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Cellulose Paper Sorptive Extraction (CPSE) Combined with Gas Chromatography–Mass Spectrometry (GC–MS) for Facile Determination of Lorazepam Residues in Food Samples Involved in Drug Facilitated Crimes

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Abstract: Reports related to incidences of drug facilitated crimes (DFCs) have notably increased in recently. In such cases, victims report being assaulted or robbed while under the influence of drugs. Lorazepam (LZ) is frequently used in DFCs as it can easily make victims docile owing to its potent numbing effect. Therefore, a straightforward and green analytical method to analyze LZ in spiked food matrices in connection with criminal acts becomes important. The current study reports a simple, green, and high sample throughput analytical method for determining LZ in food and drink matrices commonly encountered in DFCs, based on recently introduced cellulose paper sorptive extraction (CPSE). For the extraction of LZ from food matrices, pristine cellulose paper (CP, commonly used laboratory filter paper) was used as a sorptive medium. Five pieces of CP (1.5″ × 1.5″ each) were dipped into diluted food matrices (cream biscuits and tea) and stirred on a rotary shaker for 30 min at 200 rpm. The CPs were then dried, and the adsorbed LZ was back-extracted into 2 mL of methanol. The extract was then subjected to GC–MS analysis in selected ion monitoring (SIM) mode. Several parameters, including CP size and number, back-extraction solvent type and volume, sample volume, extraction time and stirring speed, pH, ionic strength, elution time and speed, were thoroughly screened and optimized. Under the optimized conditions, the method was found to be linear in the range of 0.2–10 $\mu g \cdot mL^{-1}$ (or $\mu g \cdot g^{-1}$) with a coefficient of determination (R^2) ranging from 0.996–0.998. The limit of detection and limit of quantification for cream biscuits were 0.054 and 0.18 $\mu g \cdot g^{-1}$ whereas they were 0.05 and 0.16 $\mu g \cdot mL^{-1}$ for tea samples. For all measurements, the relative standard deviations (%RSD) were always below 10%. Two mL of methanol per sample was used during the entire sample preparation process. The greenness of the proposed procedure was evaluated using Analytical Eco-Scale and GAPI greenness assessment tools. Finally, the CPSE–GC–MS method has been applied for the determination of LZ in forensic food samples which were used in DFCs.

Keywords: cellulose paper sorptive extraction; drug facilitated crime; lorazepam; gas chromatography-mass spectrometry; forensic chemistry

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1. Introduction

Drug facilitated crimes (DFCs), which involve criminal acts such as robbery, rape, murder, child abuse, extortion of money and sexual assaults; are committed when a victim is under the serious influence of some drugs e.g., benzodiazepines (BZDs). Due to the cheap and easy access to these medications, the instances of DFCs are rising significantly in many countries [1]. BZDs are frequently documented in DFCs where they are mixed in food or drinks and given to victims to intoxicate them and render them helpless against an assault, shock them, and, in most instances, erase their memories of the criminal activity. LZ is a drug of choice among criminals for this purpose due to its amnesic properties, rapid absorption in the body (with a bioavailability of ~95%), and ability to rapidly impair an individual [2,3].

The confirmation of DFC is generally achieved by analyzing biological samples such as blood, urine and hair [4–7]. Collection of samples in such cases plays a critical role in establishing DFC. Blood and urine samples should be collected as soon as possible after the crime, and hair samples are recommended to be collected after one month of the incident. However, due to their negligible concentration; detection of sedative and hypnotic drugs without the use of sophisticated analytical techniques is difficult [8]. Additionally, due to their short half-lives and rapid excretion from the body, sedative drugs and their metabolites can be difficult to detect in biological samples such as blood and urine if they are obtained many hours after the assault [9]. In such cases, it is extremely important to prove DFC using the food sample evidence collected from the crime scenes. These samples frequently have drug concentrations above the recommended therapeutic dose, therefore it is simple to identify pure drug in these samples.

There are many methods for determining BDZ for forensic and clinical purposes that may be found in the literature. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) in combination with liquid–liquid extraction (LLE) for determination of BZDs in biological samples has been frequently reported [4,5,7]. For non-biological samples such as alcoholic/non-alcoholic beverages and cream biscuits; LLE has been used as a sample preparation for their determination by gas chromatography–mass spectrometry (GC–MS), paper spray–mass spectrometry (PS–MS) and high-performance liquid chromatography (HPLC) [10–12]. In a recent report, LLE in combination with microwave assisted extraction (MAE) was used for extraction of BZDs spotted on a DBS card and analyzed by LC–MS [13]. However, LLE is a time-consuming and arduous process that requires large quantities of toxic organic solvents. Additionally, sample cleanup is a serious issue in LLE. Other approaches include dispersive liquid–liquid microextraction (DLLME) combined with high performance liquid chromatography–ultraviolet detection (HPLC–UV), QuEChERS extraction in combination with GC–MS and microextraction by packed sorbents (MEPS) followed by UHPLC–UV techniques [14–17]. Although these sample preparation strategies are more effective than LLE, they are multi-step procedures that necessitate the use of specialized kits and syringes. (e.g., in the case of QuEChERS and MEPS).

Recently, unmodified cellulose paper (CP) which is commonly known as laboratory filter paper has emerged as a natural and green sorptive material for extraction of various analytes such as biogenic amines, antidepressants and BZD from complex samples such as beer and human blood [18]. CP is generally used in qualitative analytical techniques for detection of analytes; is a thin, lightweight, and malleable substance which is produced by pressing multiple cellulose fibers together to allow their random overlapping over each other [19,20]. As a naturally occurring component of plant cell walls, cellulose, which has the chemical formula $(C_6H_{10}O_5)n$, is regarded as the most prevalent naturally occurring biopolymer. Cellulose is a long chain polysaccharide formed by joining multiple β-D-glucopyranose units together via β-(1,4)-glycosidic linkages at various degrees of polymerization ranging from hundreds to thousands. Owing to its non-toxicity, biodegradability, increased availability, flexibility, biocompatibility, and environmental friendliness; cellulose is gaining popularity as a natural sorbent for analytical extractions [21,22].

The chemistry community is developing green analytical processes due to concerns about environmental degradation and resource depletion. Green analytical chemistry (GAC) emphasizes the use of eco-friendly solvents, minimal waste production, and risk reduction [23]. Going further, the concept of white analytical chemistry (WAC) has been proposed and added the aspects of analytical efficiency and economic considerations to balance practicality and environmental sustainability [24]. The use of natural sorbents for sample preparation is a sustainable and cost-effective way to make analytical methods greener.

In the present study, we propose a simple, straightforward, green, high sample throughput and affordable sample preparation method i.e., CPSE for the analysis of LZ in forensic food samples used for the purpose of DFC. The protocol involves incubation of diluted food sample with pristine CP for a predetermined time and stirring speed in order to allow LZ adsorption on CP. This is followed by back-extraction of adsorbed LZ in methanol and subsequent analysis by GC–MS. Several significant parameters which can affect the extraction efficiency of CPME were thoroughly optimized. Finally, the greenness of the proposed method has been evaluated using Analytical Eco-Scale and GAPI greenness assessment tools.

2. Results and Discussion

2.1. Mechanism of Extraction

Cellulose, the basic building block of pristine cellulose filter paper, is a hydrophilic linear polymer of β-D-glucopyranose [25]. Each dimer possesses three hydroxyl functional groups. β-1,4-glycosidic bonds connect the dimers. In addition to the hydroxyl functional groups, β-D-glucopyranose contains an oxygen atom in its cyclic structure. All these hydroxyl and oxide functional groups can form hydrogen bonds (both as hydrogen bond donors and acceptors). As a result, pristine cellulose filter papers may interact with compounds possessing hydrogen bond donors and/or hydrogen bond acceptors in their molecular structures. The hydrophilic surface property of cellulose filter paper exerts a high affinity towards water molecules. Consequently, hydrated analytes (preferentially polar and medium polar compounds) come in close contact with the cellulose surface and interact with the analytes via hydrogen bonding. LZ is a moderately polar analyte (log K_{OW} 2.39) possessing two hydrogen bond acceptors. Due to its medium polarity and hydrogen bonding capability, it demonstrates a high affinity towards LZ molecules. The potential interactions between cellulose paper and LZ are shown in Figure 1.

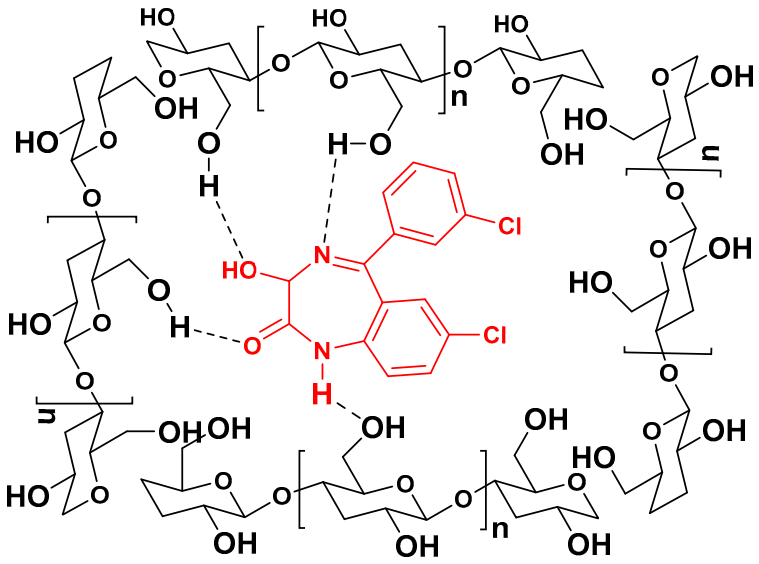


Figure 1. Schematic representation of pristine cellulose sorbent–analyte interactions.

2.2. Size and Quantity of Cellulose Paper

Our study used pristine CP, which had never been modified or chemically treated, as the sorptive phase for the extraction of LZ. Efficiency of CPSE extraction depends on the primary contact surface area (PCSA) available for LZ adsorption on CP. The surface-to-volume ratio and extraction efficiency increases with increasing PCSA. In light of this, the extraction of LZ from three various sizes of uniformly cut filter paper (0.5″ × 0.5″, 1″ × 1″, and 1.5″ × 1.5″) was investigated. Due to the availability of greater PCSA for adsorption, the enrichment factor (EF; ratio of peak area of LZ in FPSE extract to the peak area of standard) were likewise raised as the size of the CP increased (Figure 2a).

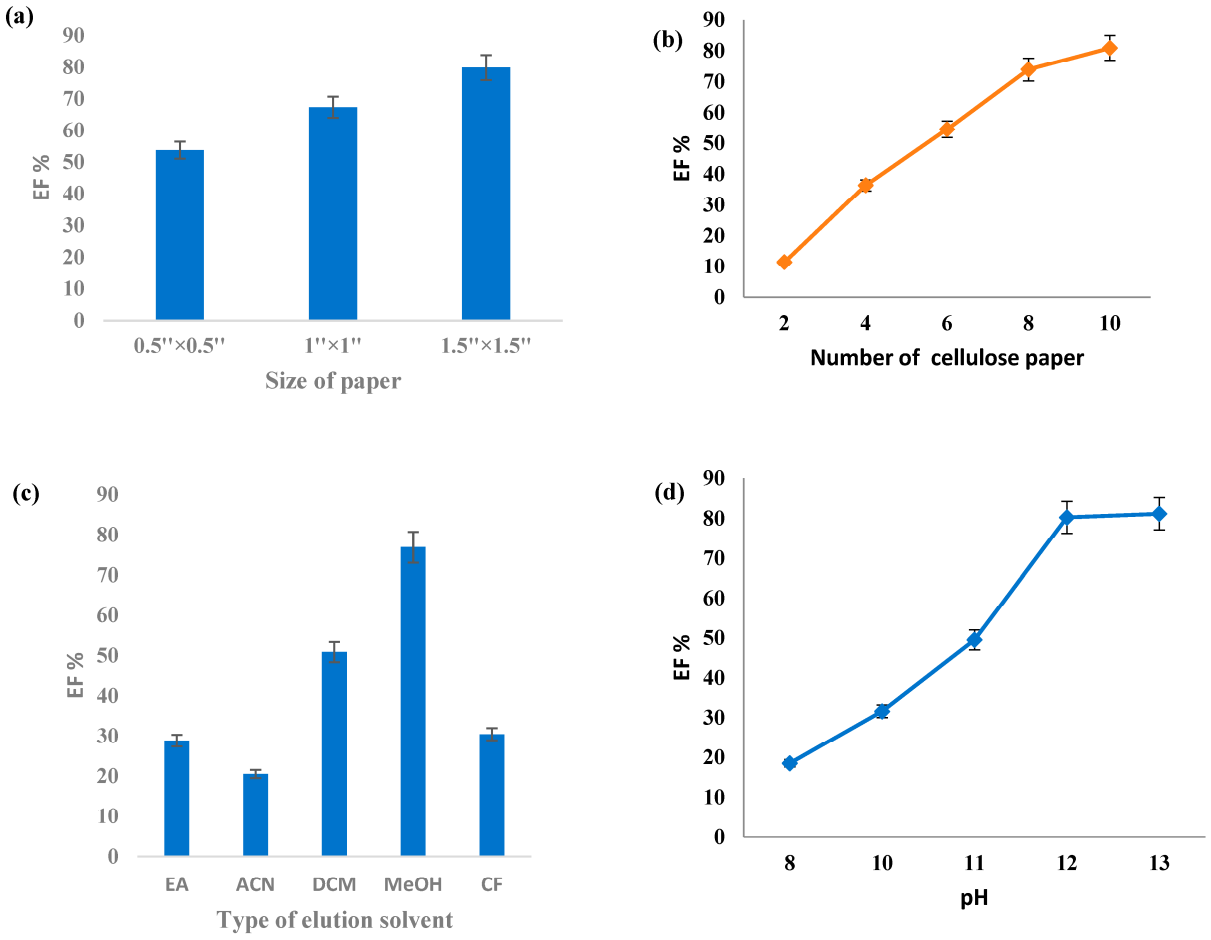


Figure 2. The effect of various parameters on the extraction efficiency of the CPSE procedure for LZ: (**a**) effect of size of CPs, (**b**) effect of numbers of CPs, (**c**) screening of back-extraction solvent, and (**d**) effect of sample pH.

The quantity of CP was examined in addition to its size. To achieve this, an experiment was carried out using various numbers of CPs (e.g., two, four, six, eight and ten) that were all the same size (i.e., 1.5″ × 1.5″). It is worth noting that up to six numbers of CP were used; the volume of back extraction solvent used was 2 mL. However, 3 mL of back extraction solvent was required for CPs greater than six. In each instance, the extract was dried by evaporation before being reconstituted in 200 µL MeOH. Figure 2b makes it clear that the EF of LZ increases as the number of CPs increases. However, CPs greater than six needs a larger quantity of back-extraction solvent, which reduces the protocol's greenness. As a result, six CP were utilized in all further studies.

2.3. Type and Volume of Back-Extraction Solvent

The role of back-extraction solvent is to desorb the adsorbed analytes on CP. Therefore, polarity of the back-extraction plays a key role in this process. All back-extraction solvents

may not be equally efficient for desorption of adsorbed analytes on cellulose paper due to polarity differences. Five commonly used back extraction solvents, including chloroform (CF, relative polarity 0.259), methyl alcohol (MeOH, relative polarity 0.762), acetonitrile (ACN, relative polarity 0.46), dichloromethane (DCM, relative polarity 3.1), and ethyl acetate (EA, relative polarity 0.228), were evaluated in a series of experiments to determine the best back-extraction solvent for LZ from CP. It is evident from Figure 2c that the highest EF for LZ was obtained when MeOH was used as back-extraction solvent. Therefore, MeOH was chosen as the back-extraction solvent in all subsequent analyses.

In order to evaluate the volume of back-extraction solvent i.e., MeOH; an experiment was conducted where the volume of MeOH was varied in the range of 2–5 mL. As the volume of MeOH increases, the peak areas of LZ should decrease due to the dilution factor. However, because the extract was evaporated and reconstituted in 200 µL of MeOH in each case, no significant variation in EF was observed. Therefore, to avoid using a larger volume of MeOH and to retain the method's green character, 2 mL of MeOH was used for all subsequent experiments ().

2.4. Optimization of pH and Ionic Strength

The extraction of analytes by CPSE depends critically on the pH of the sample solution. The optimal pH for analyte extraction is one where the analyte remains undissociated in the solution, allowing for an easier mass transfer to the sorbent phase. LZ is a highly basic and moderately polar compound with a pKa of 10.61 and logP of 2.4, respectively [26]. In light of this, the sample pH was adjusted using 0.1 mol·L^{-1} NaOH to range from 8 to 13 (i.e., 8, 10, 11, 12 and 13). The extraction efficiency of CPSE for LZ was improved as the pH of the sample solution rose from 8 to 12 and reached a plateau (Figure 2d). When the pH was lower than the pKa of LZ, it remained ionized, resulting in lower extraction efficiency. However, as the pH increased 1.5 units above its pKa, the extraction efficiency substantially increased. This was also demonstrated by the fact that basic chemicals remain in their molecular form (~99%) at two full pH units above their pKa values [27]. Therefore, in all further experiments, the pH of the sample solution was adjusted to 12.

The solubility of the analyte typically reduces as the amount of salt in the solution increases, which makes it easier for it to move from the aqueous phase to the sorbent phase. Salt addition, on the other hand, increases the viscosity of the solution and influences the rate of analyte diffusion. Because both factors potentially counter-balance each other, an experiment was carried out by adding NaCl in the range of 0–10% (w/v), the impact of ionic strength on the extraction efficiency of CPSE was investigated. There were no significant changes in the extraction efficiency of CPSE for LZ noticed by adding salt in any proportion (), hence no salt was added to subsequent experiments to limit the use of chemicals and make the protocol greener and simpler.

2.5. Optimization of Adsorption Conditions

Optimum adsorption of target analytes on CP depends on several factors such as extraction time, stirring speed and volume of sample. Since CPs are immersed directly in the sample solution, the extraction mechanism is based on the analyte's equilibrium between sample solution and adsorbent phase (i.e., CP) and therefore, finding the ideal extraction time at which the mass transfer of the target analyte from the sample solution to the CPs reaches an equilibrium is critical. Initially, four different time spans i.e., 15, 30, 45 and 60 min were investigated. Figure 3a summarizes the findings and demonstrates that stirring the sample for 30 min yielded the highest extraction efficiency of CPSE for LZ. The decrease in the recovery of LZ after 30 min of extraction time can be attributed to the fact that there is possibility of back-extraction of LZ absorbed on CP into the sample matrix, which is also evident from published literature [28]. Clearly, 30 min is sufficient for the equilibrium to be reached and therefore was selected as the extraction time for all further experiments in order to provide the highest sensitivity and sample throughput.

The stirring speed which is actually the stirring rate of the rotary shaker has been examined in the range of 100–300 rpm at an interval of 50 rpm. Stirring promotes the sample to flow continuously toward the porous substrate i.e., CP, increasing the mass transfer rate of the analyte to the CP and bringing it to equilibrium. The results of the effect of stirring rate on the CPSE procedure for LZ are presented in Figure 3b. As can be observed, by increasing the rate of stirring from 100 to 200 rpm; extraction efficiency also increased, however they remained stable from 250 to 300 rpm. Based on the obtained results, a stirring speed of 200 rpm was selected as optimum for subsequent experiments.

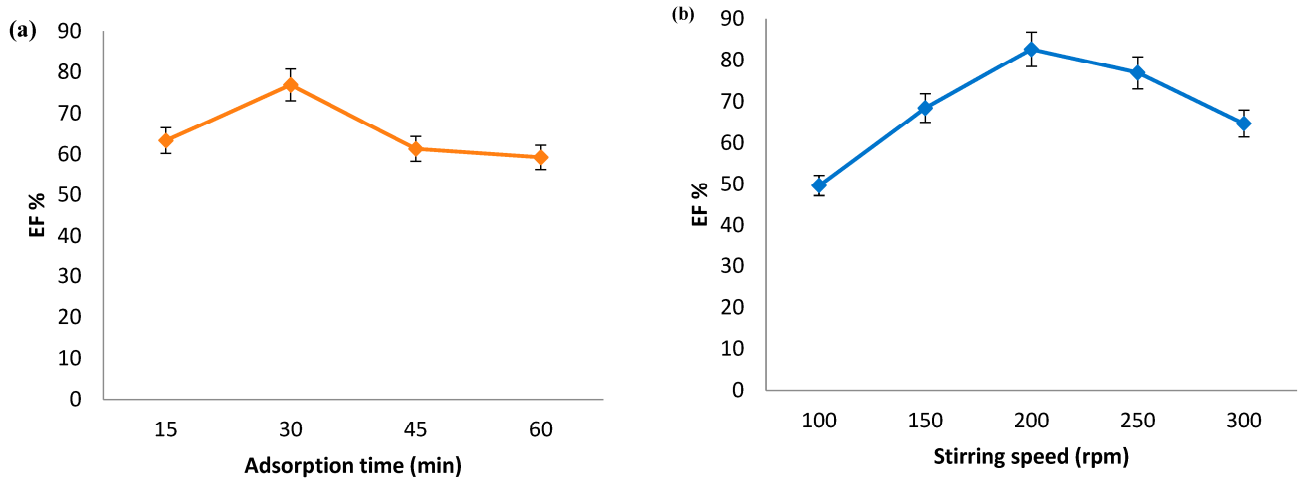


Figure 3. The impact of (**a**) extraction time, and (**b**) stirring speed on the CPSE procedure.

Furthermore, the effect of sample volume on the extraction behavior was investigated in the range of 5–20 mL. Sample volume plays a significant role in the EFs of target analytes. As can be observed, higher EFs for LZ were observed when the sample volume was kept at 10 mL. However, there was no significant improvement in EFs beyond this volume; therefore, 10 mL of sample volume was selected for further studies as represented in Figure S1.

2.6. Optimization of Back-Extraction Mode

The back-extraction of adsorbed LZ on CPs was carried out with 2 mL of MeOH. The CPs following the CPSE procedure were dipped in MeOH and then gently stirred on a rotary shaker for 10 min in order to determine whether there is any effect of external diffusion mechanisms on the elution of LZ. In a different experiment, the CPs were immersed in MeOH for a set period of time without being stirred. After being evaporated to dryness under a gentle stream of nitrogen, the back-extraction solution resulting from the experiments that contained LZ was reconstituted in 200 µL of MeOH and analyzed by GC–MS. Since it was found that LZ could be eluted most effectively under standard conditions without the use of an external diffusion mechanism, it was decided not to stir the extract in any subsequent experiments as depicted in Figure S2.

3. Analytical Performance

The suggested CPSE–GC–MS method's analytical performance in terms of linearity, sensitivity (LOD and LOQ), precision (intra-day and inter-day), enrichment factors (EF%), enrichment recovery (ER%), and accuracy (relative recovery, RR%) were evaluated under the optimized conditions (sample volume: 10 mL; number of CPs: 6 of 1.5″ × 1.5″ each; extraction time: 30 min, stirring speed: 200 rpm; pH 12; back extraction with 2 mL of MeOH for 10 min). A matrix-matched calibration curve by spiking different amount of LZ in food matrices (tea and cream biscuits) was constructed ranging from 0.2–10 $\mu g \cdot mL^{-1}$ or $\mu g \cdot g^{-1}$. For each matrix, the slopes, intercepts, and determination of coefficient (R^2) were determined using least squares linear regression analysis (Table 1). A good correlation between concentration of LZ and its corresponding peak area was obtained for biscuits and

tea samples (R^2 = 0.996 and 0.998, respectively). A signal to noise ratio of 3:1 and 10:1 was utilized for calculated LOD and LOQ, respectively. The LOD and LOQ for cream biscuits were 0.054 and 0.18 $\mu g \cdot g^{-1}$, whereas they were 0.05 and 0.16 $\mu g \cdot mL^{-1}$ for tea samples.

Method accuracy and precision were assessed by analyzing fortified food matrices at three different concentration levels of calibration graph (low QC, mid QC and high QC) as shown in Table 2. Accuracy is defined as the ratio of analyte concentration found by the CPSE method to its nominal concentration in food matrices. Satisfactory values in the range of 72.9–108.1% were obtained [29]. By comparing the analyte concentration of extracted samples spiked before extraction with similar extracts of blank samples spiked after extraction, relative recoveries (RR%) were assessed and were determined to be in the range of 72.6–91.1% (Table 2). The repeatability and reproducibility of the CPSE method were evaluated as intra-day and inter-day precisions and expressed as percent relative standard deviation (%RSD). The intra-day and inter-day precisions for both matrices were always lower than 5 and 10%, respectively. For the complete evaluation of the extraction efficiency of the proposed CPSE method, EF% and ER% were studied. The EF% is defined as the ratio the percentage of the peak area enhancement compared to the area of the reference standard solutions. On the other hand, ER% was calculated by dividing EF% by a theoretical preconcentration factor (PF) and multiplying with a factor of 100 [30]. In this case theoretical PF was calculated to be 50 (volume of sample: 10 mL; final sample volume: 0.2 mL). The values obtained for EF% and ER% are shown in Table 2. Furthermore, the matrix effect for biscuit and tea samples were obtained in the range 99–106% and 84–90%, respectively. The %RSD of <5% indicated that there were no significant matrix effects in different biscuit and tea samples.

The proposed CPSE–GC–MS method for determining LZ has been compared with previously reported methods for similar analytes in similar matrices. The comparison was performed in terms of sensitivity, precision, and greenness, as shown in Table 3, demonstrating that the proposed CPSE–GC–MS approach provided comparable sensitivity and recoveries to those previously described methods for similar analytes in analogous matrices. The LOD and LOQ reported in ref. [13] are much lower than in the current work, which is reasonable considering that they used LC–TOF–MS for analysis. Furthermore, the proposed approach has yielded LODs and LOQs that are notably lower than those achieved by prior published methods [10,12]. This could be ascribed to the limited enrichment obtained via sample preparation procedures (i.e., LLE) utilized in these approaches, as well as the usage of GC–MS which has demonstrated greater sensitivity compared to the method cited in reference [14]. As the concentration of LZ spiking by the offenders to commit DFC is greater, it can be said that the sensitivity produced by the suggested approach is fit-for-the-purpose. In such cases, the expected concentration of LZ in biological matrices (e.g., blood) and food samples is far above its therapeutic levels which is 0.05–0.24 $\mu g \cdot mL^{-1}$. However, it is difficult to anticipate the level of sedative drugs in cases of DFC, as the sole purpose of the perpetrator is to sedate the victim immediately in order render him unconscious.

In terms of sample preparation ease and greenness, the proposed method is simple and straightforward and outperforms previously reported methods that involve multi-step procedures and use a large amount of toxic organic solvents [11,12,15].

Since, LLE is still a widely used extraction method in forensic laboratories for routine analysis; we have compared the extraction and sample clean-up efficiency of the proposed CPSE–GC–MS method with LLE. The total ion chromatograms (TIC) obtained for tea samples by LLE and CPSE are compared in Figure 4. Caffeine and LZ were identified by GC–MS when the sample was extracted using the LLE approach, and seven extra prominent peaks were observed in the TIC of the tea sample, indicating poor sample cleanup by LLE. When the sample was extracted using CPSE however, only two major peaks of caffeine and LZ were observed in the TIC of the tea sample. Additionally, the peak areas of LZ obtained by CPSE were 3.1 times greater than LLE. These results indicate that CPSE is superior to LLE in terms of extraction and sample clean-up efficiencies.

Table 1. Analytical characteristics of the CPSE–GC–MS method for LZ drug.

Sample	LOD	LOQ	R^2	Linearity	Calibration Curve	Precision (%RSD)					
						Intra-Day			Inter-Days		
						0.5	5	10	0.5	5	10
Tea [a]	0.05	0.016	0.998	0.2–10	y = (12,733 ± 191.9) × − (1673.6 ± 1024.1)	3.12	2.37	1.28	8.9	8.78	6.5
Biscuit [b]	0.054	0.018	0.996	0.2–10	y = (10,283 ± 258) × + (1703.6 ± 1376.6)	4.44	3.5	2.59	9.5	8.1	7.3

[a] = concentration expressed in $\mu g \cdot mL^{-1}$; [b] = concentration expressed in $\mu g \cdot g^{-1}$.

Table 2. Extraction efficiency parameters of proposed method.

Matrix	RR%			Accuracy			EF	ER (%)
	0.5	5	10	0.5	5	10		
Tea [a]	91.1	88.4	86	108.1	96.2	100.1	46	95.8
Biscuit [b]	89.6	81.5	72.6	72.9	99.0	95.9	41	83.2

[a] = concentration expressed in $\mu g \cdot mL^{-1}$; [b] = concentration expressed in $\mu g \cdot g^{-1}$.

Table 3. Comparison of the CPSE–GC–MS method with previously reported analytical methods for similar analytes in food matrices.

Analyte(s)	Sample Matrix	Sample Preparation Method	Analytical Technique	Sensitivity LOD	LOQ	Recovery (%)	Ref.
Flunitrazepam, clonazepam, alprazolam, diazepam and ketamine	Alcoholic and non-alcoholic beverages	LLE with CF: IPA (1 mL)	GC–MS	1.3–34.2 $\mu g \cdot mL^{-1}$	3.9–103.8 $\mu g \cdot mL^{-1}$	73–112.6	[10]
Diazepam, alprazolam, bromazepam, clonazepam and cloxazolam	Alcoholic beverages	LLE with ACN (5 mL)	PS–MS	0.05 $\mu g \cdot mL^{-1}$ of diazepam	-	90–100	[11]
Diazepam	Cream biscuit	LLE with EtOH (50 mL)	HPLC	0.6 $\mu g \cdot mL^{-1}$	1.75 $\mu g \cdot mL^{-1}$	95	[12]
Ketamine, flunitrazepam, and diazepam	Alcoholic and non-alcoholic beverages	MAE at 50 °C for 15 min with EA (3 mL)	LC–TOF–MS	25–100 $ng \cdot mL^{-1}$	-	-	[13]
Chlordiazepoxide, flurazepam, bromazepam, oxazepam, lorazepam, clobazam, clonazepam, and flunitrazepam	Non-alcoholic and light alcoholic beverages	UA-DLLME with 0.2 mL of DCM and 0.3 mL of ACN	HPLC–UV	0.86–1.75 $\mu g \cdot mL^{-1}$	2.88– 5.83 $\mu g \cdot mL^{-1}$	14.30–103.28	[14]
Diazepam, chlordiazepoxide, clobazam, flunitrazepam, bromazepam, flurazepam, nitrazepam and clonazepam	Milk-based alcoholic beverages	QuEChERS with 10 mL of ACN	GC–MS	0.02–0.1 $\mu g \cdot mL^{-1}$	0.1–0.5 $\mu g \cdot mL^{-1}$	8.89–66.24	[15]
Chlordiazepoxide; lorazepam; diazepam; oxazepam; medazepam	Alcoholic beverages	MEPS with 0.2 mL of ACN	HPLC–UV	0.5–1 $\mu g \cdot mL^{-1}$	1–2 $\mu g \cdot mL^{-1}$	60.5–90.6	[16]
Lorazepam	Cream biscuit and tea	CPSE with 2 mL of MeOH	GC–MS	0.054 $\mu g \cdot g^{-1}$ and 0.05 $\mu g \cdot mL^{-1}$	0.18 $\mu g \cdot g^{-1}$ and 0.16 $\mu g \cdot mL^{-1}$	72.6–91.1% (RR%) and 72.9–108.1% (Accuracy)	Present Work

Abbreviations: LLE—Liquid liquid extraction; CF—Chloroform; IPA—Isopropyl alcohol; ACN—Acetonitrile; EtOH—Ethanol; MAE—Microwave-assisted extraction; EA—Ethyl acetate; UA-DLLME—Ultrasound assistance -dispersive liquid–liquid microextraction; MEPS—Microextraction by packed sorbent; CPSE—Cellulose paper sorptive extraction; GC–MS—Gas chromatography–mass spectrometry; PS–MS—Paper spray-mass spectrometry; HPLC—high-performance liquid chromatography.

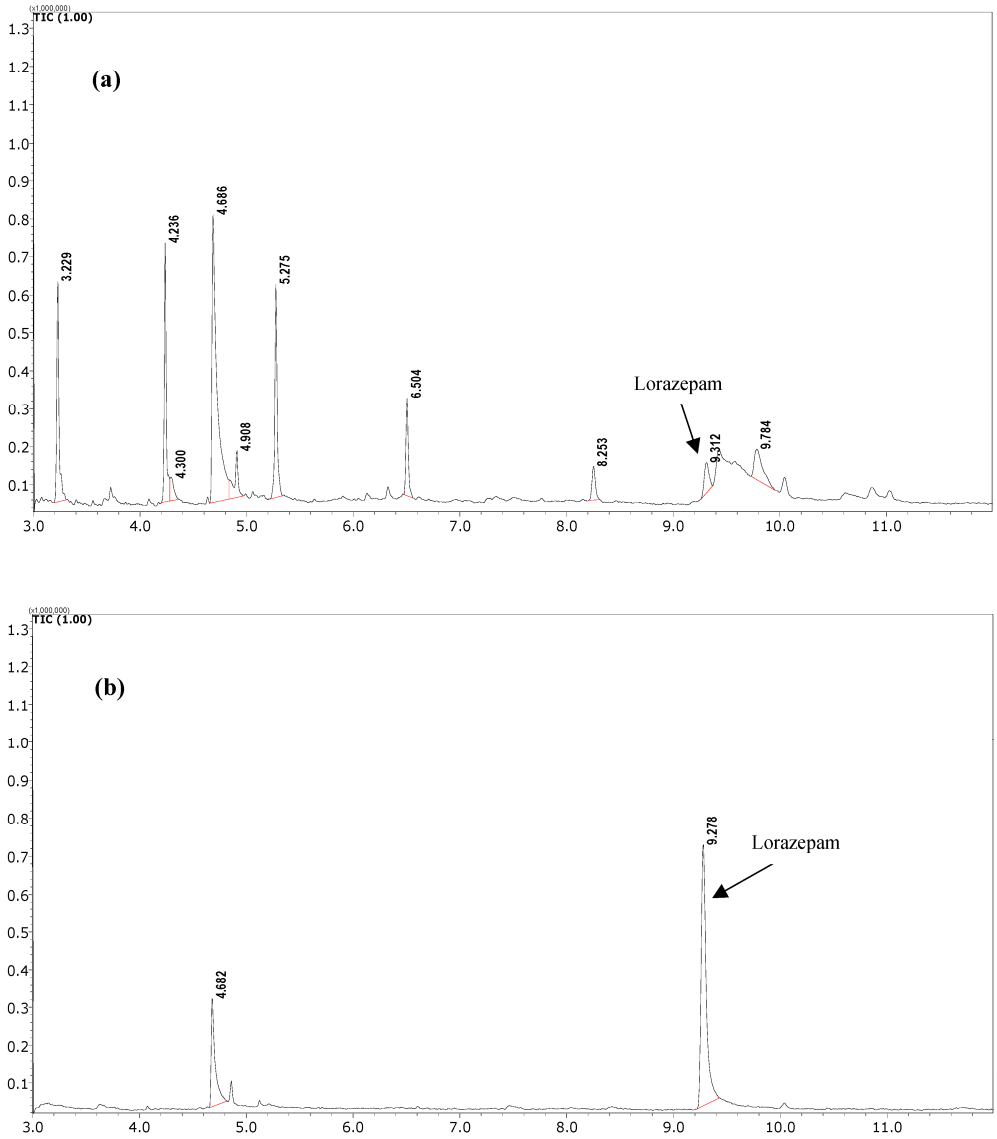


Figure 4. Comparison of sample clean-up capacity of (**a**) LLE and (**b**) CPSE in a real forensic sample (tea sample from case 1) in TIC mode. Peak identification: Caffeine at 4.68 min; Lorazepam at 9.29 min.

4. Application to Real Forensic Samples and Pharmacological Interpretation

The suggested CPSE–GC–MS method has been used to determine LZ in actual forensic samples from cases 1 and 2 after proper optimization and validation, as mentioned in the case history section. The tea sample from case 1 was found to contain LZ at 478 $\mu g \cdot mL^{-1}$ by the proposed method. The sample was also subjected for routine alcohol analysis which revealed that ethyl alcohol was also added in the sample at 317 $\mu g \cdot mL^{-1}$. In the same case, we have also received blood samples of the victim which was found to contain 2.9 $\mu g \cdot mL^{-1}$ of LZ during systematic toxicological analysis.

LZ is a benzodiazepine with a high oral bioavailability (~95%), similar to intravenous administration [2,3]. The peak concentration is reached within two hours. United States Drugs and Food Administration (USFDA) has approved LZ for treating status epilepticus and short-term use in anxiety disorders and insomnia [30]. LZ exerts its effect by binding to the benzodiazepine receptor of the post-synaptic gamma-aminobutyric acid A (GABA-A)-chloride ion channel complex. It facilitates the action of GABA, the primary inhibitory neurotransmitter in the central nervous system (CNS), which explains LZ's anxiolytic,

sedative, and anti-epileptic effects [31,32]. The adult maximum recommended oral dose is 10 mg daily [33]. The usual therapeutic concentration of lorazepam is 0.05 to 0.24 $\mu g \cdot mL^{-1}$.

In case 1, the blood level measured after ~3–5 h was 2.9 $\mu g \cdot mL^{-1}$ (not the peak concentration), more than ten times higher than the therapeutic concentration. The peak serum concentration must have been significantly higher than the toxic range of greater than 0.30 $\mu g \cdot mL^{-1}$ [34]. This high serum level was achieved by ingesting ~48 mg of lorazepam (assuming the victim drank ~100 mL of tea spiked with lorazepam in a concentration of 478 $\mu g \cdot mL^{-1}$). There was concomitant use of alcohol, which might have added to the CNS and respiratory depressant effects of lorazepam. The CNS depressant effect was manifested as profound sedation, disorientation, and confusion, whereas the low respiratory rate (eight per minute) indicated respiratory depression [35].

BDZs overdose is usually non-fatal; however, concomitant use of other sedatives, such as alcohol and opioids, might cause fatalities [36]. The US emergency department data showed that more than one in five BDZ related deaths were associated with the combined use of alcohol [37]. Binge alcohol use, defined as the ingestion of four standard drinks (~150 mL of 40% alcohol *v/v*) in 1–2 h, carries a higher risk of overdose [38]. In case 1, the victim consumed ~30 mL of alcohol (assuming she drank ~100 mL of tea spiked with alcohol in a concentration of 317 $\mu g \cdot mL^{-1}$). Ingestion lesser than the binge amount might have prevented the imminent fatality with concomitant LZ.

In case 2, the victim has consumed biscuits spiked with LZ by the perpetrator. It is important to note that the cream sandwiched between the two biscuits was mixed with LZ. The amount of LZ in the biscuits was found to be quite high i.e., 1.7 $mg \cdot g^{-1}$. As per the statement of the victim, he has consumed four biscuits in a single instance which were equal to ~10 g cream portion and corresponded to ~17 mg of LZ. This high dose of LZ resulted in immediate unconsciousness and prolonged sedation of around 15 h.

LZ is mentioned in Schedule H of the Drugs and Cosmetics Rules, 1945. Therefore, it cannot be sold or purchased over the counter without a prescription from a registered medical practitioner. In this case, the perpetrator must have procured many lorazepam tablets illegally. These unfortunate events indicated a need to tighten the legal control to reduce non-medical access to benzodiazepines.

5. Assessment of the Green Character of the Method

The widely used "Analytical Eco-Scale" approach and "Green Analytical Procedure Index" (GAPI) have been utilized to compare the greenness profiles of the new method to previously reported methods (Table 4) for similar analytes in food matrices [39–47]. The Analytical Eco-Scale provides an ideal analytical method that satisfies all greenness criteria 100 points. Subsequently, penalty points are deducted for various factors, including the type and amount of chemicals used, energy used, waste produced, and occupational hazards. If the procedure receives points >75, it is regarded as an excellent green analysis; points >50, an acceptable green analysis; and 50 points, an inadequate green analysis. The proposed CPSE–GC–MS has obtained a score of 92 which shows that the method offers an excellent green protocol for analysis of LZ in food matrices (Table 4).

The second tool used to evaluate the suggested method's greenness is GAPI, which offers a comprehensive picture of the various analytical stages through five pentagrams (P1–P5) made up of 15 zones in three distinct colors (red, yellow, and green). The color of each zone describes the degree of impact of each concerned step of the analytical method on the environment. If the zones in the pentagram are green or yellow, the respective greenness criteria are met. P1 has two red zones that correspond to off-line sample collection and transportation to the laboratory. The red zone in P2 is caused by the extraction required for LZ analysis in food matrices. The red zone in P4 is caused by the energy consumption (1.5 KWh) of the GC–MS instrument used for LZ analysis. Since the method is both qualitative and quantitative in nature, P5 is red. As a result, only five of the 15 parameters are in the red zone and cannot be avoided in forensic analysis in such cases. GAPI pictogram created for the suggested procedure is shown in Figure 5.

Table 4. Comparison of greenness of CPSE–GC–MS with previously reported analytical methods.

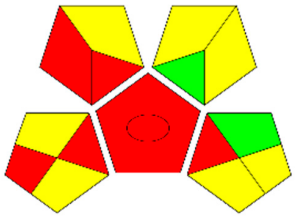
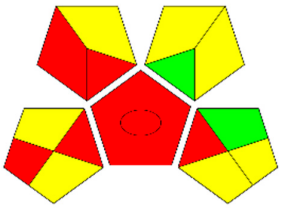
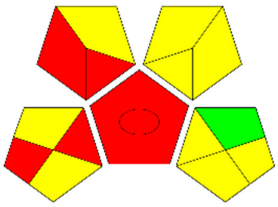
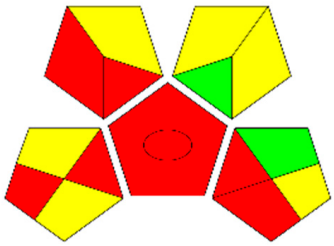
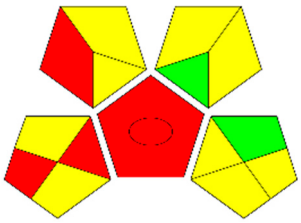
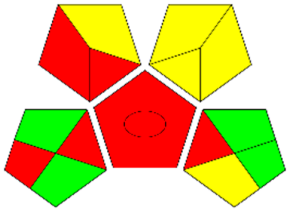
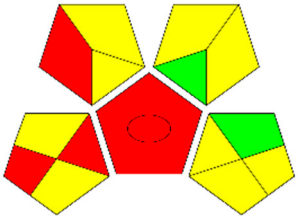
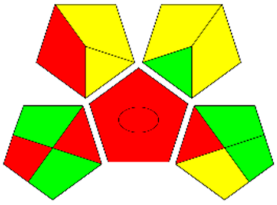
Methods	ECO-SCALE		GAPI	Reference
LLE–GC–MS	**Reagents**	**Penalty Points**		[10]
	CF	6		
	ISA	4		
	Instrument			
	Energy used	2		
	Waste	3		
	Total	12		
	Score	85		
LLE–PS–MS	**Reagents**	**Penalty Points**		[11]
	ACN	4		
	Instrument			
	Energy used	2		
	Waste	3		
	Total	9		
	Score	91		
LLE–HPLC	**Reagents**	**Penalty Points**		[12]
	EtOH	8		
	Instrument			
	Energy used	1		
	Waste	3		
	Total	12		
	Score	88		
LC-MS/MS	**Reagents**	**Penalty Points**		[13]
	MeOH	6		
	ACN	4		
	Instrument			
	Energy used	2		
	Occupational hazard	3		
	Waste	3		
	Treatment	3		
	Total	15		
	Score	79		
UA–DLLME–HPLC–UV	**Reagents**	**Penalty Points**		[14]
	ACN	4		
	DCM	4		
	Instrument			
	Energy used	1		
	Waste	3		
	Total	12		
	Score	88		
QuEchERS–GC–MS	**Reagents**	**Penalty Points**		[15]
	ACN	8		
	Instrument			
	Energy used	2		
	Total	10		
	Score	90		
MEPS–HPLC–UV	**Reagents**	**Penalty Points**		[16]
	ACN	4		
	Instrument			
	Energy used	1		
	Waste	3		
	Treatment	2		
	Total	10		
	Score	90		

Table 4. *Cont.*

Methods	ECO-SCALE		GAPI	Reference
CPSE–GC–MS	**Reagents**	**Penalty Points**		Present study
	MeOH	6		
	Instrument			
	Energy used	2		
	Total	8		
	Score	92		

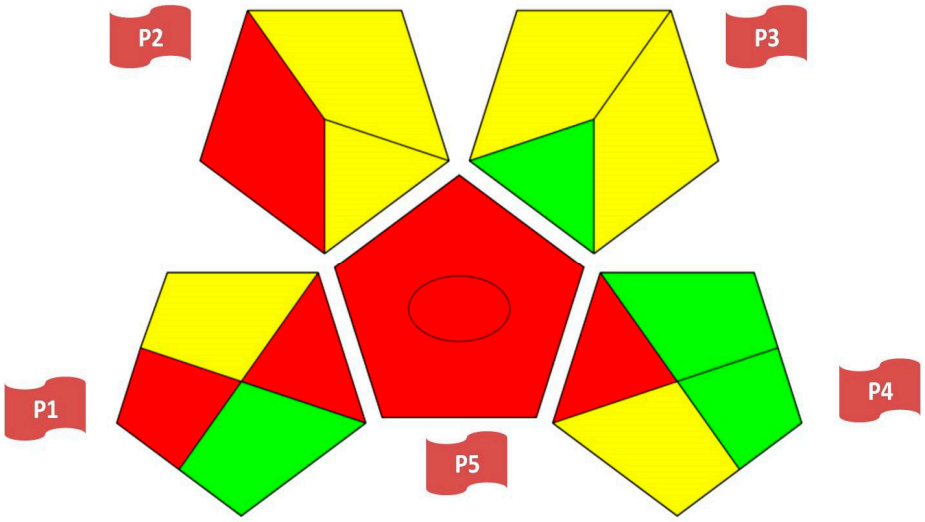


Figure 5. GAPI pictogram for proposed CPSE–GC–MS method.

6. Experimental

6.1. Material and Methods

All of the chemicals and reagents used in this investigation, unless otherwise noted, were of analytical grade. The lorazepam (LZ) standard was provided by Indian Pharmacopeia Commission, Ghaziabad, India. The following chemicals were purchased from LobaChemie (Mumbai, India): methanol (MeOH), chloroform (CF), acetonitrile (ACN), dichloromethane (DCM), ethyl acetate (EA), hydrochloric acid (HCl), and sodium chloride (NaCl). Ultra-pure water was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA) in the biology division of the laboratory. To extract the LZ from food samples, a Remi RS 12-Plus rotary shaker with a 50–400 rpm capacity was used. The adsorption of the target analytes was carried out using Whatman^{TM} circular qualitative filter paper of grade 1 (standard filter paper) with a diameter of 150 mm. Stock solution of LZ were prepared in MeOH at 1 mg·mL^{-1} and stored at ~4 °C until further use. They were diluted at desired concentration before each experiment. Drug free food samples (cream biscuits and tea) were fortified with the stock solution of target analyte for the purpose of method optimization and validation. Food samples that were collected from crime scenes by law enforcement agencies were forwarded to our laboratory for analysis. The samples were kept at room temperature and analyzed as soon as the case was opened. Five different brands of cream biscuits and tea samples were procured from the local market for the purpose of method development and validation.

6.2. Cellulose Paper Sorptive Extraction (CPSE) Procedure

The procedure is fairly straightforward and consists of just two steps: (i) adsorption of the target analyte on CP and (ii) desorption/back-extraction of the adsorbed analyte on CP in MeOH. Initially, one mL of tea sample or one gram of cream biscuit sample were

fortified with LZ a concentration of 1 μg·mL^{-1} or 1 μg·g^{-1}, respectively. The tea sample without any pretreatment was diluted up to 10 mL with ultrapure water maintained at pH 12 with the help of 0.1 M NaOH. Six pieces of equally cut CP (size 1.5″ × 1.5″) were dipped in the sample and gently rotated on a rotary shaker at 200 rpm for 30 min. This facilitated the adsorption of LZ in the sample on CPs. The pieces of CPs were then taken out and dried at room temperature. Furthermore, they were dipped in 2 mL of MeOH for back extraction of LZ and allowed to stand for 15 min without any agitation. Following back extraction, the CPs were discarded and the extract was evaporated to dryness under a gentle stream of nitrogen, reconstituted with 200 μL of MeOH and subjected for GC–MS analysis. For solid samples (i.e., cream biscuits), one gram of sample was thoroughly mixed with 10 mL of ultrapure water maintained at pH 12 and centrifuged at 5000 rpm for 3 min. As previously stated, the obtained supernatant was subjected to CPSE–GC–MS analysis. A graphical representation of the proposed protocol is shown in Figure 6.

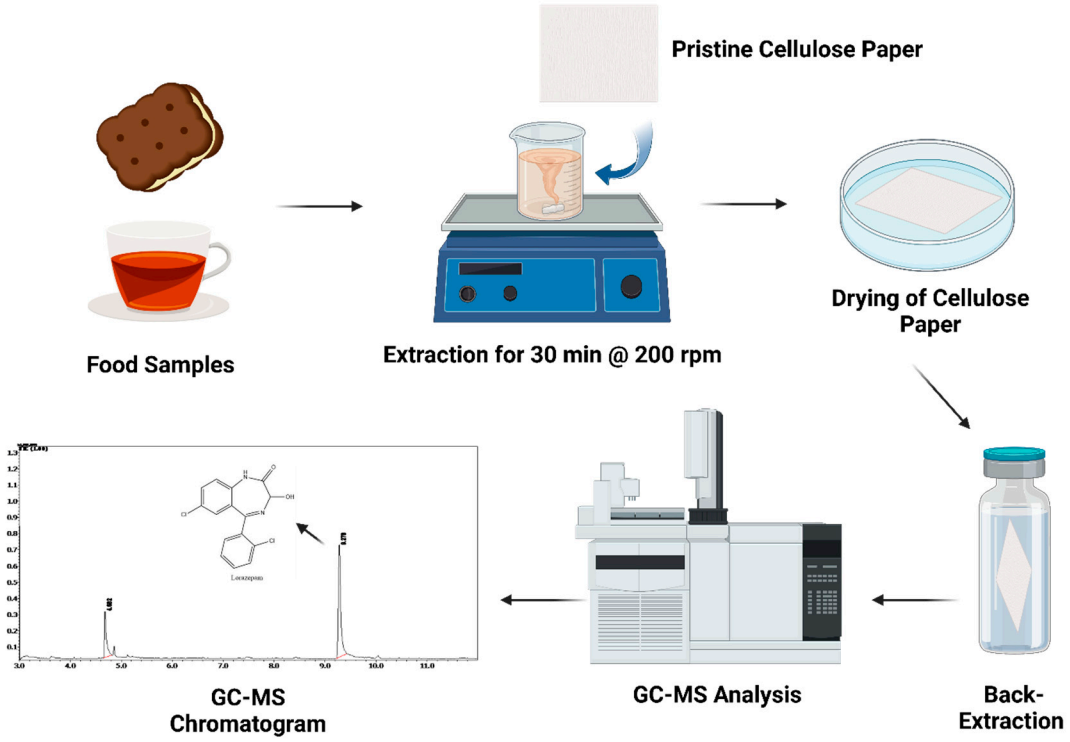


Figure 6. Graphical representation of proposed CPSE–GC–MS procedure for determination of LZ.

6.3. Gas Chromatography—Mass Spectrometry (GC–MS) Analysis

A Shimadzu Nexis GC-2030 hyphenated with QP-2020 NX mass spectrometer was used for the GC–MS analysis of the CPSE extract. An SH-Txi-5Sil MS capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness) with a stationary phase of 95% dimethylpolysiloxane and 5% phenyl was installed in the GC. Using an AOC-20i Plus Auto-sampler, a precise 2 μL of the extract was injected in split mode (split value 10) while the injection port temperature of the GC–MS was kept at 280 °C. With a flow rate of 1 mL·min^{-1}, helium was used as carrier gas. The oven's temperature was programmed to start at 150 °C, increase to 300 °C at a rate of 20 °C/min, and then hold there for 10 min (total runtime: 17.50 min). With an electron energy of 70 eV, the MS was run in electron ionization (EI) mode. The ion source and interface temperatures were maintained at 200 °C and 250 °C, respectively. Mass spectra were initially recorded in full scan mode in the 50–500 amu range for identification purposes. For the purpose of quantitative analysis, selected ion monitoring (SIM) mode was utilized with the ions of *m/z* 275, 303, 239 for LZ. The chromatograms of blank, standard and real forensic samples are shown in Figure 7.

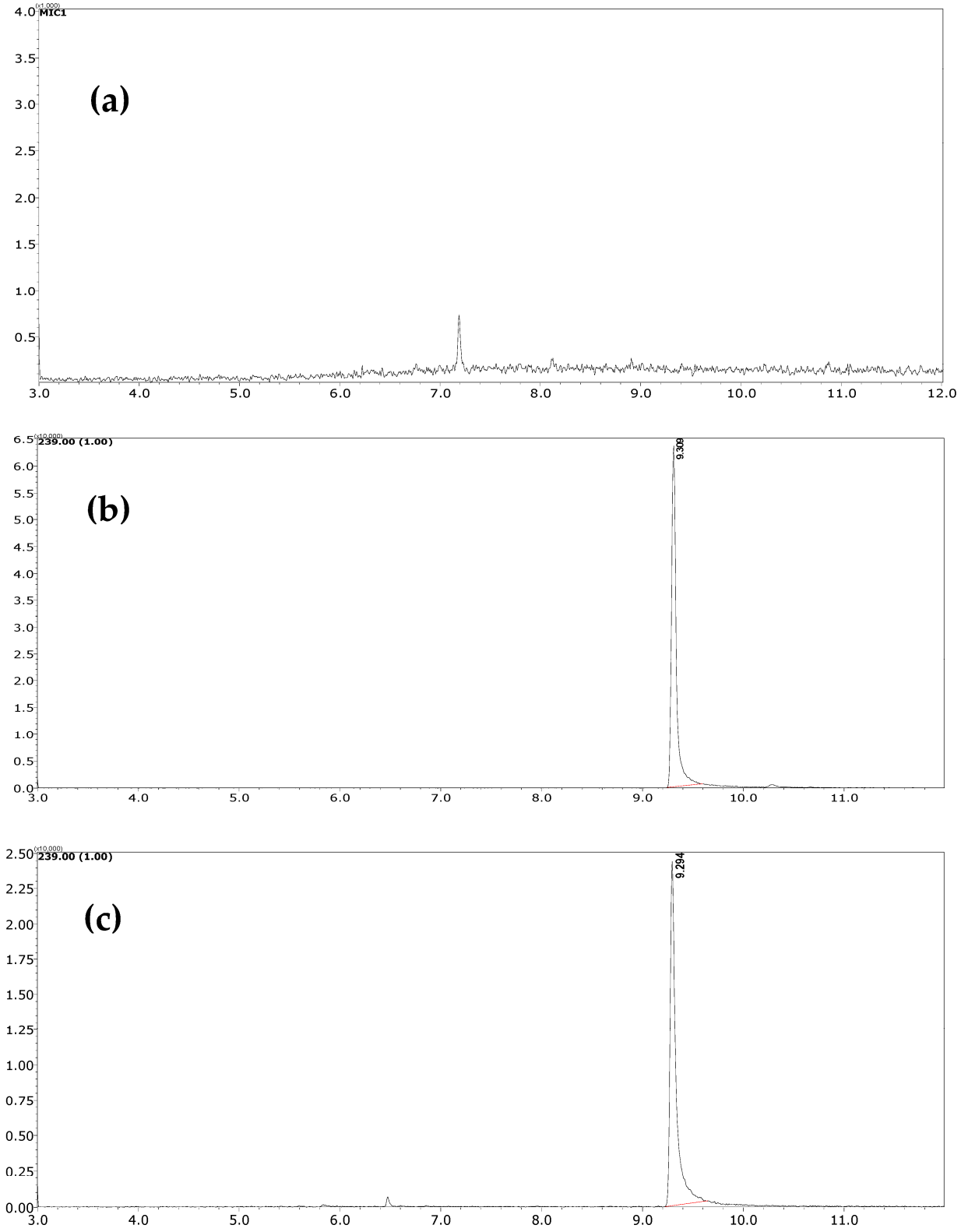


Figure 7. The selected ion chromatograms (SIM) of (**a**) blank, (**b**) standard (10 μg·mL^{-1}), and (**c**) real forensic samples (tea sample from case 1). Peak identification: Lorazepam at 9.29 min.

6.4. Method Validation

The proposed method's linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), recoveries (absolute recovery and enrichment recovery), and enrichment factors (EFs) were evaluated under optimal conditions. The peak area of LZ on the *y*-axis and the respective concentrations on the *x*-axis were taken into account while plotting matrix matched calibration curves for each matrix (tea and cream biscuit). The proposed method's LOD and LOQ were assessed using the well-known signal-to-noise ratio approach. Precision (intra-day and inter-day) were used to represent repeatability and reproducibility, and they were reported as a percentage relative standard deviation (% RSD). The percentage of the peak area enhancement compared to the area of the reference standard solutions was used to calculate the percent EF (EF%). These were calculated according to following formula:

$$\text{EF\%} = \frac{Cp}{Ci} \times 100$$

where, *Cp* is the analyte's concentration in the processed sample and *Ci* is the analyte's initial concentration in the untreated sample [48]. Additionally, the matrix effect (ME) was evaluated using five different samples of tea and biscuit which were purchased from various local grocery stores/brands. ME was assessed with the following equation in accordance with FDA guidelines [49]:

$$\text{\%ME} = \frac{\text{Area of post extracted fortified sample}}{\text{Area of neat standard}} \times 100$$

6.5. Sample Stability

Under optimal conditions (i.e., at pH 12), the stability of LZ in the processed sample was studied for 10 days at room temperature. The concentration of the detected analyte was established at 100% on the first day, and a notable decrease of <5% was observed in the concentration of LZ after 10 days, which means that LZ was stable (with a degradation of <5%) until 10 days under optimized conditions. This finding is evident from previous published literature as well [50].

7. Case History

Case 1:

The 44-year-old victim was a married woman who had employed a servant. Before midday, the maid served her egg, toast, and tea at breakfast. She fell unconscious shortly after eating the breakfast and her servant stole valuables, including money and jewels. She was found unconscious in the house by her sister after some time, who then took her to the closest hospital, where medical staff deemed her unsuitable for a statement and started her treatment. The victim's tea, which was seized during the investigation of the crime scene, was taken and sent to our laboratory for analysis together with samples of the victim's blood.

Case 2:

Around 09:30 p.m., a 32-year-old auto driver was driving his passenger to his destination. During the trip, the passenger offered him some biscuits that had been spiked with some intoxicating substance. The auto driver became unconscious after eating these biscuits, and the passenger stole his cash and important documents. The next morning when he woke up around 12:45 p.m., he discovered that his valuables had been stolen by the passenger and filed a police report. The police apprehended the culprit and seized some biscuits and unknown tablets in large quantities which were submitted to our laboratory for analysis. Photographs of biscuits seized by the law enforcement agency and forwarded to our laboratory for analysis are shown in Figure 8.

Figure 8. Photographs of forensic sample (cream biscuits) forwarded by law enforcement agencies in case 2.

8. Conclusions

The proposed CPSE–GC–MS method uses pristine CPs without any modification as an extraction medium to determine LZ in food samples used in DFCs. The suggested approach for extracting LZ from complex samples is simple, straightforward and does not require multiple steps. Moreover, the risk of sample carryover is removed as the CPs are discarded after each extraction. As the CP is composed of biopolymer, it does not have any adverse effects on the environment. These characteristics of the suggested method align it to the GAC's guiding principles. The method offers high sample throughput since 16 samples can be prepared simultaneously in 45 min using a simple rotary shaker. In conclusion, the proposed CPSE–GC–MS can be routinely utilized for the simple and reliable analysis of LZ in complex food matrices received in forensic science laboratories in cases of DFCs. The limitation of the proposed method is the selectivity towards polar and semi-polar analytes; as CP is not treated by any substances to enhance its selectivity towards non-polar analytes. Future studies will be focused on treating the pristine CP in order to enhance its adsorption capabilities towards a wide range of analytes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10050281/s1, Figure S1: The effect of volume of sample on the EF of LZ by CPSE procedure; Figure S2: Effect of stirring on elution of LZ from CPSE procedure.

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