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Development and Validation of Green CPSE-LC-MS/MS Analytical Method for Forensic Identification of Zolpidem in Beverage Matrices

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Zolpidem, a broadly prescribed Z-sedative hypnotic drug used for short-period management of insomnia. Recently, zolpidem gained forensic importance due to its misuse in drug facilitated sexual assault cases, which necessitates the development of reliable analytical method. In present study, the eco-friendly analytical method based on cellulose paper sorptive extraction coupled to liquid chromatography tandem mass spectrometry (CPSE-LC-MS/MS) was developed for the detection of zolpidem in water, alcoholic and non-alcoholic beverages. The ideal optimised extraction parameters include: pH 9, vortex and ultrasonication time 90 sec and 12.5 min, extraction time - 6 min, elution solvent (isopropanol) 1.5 mL and 7 pieces of filter paper (1.5 cm × 1.5 cm). The chromatographic separation performed using binary mobile phase: 0.1% formic acid in Milli-Q (solvent A) and 0.1% formic acid in acetonitrile (solvent B). As a result, excellent linearity from 1.325-1000 ng mL^{-1}, (R^2 = 0.9992), LOD and LOQ of 0.095 and 0.317 ng mL^{-1}, respectively. The intra-day and inter-day precision were below 7.26% and recovery ranged from 84.46 to 108.02%. Additionally, the environmental sustainability of the method was assessed through MoGAPI, BAGI and RGB 12 model, confirming the greenness of the method. Developed analytical method is more accurate, reliable and green(er) for the forensic investigations of zolpidem in the samples.

Keywords: Zolpidem, Micro-extraction, Drug facilitated crimes, LC-MS/MS.

INTRODUCTION

Zolpidem, (*N*,*N*-6-trimethyl-2-(4-methylphenyl)imidazo-[1,2-α]pyridine-3-acetamide) also known as designer benzodiazepine's is a short acting Z-sedative hypnotic drug. It belongs to the class of imidazopyridines and is commonly prescribed for the treatment of insomnia due to its rapid onset of action and sedative properties [1,2]. Zolpidem is a short-acting hypnotic agent that is rapidly absorbed, with onset of action typically occurring within 20-40 min. It exhibits approximately 90% plasma protein binding and has a relatively low volume of distribution. The drug is extensively metabolised in the liver by cytochrome P450 (CYP450) enzymes into pharmacologically inactive metabolites and has an elimination half-life of about 2-3 h. Zolpidem promotes sleep by selectively binding to the ω_1 (BZ1) subtype of the $GABA_A$ receptor complex, thereby enhancing inhibitory GABAergic neurotransmission [2-4]. In recent years, the involvement of Z-drugs in criminal activities such as sexual assaults, driving under the influence of drugs (DIUD) leads to road accidents, organ theft, property theft and robberies has drawn significant public and media attention [5,6].

Among various Z-drugs, zolpidem, have gained particular attention due to their widespread availability, psychoactive effects and potential for misuse. Consequently, they have become frequent subjects of forensic and clinical investigations [7]. From a legal perspective, zolpidem is categorised as a prescription only drug and is controlled as a psychoactive substance regulation. Furthermore, it has been listed by the Society of Forensic Toxicologists (SOFT) among the commonly identified agents involved in drug facilitated sexual assault (DFSA) incidents [8].

Drug-facilitated sexual assault (DFSA) refers to the use of a psychoactive substance to impair an individual's consciousness, judgement or ability to resist, thereby facilitating sexual assault. Alcohol is the drug most frequently associated with DFSA cases, but the involvement of other drugs has been increasingly reported. Besides alcohol, Z-drugs are among the most commonly detected agents these days in such cases [9]. Zolpidem is known to produce central nervous system

depression, impair psychomotor skills, reduce alertness and in high doses may lead to memory lapses or anterograde amnesia. Offenders exploit its pharmacokinetics properties, as the drug is colourless, tasteless and odourless when covertly spiked in beverages, making it difficult for victim to detect. Once consumed unknowingly, its sedative and amnesic actions impair individual's ability to resist, recall events or seek immediate help, thereby creating opportunity for exploitation often in the context of sexual assault [5]. Thus, zolpidem exemplifies a dual role, first act as a vulnerable therapeutic agent and second act as a potential facilitator of criminal activity like DFSA. This duality highlights the importance of advanced analytical detection method and strict regulations to prevent its misuse. To combat these issues, a number of drug testing labs are very interested in developing quick, repeatable and affordable techniques for detecting and validating these medications and their metabolites in complex biological such as blood and urine samples [10]. Traditionally the extraction of analyte was relied on liquid-liquid extraction (LLE) and solid-phase extraction (SPE). While SPE is widely employed, it is associated with limitations such as prolonged extraction times, multi-step procedure, the use of costly cartridges and less eco-friendly. In contrast, LLE offers relatively simpler and fast processing though it is labour-intensive and requires large volumes of volatile organic solvents. Recent developments in sample preparation have shifted towards microextraction techniques, which required minimum amount of solvent typically in microliter range for the extraction of analyte [2].

Various chromatographic approaches have been documented for the determination of zolpidem from biological matrices. These include liquid-liquid extraction combined with ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) [11,12], liquid chromatography tandem mass spectroscopy (LC-MS/MS) coupled with liquid-liquid extraction (LLE) for the detection zolpidem in urine [5], liquid chromatographic/mass spectrometric assay with atmospheric pressure chemical ionisation (LC/APCI-MS) in plasma sample [13]. Elkheir *et al.* [14] used first derivative synchronous spectrofluorimetry (FDSFS) and HPLC with fluorescence detector (HPLC-FD) for the quantification of zolpidem in pharmaceuticals, zirconia-based SPE combined with LC-MS/MS for zolpidem detection in hair [15], magnetic solid phase extraction (MSPE) followed by RP-HPLC for detection of analyte in apple juice [16]. Extraction techniques stand out because of their special characteristics such as they are quick, affordable, automated, eco-friendly and provide good extraction results [17].

In past few years, the concept of green analytical chemistry (GAC) has attracted considerable research interest, as it emphasises on minimising the environmental impact associated with analytical practices, seeks to minimise waste output, reduce energy consumption and eliminate hazardous compounds used in analytical procedures without compromising the method's analytical effectiveness [18]. As an extension of GAC, white analytical chemistry (WAC) emerged recently as a modern analytical concept to incorporate analytical efficiency and economic factors, aiming to develop a balance between practical applicability and ecological sustainability. Greener sample preparation techniques are gaining significant attention due to their easy integration with various instrumental techniques [19,20].

Nowadays, cellulose paper (CP) has emerged as a highly attractive material due to its excellent porosity, biocompatibility, biodegradability, adaptability, operability, affordability, convenience of use and non-toxicity [21]. But it also has a drawback that cellulose paper can only bind with polar and semi polar analytes [22]. Due to Its high cellulose content, it interacts with the target analyte by cation exchange and hydrogen bonding, only if polar molecule is accessible. Cellulose is an inexpensive, biodegradable polymer that occurs naturally is found in large quantities as a component of plant cell walls. A long chain polysaccharide called cellulose is created chemically through the linkage of repeated β-D-glucopyranose units connected by β-1,4-glycosidic bonds [23]. The high density of hydroxyl groups present on the outer surface of cellulose polymers contributes to its strong adsorption capability [24]. Cellulose paper sorptive extraction method comprises of two basic steps: firstly, there will be adsorption of targeted analyte from a particular biological matrix on to laboratory filter paper and then secondly there will be elution of targeted analyte from laboratory filter paper into an organic solvent. CPSE offers several benefits such as minimum solvent consumption, less time taking process, low cost and more extraction efficiency [25].

In this work, a straightforward yet advanced cellulose paper sorptive extraction (CPSE) technique was established for isolating zolpidem, followed by LC-MS/MS analysis for its quantitative measurement. The different sizes of laboratory filter paper and elution solvent were examined and optimised. Additional factors influencing extraction efficiency such as the volume of extraction solvent, number of filter papers, sample pH and duration of vortex and ultrasonication were systematically optimised using PBD and CCD. After finalising these conditions, the performance of the optimised CPSE-LC-MS/MS method was thoroughly assessed.

EXPERIMENTAL

All the reagents and chemicals used in this study were of analytical grade. The zolpidem standard was purchased from Indian Pharmacopeia Commission Ministry of Health and Family Welfare, Government of India, Ghaziabad, India. All solvents utilised in this study were of high purity analytical grade and sourced from SRL, India. The solvents included methanol (MeOH), acetonitrile (ACN), isopropanol (IPA) and ethanol (EtOH). For all aqueous preparations and dilutions, ultra-pure water was employed, generated using a Milli-Q water purification system present in the chemistry division laboratory of the institution. To facilitate analyte absorption, Cytiva Whatman™ quantitative filter paper was used. This ashless, circular grade 42 filter paper, measuring 125 mm in diameter with a pore size of 2.5 μm, was manufactured from high grade cotton linters, ensuring minimal background contamination and excellent adsorption quality.

Preparation of stock standard and working standard solution: The primary stock solution of zolpidem was initially prepared in ACN at a concentration of 1.0 mg mL^{-1}. This stock solution served as the basic for all subsequent dilutions.

To obtain a working solution, an aliquot of the primary stock was further diluted in ACN to achieve a final concentration of 10.0 μg mL^{-1}, which was used for method calibration and validation purposes. Additional dilutions were performed in ACN to prepare calibration standards across the desired concentration range for linearity studies and quantification. Both the stock and working solutions were stored at a controlled temperature of 4 ºC to maintain the chemical integrity and prevent any potential degradation of zolpidem. Moreover, the stability of prepared solutions was periodically verified to ensure consistent performance throughout the analytical runs.

LC conditions: The quantitative analysis of zolpidem was carried out using I-Perkin-Elmer-QSight LX50 liquid chromatography equipped with the precision sampling module. Chromatographic separations were achieved using a QUASAR AQ column (dimensions: 100 mm × 4.6 mm, particle size: 3 mm; Cat Number: N9308845, Serial No. PE050810-50), which proved to be the most effective among several columns tested. Following a comprehensive evaluation of method performance, the QUASAR AQ column demonstrated superior outcomes related to the extent of analyte retention, sensitivity, signal intensity (peak area) and the minimisation of carryover and ghost peaks. However, to address the higher backpressure observed during analysis due to the fine particles size and column dimensions, the mobile flow rate was carefully optimised to maintain system stability without compromising resolution. The chromatographic method employed a binary solvent system consisting of 0.1% formic acid in milli-Q water (solvent A) and 0.1% formic acid in ACN (solvent B). The gradient flow as shown in Table-1, was used to achieve optimal separation conditions. Each sample was introduced with a 10.0 μL injection volume. To ensure analyte stability and prevent degradation of zolpidem the autosampler was maintained throughout the analytical run.

TABLE-1
OPTIMISED GRADIENT PROFILE FOR LC ANALYSIS

Time (min)	Flow rate (mL min^{-1})	%A (mL min^{-1})	%B (mL min^{-1})	Shape
0.00	0.800	80	20	6
0.80	0.800	80	20	6
1.90	0.800	55	45	6
2.90	0.800	5	95	6
4.40	0.800	5	95	6
5.50	0.800	80	20	6

MS conditions: Samples were analysed using a previously validated liquid chromatography (LC) method coupled with tandem mass spectrometry (MS/MS). The analytical system consisted of a LX50® LC coupled to a QSight 220® spectrometer (Perkin-Elmer, Milan, Italy). Analysis was carried out under positive electrospray ionisation (ESI^+) mode, with ionisation performance and method repeatability assessed by monitoring the response of zolpidem at a fixed concentration of 1000 ng/mL. High purity gasses (> 99.9%) were generated were generated using a Peak Scientific Genius XE QSD range gas generator (Scotland). Zero-Air (dry air) served as both the nebulizing and heating gas, while nitrogen was employed as the drying and collision gas. Mass spectrometry conditions for positive ionisation were optimised as follows: electrospray voltage of 5500 kV, source temperature of 300 ºC, nebulizing gas flow of 100 L/h, drying gas flow of 80 L/h, heated surface induced desolvation (HSID) temperature of 320 ºC. To fine tune the mass spectrometric parameters, a standard solution of zolpidem at 1 ppm in ACN was continuously infused. Zolpidem produced two characteristic MRM transitions: 308.00 > 219.00 *m/z* and 308.00 > 235.00; the first transition served for quantification (primary ion trace), while the second transition was employed to confirm peak identity (secondary ion trace). Both transitions were acquired with an entrance voltage of 30 V and a collision energy of -50 V. The dwell time for zolpidem was set to 100 ms, ensuring adequate sensitivity and selectivity for accurate detection as shown in Table-2.

Extraction of zolpidem from alcoholic drinks, non-alcoholic beverages and water samples: The quantitative analysis of zolpidem in alcoholic drinks, non-alcoholic beverages and water was performed using sorbent-based method, specifically the CPSE approach, followed by LC-MS/MS detection. For sample preparation, zolpidem was spiked into 10 mL water (100 ng mL^{-1}) and transferred into a tube. The pH of the final solution was carefully adjusted to 9 using 0.1 M NaOH. Subsequently, 7 pieces of filter paper, each measuring 1.5 cm × 1.5 cm were immersed in the prepared solution and subjected to vortex agitation for 90 sec. This was immediately followed by ultrasonication for 12.5 min under continuous gentle agitation to facilitate efficient analyte absorption onto the paper matrix. After the extraction step, the remaining aqueous phase was discarded and the filter papers were gently taken out and allowed to air-dry at room temperature in a petri dish, once completely dried, the papers were finely chopped into small fragments using sterile scissors and then transferred into a 2 mL Eppendorf tube. To elute the adsorbed analytes, 1.5 mL of IPA was added to Eppendorf tube. The tubes were then left to stand undisturbed for 6 min, allowing desorption of the analytes into the solvent without any external agitation. The resulting eluate was filtered through 0.22 μ nylon syringe filter and collected into 2 mL vials for subsequent LC-MS/MS analysis.

Design of experiment (DoE): Minitab statistical software (version 22.0) was employed for method optimisation. A minimal number of experimental runs were systematically designed to support efficient method development and the

TABLE-2
OPTIMISED MASS PARAMETERS ASSIGNED TO EACH COMPOUND FOR MS ANALYSIS

Analyte name	Polarity	Q1 Mass (Da)	Q1 Mass (Da)	Dwell time (DT)	Collision cell lens 2 (CCL2)	Collision energy (CE)	Entrance voltage (EV)
Zolpidem	Positive	308.00	219.00	100	-100	-50.0	30.0
		308.00	235.00	100	-100	-50.0	30.0

resulting data were subjected to comprehensive statistical analysis. Placket-Burman design (PBD) and Central composite design (CCD) was used for the optimisation of various parameters used for method development of zolpidem drug.

RESULTS AND DISCUSSION

In order to isolate zolpidem from alcoholic drinks, non-alcoholic beverages and water samples, sample preparation plays a vital role, as it directly influences the sensitivity and accuracy of analyte detection and quantification. In CPSE method, first the screening of size of filter paper and type of elution solvent was done, then followed by optimisation of other parameters which includes, volume of elution solvent, number of filter papers, vortex time, ultrasonication time, elution time and pH of the sample solution using intricate statistical experimental designs such as PBD and CCD.

Selection of elution solvent: The choice of elution solvent is a critical factor in the effective desorption of analytes adsorbed onto cellulose paper, as solvents differ in their polarity and consequently their ability to release the target compounds. To identify the most suitable solvent, five commonly used solvents were compared *i.e.* MeOH (relative polarity 0.762), ACN (relative polarity 0.46), IPA (relative polarity 3.9) and EtOH (relative polarity 0.654) and milli-Q. These solvents were systematically tested in a series of experiments to determine their efficiency in eluting the analyte. Results indicated that IPA was the optimal elution solvent, yielding the highest analyte peak area while minimizing matrix interference. This enhanced performance is likely due to IPA moderate polarity, which offers an optimal balance, strong enough to effectively dissolve the analyte while limiting the extraction of undesired matrix constituents. As a result, the use of IPA contributes to greater analytical sensitivity and more consistent reproducebility in the final measurements as shown in Fig. 1.

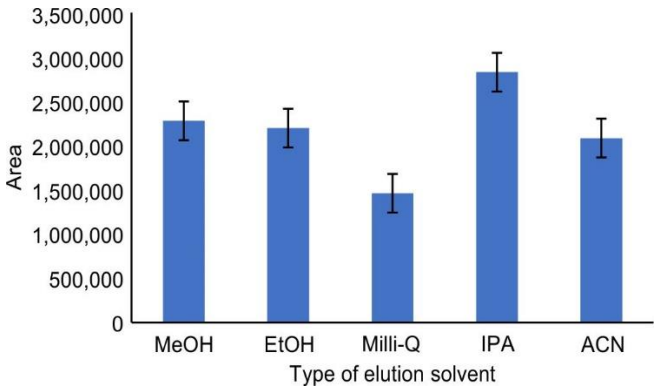


Fig. 1. Screening of elution solvent used in extraction of CPSE. Experimental conditions: Sample volume-10 mL; spiking concentration-100 ng mL^{-1}; Size of paper-1.5″ × 1.5″ cm; Quantity of paper-7, pH-9; Elution solvent-MeOH, EtOH, Milli-Q, IPA, ACN; Elution solvent volume-1.5 mL; Vortex time-90 sec; ultrasonic-cation time-12.5 min; Elution time-6 min

Selection of size of filter paper: In this study, cellulose filter paper was used in its native form, without any chemical treatment or surface alteration. Owing to its relatively large size and high surface to volume ratio, the filter paper provided a significant paper-based contact surface area (PCSA), which played a crucial role in enhancing the efficiency and sensitivity of analyte extraction. To determine the optimal size for maximum analyte adsorption, three different filter paper dimensions were evaluated *i.e.* namely 0.5″ × 0.5″, 1″ × 1″ and 1.5″ × 1.5″ cm. The findings showed that enlarging the surface area led to a proportional rise in the peak area of the target analyte, confirming improved extraction efficiency. Among various sizes, 1.5″ × 1.5″ cm filter paper exhibited the most efficient performance, delivering the highest analyte peak area (Fig. 2). This size was selected for subsequent experiments due to its superior adsorption capability, making it the most effective choice for enhancing extraction yield.

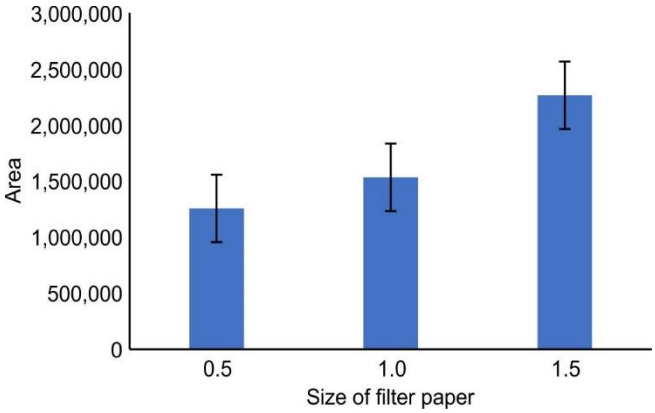


Fig. 2. Screening of size of filter paper used in extraction of CPSE. Experimental conditions: Sample volume-10 mL; spiking concentration-100 ng mL^{-1}; Size of paper-0.5″ × 0.5″, 1″ × 1″,1.5″ × 1.5″ cm, Quantity of paper-7; pH-9; Elution solvent- IPA; Elution solvent volume-1.5 mL; Vortex time-90 sec; ultrasonication time-12.5 min; Elution time-6 min

Design of experiment (DoE): There are various modelling and optimisation approaches, ranging from straightforward models like one factor at a time (OFAT) to intricate statistical designs like Box-Behnken design (BBD), Plackett–Burman design (PBD) and central composite design (CCD). More process efficiency and lesser number of laboratory experiments are provided by the design of experiment (DoE) [26,27]. Statistical experimental designs such as PBD and CCD have been successfully employed to optimise several parameters used for method development of zolpidem sedative drugs. After the selection of elution solvent and size of filter paper, several experimental parameters that could affect the efficacy of CPSE methods were evaluated using the experimental design technique. To achieve the high selectivity for Zolpidem drug, optimisation of the various parameters with LC-MS/MS was done where a statistical technique PBD and CCD was used.

Placket-Burman design (PBD) for factor optimisation: The Placket-Burman Design (PBD) is a, a two-level fractional factorial statistical screening tool frequently employed in experimental research when multiple variables need to be assessed simultaneously. It allows rapid identification of the most influential factors affecting a response variable while minimising the number of required experimental trials [28-31].

This design incorporates the effect of interactions among variables and helps to determine the optimal conditions efficiently with fewer runs [32]. In this study, the PBD was applied

to recognise the key parameters impacting the performance of the CPSE method. A 2^{6-3} design was implemented, encompassing six experimental factors: sample pH, elution time, elution solvent volume, vortex duration, ultrasonication time and the quantity of filter paper utilised. The specific upper and lower levels assigned to each parameter are summarised in Table-3. According to the PBD principle, which requires k+1 runs for (k) factors, a total of 13 experimental trials (12 factorial runs plus one centre point) were performed. Each trial was conducted in triplicate to ensure reproducibility and minimise experimental variability, resulting in 39 total runs for the analysis of zolpidem.

TABLE-3
PARAMETERS AND LEVELS TESTED FOR THE PBD OPTIMISATION

Parameters	Symbol	Low (–1)	High (+1)
pH of sample	A	4	12
Quantity of filter paper	B	1	10
Vortex time (sec)	C	30	150
Ultra-sonication time (min)	D	5	20
Elution solvent volume (mL)	E	0.5	2
Elution time (min)	F	2	10

The sum of zolpidem peak areas was considered the response variable. The Pareto chart (Fig. 3) illustrated the relative significance of each factor, where the red dotted line denoted the threshold of statistical relevance. Subsequently, analysis of variance (ANOVA) was performed at a 95% confidence level to identify statistically significant parameters. Among the six investigated variables, filter paper quantity, back-extraction solvent volume and sample pH were found to have a substantial impact on the extraction efficiency and were selected for further optimisation using central composite design (CCD). Conversely, vortex time, ultrasonication time and elution time were statistically insignificant and were therefore maintained at their respective centre point values (90 sec vortex time, 12.5 min ultrasonication and 6 min elution time) in subsequent experiments.

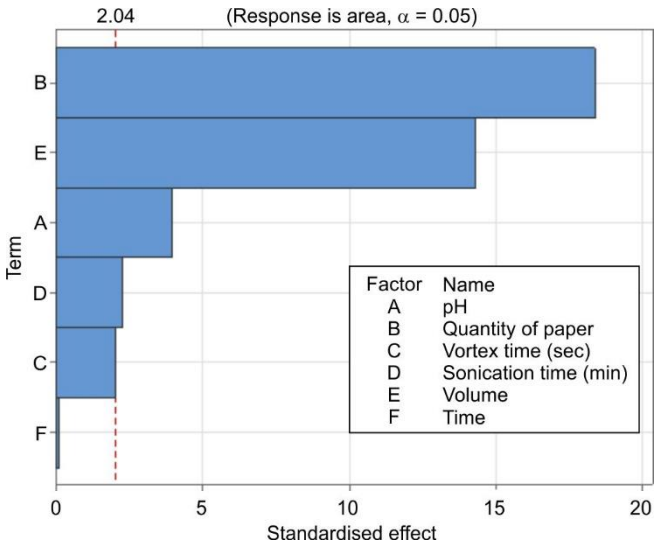


Fig. 3. Pareto chart of the standardised main effect for the Placket-Burman design (PBD) for zolpidem drug

Central composite design (CCD) for factor optimisation: In CCD, significant variables are optimised by establishing a relationship between dependent and independent factors to develop a predictive model [33,34]. The designs incorporate factorial points, axial points and centre points to fit polynomial equations that describe the system behaviour. Based on PBD screening, three critical factors were identified for optimisation: quantity of paper, elution solvent volume and pH of the sample. Each of these factors were evaluated at three levels, which were chosen at low (1), middle (0) and high (+1) levels, to capture their influence on the response. The experimental layout of these parameters and their levels is presented in Table-4. In CCD, the number of axial points is given as 2k, where k represents the number of independent variables, while the total number of runs (N) is determined by the eqn. 1:

$$N = 2^K + 2k + Cp \quad (1)$$

where 2^K corresponds to factorial points, 2k represents axial points and Cp indicates number of center points (here, Cp = 3). For the present study, since the number of factors was three (k = 3), the total runs calculated as 20 ($N = 2^3 + 2*3 + 6 = 20$), which were conducted in a randomised order to minimise systematic error. The outcome responses of CCD trials were represented by the individual peak areas of zolpidem, allowing for a comprehensive evaluation of how the chosen parameters affect the extraction efficiency.

TABLE-4
PARAMETERS AND LEVELS SCREENED FOR THE CCD OPTIMISATION

Parameters	Symbol	Low (-1)	Medium (0)	High (+1)
Quantity of filter paper	B	1	6	12
Elution solvent volume (mL)	E	0.5	1.5	2.5
pH of the sample	A	4	8	12

Analysis of variance (ANOVA) was applied to the CCD data to evaluate the suitability of different regression models including linear, quadratic and cubic, at a confidence level of $p < 0.05$. The statistical outcomes presented in Table-5 for the zolpidem drug, revealed strong model reliability. The corelation coefficient (R^2) and adjusted R^2 were calculated as 0.9123 and 0.8965, respectively. The high adjusted R^2 value confirms a close relationship between the experimental observations and the fitted model, indicating that the selected model effectively explains the variability in the data. To further interpret these results, the optimised CCD model was visualised through response surface plots and contour plots. These graphical tools not only validate the statistical findings but also provide a practical and intuitive understanding of the experimental design by highlighting the conditions that maximise performance.

A response surface plot is a three-dimensional graphical representation that illustrates how two independent variables simultaneously influence a dependent response. It allows a clear visualisation of interactions between variables. Such plots are particularly powerful in optimisation studies, as they highlight both the magnitude and direction of change in the response with respect to variations in experimental para-

TABLE-5
ANALYSIS OF VARIANCE (ANOVA) FOR THE REGRESSION COEFFICIENTS OF THE PROPOSED MODEL EQUATION

Source	DF	Adj SS	Adj MS	F-Value	*p*-value
Model	9	2.46	2.74	57.65	0.00
Linear	3	1.83	6.10	128.4	0.00
pH of sample	1	4.00	4.00	84.20	0.00
Vortex time (sec)	1	1.43	1.43	301.0	0.00
Elution time (min)	1	3.74	3.74	0.007	0.96
Square	3	2.67	8.90	18.7	0.01
pH of sample × pH of sample	1	5.12	5.12	10.8	0.12
Vortex time (sec) × Vortex time (sec)	1	1.43	1.43	0.32	0.79
Elution time (min) × Elution time (min)	1	6.93	6.93	14.6	0.07
2-Way interaction	3	3.67	1.22	25.8	0.00
pH of sample × Vortex time (sec)	1	3.14	3.14	0.66	0.70
pH of sample × Elution time (min)	1	3.61	3.61	7.59	0.00
Vortex time (sec) × Elution time (min)	1	3.33	3.33	0.70	0.69
Error	50	2.37	4.75		
Lack-of-Fit	5	7.08	1.41	59.70	0.00
Pure error	45	1.06	2.37		
Total	27	2.70			

meters. In present study, it was found that the maximum extraction yield was produced when the pH lies between 8 to 10, which clearly indicates that neutral to slightly basic conditions favour efficient elution of analyte with minimal solvent usage. Furthermore, the results highlighted that the yield was maximised when the number of filter papers ranged between 6 to 8 as it maximises the recovery while minimising analyte loss. Ultimately, the optimisation study established that the most efficient extraction conditions correspond to an elution solvent volume of 1.5 mL, a filter paper quantity of 7 and a solution pH of 9. These conditions represent the best point ensuring high yield and reproducibility by balancing all three critical parameters simultaneously. This 3D visualisation further confirmed by contour plots pin-pointing the region of maximum response.

A contour plot provides a graphical means to illustrate the relationship among three variables, where two variables act as independent factors and one serves as the dependent response. This tool is particularly valuable for visualising how variations in the input parameters influence the outcome. In such plots, contour lines link regions of equal response values. The final optimisation suggested that the ideal operating conditions for achieving maximum extraction efficiency was an elution solvent volume of 1.5 mL, a filter paper quantity of 7 pieces and a solution pH of 9.

Analytical validation of CPSE-LC-MS/MS method: The CPSE-LC-MS/MS analytical method was validated following the EURACHEM guidelines, which include assessment of parameters such as dynamic linearity range (DLR), limit of detection (LOD), limit of quantification (LOQ), correlation coefficients (R^2), relative recovery (RR%) and Relative standard deviation (RSD) for both intra-day and inter-day repeatability of zolpidem under optimised analytical conditions. Calibration curve for the zolpidem analyte was prepared at eight concentration levels 1.325-1000 ng mL^{-1} (Fig. 4). The calibration plots were constructed by correlating the analyte peak area with its corresponding concentration, yielding a regression coefficient (R^2) of 0.9992. The LOD represents the

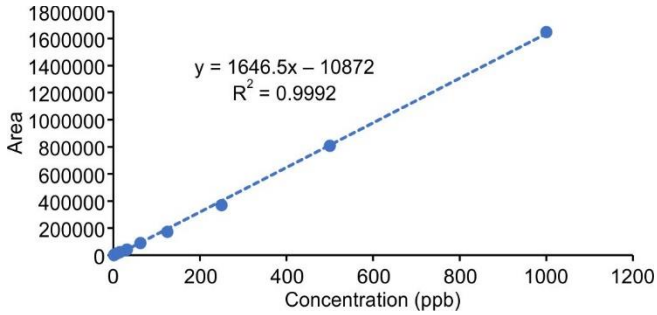


Fig. 4. Linearity plot of zolpidem drug ranging from 1.325 to 1000 ppb

minimum analyte concentration that can be detected reliably by the method, while the LOQ indicates the smallest quantity that can be quantitatively determined with acceptable precision and accuracy. These parameters were calculated using the equations LOD = 3.3 × (D/S) and LOQ = 10 × (D/S), where D denotes the standard deviation from recovery studies and S represents the slope of the calibration curve. The LOD and LOQ for the CPSE-LC-MS/MS technique were determined to be 0.095 ng mL^{-1} and 0.317 ng mL^{-1}, respectively.

Matrix effect (ME%): For the analysis of ME%, a set of five different matrices, namely water, beer, tea, frooti and limca, obtained from different sources/brands, were analysed at two quality concentration levels (1.3 ppb and 1000 ppb). The results are summarised in Table-6. The matrix effect (ME) was evaluated using the standard equation:

$$\text{ME (\%)} = \frac{\text{Peak area of extracted sample}}{\text{Peak area of standard sample}} - 1 \times 100$$

According to the obtained data, a slight ion enhancement was observed in the water samples, with ME values of 1.00% and 0.94% at low and high concentrations, respectively. In contrast, ion suppression was observed in all other matrices, including beer (-6.97% to -7.54%), tea (-8.30% to -8.99%), frooti (-7.52% to -7.99%) and limca (-5.47% to -6.15%). The ME values were found in the range of -8.99%–1.00%. These results indicate that the matrix effect is minimal and within

TABLE-6
ME% OF THE PROPOSED METHOD IN THE FOOD MATRICES

Analyte	Matrices	Concentration (ppb)	ME%
Zolpidem	Water	1.3	1.00
		1000	0.94
	Beer	1.3	-6.97
		1000	-7.54
	Tea	1.3	-8.30
		1000	-8.99
	Frooti	1.3	-7.52
		1000	-7.99
	Limca	1.3	-5.47
		1000	-6.15

acceptable limits, demonstrating that the developed method is not significantly affected by matrix interferences.

Real sample analysis: The proposed method was successfully applied for the quantification of zolpidem in fortified beverage matrices, including water, beer, frooti, tea and limca. The results obtained from the application study are shown in Table-7. The calculation for recovery was performed using the formula provided below. Moreover, the representative chromatogram of blank and standard of zolpidem are shown in Fig. 5.

$$\text{Recovery (\%)} = \frac{\text{Spiked conc.} - \text{Non-spiked conc.}}{\text{Added std. conc.}} \times 100$$

Assessment of the proposed analytical method using BAGI, RGB 12 model and MoGAPI: The environmental

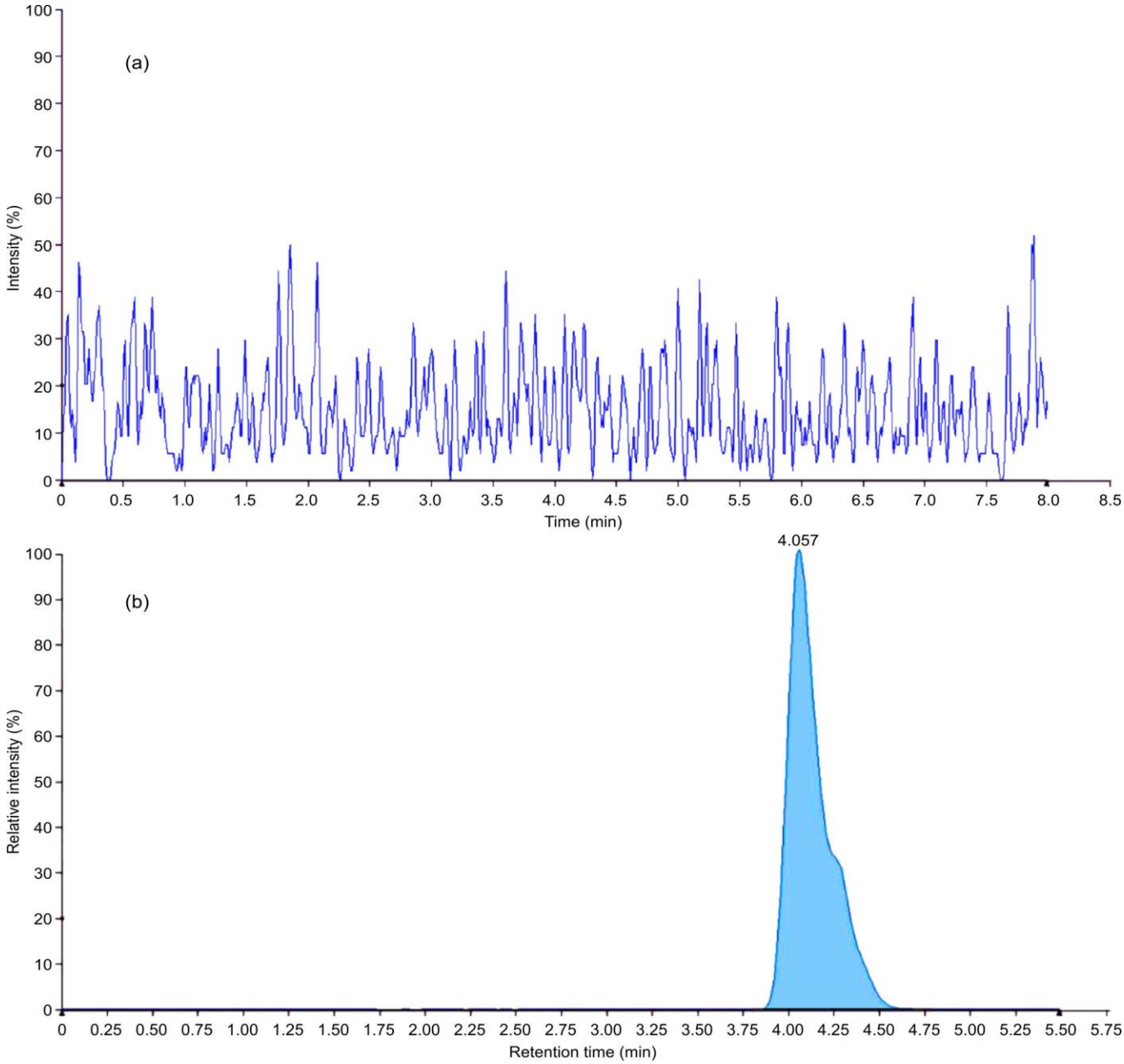


Fig. 5. (a) Representative chromatograms of blank (a) and zolpidem hypnotic sedative drugs standards (100 ng mL^{-1}) (b)

TABLE-7
DETERMINATION OF ZOLPIDEM DRUG FROM ALCOHOLIC DRINKS, NON-ALCOHOLIC BEVERAGES AND WATER SAMPLES

Analyte	Matrix	50 ng mL^{-1}			100 ng mL^{-1}			200 ng mL^{-1}		
		Mean	RSD (%)		Mean	RSD (%)		Mean	RSD (%)	
		R %	Intra-day	Inter-day	R %	Intra-day	Inter-day	R %	Intra-day	Inter-day
Zolpidem (ZOL)	Water	108.02	7.26	4.48	99.925	3.85	4.28	95.720	2.10	3.38
	Beer	95.515	2.85	4.88	92.14	5.20	3.92	88.895	3.41	1.01
	Tea	89.915	2.10	2.95	85.075	1.31	6.73	84.465	3.15	3.58
	Frooti	93.495	4.59	5.99	90.105	3.32	2.87	86.755	2.20	3.97
	Limca	92.870	3.55	4.77	88.120	6.26	2.45	87.540	3.81	3.42

RSD = relative standard deviation

sustainability of the developed cellulose paper sorptive extraction method for the determination of zolpidem was assessed using three established evaluations tools, for example, modified green analytical procedure index (MoGAPI), the blue applicability grade index (BAGI) and the RGB 12 model. These complementary models provide a structured approach to evaluate both the practical applicability and greenness of analytical methodologies in accordance with the principles of GAC and WAC [35-37].

The blue applicability grade index (BAGI) is a newly developed metric designed to evaluate the applicability domain of an analytical method by assigning scores to multiple methodological features. These includes the type of analysis, which is classified into qualitative (white), screening (light blue), quantitative (blue) and quantitative with confirmatory capability (dark blue). Bagi also considers the number of analytes measured simultaneously, the sample throughput per hour, the nature of reagents and materials utilised, the instrumentation required and the extent to which samples can be processed in parallel. Additional factors assessed are the need for preconcentration, the level of automation, the sample preparation strategy and the quantity of sample needed. By integrating these criteria, BAGI produces a corresponding score along with an asteroid shape pictogram that visually represents the method's applicability profile [38,39]. In the present work, the developed method achieved a high BAGI score of 77.5%, attributed to its straightforward operation, minimal sample preparation, use of readily available materials (cellulose paper) and low cost, safe solvent (isopropanol). These factors confirm the methods strong practical applicability and suitability for routine analysis.

The RGB 12 model (red-green-blue greenness model) is used to provide a comprehensive visual and numerical assessment of the methods performance, integrating three critical dimensions *i.e.* red (analytical efficiency and performance), green (environmental sustainability) and blue (practicality, operational safety and economical aspects) [38]. The RGB 12 analysis indicates a balanced colour profile, with a dominant green component, reflecting the methods environmental friendliness and a significant blue contribution highlights its strong applicability and low operational complexity. The use of a biodegradable extraction medium and a low-toxicity solvent align with the key principles of GAC, including minimisation of hazardous reagents, waste reduction and enhancement of operator safety [36,39-42]. As a result, white score of 104.0% obtained through the RGB 12 model indicated an excellent balance among the red, green and blue sustainability parameters.

The modified green analytical procedure index (MoGAPI) is an enhanced and more comprehensive version of the traditional green analytical procedure index (GAPI), developed to provide a more accurate and semi-quantitative evaluation of the environmental impact of analytical methods. Unlike the original GAPI, which primarily relies on a qualitative colour-coded pictogram, MoGAPI incorporates refined criteria to minimise subjectivity and improve the reliability of assessment across the entire analytical workflow. It systematically evaluates each step of the procedure including sample collection, sample preparation, type and volume of reagents and solvents used, energy consumption, instrumentation, occupational hazards and waste generation [43]. The proposed method obtained greenness score of 66, which indicates acceptable green method.

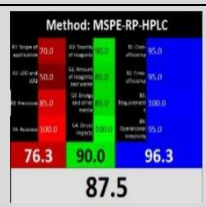
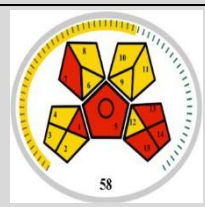
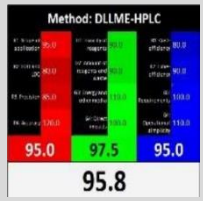
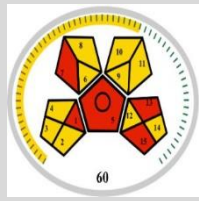
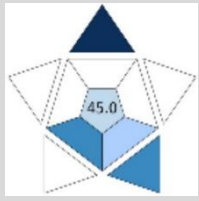
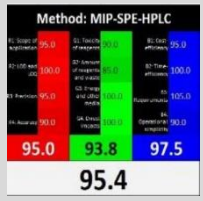
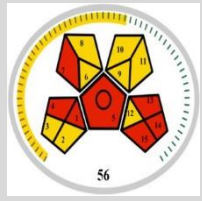
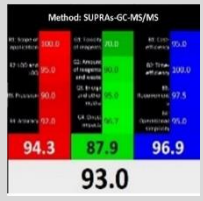
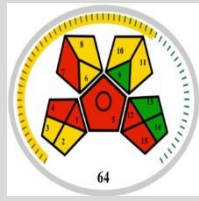
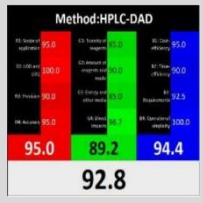
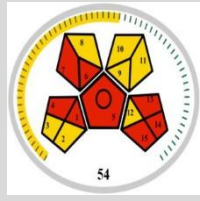
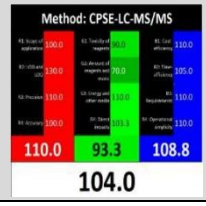
The eco-friendliness and practicality of the proposed protocol were evaluated using MoGAPI, RGB12 and BAGI tools, respectively and compared with previously reported methods, namely MSPE-RP-HPLC [16], DLLME-HPLC-PDA [2], MIP-SPE-HPLC [44], SUPRA-GC-MS/MS [45] and LLE-HPLC-DAD [46]. The observed differences in greenness and practicality scores between the developed and reported methods can be attributed to several methodological and operational factors (Table-8). Many of the published methods lack adequate waste management strategies and generate higher volumes of solvent waste, particularly those involving LC-based techniques. Furthermore, additional steps such as solvent evaporation contribute to increased solvent consumption, higher energy requirements and greater procedural complexity [2,16,44,46]. In addition, some methods involve multi-step procedures including sorbent synthesis, which are labour-intensive and time-consuming [16,44,46].

In contrast, the proposed method does not require any synthesis step and is cost-effective. It employs biodegradable cellulose paper as the sorptive phase and involves a simple two-step procedure, thereby minimising solvent consumption and enhancing inclusive sustainability.

Conclusion

In present study, CPSE sample preparation method was adopted to achieve efficient and selective extraction of zolpidem from alcoholic drinks, non-alcoholic beverages and water samples. Quantification of the extracted zolpidem was accomplished using a validated LC-MS/MS method. The developed procedure demonstrated a good recovery, suitable for detecting zolpidem concentrations typically encountered in consumer beverages. The ecological impact and sustain-

TABLE-8
A COMPARATIVE EVALUATION OF THE CURRENT METHOD WITH THE PREVIOUSLY PUBLISHED METHODS

Analyte	Samples	Extraction procedure	Analytical Parameters	BAGI	RGB 12 Model	MoGAPI	Ref.
Zolpidem tartrate	Apple juice	MSPE-RP-HPLC	LOD 1.8 µg/mL LOQ 6 µg/mL Precision < 2% Recovery 92-120% ME% NA				[16]
Zolpidem	Human plasma	DLLME-HPLC-PDA	LOD 0.05 µg/mL, LOQ 0.15 µg/mL, Precision < 9.36% Accuracy 0.84 to 14.97% Recovery 100.84-121.01% ME% NA				[2]
Zolpidem	Plasma	MIP-SPE-HPLC	LOD 0.34 ng/mL LOQ 1.04 ng/mL Precision 0.45-2.86% Recovery >90% ME% NA				[44]
Zolpidem	Urine and blood	SUPRAs-GC-MS/MS	LOD- 0.30 ng/mL LOQ- 1 ng/mL Precision < 9.53% Accuracy < 9.99% Recovery 102.76 ± 2.58% ME% NA				[45]
Zolpidem	Caffeine-based carbonate beverage	HPLC-DAD	LOD 0.17 µg/mL LOQ-0.54 µg/mL Precision 1.46-5.35% ME% NA				[46]
Zolpidem tartrate	Alcoholic drink, non-alcoholic beverages and water sample	CPSE-LC-MS/MS	LOD 0.095 ng/mL LOQ 0.317 ng/mL Precision < 7.26% Recovery 84.46-108.02% ME% 8.99-1.00				Present study

ability aspects of the proposed method were examined using the MoGAPI, BAGI and the RGB 12 model. MoGAPI score of 66%, a BAGI score of 77.5% under consideration of the blue principle confirms the method's practicality and real-world applicability. Furthermore, a white score of 104.0% obtained through the RGB 12 model indicated an excellent balance among the red, green and blue sustainability parameters. These evaluations, performed in comparison with other recently reported zolpidem detection methods, highlighted the superior greenness and sustainability of the present approach. Henceforth, further the current work can be applied for other types of beverages, as the extraction includes a simple two step procedure. Moreover, the developed method shows a strong potential for detecting various drug compounds associated with criminal and abusive activities in diverse edible matrices.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The

authors reviewed and edited the content and take full responsibility for the published work.

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