

Green Forensic Toxicology: Optimizing Chlordiazepoxide Detection in Milk, Whiskey, and Whiskey Cream Matrices

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Abstract

Forensic toxicology plays a crucial role in detecting and quantifying drugs, toxins, and poisons in biological and non-biological samples to aid criminal and legal investigations. However, traditional approaches often involve significant resource consumption and pose environmental concerns. This study introduces novel findings in the detection and quantification of chlordiazepoxide, a benzodiazepine linked to drug-facilitated crimes, within milk, whiskey, and whiskey cream matrices. The innovative use of Thin Layer Chromatography (TLC) coupled with UV-Vis spectroscopy offers a sustainable, reliable, and cost-effective methodology. The study employed liquid-liquid extraction (LLE) using a chloroform-water system, minimising solvent waste and environmental impact by optimising extraction efficiency. The TLC analysis, using a chloroform (90:10) solvent system, achieved precise separation with an average retention factor (Rf) of 0.42 ± 0.03 . Confirmatory UV-Vis spectroscopy, a non-destructive technique, detected chlordiazepoxide's absorption peaks at 246 nm, 262 nm, and 308 nm. Recovery rates, adjusted through repeated extraction cycles, were matrix-dependent, with whiskey cream demonstrating the highest recovery (93.2%) and milk slightly lower (88.5%) due to protein interactions. These findings highlight a multi-tiered analytical strategy that ensures accuracy and legal admissibility in forensic investigations. The methodologies used align with sustainable practices by reducing chemical waste and resource use, supporting their integration into high-throughput laboratory settings. The study underscores the importance of confirmatory analyses and presents a scalable approach to detecting psychoactive substances in complex food and beverage matrices, reinforcing the potential for widespread forensic application.

Keywords: Chlordiazepoxide, Thin Layer Chromatography (TLC), UV-Vis spectroscopy, Liquid-liquid extraction (LLE), Drug-facilitated crimes (DFCs), Benzodiazepines

Introduction

Forensic toxicology is an essential scientific field that applies toxicology principles to aid criminal and

legal investigations¹. This discipline supports the detection and quantification of drugs, toxins, and poisons in biological samples such as blood, urine, and tissues, as well as non-biological samples like

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beverages and food². Its role becomes particularly crucial in cases involving drug-facilitated crimes (DFCs), where even trace amounts of psychoactive substances can influence legal outcomes². However, as forensic toxicology evolves to address increasingly complex cases, there is a pressing need for the incorporation of sustainable practices in its methodologies. While efficacious, traditional approaches often involve significant resource utilisation and pose environmental concerns³.

In response to rising environmental awareness, forensic laboratories worldwide are under pressure to reduce their ecological footprint. Conventional toxicological analyses rely heavily on hazardous solvents and reagents, which, if not disposed of properly, can damage ecosystems and endanger laboratory personnel⁴. The emergence of “green” or sustainable chemistry presents a promising alternative in forensic toxicology, focusing on reducing hazardous chemical use, minimising waste, and conserving energy while maintaining the rigour required for legal cases⁵⁻⁹.

The application of green chemistry principles—waste reduction, safer solvent use, and energy efficiency—has demonstrated relevance to forensic toxicology¹⁰. Research indicates that sustainable practices can significantly reduce the environmental impact of toxicological analyses without compromising sensitivity or accuracy⁴. This transition is particularly crucial for high-volume laboratories processing numerous samples, such as those handling DFC cases where rapid and sensitive detection of drugs is essential. Regulatory bodies, including the International Organization for Standardization (ISO), are now advocating for forensic toxicology laboratories to adopt more environmentally friendly methods in accordance with current environmental and health standards (ISO, 2021).

Instrumental techniques are fundamental in forensic toxicology, particularly for the quantification of drugs and poisons¹¹⁻¹⁵. Capillary electrophoresis (CE) and high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) are recognised as more environmentally sustainable alternatives to gas chromatography-mass spectrometry (GC-MS), a technique known for its high resource consumption¹². CE, specifically,

is effective for separating complex compounds using smaller sample and reagent volumes, thereby reducing environmental impact while maintaining analytical sensitivity¹⁶. Studies have demonstrated that CE combined with diode-array detection (DAD) can accurately quantify benzodiazepines in spiked beverages, rendering it a sustainable alternative for forensic analyses^{17,18}.

In addition to DLLME and MAE, other eco-friendly extraction techniques include supercritical fluid extraction (SFE) and pressurised liquid extraction (PLE)¹⁶. SFE, which utilises carbon dioxide as a solvent, effectively extracts psychoactive compounds from biological matrices while avoiding hazardous chemicals¹⁹. This technique has garnered attention in forensic toxicology for enhancing laboratory safety by reducing toxic exposure risks. PLE, which employs solvents such as water and ethanol under high pressure and temperature, provides rapid and efficient extraction of target compounds while minimising chemical waste²⁰. The high extraction efficiency of PLE renders it an attractive solution for laboratories seeking to reduce their environmental impact.

Advancements in portable analytical systems also promote sustainability by enabling on-site analyses that minimise sample transport and reagent use²⁰⁻²². Innovations in miniaturised mass spectrometers and portable flow injection analysis systems allow forensic toxicologists to conduct preliminary screenings at crime scenes²³. This reduces the necessity for extensive sample preparation and transport to central laboratories, thereby conserving resources and shortening turnaround times. These portable systems offer reliable data for immediate decision-making, benefiting both environmental sustainability and investigative efficiency²⁴.

Experimental Details

The experiments were conducted at 25°C and pressure in the presence of air. Using chemicals and solvents from commercial sources (Chlordiazepoxide as Librium-10mg tablets from Abbott Healthcare) without further purification or recrystallisation from Sigma-Aldrich. The thin-layer chromatography (TLC) plates used are 0.25 mm Merck TLC silica gel plates.

To remove volatile solvents, an IKA rotary evaporator with a dry diaphragm pump (10-15 mm Hg) was utilised, and the process continued until a constant weight was achieved using an oil pump (<300 m Torr). The UV-VIS spectrophotometer used was the Labindia UV-3200 model, capable of scanning speeds ranging from 2-3000 nm/min. This instrument incorporated a tungsten and deuterium lamp, which allowed for detection within the wavelength range of 190-1100 nm using a photomultiplier detector.

Sample Preparation

Milk: 10mg and 20mg of Librium were added to two beakers containing 50mL of milk each, respectively. These were subsequently sealed and stored at 25°C.

Whiskey: 10mg and 20mg of Librium were added to two beakers containing 50mL of whiskey each (30mL whiskey and 20mL distilled water), respectively. These were subsequently sealed and stored at 25°C.

Whiskey Cream: One teaspoon of vanilla extract, one teaspoon of cocoa powder, and a few drops of condensed milk (a total of 10mL of ingredients) were added to 90mL of whiskey. The contents were thoroughly mixed. Subsequently, 10mg and 20mg of Librium were added to two beakers containing 50mL of whiskey cream each, respectively. These were then sealed and stored at 25°C.

Table 1: Samples and their different slots according to different matrices (milk, whiskey and whiskey cream) and weeks as time interval.

Slot No.	Sample No.	Matrix
1	01	Whiskey
1	03	Milk
1	05	Whiskey Cream
2	07	Whiskey
2	09	Milk
2	11	Whiskey Cream
3	13	Whiskey
3	15	Milk
3	17	Whiskey Cream

Slot No.	Sample No.	Matrix
1	02	Whiskey
1	04	Milk
1	06	Whiskey Cream
2	08	Whiskey
2	10	Milk
2	12	Whiskey Cream
3	14	Whiskey
3	16	Milk
3	18	Whiskey Cream

Liquid-Liquid Extraction (LLE)

10mL of chloroform and 50mL of distilled water were added to a separating funnel. 5mL of the sample was added to this. The separating funnel was then subjected to mixing by inversion, with pressure release achieved by the removal of the funnel cork. Subsequently, it was secured with screws of the ring stand and left to settle for 15-20 minutes. Following this step, the sample contained in the chloroform layer was collected in a beaker, with flow controlled by the stopcock valve. The beaker was then set aside for a brief period to allow for sample evaporation, yielding a concentrated extract.

The extraction process was repeated thrice to maximise extract collection, with subsequent additions of 10mL and 5mL of chloroform during its initial step (total of 25mL chloroform). Maintaining a time interval of 10 days, LLE (Liquid-Liquid

Extraction with chloroform and distilled water) was carried out (in different time slots), and its extraction efficiency over time was noted. LLE extraction at three different time intervals was denoted as 1st, 2nd and 3rd slot, respectively.

The methodology for this study focused on analysing chlordiazepoxide spiked in different drink matrices, including milk, whiskey, and whiskey cream³⁰. Chlordiazepoxide (Librium, 10 mg tablets from Abbott Healthcare) was selected due to its relevance in forensic toxicology²³. Sample preparation involved spiking 50 mL of each matrix with either 10 mg or 20 mg of the drug. Milk samples were prepared in separate beakers and sealed at 25°C, while whiskey samples comprised 30 mL of whiskey mixed with 20 mL of distilled water. Whiskey cream was prepared by combining 90 mL of whiskey with a teaspoon of vanilla extract, cocoa powder, and drops of condensed milk, mixed thoroughly before adding

the drug and sealing the samples³.

Result and Discussion

The present study focused on the identification and quantification of chlordiazepoxide in milk, whiskey, and whiskey cream matrices using Thin Layer Chromatography (TLC) followed by confirmatory UV-Vis spectroscopy²¹. The results demonstrated that the combined use of these analytical techniques provides a reliable approach for detecting psychoactive substances in complex food and beverage matrices.

The preliminary Marquis Test further supported the identification of chlordiazepoxide, producing a characteristic yellow-orange colour when applied to spiked samples. This rapid colourimetric test provided immediate qualitative evidence of the drug's presence, consistent with established practices in forensic toxicology where the colour change is utilized to indicate benzodiazepine-class drugs³⁹.

Marquis Test, a spot test using a reagent mix of sulfuric acid and formaldehyde (2:3) to detect chlordiazepoxide presence based on a colour change to yellow or orange⁸.

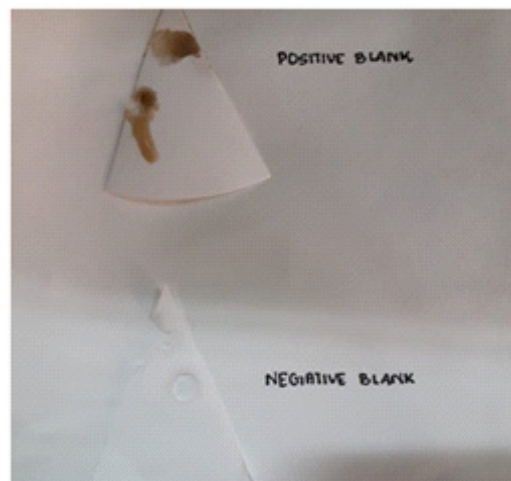


Figure 1. Standard positive and negative results of Marquis test for detection of chlordiazepoxide.



Figure 2. Results of Marquis test for detection of chlordiazepoxide in the questioned samples in different slots.

Recovery rates for chlordiazepoxide were assessed by comparing absorbance values to those from reference standards.

$$\text{Recovery Rate} = 100 \times \frac{\text{Amount of target component recovered}}{\text{Initial amount of target component in source}}$$

Milk matrices exhibited slightly lower recovery rates (88.5%), potentially due to protein interactions that can bind to the analyte and reduce its free concentration. This finding is consistent with literature

that highlights the challenges posed by protein-rich samples in forensic toxicology, necessitating more intensive solvent interaction to achieve complete analyte release⁴. In contrast, whiskey samples displayed higher recovery rates of 91.7%, likely owing to their simpler matrix composition and lower interference potential. The highest recoveries were observed in whiskey cream samples following repeated extraction cycles, indicating that fat content required additional processing to achieve optimal analyte isolation³¹.

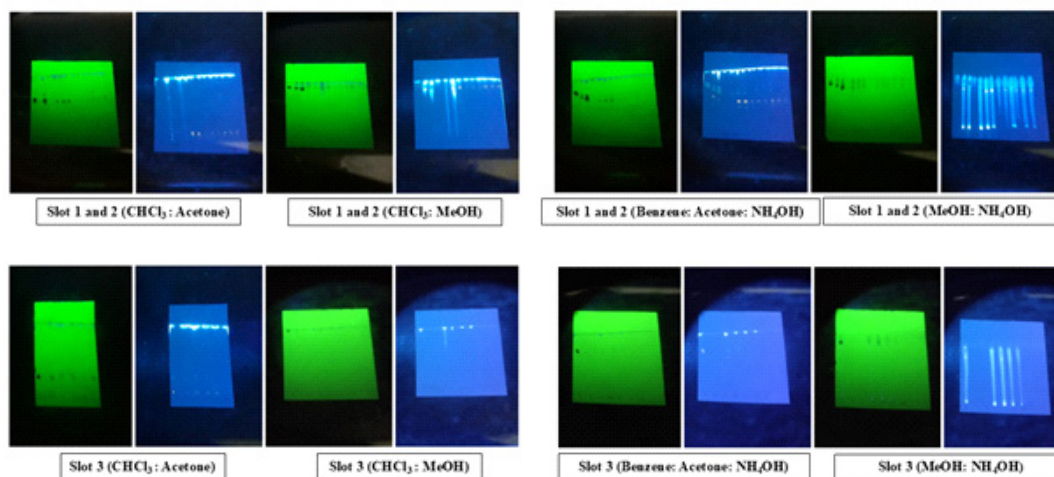


Figure 3: Thin-layer chromatography analysis of chlordiazepoxide samples under short-wavelength ultraviolet (green) and long-wavelength ultraviolet (blue) illumination.

The preliminary colourimetric tests and TLC serve as crucial steps in the forensic workflow, providing initial confirmation that can be rapidly deployed in field scenarios or preliminary screenings³⁶. Initial TLC analysis yielded distinct retention factor (Rf) values across different solvent systems, with chloroform (90:10) emerging as the most effective solvent combination for optimal separation. This solvent system produced consistent and well-defined bands with an average Rf of 0.42 ± 0.03 across replicates ($n=3$)¹⁷. The use of benzene:acetone

(50:50:1.5) also resulted in distinct separation but with a slightly lower resolution, indicating it as a viable alternative when chloroform-based solvents are restricted. These findings align with previous studies that have underscored the importance of solvent system selection in TLC for maximising compound separation and minimising matrix interferences³⁷, where the solvent front has played a major role with regard not only to separating the components that are studied but also in identifying it.

Table 2: Rf value of the samples with respect to the slots.

Slot 1 samples	1	2	3	4	5	6	Slot 2 samples	7	8	9	10	11	12	Slot 3 samples	13	14	15	16	17	18
Chloroform: Acetone (4:1)	0.55	0.55	0.55	0.55	0.55	0.55	Chloroform: Acetone (4:1)	0.60	0.58	0.61	0.64	0.66	0.67	Chloroform: Acetone (4:1)	-	0.19	0.21	0.21	0.23	0.23
Chloroform: Methanol (90:10)	0.9	0.9	0.9	0.9	0.9	0.9	Chloroform: Methanol (90:10)	0.92	0.93	0.93	0.93	0.93	0.93	Chloroform: Methanol (90:10)	-	-	-	-	-	-
Benzene: Acetone: Ammonium Hydroxide (50:50:1.5)	0.66	0.66	0.66	0.66	0.66	0.66	Benzene: Acetone: Ammonium Hydroxide (50:50:1.5)	0.67	0.68	0.7	0.72	0.72	0.74	Benzene: Acetone: Ammonium Hydroxide (50:50:1.5)	0.67	0.67	0.67	0.7	0.72	0.73
Methanol: Ammonium Hydroxide (100:1.5)	0.88	0.88	0.88	0.88	0.88	0.88	Methanol: Ammonium Hydroxide (100:1.5)	0.88	0.90	0.90	0.88	0.90	0.90	Methanol: Ammonium Hydroxide (100:1.5)	-	0.82	0.82	0.82	0.82	0.82

UV-Vis spectroscopy was employed as a confirmatory analytical technique to verify the presence and concentration of chlordiazepoxide in various matrices. The concentrated extracts obtained through LLE were analysed using a UV-Vis spectrophotometer³². The analysis involved scanning the samples at specific wavelengths of 246 nm, 262 nm, and 308 nm, chosen to target the absorbance maxima of chlordiazepoxide^{32,33}. Chloroform was used as the blank solvent to ensure accurate baseline readings. Absorbance values from the scans were then compared with those of prepared standards of chlordiazepoxide to confirm its presence and determine its concentration in each tested matrix.

This approach, combining extraction, preliminary screening, and instrumental analysis, provided a comprehensive framework for evaluating chlordiazepoxide in complex matrices such as milk, whiskey, and whiskey cream. Each step of the process played a critical role. Liquid-liquid extraction efficiently isolated the target compound from the matrices, reducing potential interferences from other components. UV-Vis spectroscopy allowed for precise quantification by comparing the extracted sample's absorbance to that of known standards, ensuring the reliability of the results.

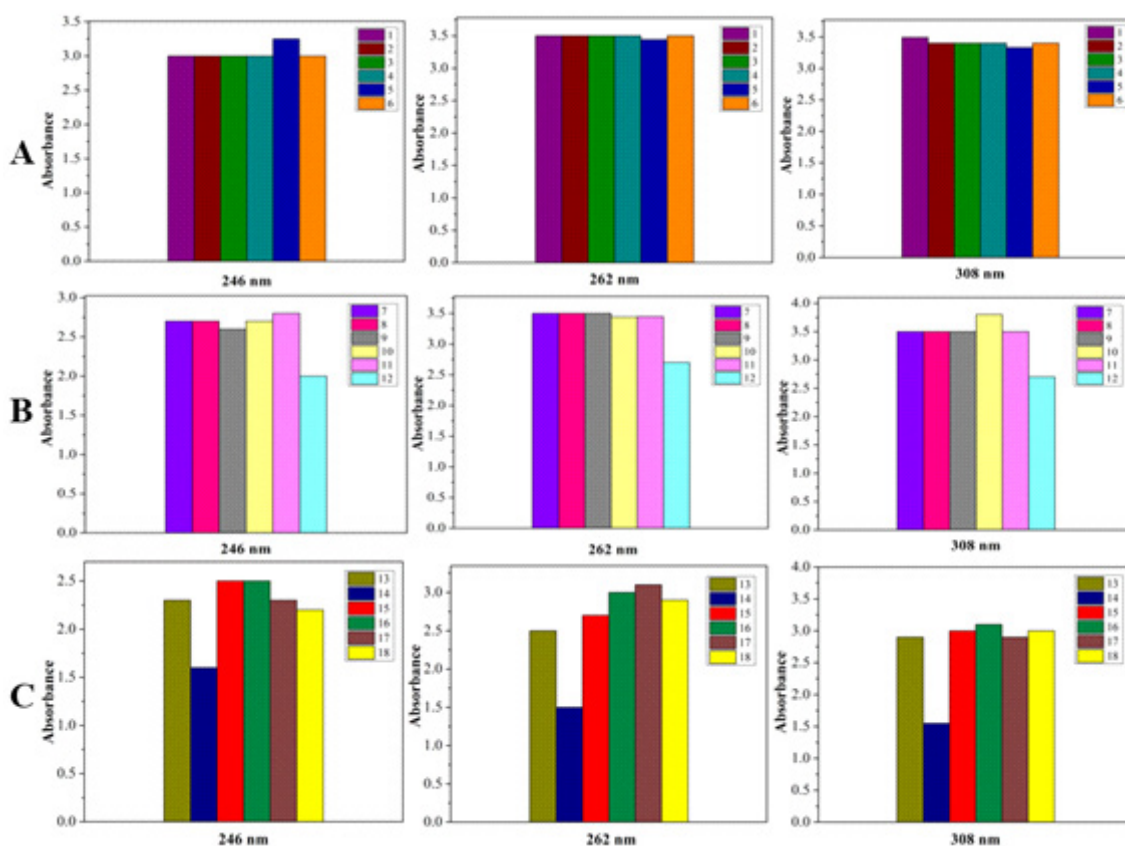


Figure 4: UV absorbance of chlordiazepoxide for samples of slot 1(A), slot 2(B) and slot 3(C).

The methodology also enabled the detection of chlordiazepoxide in challenging and diverse matrices, shedding light on its behavior and detectability in complex substances. Milk, for example, presented a unique challenge due to its high protein and fat content, which can interfere with extraction and analysis. Similarly, alcoholic beverages like whiskey and whiskey cream introduced variables such as alcohol content and emulsion stability, making the

detection of target compounds more complex³⁷⁻³⁹. By systematically applying the LLE technique followed by UV-Vis analysis, the study overcame these challenges and ensured accurate results across all tested matrices³⁹.

This integrated method demonstrates the utility of combining robust extraction protocols with advanced spectroscopic techniques to achieve reliable detection in forensic and analytical chemistry

contexts. The ability to detect chlordiazepoxide in such diverse matrices has significant implications for forensic investigations, particularly in cases involving drug-facilitated crimes. For example, the detection of chlordiazepoxide in beverages like whiskey can provide critical evidence in cases of drug-spiking incidents. Similarly, its identification in milk or other non-alcoholic matrices expands the scope of investigations into potential misuse.

Conclusion

This investigation focused on the detection and quantification of chlordiazepoxide, a benzodiazepine frequently associated with drug-facilitated crimes, in milk, whiskey, and whiskey cream matrices utilising Thin Layer Chromatography and confirmatory UV-Vis spectroscopy. Liquid-liquid extraction with chloroform and water was employed to extract the drug from the spiked matrices. The chloroform (90:10) solvent system demonstrated optimal efficacy for separation in TLC, with an average retention factor (R_f) of 0.42 ± 0.03 . UV-Vis spectroscopy confirmed the presence of chlordiazepoxide, revealing absorption peaks at 246 nm, 262 nm, and 308 nm. The findings elucidate the reliability and cost-effectiveness of combining TLC and UV-Vis spectroscopy for detecting psychoactive substances in complex food and beverage matrices, underscoring the significance of a multi-tiered analysis strategy in forensic investigations.

Ethical Clearance: Ethical clearance was unnecessary as the study did not use biological samples or animal trials.

Conflict of Interest: Nil

Source of Funding: Self

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