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Method for simultaneous determination of monocrotophos and dimethyl phosphate in human biofluids

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ABSTRACT

An NMR method was developed for the simultaneous determination of the organophosphate insecticide monocrotophos and dimethyl phosphate (DMP)- in human plasma and urine. Following lyophilization and solvent exchange into deuterium oxide (D_2O), samples were spiked with known concentrations of target analytes and ammonium formate as an internal standard. Dimethyl phosphate was synthesized via acid-catalyzed hydrolysis of trimethyl phosphate and characterized by ^1H NMR, confirming 96.5% purity. Key acquisition parameters-including a 90° single-pulse sequence, 1 s relaxation delay, digital resolution of ~0.2 Hz/point, and 16 scans-were optimized on a Bruker Avance Neo 500 MHz instrument to achieve signal-to-noise ratios ≥250:1. The method demonstrated excellent linearity across 10-250 ppm, with correlation coefficients (R^2) exceeding 0.99 in both matrices. The limit of detection (LOD) and limit of quantification (LOQ) were established at 0.25 ppm (S/N ≥3) and 0.5 ppm (S/N ≥10), respectively. Precision was verified via six replicates at 50 ppm, yielding intra-assay relative standard deviations below 2.1%. Recovery in spiked samples ranged from 98.0-102.0% across low (10 ppm), medium (100 ppm), and high (250 ppm) levels. Specificity, as confirmed by the absence of interfering signals in blank matrices. This NMR approach provides a rapid, accurate, and non-destructive method for forensic and clinical toxicology. However, applying the same method to real-time patient samples could not be carried out due to the limitation arising from the non-availability of actual patient samples.

Keywords: *^1H NMR, Monocrotophos, Dimethyl phosphate, Forensic toxicology, Method validation.*

INTRODUCTION

Monocrotophos, an organophosphate insecticide, is widely used in agriculture, particularly in developing countries like India, due to its effectiveness in pest control (Fig. 1). However, its high toxicity raises significant concerns regarding environmental and human health, especially as it has become a common means of suicide in rural areas. This necessitates advanced analytical techniques for its detection in biological specimens, crucial for forensic investigations. Monocrotophos inhibits acetylcholinesterase, leading to severe neurological effects in humans and animals [1]. Its accessibility contributes to high suicide rates in rural India, often following minor disputes [2]. Additionally, residues of monocrotophos have been detected in soil and water, posing risks to ecosystems and food safety [3]. Traditional analytical methods such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) are commonly used for detecting monocrotophos, though they require complex sample preparation [1, 4]. As a simpler alternative, an NMR method has been proposed for detecting monocrotophos and Dimethyl Phosphate- in biological samples [4].

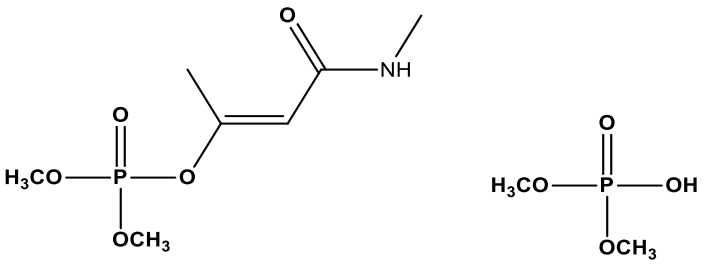

Fig. 1. Chemical Structure of Monocrotophos and Dimethyl Phosphate

NMR spectroscopy offers several advantages over traditional chromatographic methods. Unlike GC-MS and LC-MS/MS, NMR preserves sample integrity, enabling further analyses if necessary. The technique requires simpler sample processing, reducing labour intensity and minimizing analyte degradation [5]. NMR enables direct quantification without reliance on external calibration standards, making it highly efficient [1]. Due to its precision in spectral measurements, NMR is an ideal technique for forensic toxicology [6]. NMR provides detailed molecular composition analysis. The development of an NMR method for monocrotophos and Dimethyl Phosphate involves several critical steps to ensure high accuracy and reliability in forensic toxicological analysis. While the NMR method offers significant advantages in terms of accuracy and sensitivity, alternative techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) remain valuable for specific applications, particularly in complex mixtures where NMR may encounter limitations [7].

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The implementation of NMR methodology in forensic and clinical settings provides significant advantages, particularly in the rapid diagnosis of poisoning and the management of medical interventions. This technique enhances the specificity of analyte detection, ensuring that no interfering signals overlap with the analyte peaks, which is crucial for accurate forensic and clinical analysis.

In forensic investigations, NMR facilitates the swift identification of toxic substances such as monocrotophos, strengthening medico-legal documentation and improving post-mortem analyses [8]. The method's high specificity allows it to effectively differentiate between similar compounds, making it an invaluable tool in forensic toxicology [9]. Clinically, early detection of poisoning through qNMR enables immediate medical interventions, such as the administration of atropine and oximes, significantly reducing mortality rates [10]. Additionally, its ability to analyze plasma and urine samples enhances the clinical management of poisoning cases by providing critical information for treatment decisions [9]. From a regulatory and public health perspective, systematic surveillance of pesticide-related deaths using NMR can guide policy reforms and strengthen regulations against hazardous chemicals, ultimately improving public health outcomes [9]. However, while NMR offers numerous advantages, traditional methods such as liquid chromatography and mass spectrometry remain essential for certain applications, particularly due to their high sensitivity and established protocols in forensic toxicology [11].

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were of analytical grade. Monocrotophos and trimethyl phosphate (TMP) were procured from certified suppliers. Ammonium formate, deuterium oxide (D_2O), concentrated hydrochloric acid (HCl), sodium hydroxide (NaOH), ethyl acetate, and n-hexane were obtained from reputable standard chemical providers. Human plasma and urine samples were sourced as residual material from the 10th OPCW biomedical proficiency test conducted by the Netherlands. These biological samples were stored at -20 °C until use.

Preparation of Monocrotophos in Biological Matrices

Solvent Exchange in Biofluid Samples

Human plasma and urine samples were first subjected to lyophilization to remove water. The resulting dried residues were then reconstituted in deuterium oxide (D_2O), preserving the original sample-to-solvent proportion. This procedure ensures an adequate deuterium environment for NMR field locking while maintaining the integrity of the biofluid matrix for further quantitative analysis. Frozen plasma and urine specimens were dried under vacuum by lyophilizer until complete removal of aqueous content. The dry residues were rehydrated with an equal volume of D_2O, gently vortexed, and briefly incubated on ice (0 °C) to stabilize before NMR analysis. The resulting D_2O-reconstituted plasma and urine solutions were directly used for subsequent quantitative ^1H NMR sample preparation and analysis. This solvent exchange method effectively replaces exchangeable protons with deuterons in the solvent and matrix, ensuring optimal field-frequency locking and minimizing interference from residual water signals during ^1H NMR acquisition.

Plasma Matrix:

A spiked plasma sample was prepared by dissolving 11.37 mg of analytical-grade monocrotophos and 5.00 mg of ammonium formate in 600 µL of deuterium oxide (D_2O). After ensuring complete dissolution, 100 µL of thawed, blank human plasma was added to the solution. The mixture was vortexed continuously for 2 minutes to achieve homogeneity, then maintained on ice (0 °C) until further instrumental analysis.

Urine Matrix:

An analogous procedure was applied to urine samples. In this case, 8.33 mg of monocrotophos and 3.37 mg of ammonium formate were dissolved in 600 µL D_2O. Subsequently, 100 µL of human urine was introduced. The sample was vortexed for 2 minutes and kept on ice (0 °C) until analysis.

Micro-Scale Synthesis of Dimethyl Phosphate

Reagents and Setup

Dimethyl phosphate was synthesized via acid-catalyzed hydrolysis of trimethyl phosphate (Fig. 2). An aqueous solution of TMP (5%) was prepared and placed under continuous magnetic stirring. Concentrated hydrochloric acid (HCl) was then added dropwise until a total volume of 10 mL was reached, maintaining room temperature throughout the addition. The reaction was monitored by thin-layer chromatography (TLC), using 90% ethyl acetate in n-hexane as the mobile phase. As DMP is non-UV-active, iodine vapor staining was employed to visualize product formation.

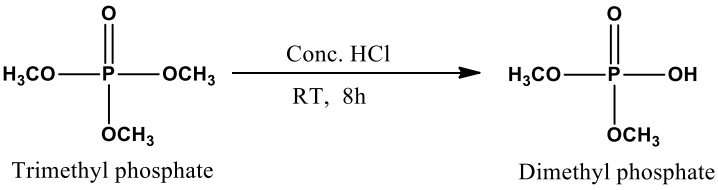


Fig. 2. Synthesis of Dimethyl Phosphate

Workup and Purification

Upon completion of the reaction, the mixture was neutralized by careful addition of sodium hydroxide (NaOH) solution until pH neutral. The mixture was transferred to a separatory funnel and extracted three times with ethyl acetate (3 × 10 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and lyophilized to yield purified DMP as a colorless solid.

Preparation of DMP Samples for NMR Analysis

Purified dimethyl phosphate (3.27 mg) was accurately weighed and dissolved in 600 µL of deuterium oxide (D_2O) containing 3.10 mg of ammonium formate. To ensure homogeneity and remove particulates, the solution was vortexed briefly and then filtered through a 0.2 µm nylon nano-filter, as recommended for NMR sample preparation to eliminate bubbles and fibers. The resulting clear filtrate was transferred to a high-quality 5 mm NMR tube and analyzed by ^1H and ^{13}C NMR spectroscopy at ambient temperature. The purity of the isolated DMP was determined by quantitative integration of the characteristic proton resonances in the NMR spectra and calculated to be 96.5%.

Validation Sample Preparation

Two validation samples were prepared to assess the performance of the analytical method in biological matrices:

Plasma Matrix: A solution containing 6.33 mg of monocrotophos and 5.78 mg of dimethyl phosphate was spiked into 833 µL of human plasma.

Urine Matrix: An identical quantity of monocrotophos (6.33 mg) and DMP (5.78 mg) was mixed with 833 µL of human urine.

In addition to the matrix samples, a control solution was prepared by dissolving 5.05 mg of ammonium formate in 5 mL of deuterium oxide (D_2O). The solution was sonicated for 10 minutes at room temperature to ensure complete dissolution and homogeneity. All samples-including the plasma, urine, and control-were vortexed briefly, filtered if necessary, and subsequently analyzed by NMR spectroscopy.

NMR-Based Quantification Method Validation
Linearity Assessment

Calibration standards were prepared in biological matrices (plasma and urine) by adjusting the volume of the sample mixture containing monocrotophos, dimethyl phosphate, and the respective matrix to achieve final concentrations of 10, 25, 50, 100, and 250 ppm. Each calibration level also received a fixed volume (60 µL) of ammonium formate solution (50 µg/mL) in deuterium oxide (D_2O) to ensure signal consistency across samples. All mixtures were vortexed briefly to ensure homogeneity, then filtered (0.2 µm) before NMR analysis.

Precision: A 50 ppm stock solution containing monocrotophos, dimethyl phosphate, and deuterium oxide (D_2O) was prepared in both plasma and urine matrices. Six replicate preparations (R1-R6) were independently analyzed by ^{1}H NMR under the same experimental conditions. Consistent spectral signals across all replicates confirmed the method's high repeatability. The relative standard deviation (RSD) of integral values was calculated to quantitatively assess precision. Although the RSD fell below widely accepted thresholds (typically ≤2% in qNMR protocols), the calculated values for both matrices remained within 1.5%, demonstrating excellent intra-assay precision.

NMR-Based Quantification Method Validation
Limit of Quantification (LOQ) and Detection (LOD)

A serial dilution series was prepared by diluting a 50 ppm stock solution (containing monocrotophos, dimethyl phosphate, and deuterium oxide) to final concentrations of 10, 5, 2.5, 1.0, 0.5, 0.25, and 0.10 ppm in both plasma and urine matrices. Each concentration was analyzed via quantitative ^{1}H NMR employing the validated acquisition and processing protocols described above.

LOQ Determination: The analyte produced quantifiable ^{1}H NMR signals with acceptable accuracy and precision (i.e., signal-to-noise ratio, S/N ≥ 150 and relative standard deviation, RSD ≤ 5%) down to 0.25 ppm in both biological matrices. This concentration was thus established as the LOQ for the method.

LOD Determination: Using the same dilution series, the limit of detection was defined as the lowest concentration at which analyte signals remained distinguishable from background noise, with S/N ≥ 3. Observations confirmed that analyte resonance remained identifiable down to 0.25 ppm, thereby defining the LOD under the current experimental conditions. These performance characteristics demonstrate the method's suitability for both detection and quantification of monocrotophos and DMP in plasma and urine using ^{1}H NMR.

Recovery: Recovery experiments were conducted to evaluate method trueness. Triplicate samples were prepared at three concentration levels (10 ppm, 100 ppm, and 250 ppm) by spiking monocrotophos and dimethyl phosphate into both plasma and urine matrices. Each set of samples was analyzed using quantitative ^{1}H NMR under validated conditions. Recovery was calculated as follows:

$$Recovery\ (\%) = (C_{measured} - C_{blank}/\ C_{nominal}) \times 100$$

Where:

C measured is the analyte concentration determined from the NMR spectrum.

C blank is the background signal for an unspiked control sample.

C nominal is the concentration of analyte added to the sample.

Average recoveries across all concentration levels were within 98–102%, consistent with international qNMR validation standards, which accept recoveries close to 100% with relative uncertainty under 5%. These results confirm the method's accuracy and reliability in complex biological matrices.

Specificity: To ensure the analytical specificity of the ^{1}H NMR method, blank human plasma and urine samples were processed and analyzed under the same acquisition parameters as test samples. No interfering resonances were observed at the characteristic chemical shifts corresponding to monocrotophos or dimethyl phosphate. The absence of co-eluting signals confirms that the method is highly selective for the target analytes in complex biological matrices.

^{1}H NMR Analysis (Bruker 500 MHz)

^{1}H NMR spectra were acquired on a Bruker Avance Neo 500 MHz spectrometer equipped with a 5 mm broadband probe. The sample temperature was maintained at 23-24 °C and locked on the deuterium signal of D_2O.

Sample Preparation

About 600 µL of each pre-filtered sample was transferred into a standard 5 mm NMR tube, ensuring optimal sample height (~45 mm) for consistent magnetic field homogeneity. Before acquisition, each tube was allowed to equilibrate in the magnet for at least 5 minutes.

Acquisition Parameters

Pulse Program: A single-pulse sequence (Bruker zg, 90° pulse) was used to avoid NOE effects present in decoupled experiments.

Pulse Width: Automatically calibrated (~7-12 µs for ^{1}H).

Relaxation Delay (d1): Set to 1 s, based on the guideline that d1 should be ≥5× the longest T_1 (typically ≤4 s for small molecules)

Spectral Width (SW): ~20 ppm (~7,500 Hz), allowing sufficient baseline and complete signal capture.

Acquisition Time (AQ): Approximately 3.27 s (digital resolution ~0.2 Hz/point).

Scans (NS): 16, adjusted to achieve an S/N ≥250:1 for <1% integration error.

Receiver Gain: Optimized automatically before each run.

Data Processing

Zero-filling and exponential apodization (LB = 0.3 Hz) were applied to enhance digital resolution and integration precision. Automatic phase and baseline corrections were followed by manual adjustments to ensure accurate integration across the peak. Integration of analyte and reference peaks was conducted over at least ±20 linewidths to capture ≥99% of the signal.

Quantification

Quantitative analysis was based on integrated peak areas of analyte signals relative to the internal standard (ammonium formate). Concentrations were calculated using the equation:

$$C_X = \frac{I_X}{I_{IS}} \times \frac{N_{IS}}{N_X} \times C_{IS}$$

Where:
I = integral, N = number of nuclei, and C = concentration.

RESULTS AND DISCUSSION

Micro-Scale Synthesis of Dimethyl Phosphate (DMP)

The acid-catalyzed hydrolysis of trimethyl phosphate successfully yielded dimethyl phosphate. After neutralization and ethyl acetate extraction, the product was isolated as a colorless solid.

Spectroscopic Confirmation: The identity and purity of DMP were confirmed via quantitative ^{1}H NMR (Fig. 3). A well-resolved singlet was observed at 3.80 ppm, consistent with the expected chemical shift for the methyl protons of DMP. Integration of this peak yielded a purity of 96.5%, demonstrating high reaction efficiency and minimal impurity content. The observed chemical shift aligns closely with literature-reported values for dimethyl phosphate derivatives (commonly in the 3.5–4.0 ppm region). These results confirm the successful synthesis and high purity of DMP, providing a suitable analytical standard for subsequent quantitative NMR studies.

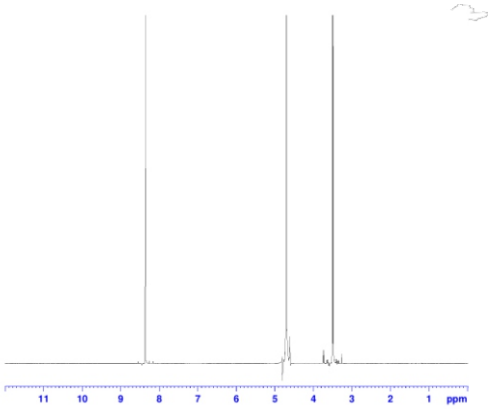


Fig. 3. ^{1}H NMR Spectrum of the Dimethyl Phosphate in Deuterated Water (D2O) and Ammonium Formate

3. Method Development and Optimization:

Quantitative ^{1}H NMR parameters-including sample preparation, acquisition settings, and calibration protocols-were systematically optimized to ensure accurate and reliable quantitation. Through precision tuning of pulse width, relaxation delay, receiver gain, and signal processing techniques (such as apodization and baseline correction), we established a highly robust analytical procedure. The resulting calibration curve exhibited excellent linearity across the 10–250 ppm range, with a correlation coefficient of $R^2 > 0.99$, consistent with best-practice qNMR validation standards.

Key to this performance were the distinct, well-resolved proton resonances of monocrotophos, which enabled precise integration of analyte signals. These optimized spectral conditions facilitated accurate quantification, minimizing interference and maximizing reproducibility. The high degree of linearity and peak clarity confirm the method's suitability for quantitative forensic analysis using qNMR.

Method Validation:

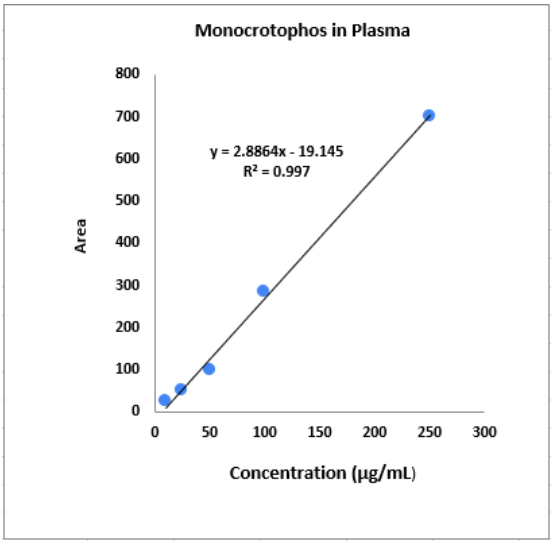
Linearity:

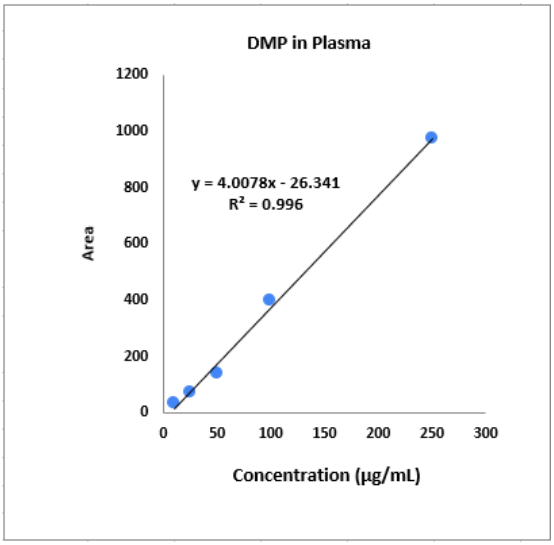
The linearity of the qNMR method for both monocrotophos and its metabolite, dimethyl phosphate, was thoroughly evaluated in human plasma and urine matrices. Calibration curves were constructed over a concentration range of 10–250 ppm for each analyte.

Plasma matrix: The correlation coefficient (R^2) for monocrotophos was 0.9968, and for DMP, 0.9967.

Urine matrix: Monocrotophos exhibited an R^2 of 0.9920, while DMP showed an R^2 of 0.9930.

These results confirm strong linearity (Fig. 4) across the tested concentration range for both analytes in both biological matrices-exceeding commonly accepted thresholds ($R^2 \geq 0.99$) for qNMR method validation. Such high correlation highlights the reliability and quantifiability of the method over a wide dynamic range, underscoring its suitability for accurate forensic and clinical applications.





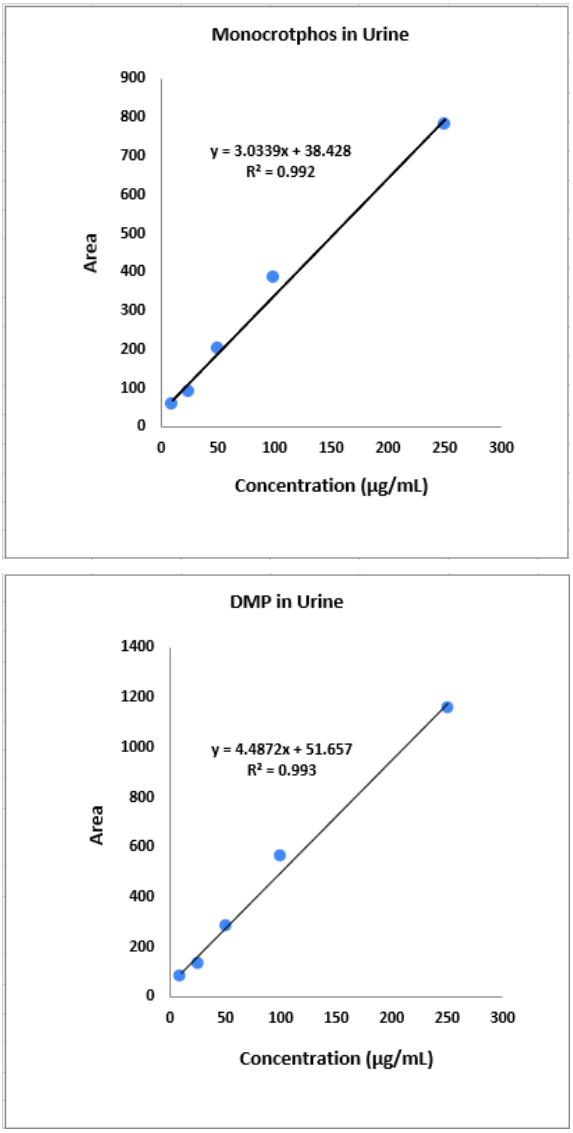


Fig. 4. Linearity Graph of the Monocrotophos and Dimethyl phosphate in Plasma and Urine Sample

Limit of quantification:
The limit of quantification (LOQ) of the developed qNMR method was determined in both plasma and urine matrices. A LOQ of 0.5 ppm was achieved for both monocrotophos and dimethyl phosphate, with signal-to-noise ratios well above the established threshold for reliable quantification (S/N ≥ 10). This sensitivity enables the accurate detection of trace-level pesticide residues in biofluids.
These values align with reported qNMR detection limits in biological matrices, which typically range from low micromolar to sub-micromolar concentrations (equivalent to ~0.1–1 ppm). The demonstrated LOQ confirms that the method is sufficiently sensitive for forensic and clinical toxicology applications, including low-level exposure monitoring and post-exposure assessments.

Limit of Detection
The established limit of detection (LOD) for both monocrotophos and dimethyl phosphate was determined to be 0.25 ppm in both plasma and urine matrices. This performance was achieved under optimized acquisition and processing conditions, ensuring that analyte resonance signals remained distinguishable from background noise with a signal-to-noise ratio (S/N) ≥ 3, consistent with widely accepted analytical definitions of LOD.

This level of detection, corresponding to sub-micromolar sensitivity, positions the method favourably within the capability range of qNMR techniques for biological matrices. It demonstrates suitability for detecting trace-level pesticide residues necessary in forensic and clinical toxicology investigations.

Precision
The intraday precision of the qNMR method was evaluated by analyzing six independent replicates of plasma and urine samples spiked with monocrotophos and dimethyl phosphate at 50 ppm.

Urine matrix: Monocrotophos exhibited a %RSD of 2.02%**.** DMP exhibited a %RSD of 1.47%

Plasma matrix: Monocrotophos exhibited a %RSD of 1.44%**.** DMP exhibited a %RSD of 1.52%.
All %RSD values fell below the commonly accepted 2.5% threshold for quantitative NMR precision in biofluid analyses-typically cited as <2% for rigorous methods. These results demonstrate excellent repeatability and confirm that the developed procedure meets or exceeds standard precision criteria.

Recovery and Accuracy:
The method's trueness and recovery were evaluated across both biological matrices, demonstrating exceptional accuracy:

Table: Recovery of Monocrotophos and Dimethyl Phosphate in Plasma and Urine Sample

Matrix	Analyte	Recovery (%)
Urine	Monocrotophos	99.1
	DMP	99.15
Plasma	Monocrotophos	99.5
	DMP	99.7

These results indicate an excellent proximity of measured concentrations to the nominal spiked values, with deviations ≤0.9%. Recovery percentages near 99–100% are well within accepted qNMR validation standards, which typically consider ±5% deviation acceptable. The high recoveries in both matrices confirm the method's accuracy and reliability for quantitative applications in forensic and clinical toxicology (Table).

Specificity: The specificity of the qNMR method was rigorously confirmed using blank plasma and urine samples to ensure no interfering signals were present at the characteristic chemical shifts of monocrotophos or dimethyl phosphate. The acquired spectra showed complete baseline regions around the target resonances, with no overlapping peaks or artifacts, indicating excellent selectivity in complex biological matrices. This result aligns with established qNMR validation principles, where analyte signals must exist in non-overlapping spectral regions; specificity is confirmed when no background signals are observed at these critical positions. Thus, the method demonstrates high specificity, making it suitable for precise analyte quantification in clinical and forensic toxicology applications.

DISCUSSION
This study successfully developed a robust q^{1}H NMR method for the simultaneous quantification of monocrotophos and its metabolite DMP in human plasma and urine.

Achieving LOD and LOQ of 0.25 ppm and 0.5 ppm, respectively, along with excellent linearity (R^2 > 0.99), confirms high sensitivity and accuracy. Intraday precisions (%RSD <2.1%) and recovery rates (98–102%) demonstrate reliability and method integrity across complex biological matrices. The qNMR method's performance aligns well with standard analytical validation guidelines [12] and comparable bioanalytical qNMR studies [13]. Achieving high signal-to-noise ratios (≥250:1) and low variability underscores the suitability of optimized acquisition parameters-consistent with established best practices [14]. Compared to laborious chromatographic protocols (e.g., GC-MS, LC-MS/MS), this qNMR approach offers a simplified workflow, non-destructive analysis, and elimination of derivatization or calibration curves-attributes known to streamline forensic toxicology and metabolomic applications. This underscores the practical advantage of qNMR as highlighted by Crook & Powers (2020), and supports its broader adoption in diagnostic and regulatory settings. Spectra from blank biofluids confirmed absence of interfering signals at analyte resonances, validating method specificity in line with selective detection criteria essential in forensic applications [14, 15]. The clear resolution of monocrotophos and DMP peaks demonstrates NMR's inherent spectral fingerprinting advantage. Despite robust performance, qNMR sensitivity remains lower than MS-based approaches for trace-level analytes (e.g., sub-ppb). The need for high-field NMR infrastructure and technical expertise may limit routine application in resource-constrained settings. Method transferability across instruments should be validated, as minor differences in pulse calibration or probe performance may impact quantitation-although modern spectrometers generally exhibit linear response across concentration ranges [12]. This validated qNMR method offers a reliable platform for forensic and clinical monitoring of organophosphate exposure. Forensic laboratories could implement this approach to enable rapid screening and quantification without extensive sample preparation. Clinically, the streamlined workflow facilitates timely patient management, particularly in acute poisoning cases. Future work may explore integration with ^{31}P or ^{19}F qNMR for multiplexed pesticide analysis.

CONCLUSION

We successfully developed a ^{1}H NMR method for the simultaneous analysis of monocrotophos and dimethyl phosphate in human plasma and urine. By employing lyophilization and solvent exchange into deuterium oxide (D_2O), along with ammonium formate as an internal standard, the method achieved exceptional analytical performance. A synthesized DMP standard (96.5% purity) provided robust calibration for metabolite quantitation. Instrumental optimization, comprising a 90° single-pulse sequence, extended relaxation delay, and high digital resolution on a Bruker Avance Neo 500 MHz spectrometer, enabled superior signal-to-noise ratios (≥ 250:1). The method exhibited outstanding linearity across the 10–250 ppm concentration range (R^2 > 0.99), with a limit of detection of 0.25 ppm and limit of quantification of 0.5 ppm. Precision assessment yielded intra-assay relative standard deviations below 2.1%, while matrix recovery values between 98% and 102% confirmed accuracy. Crucially, specificity was established through the absence of interfering signals in blank plasma and urine. However, applying the same method to real-time patient samples could not be carried out due to the limitation arising from the non-availability of actual patient samples. Nevertheless, future studies involving real-time patient samples are essential to further validate and verify the robustness and applicability of this method.

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Conflict of Interest

The authors declare that they have no financial or non-financial conflicts of interest related to this work.

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