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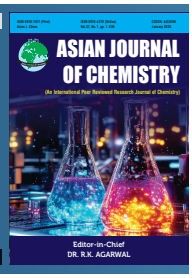

Optimization of QuEChERS for High-Sensitivity Pesticide Detection in Simulated Biological Matrices Using UPLC-MS/MS

PRATIK SINGH[1], ARVIND KUMAR MAURYA[2,3], PRIYANKA GOPI[1], JASJEET KAUR[4], SANJEEV KANOJIYA[2,3] and PRATEEK PANDYA[1,*]

[1]Amity Institute of Forensic Sciences, Amity University, Noida-201313, India
[2]Sophisticated Analytical Instrument Facility & Research, CSIR-Central Drug Research Institute, Lucknow-226031, India
[3]Academy of Scientific and Innovative Research (AcSIR), Ghaziabad-201002, India
[4]Department of Chemistry, Shaheed Rajguru College of Applied Sciences for Women (University of Delhi), Delhi-110096, India

*Corresponding author: E-mail: prateekpandya@gmail.com; ppandya@amity.edu

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This study presents an analytical method for detection and identification of pesticides in simulated biological matrices, *i.e.*, simulated gastric fluid (SGF) and simulated urine sample (SUS). The approach combines the QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation technique with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for pesticide analysis. A modified QuEChERS extraction protocol was developed to enhance pesticide recovery, improving both accuracy and reproducibility in pesticide quantification in seven different concentrations ranging from 2.5 ng/mL to 160 ng/mL. This combined methodology offers a robust tool for the precise detection and characterization of pesticide residues in biological fluids, with promising applications in toxicological analysis and forensic investigations. The developed method demonstrates the ability to reliably detect low concentrations of pesticide residues, establishing a strong foundation for comprehensive pesticide detection in complex biological matrices. As a supporting analysis, the molecular docking simulations were employed to explore the interaction dynamics of selected pesticides with key proteins unique to each matrix–pepsin (PEP) in SGF and Tamm-Horsfall (THF) protein in SUS. These simulations revealed the binding affinities and interaction strengths of the pesticides, providing further insight into their stability and persistence within different biological environments. This molecular perspective enhances the interpretation of pesticide residue behaviour, complementing the analytical results and deepening our understanding of pesticide dynamics in biological systems.

Keywords: Tetramethrin, Dimethoate, Pesticides, Molecular docking, Modified QuEChERS, UPLC-MS/MS.

INTRODUCTION

Pesticides are substances that tend to restrict, eradicate or prevent the growth of any organism that causes an allergen to a crop. Pesticides, insecticides, herbicides and fungicides are some of the classifications based on the organisms they manage [1,2]. Pesticides containing organophosphate compounds have been used for over 50 years. Nearly 34% of organophosphate pesticides are produced and sold for usage in agricultural sector worldwide [3]. Insecticides are still used, along with some fungicides and herbicides, although their widespread usage has resulted in numerous poisonings of non-target species, including numerous fatalities among humans, they have been and continue to be enormously beneficial in agricultural pest management throughout the world [4]. The toxicity of organophosphorus pesticides depends on the amount of sulfur present and the valency of phosphorus [5-7]. On the other hand, pyrethroid toxicity implies the sustained triggering of voltage-gated sodium channels in neural cells. This disturbance leads to constant tachycardia and increases neural excitability [8].

The detection and quantification of pesticide residues in biological matrices are crucial for protecting public health, particularly in light of the increasing use of pesticides in agriculture. This study focuses on optimizing the QuEChERS (quick, easy, cheap, effective, rugged and safe) method for the high-sensitivity detection of two specific pesticides: tetramethrin and dimethoate are widely used insecticides with distinct modes of action and applications. Tetramethrin, a synthetic pyrethroid, disrupts nervous systems by affecting sodium channels, causing paralysis and death. It is valued for its rapid action and mamm-

alian toxicity but raises concerns about environmental persistence and potential accumulation posing risks to non-target organisms and human health [9,10]. Dimethoate, an organophosphate, inhibits acetylcholinesterase (AChE), leading to acetylcholine buildup and nervous system overstimulation. While effective against various pests, it carries significant health risks, with acute exposure causing symptoms like headaches and respiratory distress and chronic exposure linked to long-term neurological damage. Its widespread agricultural use necessitates strict monitoring of residues in food for safety [11,12].

The QuEChERS method is widely recognized for its efficiency in extracting pesticide residues from complex biological matrices. It involves a simple two-step process: extraction with organic solvents and cleanup *via* dispersive solid-phase extraction (dSPE). Known for its speed and high pesticide recovery rates, QuEChERS is a preferred approach in residue analysis [13-17]. However, optimizing the method is essential to improve sensitivity and selectivity for detecting low concentrations of pesticides like tetramethrin and dimethoate. Ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) is a highly sensitive and precise tool for pesticide detection. UPLC ensures efficient separation of compounds with similar mass-to-charge ratios, ideal for analyzing complex matrices with multiple residue [18]. Tandem mass spectrometry enhances detection sensitivity through multiple reaction monitoring (MRM), enabling trace-level pesticide analysis in biological samples [18,19]. Combining QuEChERS with UPLC-MS/MS offers a robust and reliable approach for high-sensitivity pesticide detection.

EXPERIMENTAL

All chemicals used in this study were of analytical grade. Methanol and acetonitrile were purchased from Thomas Baker. while formic acid was purchased from Finar (Ahmedabad, India). Milli-Q water was produced using a Milli-Q system (Merck Millipore, India) and the chemicals used to prepare simulated biological matrices were purchased from SRL (Sisco Research Laboratories Pvt. Ltd., India). The DisQue™ CEN-QuEChERS salt pouch was purchased from Waters India. Ltd. Merck supplied both tetramethrin and dimethoate, the pesticides PESTANAL® analytical standard.

Preparation of simulated gastric fluid (SGF) and simulated urine sample (SUS) samples: SGF was prepared based on the procedure described by Wang *et al.* [20]. The formulation involved dissolving 0.03 M NaCl, 0.084 M HCl and 0.32% (w/v) pepsin in distilled water, with the final volume adjusted to 100 mL and by referring to other related studies. SUS were prepared based on the procedure reported by Stolarz *et al.* [21]. The composition included 16 g/L urea, 9.60 g/L chlorine, 5.40 g/L sodium, 1.35 g/L sulfate, 0.65 g/L magnesium, 0.20 g/L calcium and 0.20 g/L potassium, all dissolved in 1.0 L of distilled water. The pH was adjusted to 6, following the specifications provided by Sarigul *et al.* [22].

QuEChERS extraction: The QuEChERS extraction method was further optimized using a two-phase approach, enhancing its efficiency and effectiveness.

Step-1 Partitioning: A falcon tube was prepared with 5 mL each of SGF and SUS spiked with 7 different concentrations ranging from 2.5 to 160 ng/mL. To this, 10 mL of methanol was added as diluent and the mixture was thoroughly homogenized by vortexing for 7 min to ensure complete mixing.

Step-2 Clean-up: The obtained mixture underwent a purification process to remove moisture and water content using a Waters DisQue™ Pouch containing CEN-QuEChERS salt. The mixture was then centrifuged at 5000 rpm for 12 min at 2-8 ºC. The supernatant was transferred to a falcon tube containing 250 mg of $MgSO_4$ to eliminate residual moisture and water molecules. Subsequently, 250 mg of primary and secondary amine (PSA) was added to ensure compound uniformity. The sample was vortexed for 1 min and centrifuged again under the same conditions. The resulting extract was transferred to another falcon tube containing 150 mg of $MgSO_4$ and centrifuged for 12 min at 5000 rpm and 2-8 ºC. Finally, the supernatant was transferred into individual vials. A 5 µL aliquot of the prepared sample was directly injected into the LC-UPMS/MS instrument for analysis.

Calibration and quality control standards: The primary stock solution of tetramethrin and dimethoate was prepared by dissolving 50 µg of each compound in 1 mL of solvent, resulting in a concentration of 1000 µg/mL. This stock solution was then serially diluted in methanol to prepare calibration standards at seven different concentrations: 2.5, 5, 10, 20, 40, 80 and 160 ng/mL SGF and SUS samples were prepared using distilled water [23].

UPLC-MS/MS conditions: The qualitative and quantitative analysis was performed using a Waters H-Class Acquity UPLC system coupled with a Waters Xevo Tandem Quadrupole detector (Waters Co., Milford, USA) equipped with an electrospray ionization (ESI) source. Using a Waters Acquity BEH C_{18} column (50 mm × 2.1 mm, 1.7 µm) at a flow rate of 0.5 mL/min and the separation was performed. Sample (5 µL) were injected using an autosampler at 25 ºC. The mobile phase was made up of two solutions: acetonitrile (A) and a buffer containing 0.1% formic acid (B), which was a mixture of 95% water and 5% acetonitrile. The ratio of acetonitrile to buffer was set at 80:20 and stayed the same for 10 min. Multiple reaction monitoring (MRM) for tetramethrin and dimethoate was used in positive ion mode MS/MS analysis. A capillary voltage of 3.5 kV and a cone voltage of 30 V were used to operate the ESI source. The gas used for nebulizing and drying was nitrogen, which flowed at 50 L/h and 950 L/h, consequently at 120 ºC and 350 ºC, as well, the source and desolvation temperatures were maintained constantly. The collision-induced dissociation (CID) gas used was argon. Mass Lynx V4.1 software was used for data acquiring and processing, ensuring precise interpretation.

Molecular docking study: The binding mechanism of tetramethrin and dimethoate with PEP and THF was determined by molecular docking simulations using Auto Dock Vina [24,25]. The crystal structures of PEP (PDB 1PSN, with a resolution of 2.20 Å) and THF (PDB 7Q3N, with a resolution of 7.40 Å) were taken from the Protein Data Bank [18]. The 3D structure of tetramethrin and dimethoate was obtained in sdf format from PubChem and then converted into pdb format with

Biovia Discovery Studio [19]. A minimization was performed for tetramethrin and dimethoate, as well as PEP and THF for employing the steepest descent and conjugate gradient algorithms with the MMFF94 force field [26]. This minimization was carried out with the help of Avogadro software and then the prepared files were added to the system and pdbqt files were generated. The docking grid boxes were 58.712 × 80.945 × 44.149 Å for PEP and 88.202 × 87.801 × 64.541 Å for THF. The docking calculations were set to an exhaustiveness of 64 for the acquirement of accurate results. After completing the docking process, the resulting poses were re-ranked based on their docking scores. The top-ranked conformation was identified as the best binding pose and the corresponding site was designated as the optimal binding site for the ligand and analyzed using BIOVIA Studio Visualizer solftware [27,28].

RESULTS AND DISCUSSION

Sample preparation: Simulated biological matrices, including simulated gastric fluid (SGF) and simulated urine sample (SUS) were prepared. To develop calibration standards, these matrices were spiked with pesticides at varying concentrations (1.25-160 ng/mL). Upon the QuEChERS extraction, the final extract was filtered with a 0.22 µm syringe filter, transferred to LC vials and subsequently injected (5 µL) into the UPLC-MS/MS system for analysis. All samples were kept at 4 ºC until analysis and blank matrices and quality control samples were included to ensure reliability and precision.

Prerequisites for implementing LC-MS/MS condition: Spectrometric and chromatographic parameters were tuned for the detection of tetramethrin and dimethoate optimized for the detection of tetramethrin and dimethoate through the direct injection of spiked standard solutions into the UPLC-MS/MS. Immediately after the first identification of each precursor ion, different collision energy voltages have been employed to differentiate between the two different product ions. Particularly, the qualifier ion and quantifier ion were selected for two transitions. In the end, the parameters were modified employing dual timing and multiple reaction monitoring (MRM) transitions. Under ideal UPLC conditions, the tetramethrin and dimethoate retention times for SGF and SUS were obtained. The MS parameters for both positive and negative ionization modes were modified using a tuning solution (100 ng/mL). When compared to the negative mode, the positive ionization mode showed a substantially higher sensitivity for detecting tetramethrin and dimethoate, with less background noise. At *m/z* 332 and 230, respectively, the precursor ion mass transitions for tetramethrin and dimethoate were identified. Tetramethrin and dimethoate were identified at *m/z* 332 and 230, respectively, for the product (daughter) ions.

Method validation: The validation process, conducted in accordance with SWGTOX guidelines [29], was thoroughly evaluated by key performance parameters including specificity, accuracy, recovery and matrix effects. Matrix effects, defined as the influence of sample components other than the analytes on the quantification process, were carefully assessed to ensure precise and reliable data acquisition.

Specificity and sensitivity: The detection of pyrethroid and organophosphorus pesticides in SGF and SUS samples were found to be rapid and highly precise approach which showed exceptional sensitivity and accuracy. The efficiency of method for routine pesticide detection was shown by the exceptional chromatographic performance, including distinct peak forms and decreased retention times. The chromatograms exhibited the pesticide peaks clearly showing that the proposed QuEChERS method obtained clean separations. The excellent specificity of method has been confirmed by specificity analysis at seven different concentrations, which showed no interference peaks at the target retention times (Fig. 1).

Linearity: Linear regression analysis and analysis of variance were used to analyze the pesticides at seven various concentrations, the method's linearity was evaluated using the slope-intercept equation, response towards concentration was plotted to generate calibration. According to the correlation coefficient (R^2), the generated plots indicated great linearity and an excellent correlation. With R^2 values close to 1, the calibration curves for tetramethrin and dimethoate were linear across the 2.5 ng/mL to 160 ng/mL range, showing the great precision and consistency of the method curves as shown in Fig. 2. The obtained values are summarized in Table-1.

LOD, LOQ, recovery: The QuEChERS technique was validated by evaluating the recovery, limit of quantification (LOQ) and limit of detection (LOD). The limits of detection (LOD) for tetramethrin and dimethoate were determined to be 5.62 ng/mL and 4.80 ng/mL in SGF and, 3.35 ng/mL and 5.53 ng/mL in SUS, respectively. The LOQ in SGF ranged from 17.03 ng/mL to 14.56 ng/mL and SUS ranged from 10.15 ng/mL to 16.77 ng/mL, while in. Both tetramethrin and dimethoate achieved an adequate recovery rate of 70-120% as shown in Table-1. No reported studies have shown the identification of tetramethrin and dimethoate in simulated SGF and SUS. As a result, this study led to the development of a robust UPLC-MS/MS method with a minimal total analysis time.

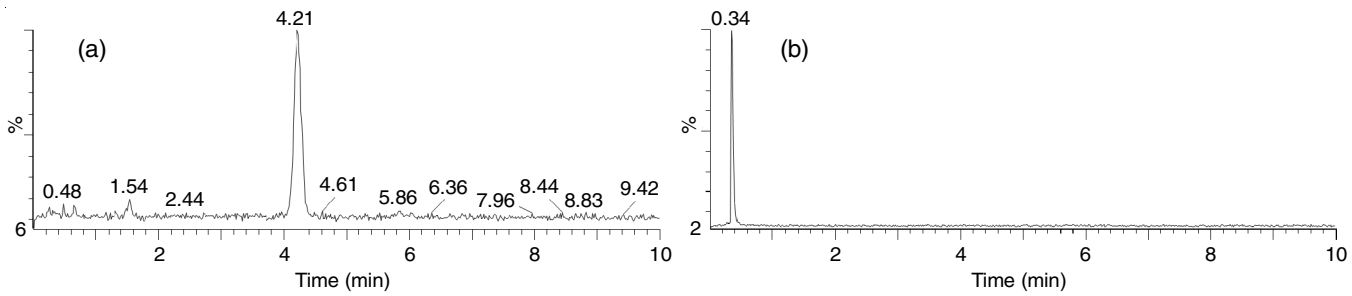

Fig. 1. Full scan chromatograms of pyrethroid (TMT) (a) and organophosphate (DMT) (b)

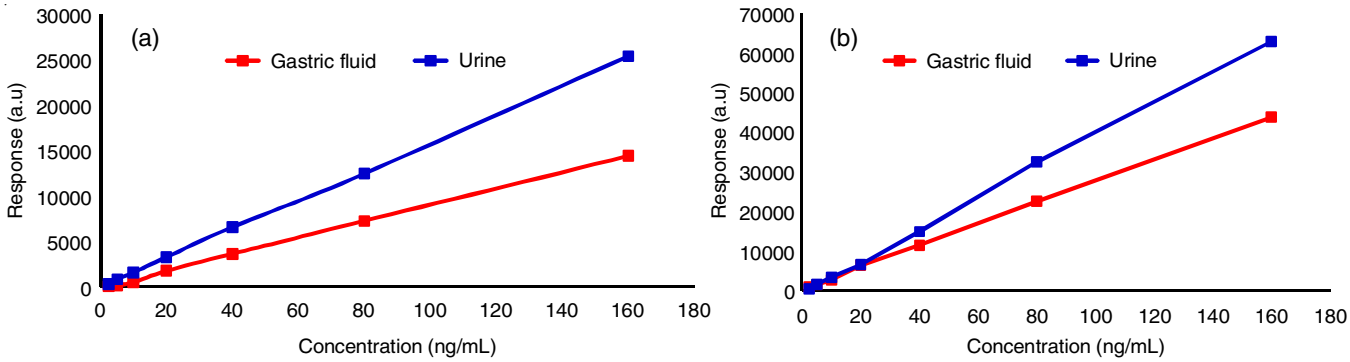

Fig. 2. Linearity of pyrethroid (TMT) in (SGF) and (SUS) (a) and organophosphate (DMT) in (SGF) and (SUS) (b)

TABLE-1
CALIBRATION CURVES, LINEARITY, LOD, LOQ, INTERCEPT AND SLOPE
FOR PYRETHROID (TMT) AND ORGANOPHOSPHORUS (DMT)

Analyte	m.w. (g/mol)	Conc. range (ng/mL)	Linearity (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Intercept	Slope	Precursor ion	Product ion
TMT (SGF)	331.406	2.5-160	0.9993	5.62	17.03	91.567	-224.6	332	135
TMT (SUS)	331.406	2.5-160	0.9991	3.35	10.15	157.988	-10.2	332	135
DMT (SGF)	229.260	2.5-160	0.9995	4.80	14.56	332.399	272.8	230	199
DMT (SUS)	229.260	2.5-160	0.9993	5.53	16.77	-685.603	399.3	230	199

Matrix effect and interference: All analytes were analyzed for two different concentration levels of matrix effects (lower quality control, LQC; 2.5 ng/L; higher quality control, HQC; 160 ng/L). The analyte variations from matrix effects of less than 20% for both quality control levels were found. The results meet the validation standards and are therefore considered acceptable. Since the interference analysis failed to identify a substance, which was equivalent to the simulated samples, it proved that the method is unique to tetramethrin and dimethoate. Tetramethrin and dimethoate can be detected in a single analysis using present method used UPLC-MS/MS method at a detection limit of 2.5 ng mL^{-1}.

Several methods were used for biological material using various solvents, salt combinations and cleanup processes. However, there is limited research available that has utilized a design of experiments and statistical data to optimize the conditions for the detection of tetramethrin and dimethoate. Overall, the reported recovery ranges for tetramethrin and dimethoate indicate that the QuEChERS extraction method was effective in extracting these drugs from SGF and SUS, with recovery percentages falling within the acceptable range of 70-120% for analytical methods as shown in Fig. 3.

Molecular docking interaction studies: To identify the preferred binding site and interactions of tetramethrin with PEP and THF, molecular docking studies were conducted. For the tetramethrin-PEP complex, the results revealed that tetramethrin binds within the connecting domain near the Asp32 residue, exhibiting the lowest binding affinity of -8.1 kcal/mol. The stabilization of complex was primarily attributed to one hydrogen bond and 13 van der Waals interactions. Specifically, the acetic acid group of methyl 2,2-dimethyl-3-(2-methylprop-1-en-1-yl)-cyclopropane-1-carboxylate moiety in tetramethrin formed a hydrogen bond with Thr77 and an alkyl bond with Phe111. Additionally, the 4,5,6,7-tetrahydro-1*H*-isoindole moiety containing a cyclohexane ring established a pi-alkyl interaction with Tyr189 and Ile300, while its cyclopentene ring formed a pi-anion bond with Asp215 and forms an electrostatic attractions Asp215 as shown in Fig. 4. Overall, the tetramethrin-PEP complex stabilized primarily by hydrogen bonding and van der Waals forces. For THF, tetramethrin binds to the GPI (glycosylphos-

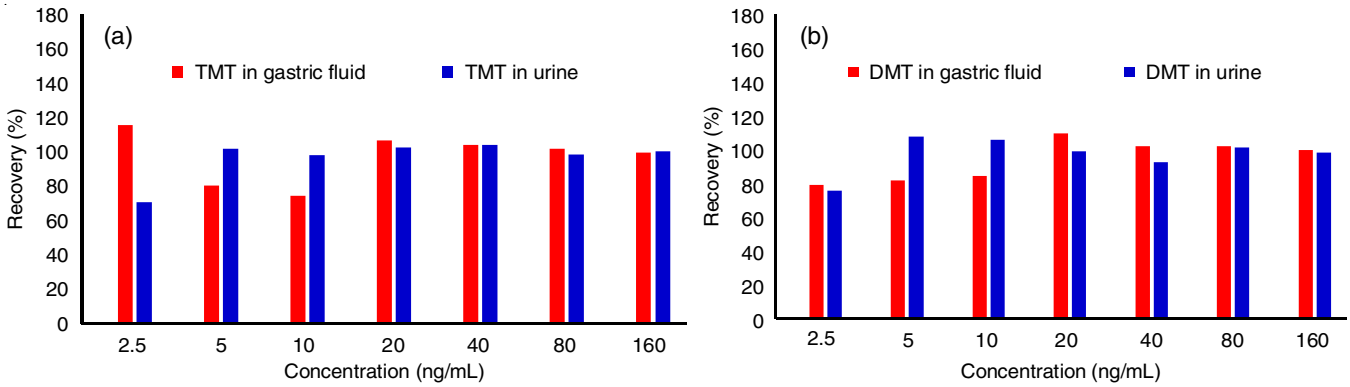

Fig. 3. The recovery percentage of pyrethroid (TMT) in (SGF) and (SUS) (a) and organophosphate (DMT) in (SGF) and (SUS) (b)

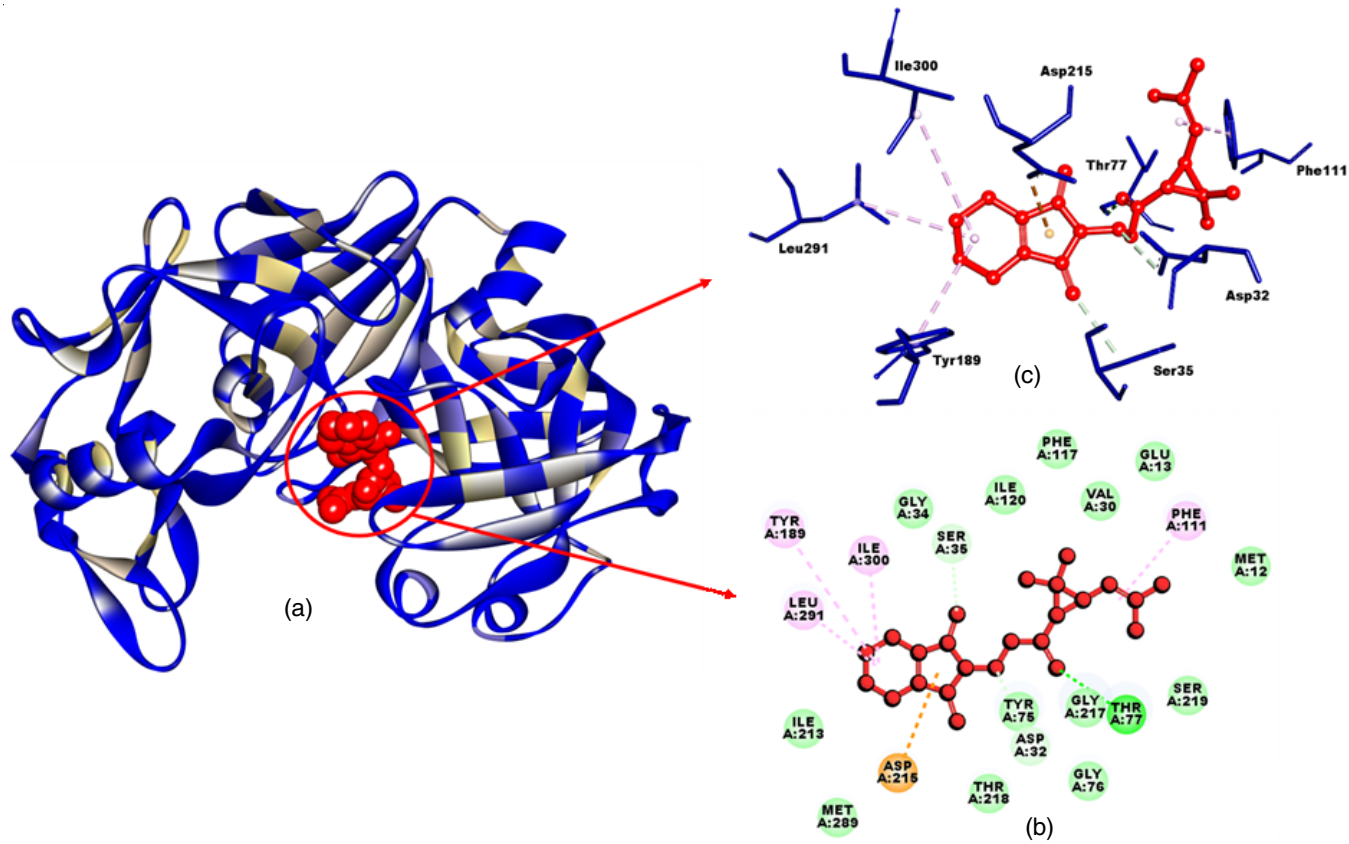

Fig. 4. (a) TMT binding near the ASP 32 active binding site at the connecting domain of PEP (b) three-dimensional (3D) atom-to-atom representations of the TMT_PEP complex and (c) two-dimensional (2D) interactions between the TMT_PEP complex

phatidylinositol) site at the C-terminal, exhibiting the lowest binding affinity of -5.8 kcal/mol. The tetramethrin-THF complex is stabilized by 1 hydrogen bond and 10 van der Waals interactions. Specifically, the cyclopentene ring of tetramethrin forms a hydrogen bond with Cys315. Additionally, the isoindole moiety containing the cyclopentene ring and methyl group engages in alkyl, pi-alkyl and pi-pi stacking interactions with Trp313 and Pro290 residues (Fig. 5). The TMT-THF complex is predominantly stabilized by hydrogen bonds and van der Waals forces. These interactions significantly contribute to the overall stability and specificity of both the tetramethrin-PEP and tetramethrin-THF complexes.

For dimethoate, the molecular docking studies revealed its binding interactions with PEP and THF. In case of PEP, dimethoate binds within the connecting domain near the Asp32 residue, with a minimum binding affinity of -4.1 kcal/mol. The dimethoate-PEP complex is stabilized by three hydrogen bonds and five van der Waals interactions. Specifically, the phosphonodithioate moiety of dimethoate forms two hydrogen bonds with Gly76 and exhibits an electrostatic interaction with Asp215. Additionally, this moiety engages in a pi-sulfur interaction with Tyr75. The N-methylpropionamide group of dimethoate forms a hydrogen bond with Gly217 (Fig. 6). Overall, the dimethoate-PEP complex is primarily stabilized by hydrogen bonds and van der Waals forces. Whereas for THF, dimethoate binds to the glycosylphosphatidylinositol (GPI) site at the C-terminal, exhibiting a lowest binding affinity of -4.3 kcal/mol. The dimethoate-THF complex is stabilized by four hydrogen bonds and five van der Waals interactions. Specifically, the N-methylpropionamide group of dimethoate forms two hydrogen bonds with Ser291 and Ser292, while the phosphonodithioate moiety forms hydrogen bonds with Ser186 and Glu188 and engages in an electrostatic interaction with Glu188. Additionally, both the phosphonodithioate moiety and the N-methylpropionamide group form carbon-hydrogen bonds with Gln208 and Glu294 (Fig. 7). The dimethoate-PEP and dimethoate-THF complexes are predominantly stabilized by hydrogen bonds and van der Waals forces, which significantly contribute to their overall stability and specificity.

Conclusion

The QuEChERS extraction method, combined with UPLC-MS/MS analysis, presents a promising approach to addressing the challenges of pesticide detection in simulated biological matrices. By optimizing extraction conditions and leveraging advanced analytical techniques, this study aims to improve the accuracy and reliability of pesticide analysis, thereby supporting public health and environmental sustainability initiatives. A novel method using QuEChERS extraction and UPLC-MS/MS was developed for the quantification of pyrethroid and organophosphorus pesticides, specifically tetramethrin and dimethoate, in simulated gastric fluid (SGF) and simulated urine sample (SUS) samples. The method demonstrated reliable detection of tetramethrin and dimethoate across 7 different concentrations and was validated as precise and accurate. Recovery rates ranged from 70% to 120%, in accordance with SWGTOX guidelines.

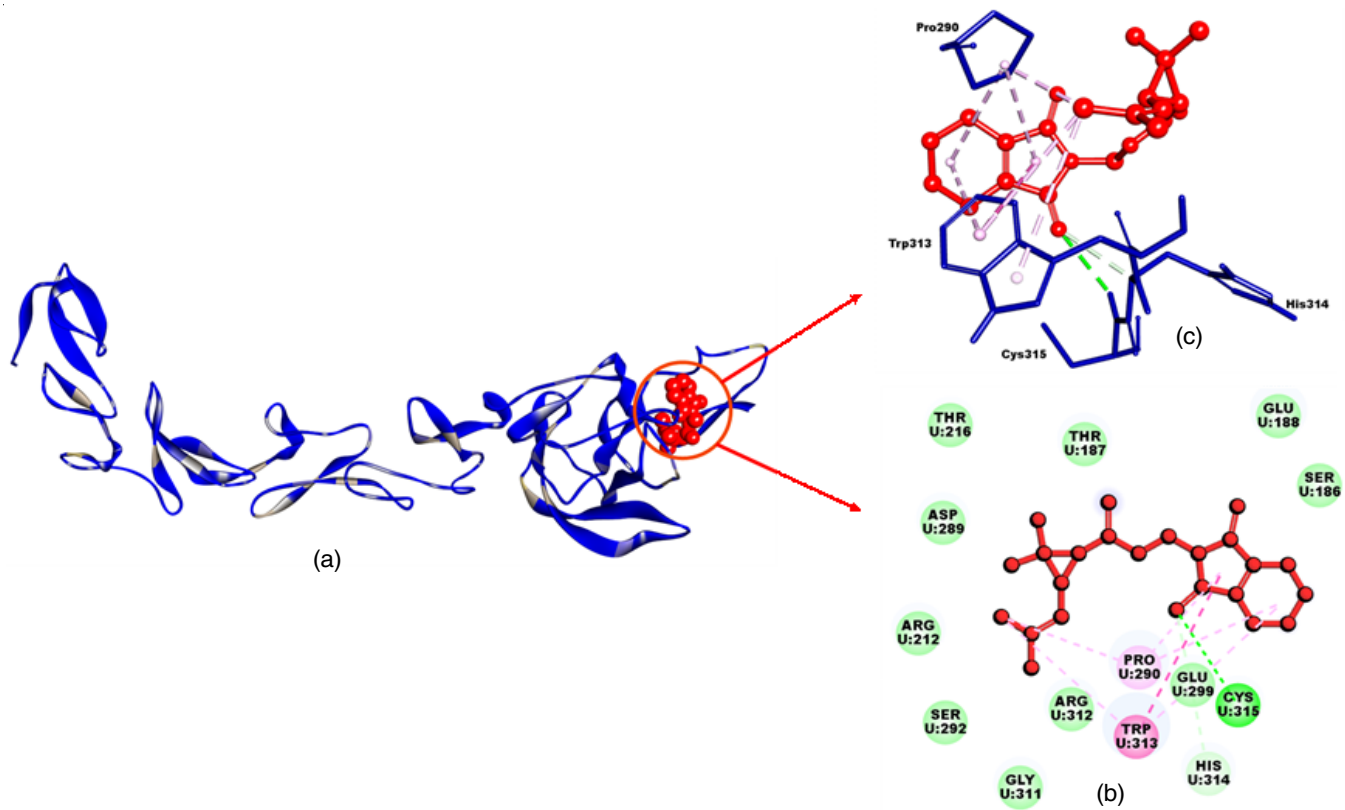

Fig. 5. (a) TMT is binding in the ZP domain at C-terminal of THF is depicted in the docked structure, in (b) (3D) representations of the TMT_THF complex and (c) (2D) interactions between the TMT_THF complex

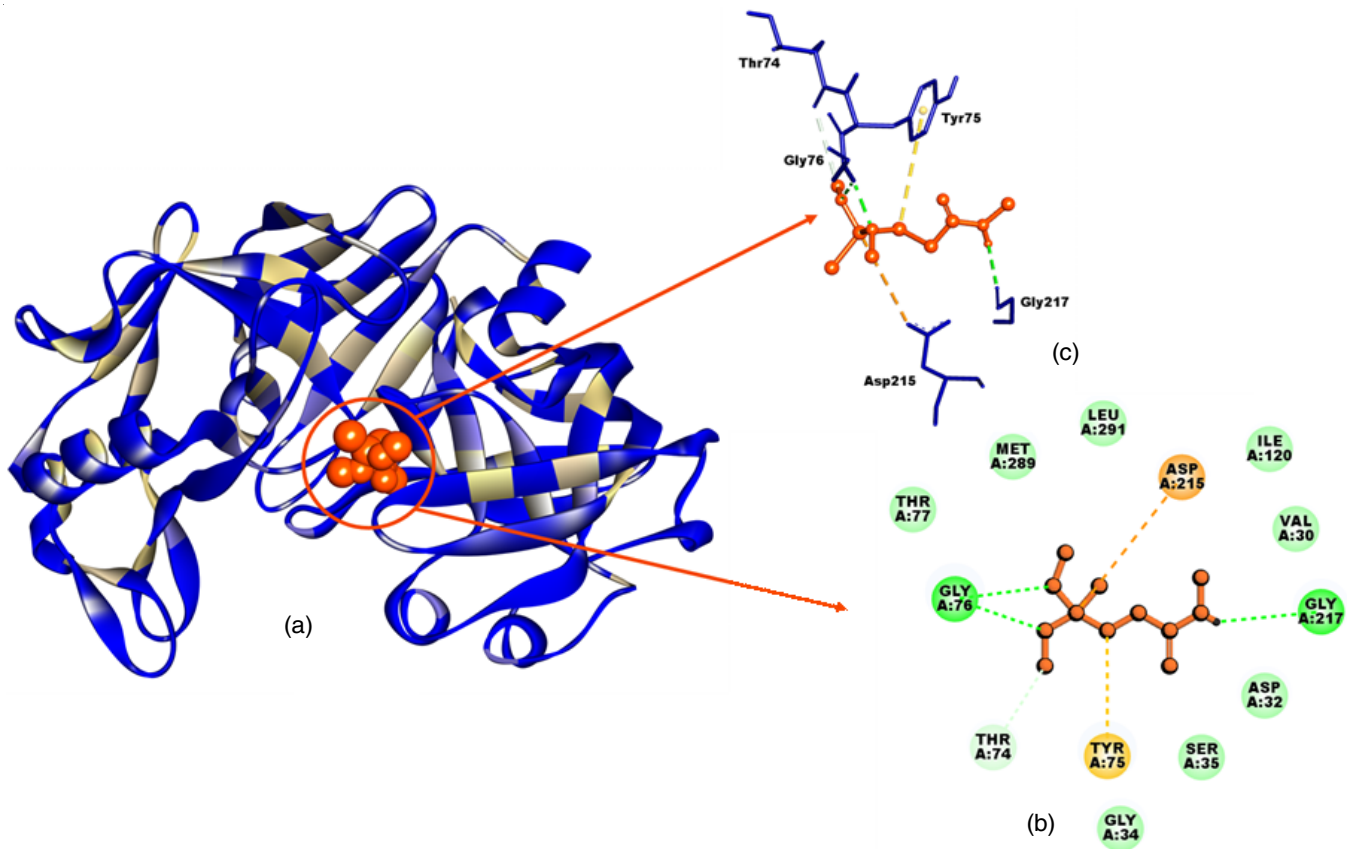

Fig. 6. (a) DMT binding near the ASP 32 active binding site at the connecting domain of PEP (b) three-dimensional (3D) atom-to-atom representations of the DMT_PEP complex and (c) two-dimensional (2D) interactions between the DMT_PEP complex

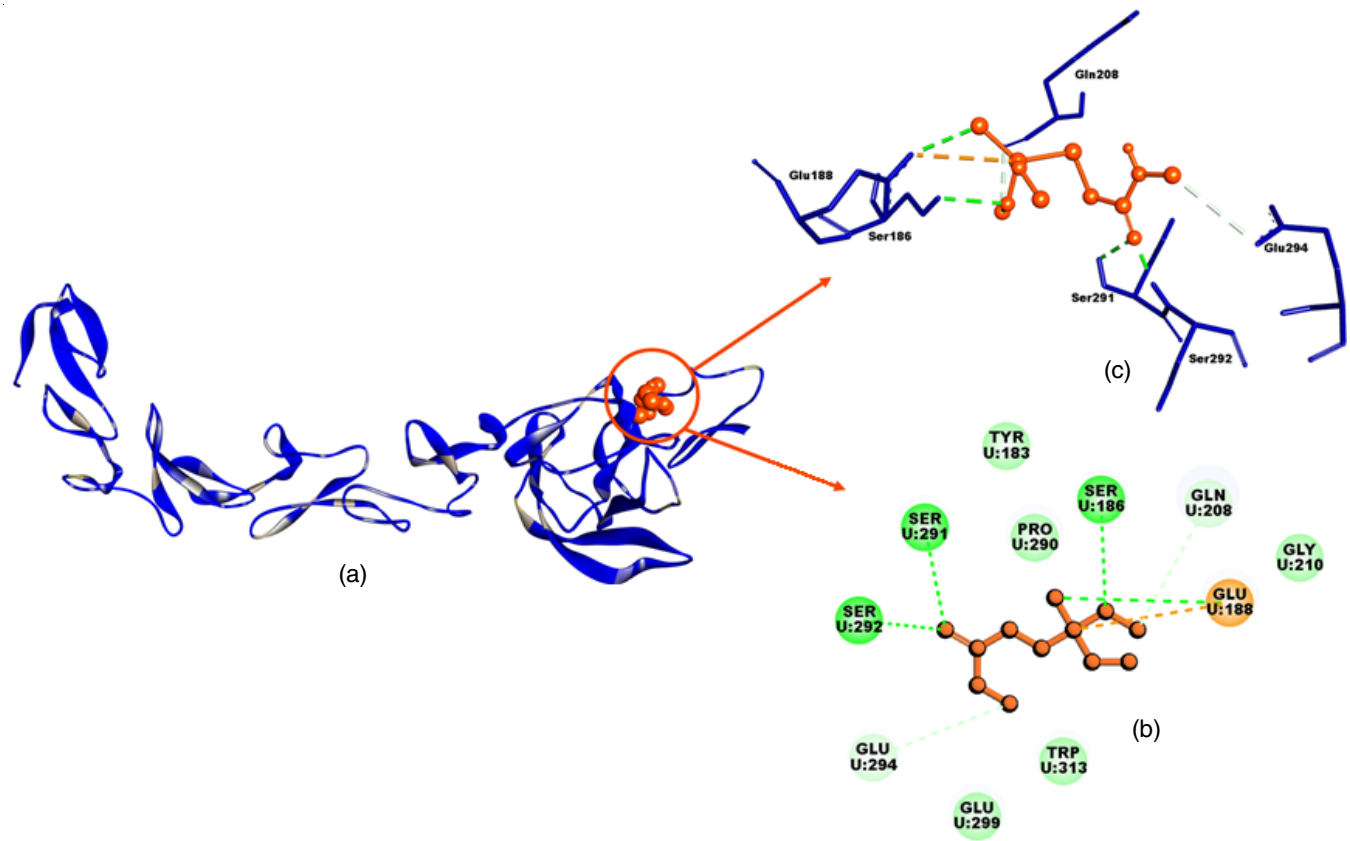

Fig. 7. (a) DMT is binding in the ZP domain at C-terminal of THF is depicted in the docked structure, in (b) (3D) representations of the DMT_THF complex and (c) (2D) interactions between the DMT_THF complex

The docking study revealed that the pesticides tetramethrin and dimethoate exhibited a preference for binding to the connecting domain near the Asp32 residue of PEP. The binding affinities were -8.1 kcal/mol for tetramethrin and -4.1 kcal/mol for dimethoate, indicating a stronger interaction for tetramethrin with PEP. Conversely, both tetramethrin and dimethoate displayed a preference for binding at the GPI site at the C-terminal of THF, with binding affinities of -5.8 kcal/mol for tetramethrin and -4.3 kcal/mol for dimethoate. The higher binding affinity of tetramethrin to PEP and THF suggests stronger pesticide-protein interactions, which correlates with their lower recovery from PEP during extraction, likely due to matrix effects. The validation results confirmed excellent linearity, precision and accuracy. Moreover, the method achieved satisfactory limits of detection (LOD) and quantification (LOQ), enabling the sensitive detection of tetramethrin and dimethoate at concentrations as low as 2.5 ng/mL in simulated SGF and SUS. The optimized protocol was successfully validated for linearity and trueness, yielding accurate and reproducible results within a short analytical time frame. This versatile method is not only effective for detecting tetramethrin and dimethoate in simulated matrices but also offers potential for adaptation to other pyrethroid and organophosphorus pesticides in human matrices with appropriate modifications.

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CONFLICT OF INTEREST

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

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