156
Nitrazepam	0.0494	0.0017	0.9992	4.0	13.2	85.9	9.7	143
Temazepam	0.0219	0.0008	0.9991	4.2	13.9	90.9	12.0	152
Diazepam	0.1103	0.0030	0.9994	3.9	12.9	95.9	15.9	160

Analysis of spiked and real samples by SBME

After optimizing the parameters of this novel microextraction technique, it was applied to real samples, yielding high performance (recoveries >85%) for both forensic and spiked sample analysis. This method allowed for the detection of these compounds using a simple, fast, robust, and cost-effective technique compared to the conventional SPE methods currently used in all Colombian forensic laboratories.

A standard mixed solution at 1000 ng mL^{-1} was injected as a reference to determine the recovery percentages for each microextraction. Subsequently, a 6 ng mL^{-1} standard solution mixture was prepared, considering that the membrane has a concentration factor of 167 (50 mL/0.03 mL). Therefore, the final concentration after extraction should be approximately 1000 ng mL^{-1} (167 × 6 ng mL^{-1}). Three beakers were prepared, each containing 5 mL of the 6 ng mL^{-1} standard solution mixture. Additionally, 0.5 g of NaCl was added to each beaker to enhance extraction (10% w/v). A direct injection of the 1000 ng mL^{-1} standard mixture was performed to calculate the recovery percentage for the microextracted samples.

Analysis of forensic samples

An analysis was performed on forensic urine samples containing the following substances: 1) Diazepam-Morphine FAB1; 2) Diazepam-Morphine FAB2; and 3) Interlaboratory Diazepam 20 µg mL^{-1}. The samples were first hydrolyzed and then extracted using SBME. A calibration curve was prepared using hydrolyzed spiked samples at six concentration points: 100, 300, 500, 700, 900, and 1100 ng mL^{-1}.

Chromatographic runs of forensic urine samples containing diazepam were performed to confirm the mass spectra of the peak eluting at the retention time of diazepam. Figures 5 and 6 show the chromatogram and mass spectrum of a sample, where the molecular ion (*m/z* 284), the base peak (*m/z* 256), and characteristic ions (*m/z* 242 and 165) are observed. To determine the sample concentrations, the area results were interpolated on the calibration curve. The calculated concentrations of the forensic samples are shown in Table III.

Table III. Concentration of Forensic Diazepam Samples in (Urine) Microextracted and Hydrolyzed

Forensic urine samples	Peak areas (mAU)	Interpolated value x	Concentration (µg mL^{-1})
ME-ILAB 1	0.419	2005.62	17.19
ME-FAB 13	0.213	718.125	6.15

(continued on next page)

Table III. Concentration of Forensic Diazepam Samples in (Urine) Microextracted and Hydrolyzed (continued)

Forensic urine samples	Peak areas (mAU)	Interpolated value x	Concentration (μg mL^{-1})
ME-FAB 21	0.122	149.375	1.28
ME-FAB 22	0.226	799.375	6.85

ILAB means Interlaboratoryal sample, and FAB means Forensic Analysis Batch; both correspond to an internal identification laboratory code for anonymized samples.

According to Table III, the calculated diazepam concentration for the interlaboratory sample using the developed SBME method is close to the certified concentration value (20 μg mL^{-1}), which demonstrates the good recovery performance of the method. The diazepam concentration values for the samples from the oncology patient show good precision, with the exception of sample FAB 21.

Confirmation analysis by GC-MS and LC-MS

To confirm the presence of diazepam in the forensic samples, they were injected into a GC-MS system. The SBME method was applied under the previously optimized conditions, and four trials were conducted. A diluted reference mixture solution (1000 ng mL^{-1}) was used to determine the recovery percentage. The SBME method was performed on urine samples diluted to 6 ng mL^{-1}, considering the theoretical concentration factor of 167. The results were then compared with a 1000 ng mL^{-1} diluted mixture solution of the four standards, yielding the recovery percentages shown in Table IV. The analysis of the forensic urine samples by GC-MS showed a peak with a retention time of 22.3 minutes, which corresponds to the signal of diazepam.

Table IV. Recovery Percentage of Urine Samples Microextracted by SBME GC-MS

BDZ	Diazepam MIX area 1000 ng mL^{-1}	Retention time (min)	Microextracted area (mAU)	Recovery (%)
DIAZEPAM 1	5168198	22.3	4562589	88
DIAZEPAM 2		22.3	4632300	90
DIAZEPAM 3		22.3	3898610	75
DIAZEPAM 4		22.3	3953481	76

To further confirm the presence of diazepam, sample injections were performed using an LC-QTOF system. The identification of diazepam by LC-MS/MS showed a signal for this analyte with a retention time of 6.04 minutes (Figure 5).[8]

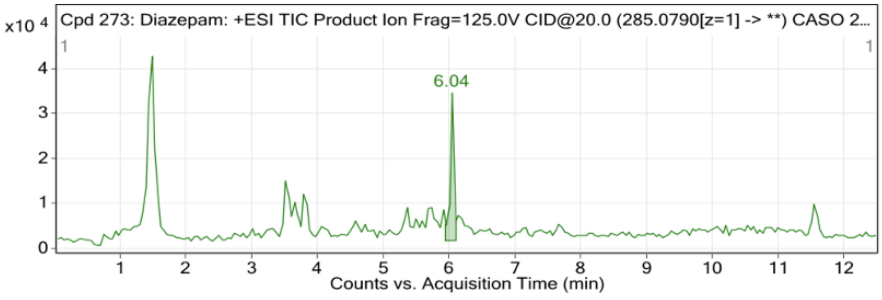

Figure 5. Chromatogram of Diazepam by LC-MS.

The mass spectrum of the diazepam molecule was obtained (Figure 6), confirming that the fragments correspond to the analyte of interest. These analyses confirm that the developed methodology is appropriate for the identification of diazepam, as it allows for differentiation between the analyte and other matrix compounds. Therefore, this method is reliable for use in future studies. The mass spectra of classic drugs and BZD monitored in the study, corresponding to central nervous system depressants and stimulants, which could have been consumed together with benzodiazepine-type drugs, are displayed in the Supplementary Material.

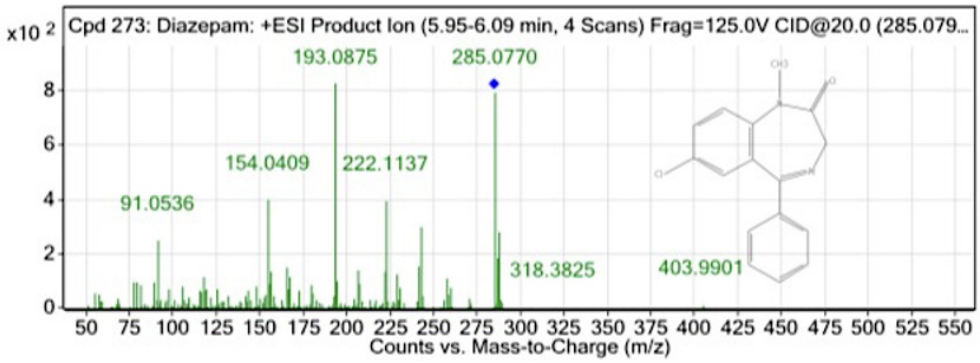

Figure 6. Mass spectrum of diazepam obtained by LC-MS.

In today's society, the analysis of BZD has gained great importance, making analytical testing increasingly significant. This is the case for the hollow fiber solvent bar microextraction technique, which allows for rapid analysis with small sample quantities, low costs, high recovery rates, low detection and quantification limits, more efficient results, and greater eco-efficiency compared to conventional techniques such as solid-phase extraction, which require longer response times. This provides clear limits for consumers or aids in clarifying criminal cases.[9]

For the identification of BZD, a robust technique such as high-performance liquid chromatography with ultraviolet detection (HPLC-UV) is used,[25] and it is further confirmed with even more robust techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS).[25] The extracted ion chromatogram for diazepam is shown in Figures 5-6, while the chromatograms for all other analytes can be found in the Supplementary Information (Figure SI-02).

CONCLUSIONS

The implementation of hollow fiber solvent bar microextraction (SBME) represents a significant advancement in the detection of BZDs in forensic samples. This method improves recovery rates and reduces interference, making it a viable alternative to more costly and labor-intensive sample preparation methods, such as solid-phase extraction (SPE). The ease of integration of SBME with chromatographic techniques like HPLC and GC-MS makes it a promising option for routine analysis in forensic toxicology laboratories.

SBME offers a more cost-effective, environmentally friendly, and accessible alternative to conventional extraction methods. This advancement has great potential in forensic toxicology, as it can facilitate the detection of compounds in biological matrices with greater speed and accuracy, thereby reducing the time and costs associated with forensic analyses.

The continuous evolution of detection and analytical techniques is essential to keep pace with the development of new BZDs and their derivatives. Research into the improvement of analytical methods, including microextraction and advanced chromatography techniques, plays a crucial role in forensic investigations and contributes to a more efficient and accurate approach to the analysis of controlled substances in biological samples.

Finally, in a great effort to understand better the intearactions between the key variables in the extraction processs, the follow-up study using a response surface methodology (RSM) or a full factorial design would be the logical next step to build a comprehensive statistical model.

Conflicts of interest

The authors don´t have any conflicts of interest

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SUPPLEMENTARY MATERIAL

The Supplementary Material of this article is available at **http://dx.doi.org/10.30744/brjac.2179-3425.AR-45-2025-SM**