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Development of a Green and Robust Micellar Liquid Chromatography Method for the Simultaneous Determination of Malathion and Carbofuran

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KEYWORDS

Micellar Liquid Chromatography; Malathion; Carbofuran; Forensic Toxicology; Green Analytical Method

ABSTRACT:

Introduction: Animal poisoning linked to wildlife crime and agricultural conflicts poses significant challenges to forensic investigations and environmental safety. Malathion and carbofuran, two highly toxic pesticides commonly involved in deliberate poisoning cases, are difficult to detect due to their low concentrations in complex biological and environmental matrices. Conventional analytical methods often rely on hazardous organic solvents and extensive sample preparation, limiting their sustainability and routine applicability.

Objectives: This study aimed to develop a green, rapid, and cost-effective analytical method for the simultaneous detection of malathion and carbofuran while minimizing hazardous solvent use and sample pretreatment. The method was validated according to ICH guidelines to ensure reliability for forensic applications.

Methods: A Micellar Liquid Chromatography (MLC) method employing a sodium dodecyl sulfate (SDS)–pentanol mobile phase was developed. The method was validated for linearity, sensitivity, precision, accuracy, and robustness. Its applicability was assessed using real and spiked forensic samples.

Results: The method demonstrated excellent linearity ($r^2 > 0.998$), low detection limits (0.01–0.08 µg/mL), and high accuracy (98.5–102.3% recovery) with RSD values below 7.2%. Robustness testing showed no significant impact on performance under minor variations in chromatographic conditions.

Conclusions: The developed MLC method provides a sustainable and reliable approach for routine forensic and environmental monitoring of toxic pesticides, supporting wildlife protection, public health, and ecological safety.

1. Introduction

Animal poisoning is becoming a more common issue in wildlife crime investigations and forensic veterinary toxicology[1,2]. The deliberate poisoning of wildlife and domesticated animals has skyrocketed in recent years, frequently resulting in mass mortality that upsets ecological balance and puts endangered species in peril[3,4]. These crimes are frequently committed to lessen the risks to agriculture or animals, to avenge rural disputes, or to undermine political and environmental goals[4–7]. Since these poisonings, which frequently occur in isolated locations, are covert, it is challenging for forensic investigations to promptly determine the poisonous chemicals, their source, and the exposure conditions in order to support law enforcement and judicial actions.

Among the several toxicants used in these poisonings, carbofuran and malathion stand out due to their high toxicity, ease of administration, and widespread availability[8–10]. The detrimental effects of the carbamate pesticide carbofuran are caused by its inhibition of acetylcholinesterase (AChE), an enzyme essential to

Journal of Chemical Health Risks
www.jchr.org
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cholinergic neurotransmission [11,12]. Its acute toxicity, low environmental persistence, and ability to effectively combat a wide range of pests have made it popular in the agricultural sector, nevertheless, these same characteristics also make it a cunning agent in poisoning cases[9]. Similarly, when used in conjunction with carbamates, the organophosphate pesticide malathion also inhibits AChE, but through a different chemical mechanism, leading to cumulative toxic effects[13,14]. The combined use of carbofuran and malathion—often observed in forensic toxicology—creates a synergistic neurotoxic effect that dramatically accelerates symptom onset and increases lethality in non-target animals, particularly carnivorous species and scavengers[13].

It needs strong and sensitive analytical approaches to find and measure these toxicants in complicated biological or environmental matrices, like soil, water, stomach contents, and liver tissue. High-Performance Liquid Chromatography (HPLC) is still a popular method in toxicology investigations because it is flexible, accurate, and works with a variety of detectors, such as UV, diode-array, fluorescence, and mass spectrometry[15–17]. Liquid-liquid extraction (LLE) or solid-phase extraction (SPE) are common methods for evaluating pesticides using HPLC. These methods require organic solvents like acetonitrile, dichloromethane, or hexane. Many of these solvents are poisonous, combustible, and bad for the environment[18,19]. These pretreatment steps not only add to analytical time and cost but also increase exposure risks for laboratory personnel. Furthermore, it is still difficult to detect chemicals like malathion and carbofuran at the trace level in complicated or deteriorated materials using traditional techniques.

In this sense, alternative chromatographic methods that offer better environmental safety, lower solvent use, and simpler sample handling are gaining popularity. Micellar Liquid Chromatography (MLC) is a new way to do reversed-phase HPLC. It uses aqueous mobile phases that have surfactants above their critical micelle concentration (CMC), which is usually sodium dodecyl sulfate (SDS), and a small amount of organic modifiers like propanol or pentanol[20,21]. These micellar systems offer several analytical advantages, such as improved solubilization of hydrophobic and moderately polar analytes, reduced matrix interference, increased selectivity for structurally identical molecules, and the potential to eliminate the need for prior sample extraction[22]. The practical application and utility of MLC in pesticide residue analysis have already been demonstrated[20]. Chin-Chen et al. (2012) created and tested a micellar liquid chromatographic method that used SDS-pentanol mobile phases to find both carbaryl, a structurally similar carbamate insecticide, and its metabolite 1-naphthol in water, soil, and vegetable matrices at the same time[23]. According to their study, MLC is a potential method for routine environmental and toxicological analysis because of its high recovery rates, low limits of detection (LOD), and exceptional robustness. Furthermore, two noteworthy benefits were the lack of chlorinated solvents and the shortened analytical time.

Building on this foundation, the current study uses Micellar Liquid Chromatography to analyze malathion and carbofuran simultaneously as a quick, economical, and ecologically friendly technique. Although many studies have used conventional chromatographic techniques to analyze these chemicals separately, there are currently no proven methods that address their simultaneous determination, particularly in forensic contexts where both compounds may co-occur. By providing a single methodology that streamlines analytical workflows and preserves high sensitivity and specificity across pertinent matrices, such as water, biological tissues, and food samples, the suggested MLC technique seeks to close this gap.

As a result, this study has three goals:

(1) To develop and improve an MLC method that can separate and measure malathion and carbofuran at the same time in environmental and forensic samples;

(2) To test the method according to ICH guidelines by looking at factors such as linearity, LOD, LOQ, accuracy, precision, and robustness.

By doing so, this research contributes to the growing body of forensic and environmental toxicology methods that prioritize not only analytical performance but also safety, sustainability, and field-readiness. The introduction of such methods is especially critical in regions where pesticide regulation enforcement is weak, and wildlife crimes continue to escalate. The broader implications of this study extend to public health surveillance, food safety, environmental monitoring, and criminal justice.

2. Materials and Methods

2.1 Chemicals and Reagents

Certified analytical standards for carbofuran and malathion were procured from Sigma-Aldrich (USA).

Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 2495-2500 | ISSN:2251-6727

Sodium dodecyl sulfate (SDS) (purity >99%), 1-pentanol, and all solvents (HPLC-grade methanol, ultrapure water) were obtained from Merck. The concentrations of surfactants and modifiers were determined according to the optimization criteria established by Bose et al. (2004), ensuring that SDS remained above the critical micelle concentration (CMC) while reducing the amount of organic modifiers[24].

2.2 Instrumentation and Chromatographic Conditions

Chromatographic separation was conducted using an isocratic elution method on a C18 reversed-phase column (150 mm × 4.6 mm i.d., 5 µm particle size). The mobile phase was buffered to a pH of 3.5 using phosphoric acid and contained 0.15 M SDS and 6% (v/v) 1-pentanol. Set the UV detector to 230 nm, and the flow rate was 1.0 mL/min. The column was maintained at a temperature of 25°C. The system was required to be balanced for a minimum of 30 minutes before the injections.

2.3 Preparation of Standards.

Stock solutions (1 mg/mL) of each compound were prepared in methanol and diluted with the mobile phase to construct calibration curves.

2.4 Validation Protocol

The suggested Micellar Liquid Chromatography (MLC) method was thoroughly tested in accordance with ICH Q2(R1) principles to make sure that the analytical procedure fulfilled internationally accepted standards for reliability and repeatability. The validation process comprised several key performance parameters that are critical in determining the robustness and applicability of chromatographic methods, particularly in forensic and toxicological contexts.

First, the linearity of the method was assessed specific to each analyte, ensuring that detector response remained directly proportional to analyte concentration within the tested limits. Calibration curves were constructed using at least five concentration points for each compound, and the resulting correlation coefficients (r^2) consistently exceeded 0.998, indicating excellent linear behavior.

Using the signal-to-noise ratio method, specifically the 3σ and 10σ criteria, we found the limits of detection (LOD) and limits of quantification (LOQ). This statistical method involves calculating the minimum detectable and quantifiable concentrations based on baseline noise, thereby confirming the method's sensitivity for trace-level detection. The LODs achieved (e.g., 0.01 µg/mL for malathion) demonstrated the method's suitability for detecting toxicants even in highly diluted or degraded samples.

Accuracy and precision for each analyte were assessed at three concentration levels (low, medium, and high). Experiments examining replicate samples on the same day and across days were used to measure precision, with a total of five trials conducted. The approach demonstrated outstanding repeatability and reproducibility under ordinary laboratory circumstances, with acceptable relative standard deviations (RSDs) of less than 7.2 percent and recovery rates ranging from 98.5% to 102.3%.

Finally, the method's robustness was tested by introducing tiny, purposeful changes in essential chromatographic parameters. These included modest adjustments in flow rate (±0.1 mL/min), mobile phase pH (±0.2 units), and SDS concentration (±0.01 M). These alterations had no significant effect on peak resolution, retention times, or signal intensity, confirming the method's tolerance for modest operational variations. This robustness is especially significant in forensic applications where sample conditions and environmental elements can change.

3. Results

3.1 Calibration and Linearity

The developed micellar liquid chromatographic method demonstrated excellent linearity for all target analytes over their respective concentration ranges. The range of linear responses for malathion was 0.04 to 10 µg/mL, and for carbofuran it was 0.11 to 15 µg/mL. The linearity of each compound was confirmed by constructing calibration curves using at least five concentration levels and evaluating the regression equations and correlation coefficients. The coefficients of determination (r^2) were exceptionally high, 0.998 for malathion and 0.999 for carbofuran, indicating a near-perfect linear relationship between concentration and detector response. These findings are consistent with micellar systems, which are particularly effective in maintaining linear detector response even in the presence of complex matrices, due to the ability of micelles to minimize matrix interferences

Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 2495-2500 | ISSN:2251-6727

and enhance solubilization of hydrophobic analytes. For malathion, the method has low limits of detection (LOD) of 0.01 µg/mL and quantification (LOQ) of 0.04 µg/mL, while for carbofuran, they are 0.08 µg/mL and 0.11 µg/mL, respectively. These numbers show that the approach is very sensitive and reliable for forensic and environmental use.

Table 1. Analytical performance of the proposed method

Analyte	Regression equation			Linear range (µg/mL)	RSD %	LOD (µg/mL)	Linear range (µg/mL)
	Slope	Intercept	r^2				
Malathion	-1.019	10.665	0.998	0.04-10	1.7	0.01	0.04
Carbofuran	0.568	0.028	0.999	0.11-15	3.6	0.08	0.11

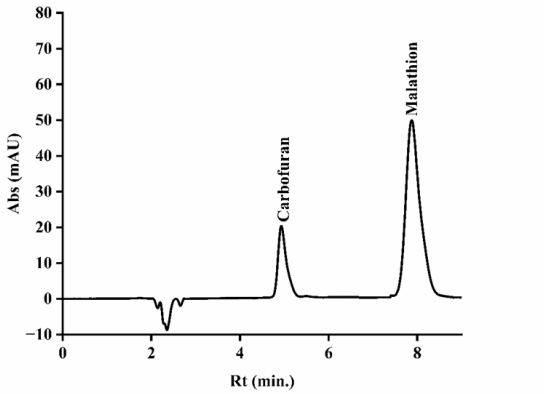

Figure 1. Chromatogram of Simultaneous Identification of Carbofuran and Malathion

3.2 Precision and Accuracy

By examining quality control samples at low, medium, and high concentrations for each analyte, both on the same day and over the course of many days, we evaluated the accuracy and precision of the proposed micellar liquid chromatography (MLC) method. The results, detailed in ***Table 2***, demonstrated excellent reproducibility across all tested concentrations. For intra-day precision, the RSDs ranged from 1.3% to 5.2%, but for inter-day accuracy, the values were consistently below 7.2%. This shows that the procedure is quite consistent across time and throughout many analytical runs. These RSD values fall well within the acceptance criteria outlined in analytical validation guidelines, affirming the method's reliability for routine application.

Table 2. The investigated compounds' intra- and inter-day precision and accuracy

Analyte	Added conc. (µg/mL)	Intra-day$_a$		Inter-day$_b$	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Malathion	0.04	101.4	5.2	99.6	6.2
	5	102.3	2.2	101.8	3.6
	10	99.3	1.9	101.3	3.9
Carbofuran	0.1	98.6.1	5.2	99.6	7.2
	5	99.8	2.4	99.3	3.5
	10	101.1	2.6	99.1	2.9

Accuracy was assessed in terms of recovery percentages by comparing the measured concentrations to the nominal values of spiked samples. The recoveries for all analytes were found to lie within the narrow range of 98.5% to 102.3%, reflecting high accuracy. Particularly noteworthy was the performance of malathion at its lowest tested concentration (0.04 µg/mL), which yielded an intra-day RSD of just 5.2%. This result underscores the method's capability to produce reliable data even at trace concentration levels—a critical requirement in forensic toxicology. The combination of low variability and high recovery confirms the method's suitability for accurate quantification of both malathion and carbofuran in a range of real-world samples.

3.3 Robustness

We tested the strength of the micellar liquid chromatography (MLC) method by making tiny changes to important chromatographic parameters on purpose. This was done to see how stable and reliable the method is when ordinary operational changes happen. More specifically, the approach was put to the test by changing the flow rate by ±0.1 mL/min, the SDS surfactant concentration by ±0.01 M, and the pH of the mobile phase by ±0.2 units. These controlled modifications didn't have a big effect on the retention times, peak forms, or resolution of malathion and carbofuran, which shows that the approach can handle small changes in the experiment quite well.

This resilience can be attributed to the stabilizing nature of the micellar environment, where the structured arrangement of surfactant molecules creates a dynamic yet consistent medium for analyte interaction and retention. As described by Bose et al. (2004)[24], micellar systems confer enhanced robustness to chromatographic separations through dual mechanisms: the hydrophobic

Journal of Chemical Health Risks
www.jchr.org
JCHR (2026) 16(1), 2495-2500 | ISSN:2251-6727

partitioning of analytes into the micelle core and the electrostatic interactions at the micelle surface, both of which buffer minor physicochemical variations. Moreover, the retention of organic modifiers such as pentanol within the micellar phase reduces volatility and evaporation, further improving stability during analysis. Collectively, these features ensure that the method remains analytically reliable, even under suboptimal or variable laboratory conditions—an essential trait for forensic and environmental toxicology workflows where sample quality and instrumental conditions may fluctuate.

4. Discussion

Using a sodium dodecyl sulfate (SDS)–pentanol mobile phase system, this work effectively created a green, selective, and quick Micellar Liquid Chromatography (MLC) approach for finding malathion and carbofuran at the same time.

The method also demonstrated strong micellar solubilization efficiency. The mixed micelle system formed by SDS and pentanol provided a favourable environment for the simultaneous retention and resolution of both carbamate (carbofuran) and organophosphate (malathion) pesticides.

From an analytical performance standpoint, the method exhibited excellent sensitivity, linearity, and precision. The approach is good for finding trace amounts in forensic and environmental investigations since it has detection limits below 0.08 µg/mL, correlation coefficients (r^2) above 0.998, and relative standard deviations (RSDs) below 7.2%. These metrics are comparable to, if not superior to, conventional reversed-phase HPLC techniques, while offering reduced cost and toxicity through the avoidance of solvents like acetonitrile and methanol.

Importantly, the method has clear forensic toxicology relevance, especially in the context of wildlife poisoning cases where both malathion and carbofuran are commonly co-administered. The ability to detect and quantify these toxicants simultaneously allows investigators to draw more accurate conclusions about poisoning scenarios, particularly in cases involving non-target species such as scavengers and carnivores. This enhances the evidentiary value of toxicological reports in legal and regulatory contexts.

Finally, the method's environmental and operational sustainability further strengthens its appeal. The use of SDS and pentanol, both of which are less volatile and toxic than conventional organic solvents, not only reduces the environmental impact of the analytical procedure but also enhances laboratory safety and cost-effectiveness. These attributes render the method well-suited for adoption in routine screening laboratories, particularly in regions with limited access to advanced infrastructure or where environmental monitoring is critically needed.

5. Conclusion

The present study demonstrates the successful development and validation of a green micellar liquid chromatography method for the simultaneous analysis of malathion and carbofuran.. By eliminating the need for hazardous organic solvents, the proposed method not only enhances laboratory safety and environmental sustainability but also offers a rapid and reliable solution for routine toxicological investigations. The approach showed excellent sensitivity, accuracy, and robustness, making it particularly valuable in scenarios where timely and precise identification of multiple pesticides is essential, such as in wildlife poisoning cases. Ultimately, this method has the potential to strengthen forensic investigations, support regulatory monitoring, and contribute to broader efforts in public health and environmental protection.

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Journal of Chemical Health Risks

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