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A Validated LLE–LTP–LC–MS/MS Workflow for Pesticide Biomonitoring in Human Plasma: Application to Occupational Exposure Assessment in Rural Workers

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HIGHLIGHTS

- Development of a robust and environmentally sustainable LLE–LTP method for pesticide biomonitoring.
- Successful application of LLE–LTP for multiresidue extraction in human plasma.
- Use of multivariate experimental design for method optimization.
- Satisfactory analytical performance with recoveries of 80–120%, RSD ≤ 15%, and limits of quantification (LOQs) ranging from 0.01 to 30 ng mL^{-1}.
- Practical application of the method for occupational exposure assessment.

ABSTRACT: Low-temperature partitioning liquid–liquid extraction (LLE–LTP) is a promising alternative for preparing samples for liquid chromatography-tandem mass spectrometry (LC–MS/MS) to determine multiresidue pesticides in biological samples. This technique enables simultaneous extraction and cleanup through phase separation at temperatures below freezing (-18 °C). This study reports the development, optimization, miniaturization, and validation of an LLE–LTP method for quantifying 30 pesticides in human plasma using LC–MS/MS. Initially, a factorial screening design was used to identify the most influential variables, followed by a central composite design (CCD) to optimize the volumes of acetonitrile (ACN), ultrapure water, and plasma. A reduced quadratic model was fitted to the experimental data, and statistically significant terms were selected through confidence interval analysis. The optimal extraction conditions were determined to be 500 µL of water, 1000 µL of ACN, and 250 µL of plasma. This was confirmed through constrained numerical optimization, response surface plots, and experimental validation. The method adheres to the validation guidelines set by Brazilian regulations (INMETRO DOQ-CGCRE-008) and international standards (EU SANTE/11312/2021). The developed methodology exhibited a matrix effect (ME < ±20 %; $p > 0.05$), excellent linearity ($r > 0.995$), a limit of quantification (LOQ) ranging from 0.01 to 30 ng mL^{-1}, accuracy of 80%-120%, and precision (RSD < 15%). A direct comparison with the micro-QuEChERS AOAC 2007.1 workflow showed equivalent or improved recovery and precision for most analytes. Finally, the LLE–LTP–LC–MS/MS method was applied to analyze real plasma samples from agricultural workers in Goiás, Brazil. Remarkably, 11 of 21 workers had at least one detectable pesticide signal, with chlorpyrifos quantified at 14 ± 4.6 ng mL^{-1} in one individual. Therefore, the validated LLE–LTP–LC–MS/MS workflow combines analytical rigor, operational simplicity, and sustainability, making it a practical tool for assessing occupational exposure and biomonitoring.

KEYWORDS: Human plasma; Pesticide residues; Sample preparation; Occupational exposure; Biomonitoring.

1. Introduction

Agriculture forms the foundation of the global food supply, representing the primary source of nourishment, both directly, through agricultural products that reach consumers' plates, and indirectly, through secondary sources such as livestock [1,2]. This is further supported by the Food and Agriculture Organization (FAO), which highlights that agrifood systems — the entire chain of production from farm to fork, including both food and non-food agricultural products — are central to the livelihoods of billions of people and constitute the backbone of global food security [3].

However, crops are naturally susceptible to attacks by pests, including insects, fungi, weeds, rodents, and other harmful agents. To mitigate these threats, various chemical compounds have been developed to protect agricultural production, enhance yields, and prevent the spread of diseases [4,5].

Besides, the efficacy of modern agrochemicals against these harmful agents is well established and rigorously evaluated by regulatory authorities worldwide; these substances inherently have the potential to cause undesirable effects on human health and/or the environment [6,7]. Various diseases are linked to pesticide exposure, including cancer, hormone disruption, asthma, allergies, and neurological disorders [7–10]. The severity of such effects depends on the extent and duration of exposure and the likelihood of their occurrence; even small doses can have adverse health effects. [6,7].

In this context, agricultural workers are the most vulnerable due to their direct involvement in farming and, often, a lack of appropriate personal protective equipment (PPE) and adequate soil treatment measures [11–13]. In Brazil, between 2000 and 2017, nearly 25,000 cases of pesticide poisoning were reported among agricultural workers. This reflects on the country's ranks among the world's largest consumers of pesticides, accounting for approximately 86% of all pesticides used in Latin America [12,14]. It highlights the significant occupational risks associated with pesticide exposure [13]. These figures underscore the urgency of addressing pesticide-related illnesses as a public health issue that requires continuous monitoring and comprehensive evaluation.

Multiresidue pesticide determination is commonly carried out using chromatographic techniques, which typically require labor-intensive sample preparation involving multiple steps, extensive solvent consumption, and prolonged processing times [15–17]. Established methods such as Solid Phase Extraction (SPE) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) are widely accepted as official procedures for this purpose and guided the extraction of pesticides throughout the last decades [18–20]. These approaches use sorbent phases or salt-based kits, various solvents, and additional cleanup steps. As an alternative, low-temperature partitioning (LTP) has been integrated into traditional liquid–liquid extraction (LLE) workflows to simplify the procedure and overcome some of its limitations [21]. LTP relies on the fundamental principles of LLE but employs extractant solvents that are fully miscible with the aqueous sample phase. Upon freezing, the aqueous phase solidifies while the organic extractant remains liquid, allowing analyte partitioning and simultaneous matrix cleanup by immobilizing several interfering compounds, especially lipids, and suspended particles, within the frozen sample phase, which are significant sources of matrix effects in LC–MS/MS analysis of biological samples [21–23].

Given its operational characteristics, LLE-LTP represents a promising strategy for the multiresidue extraction of pesticides from human plasma. This approach is particularly suitable for polar and semi-polar compounds, which are efficiently extracted using water-miscible organic solvents (such as acetonitrile) with intermediate to high polarity. These solvents exhibit strong affinity for polar analytes through dipole-dipole interactions and hydrogen bonding. The subsequent freezing step induces phase separation by crystallizing water, leaving the organic phase liquid. This promotes analyte migration into the organic layer via a thermodynamic exclusion effect while simultaneously enabling matrix clean-up by precipitating proteins and retaining lipids and other interfering components [21,23,24].

In addition to its analytical advantages, developing robust methods for pesticide determination in plasma is essential for monitoring occupational exposure, especially among agricultural workers regularly exposed to agrochemicals [25–28]. In this context, plasma serves not only as a biological matrix but also as a biomarker-based indicator of chemical exposure and potential health risks [7,26,28].

This study presents the complete development of an LLE–LTP-based sample-preparation method for the determination of 30 pesticides in human plasma, followed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis. Initially, the LLE-LTP method was thoroughly evaluated and optimized using a factorial design with a central point following a central composite design (CCD). Additionally, response surface graphs were constructed to assess the interaction effects between the extraction solvent and water volumes. Several miniaturized versions of the optimized protocol were also tested and validated in accordance with national and international guidelines. Finally, the validated methodology was applied to plasma samples from agricultural workers engaged in horticultural activities across different municipalities in the state of Goiás, Brazil, as part of a broader effort to assess pesticide exposure in the context of occupational health surveillance.

2. Materials and methods

2.1. Chemicals and Reagents.

The following pesticides were selected as target analytes: 2,4,5-T, 2,4-D, acephate, alachlor, aldicarb, ametryn, atrazine, benzidine, carbendazim, carbofuran, chlorpyrifos, cyproconazole, deisopropylatrazine, demeton, desethylatrazine, desthio-prothioconazole, dimethoate, diuron, epoxiconazole, fipronil, flutriafol, methamidophos, metolachlor, metribuzin, molinate, picloram, simazine, tebuconazole, thiamethoxam, and thiodicarb. Stock solutions of the target pesticides (100 µg mL^{-1}) were obtained from Agilent Technologies (Santa Clara, CA, USA). ACN (≥99.9%, HPLC gradient grade), formic acid (≥98%, ACS reagent grade), and ammonium formate (≥97%, analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA); ethyl acetate (HPLC grade) from Tedia (Fairfield, OH, USA); and acetone (HPLC grade) from Biograde (São Paulo, Brazil). Ultrapure water was produced using a Gehaka Master WFI system (São Paulo, Brazil). Deuterated melatonin (melatonin-d$_4$, $C_{13}D_4H_{12}N_2O_2$, 98 atom % D, purchased from Sigma-Aldrich) was used as a global internal standard and added to samples before extraction to monitor system stability and injection reproducibility. Melatonin-d4 was selected as an internal standard because it is not naturally present in biological samples, thereby avoiding interference from endogenous sources. Additionally, it elutes near the midpoint of the chromatographic run (retention time (RT) = 3.93 min; mean RT of target analytes = 4.20 min) without co-eluting with any target pesticide, thereby providing reliable normalization across the entire analytical window. The individual RT of all analytes are reported in the Supplementary Material.

2.2. Preparation of Standard Solutions.

Individual pesticide stock solutions (100.0 μg mL^{-1}) were used to prepare a mixed standard solution containing each pesticide at 0.2 μg mL^{-1}. This solution was further diluted with ACN to prepare mixed working standard solutions at lower concentrations, which were used for calibration and analytical curve construction, and method validation. A stock solution of deuterated melatonin (melatonin-d_4) at 1.0 μg mL^{-1} was prepared in ultrapure water and added as an internal standard (IS) to all calibration standards and samples.

2.3. Instrument Analysis Conditions.

LC–MS/MS analysis was carried out on an Agilent 1290 Infinity II Bio LC system coupled to an Agilent 6495D triple quadrupole mass spectrometer (Santa Clara, CA, USA). The LC system consisted of a high-speed pump (G7132A), a multisampler with thermostat (G7137A), and a multicolumn thermostat (G7116B). Chromatographic separation was achieved using an InfinityLab Poroshell 120 EC-C18 column (100 × 2.1 mm, 2.7 μm, Agilent) maintained at 40 °C. The autosampler temperature was set to 10 °C, the flow rate was 0.5 mL min^{-1}, and the injection volume was 4 μL. Mobile phase A consisted of water with 0.5% methanol, 0.1% formic acid, and 3.5 mM ammonium formate. In comparison, mobile phase B consisted of 96% ACN, 2% methanol, 2% water, 0.1% formic acid, and 3.5 mM ammonium formate. The gradient program for mobile phase B was as follows: initially 2% B held for 0.2 min, increased to 20% at 1.5 min and maintained until 2.5 min, ramped to 100% at 5.0 min and held until 6.5 min, then returned to 2% at 6.51 min. The total run time was 8.0 min, followed by a 1.0 min post-run for column re-equilibration. In the mass spectrometer, ionization of the target analytes was performed using the Agilent Jet Stream electrospray ionization (AJS-ESI) source in positive/negative switching mode. The drying gas flow was set at 13.0 L min^{-1}, the nebulizer pressure at 40.0 psi, and the sheath gas flow at 11.0 L min^{-1}. The gas temperature and sheath gas temperature were maintained at 250 °C and 350 °C, respectively. The capillary voltage was set at 3500 V in positive mode and 4000 V in negative mode. The nozzle voltages were 500 V for positive ionization and 1500 V for negative ionization. Data acquisition was performed in dynamic multiple reaction monitoring (dMRM) mode. For each compound, the most intense product ion was used for quantification (quantifier), and the second most intense for confirmation (qualifier). MRM transitions were planned based on each compound's retention time, using compound-specific detection windows. Dwell times were automatically adjusted to ensure optimal data acquisition based on the instrument's cycle time. Agilent MassHunter Workstation software version 12.1 was used for instrument control and data processing. The optimized LC–MS/MS parameters, including retention times and corresponding time windows, MRM transitions, and collision energies (CE) for each pesticide, are provided in Supporting Information.

2.4. Plasma Matrix and Ethical Compliance.

A pooled human plasma sample from six individuals was used throughout method development, optimization, and validation. The authors adopted the inclusion criterion of using only plasma from individuals with no direct occupational exposure to pesticides to minimize background contamination. Thus, this sample was used as a blank reference matrix. All procedures involving human biological material were conducted in accordance with ethical standards for research involving human participants and were approved by the Brazilian Research Ethics Committee (protocol number 7.081.319).]

2.5. Development and Optimization of Sample Preparation.

Development and Optimization of LLE–LTP usually require systematically assessing key parameters that influence extraction efficiency, phase separation, and overall analytical performance [21]. In the present study, to the LLE–LTP procedure, an initial screening of extraction solvents was conducted. Methanol, acetone, and ACN were evaluated under identical conditions. In each trial, 500 µL of pooled plasma spiked with the analytes at 100.0 ng mL-1 were mixed with 1000 µL of the extraction solvent, and then 100 µL of ultrapure water was added. The mixtures were vortexed and frozen at −18 °C for 12 hours, a time selected to ensure complete and reproducible freezing of the aqueous phase and stable phase separation, while maintaining a reasonable compromise between extraction efficiency and analytical throughput. After this period, samples were visually inspected to assess phase separation behavior. Among the tested solvents, only ACN promoted the formation of a distinct organic phase above the frozen aqueous layer and was therefore selected for subsequent method development.

To investigate the effect of modifying the extraction solvent, pure ACN was compared with a binary mixture of ACN and ethyl acetate (ACN:EtOAc, 87.5:12.5 v/v), using mean pesticide recovery as the performance metric. Plasma samples were fortified with a pesticide mixture to a final concentration of 100.0 ng mL^{-1}. Then, 500 µL of spiked plasma was mixed with 1000 µL of the extraction solvent (either pure ACN or the ACN:EtOAc mixture), followed by 100 µL of ultrapure water. The samples were vortexed and frozen at −18 °C for 12 hours. After phase separation, the organic phase was analyzed, and the absolute recoveries of each analyte were calculated using external calibration curves prepared in ACN. The arithmetic mean of individual absolute recoveries was adopted as a pragmatic global response to guide the multivariate optimization process. Using an unweighted mean recovery as the response function aims to identify compromise extraction conditions that provide balanced performance across analytes with diverse physicochemical properties. A Student's t-test was applied to determine whether the differences between the two solvent systems were statistically significant.

To identify the most influential variables affecting extraction efficiency, a full 2^3 factorial design with two central points (n = 2) was implemented. The independent variables were ultrapure water volume (100–1000 µL), ACN volume (500–1000 µL), and vortexing time (20–60 s). Each experiment received 500 µL of fortified pooled plasma containing the pesticide mixture (100 ng mL^{-1}). The analysis was performed in a randomized manner to reduce systematic error. Mean absolute recovery of pesticide was used as the response variable. The experimental matrix and factor levels are detailed in Table 1.

Table 1. Experimental design matrix of the 2^3 full factorial design with center points used for screening of LLE–LTP extraction method variables.

Experiments (assay order)	**H_2O**		**ACN**		**Vortexing Time**		**Mean Absolute Recovery of Pesticides (%)**
	Real (µL)	Code	Real (µL)	Code	Real (s)	Code	
1 (4)	100	-1	500	-1	20	-1	44
2 (6)	1000	1	500	-1	20	-1	25
3 (5)	100	-1	1000	1	20	-1	79
4 (7)	1000	1	1000	1	20	-1	37
5 (2)	100	-1	500	-1	60	1	52
6 (1)	1000	1	500	-1	60	1	27
7 (10)	100	-1	1000	1	60	1	64
8 (8)	1000	1	1000	1	60	1	38
9 (3)	550	0	750	0	40	0	31
10 (9)	550	0	750	0	40	0	30

For extraction optimization, a CCD was used to optimize the key variables affecting LLE–LTP extraction efficiency. The three independent variables selected were the ultrapure water volumes, ACN, and plasma. These variables were encoded at five levels (–α, –1, 0, +1, +α), where $\alpha = (2^k)^{0.25}$ and k is the number of factors (in this case, 3). The system response was defined as the mean absolute recovery of the pesticide mixture, with fortified plasma samples at a final concentration of 100.0 ng mL^{-1}. The design matrix, along with the experimental runs and coded/real values for each factor, is presented in Table 2. Experimental runs were conducted in randomized order to minimize the influence of uncontrolled sources of variability.

A quadratic polynomial model was fitted to the data to describe the system behavior and to predict the optimal extraction conditions. Based on the fitted equation, three-dimensional scatter plots with color-coded response values were generated to visualize the response distribution across the experimental space and identify the optimal region. In addition, response surface plots were constructed to investigate_to investigate the interactions between the variables, particularly between the extraction solvent and water volumes, while the plasma volume was fixed at its predicted optimal level.

Table 2. CCD matrix with coded and real values of the independent variables and corresponding pesticide recovery (%) results.

Experiments (assay order)	**H_2O**		**ACN**		**Plasma**		**Mean Absolute Recovery of Pesticides (%)**
	Real (µL)	**Code**	**Real (µL)**	**Code**	**Real (µL)**	**Code**	
1 (3)	180	-1	700	-1	300	-1	58
2 (16)	420	1	700	-1	300	-1	84
3 (4)	180	-1	1300	1	300	-1	90
4 (12)	420	1	1300	1	300	-1	91
5 (1)	180	-1	700	-1	450	1	57
6 (15)	420	1	700	-1	450	1	71
7 (17)	180	-1	1300	1	450	1	68
8 (2)	420	1	1300	1	450	1	76
9 (13)	100	-1.68	1000	0	375	0	66
10 (8)	500	1.68	1000	0	375	0	82
11 (5)	300	0	500	-1.68	375	0	41
12 (6)	300	0	1500	1.68	375	0	60
13 (19)	300	0	1000	0	250	-1.68	108
14 (18)	300	0	1000	0	500	1.68	77
15 (10)	300	0	1000	0	375	0	75
16 (11)	300	0	1000	0	375	0	71
17 (14)	300	0	1000	0	375	0	73
18 (7)	300	0	1000	0	375	0	69
19 (9)	300	0	1000	0	375	0	72

To assess the feasibility of miniaturizing the method while preserving the optimized solvent/sample ratios, four scaled-down conditions were tested: (i) 50 µL plasma, 200 µL ACN, 100 µL water; (ii) 100 µL plasma, 400 µL ACN, 200 µL water; (iii) 125 µL plasma, 500 µL ACN, 250 µL water; (iv) 250 µL plasma, 1000 µL ACN, 500 µL water (reference condition).

A one-way ANOVA ($\alpha = 0.05$) was used to compare mean absolute recoveries across these miniaturized protocols and to determine whether they differed significantly from the reference condition, assuming a normal distribution and homogeneity of variances.

All data processing, modeling, and graphical analyses were performed using GNU Octave and Microsoft Excel (Microsoft 365, Redmond, WA, USA).

Based on the optimization and miniaturization results, the final sample preparation protocol was established as follows: 100 µL of plasma containing melatonin-d_4 as IS at a concentration of 40 ng mL^{-1} was transferred to a 2.0 mL microcentrifuge tube and mixed with 200 µL of ultrapure water. Subsequently, 400 µL of ACN was added, and the mixture was vortexed for 20 s. As a sample clarification step before freezing, the samples were briefly centrifuged at 6,000 rpm for 1 min to promote the settling of precipitated proteins and particulate matter, thereby facilitating subsequent phase separation. The tubes were then stored at −18 °C for 12 hours to allow complete freezing of the aqueous phase. After this period, the upper organic phase was carefully collected and subjected to LC–MS/MS analysis.

2.6. Method Validation.

The analytical method for pesticide quantification was validated in accordance with the guidelines established by the Brazilian National Institute of Metrology, Quality and Technology (INMETRO) in the document DOQ-CGCRE-008 and complemented by the European Commission's SANTE/11312/2021 guideline [29,30]. The following performance parameters were assessed: matrix effect, linearity, limits of detection (LOD) and quantification (LOQ), accuracy, and precision (repeatability and intermediate precision).

To evaluate the matrix effect (ME), two analytical curves were constructed and processed using the same LLE–LTP extraction: one prepared in solvent (pesticide-spiked ultrapure water) and one prepared in spiked plasma matrix (pesticide-spiked human plasma). Each calibration curve consisted of five concentration levels, each analyzed in quintuplicate (n = 5), covering the same concentration range. The matrix effect was assessed by comparing the slopes of the calibration curves obtained in solvent and matrix. A Student's t-test was applied to the slope coefficients to determine statistical significance (p-value), with $p > 0.05$ indicating no significant difference at a 95% confidence level. In addition, the matrix effect percentage (ME%) was calculated using Equation 1, where $\mathrm{S_{mat}}$ represents the slope of the curve obtained in the fortified matrix, and $\mathrm{S_{sol}}$ corresponds to the slope of the curve prepared in solvent. A matrix effect was considered negligible when the relative variation between slopes was within ±20%.

$$\text{Matrix effect (\%)} = \left(\frac{\mathrm{S_{mat}}}{\mathrm{S_{sol}}} - 1\right) * 100 \quad \text{Equation 1}$$

To assess linearity, solvent-based calibration curves were constructed using pesticide-spiked ultrapure water, and no significant matrix effect was observed. Five concentration levels were evaluated, each analyzed in quintuplicate (n = 5), processed using the LLE–LTP extraction. The curves were generated by plotting the ratio of analyte peak area to internal standard peak area versus analyte concentration. Due to differences in ionization efficiency among the target analytes, they were grouped according to appropriate linear ranges, as detailed in Table 3. Linearity was considered acceptable when the correlation coefficient (r) exceeded 0.9950.

The limit of quantification (LOQ) was estimated using the calibration curves constructed during the linearity study in fortified plasma. It was calculated as the ratio between the standard deviation (σ) of the responses from ten blank plasma samples and the slope ($\mathrm{S_{sol}}$) of the calibration curve, as shown in Equation 2. The limit of detection (LOD) was defined as one-third of the LOQ value.

$$\mathrm{LOQ} = \frac{10 * \sigma}{S_{sol}} \quad \text{Equation 2}$$

Accuracy was evaluated through recovery experiments at three quality control (QC) levels: low (LQC), medium (MQC), and high (HQC), as defined in Table 4. These concentration levels were selected to represent the lower, middle, and upper points of the previously established linear range for each analyte, which refers to the concentrations present in plasma. For each level, five independently prepared replicates were analyzed (n = 5). Recoveries were calculated using Equation 3, where C_{found} is the concentration of the analyte in the fortified sample, $C_{original}$ is the concentration in the unfortified sample, and C_{spike} is the concentration of the analyte added. Recoveries between 80% and 120% were considered acceptable.

$$\text{Recovery (\%)} = \left(\frac{C_{found} - C_{original}}{C_{spike}}\right) * 100 \qquad \text{Equation 3}$$

Repeatability was evaluated at the LQC, MQC, and HQC levels, with five independent replicates at each level. Intermediate precision was assessed under the same conditions but on different days and by different analysts, using the same number of replicates. Precision was expressed as the relative standard deviation (RSD), with values ≤ 15% considered acceptable. To further assess intermediate precision, a one-way analysis of variance (ANOVA) was performed at a 95% confidence level. A p-value greater than 0.05 was interpreted as indicating no statistically significant differences among the replicate sets.

2.7. Comparison with QuEChERS AOAC 2007.01 Method.

For comparative purposes, plasma samples were extracted using a micro-volume QuEChERS procedure adapted from the AOAC 2007.01 method. Extractions were performed with a Micro Volume QuEChERS Kit (Shimadzu, Kyoto, Japan) containing pre-weighed salts (80 mg magnesium sulfate and 20 mg sodium acetate). To each tube, 200 µL of ultrapure water and 300 µL of ACN were added to the salt mixture, followed by 100 µL of human plasma previously spiked with the target analytes and the internal standard (melatonin-d_4 at 40 ng mL^{-1}). The mixture was vortex-mixed for 1 min, then centrifuged at 6,000 rpm for 10 min; the supernatant was collected and analyzed under the same LC–MS/MS conditions described previously. The comparative evaluation between the QuEChERS and LLE–LTP procedures was performed using plasma samples fortified at low, medium, and high quality-control levels (LQC, MQC, and HQC), each analyzed in quintuplicate (n = 5). The comparison was based on the recovery and relative standard deviation (RSD, %) for each level, which represent the accuracy and precision of both extraction workflows. This assessment aimed to demonstrate the extraction efficiency and practical applicability of the developed LLE–LTP method compared with the AOAC-based QuEChERS approach, which is commonly used for biological samples analysis.

2.8. Application to farmers' plasma samples.

To demonstrate the applicability of the developed method under real-world conditions, plasma samples from 21 agricultural workers involved in horticultural activities in the state of Goiás, Brazil, were collected and analyzed. All participants provided informed consent, and the Brazilian Research Ethics Committee (protocol number 7.081.319) approved the study protocol. Inclusion criteria comprised rural workers with direct and routine exposure to pesticides during cultivation and handling practices. Blood samples were collected via venipuncture into EDTA-coated tubes and immediately centrifuged at 3000 rpm for 10 minutes to separate plasma. The resulting plasma aliquots were stored at −80 °C until analysis. Before analysis, samples were thawed at room temperature and subjected to the final established sample preparation protocol, as described in the previous section. Each plasma sample was processed and analyzed in quintuplicate (n = 5) using the validated LLE–LTP–LC–MS/MS method.

3. Results and discussion

3.1. Evaluation of Extraction Solvents.

The choice of an appropriate organic solvent is a critical factor in LTP protocols, directly affecting extraction efficiency and the removal of matrix interferences. For optimal performance, the solvent must meet three main criteria: (i) complete miscibility with water at room temperature to ensure a homogeneous solution before cooling; (ii) a melting point below that of water to prevent the organic phase from freezing; and (iii) a density lower than water, allowing the organic layer to remain on top after freezing, which facilitates separation. Under these conditions, the frozen aqueous phase acts as a physical barrier that retains proteins and other interferences, enabling selective analyte extraction [21].

Among the solvents tested — ACN, methanol, and acetone — only ACN exhibited distinct phase separation after 12 h at –18 °C, with a liquid organic upper layer and a frozen aqueous lower phase. In contrast, acetone and methanol remained fully miscible with water under the same conditions, and no phase separation was observed, precluding their use in LTP extraction. This behavior is consistent with the physicochemical properties of the solvents. Although all possess melting points well below –18 °C (methanol: –97.8 °C; ACN: –45.0 °C; acetone: –95.0 °C), their temperature-dependent miscibility with water differs markedly [31]. ACN–water mixtures exhibit cold-induced phase separation near –16 °C, forming two liquid layers, with the upper phase enriched in ACN (~71.7%) [32]. In contrast, methanol and acetone remain fully miscible with water at below-freezing conditions (−18 °C). Takaizumi and

Wakabayashi (1997) demonstrated via differential scanning calorimetry that no liquid–liquid phase separation occurs in methanol–water systems down to –20 °C [33]. Similarly, Stephen and Stephen (1963) compiled experimental data showing that acetone is completely miscible with water across all proportions, including below 0 °C [34]. Given its unique phase separation behavior upon cooling, compatibility with a wide range of analytes, and widespread use in chromatographic techniques [32,35–37], ACN was selected as the extraction solvent for all subsequent stages of method development.

The addition of ethyl acetate (EtOAc) to the extraction solvent was evaluated to determine its potential for improving the recovery of pesticide residues from plasma samples. EtOAc has been reported to modify solvent polarity and enhance extraction selectivity when combined with ACN, especially in complex biological matrices [21]. In this study, a binary solvent system (ACN:EtOAc, 87.5:12.5 v/v) was compared with pure ACN in terms of overall extraction efficiency. Figure 1 shows the mean absolute_recoveries obtained with both solvents: 70% for pure ACN and 74% for the ACN:EtOAc mixture. Although the modified solvent system yielded a slightly higher mean absolute_recovery, a Student’s t-test revealed no significant difference between the two conditions *(critical* t = 4.30*;* p = 0.20*)*. These results indicate that adding EtOAc at the tested proportion does not significantly enhance extraction efficiency for the evaluated pesticides. Given ACN's equivalent performance, operational simplicity, and broad chromatographic compatibility, pure ACN was retained as the extraction solvent for the protocol. Additionally, adding EtOAc introduces another organic solvent, reducing the green metric of the developed method; therefore, it was not used in the following assays.

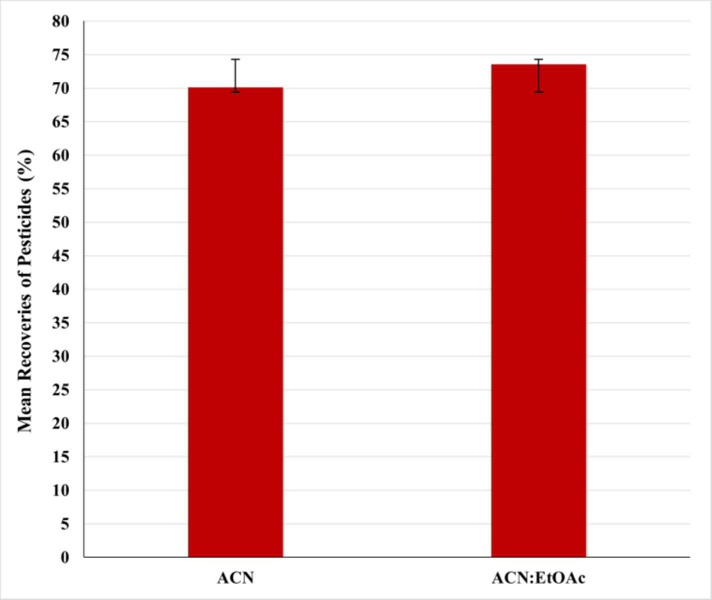


Figure 1. Evaluation of the extraction solvent in the LLE-LTP procedure. Mean absolute recovery of pesticide residues in plasma samples using pure ACN and a mixture (ACN:EtOAc, 87.5:12.5 v/v).

3.2. Experimental design for LLE-LTP sample preparation method.

To identify the most influential parameters affecting the extraction efficiency in the LLE–LTP procedure, a full factorial design 2^3 with two center points (n = 2) was employed. The independent variables investigated were the aqueous sample volume (100–1000 µL), ACN volume (500–1000 µL), and vortex time (20–60 s). The response variable was the mean absolute recovery of the spiked pesticides in plasma samples. Table 1 summarizes the experimental matrix, including the real and coded levels of the variables, the run order, and the mean absolute recoveries. The statistical significance of the main effects and their interactions was evaluated by calculating standardized effects and comparing them within a 95% confidence interval (CI) based on the pooled variance at the center points. The effect error was estimated at 0.4, and with one degree of freedom (df = 1), the critical t-value (95%) was 13, resulting in a CI of ±5. A Pareto diagram of standardized effects is shown in Figure 2.

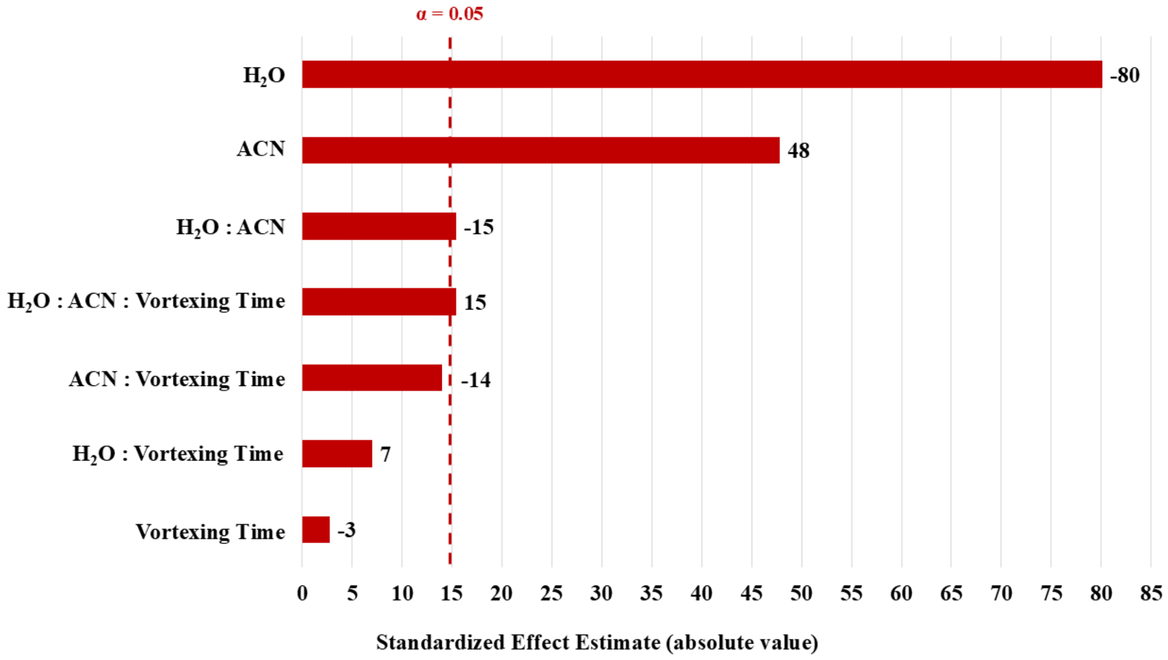


Figure 2. Pareto chart of standardized effects for the 2^3 factorial design. The dashed line represents the significance threshold.

Among the evaluated factors, the volume of water exhibited the strongest negative standardized effect (–80), while the volume of ACN showed a significant positive effect (+48). These results indicate that reducing water volume and increasing ACN volume substantially enhance analyte recovery. This suggests that the miniaturization could be promising, reducing the sample volume requirement and residue generation, making the developed method more sustainable.

The vortex time, on the other hand, showed a negligible effect (–3) and was not statistically significant within the evaluated range. A significant negative interaction between water and ACN (–15) was also observed, suggesting an antagonistic relationship in which optimal recovery is achieved when these two factors are adjusted in opposite directions. Interestingly, a third-order interaction involving water, ACN, and vortex time (+15) exceeded the significance threshold, indicating a possible synergistic effect under specific conditions.

However, because neither the main effect of the vortex nor its two-factor interactions was significant, this three-way interaction likely reflects a context-specific adjustment rather than a dominant trend. Collectively, water and ACN volumes accounted for approximately 91% of the total explained variance in recovery, underscoring their critical role. Based on these findings, the vortex time was fixed at the minimal level (20 s) for simplicity and reproducibility, while water and ACN volumes were selected for further optimization.

A CCD was employed to evaluate and optimize the LLE–LTP extraction parameters. The experimental matrix included 19 statistically designed assays comprising 8 factorial points, 6 axial points (±α, with α = 1.682), and 5 center points. The independent variables studied were the volumes of ultrapure H_2O, ACN, and plasma, and the response was defined as the mean absolute recovery of pesticides from spiked plasma (100.0 ng mL^{-1}). Table 2 presents the CCD matrix, including actual values and coded levels of the variables, along with the observed recovery values.

Initially, a full second-order polynomial model, including all linear, quadratic, and interaction terms, was fitted to the experimental data to describe the system behavior. In a subsequent model refinement step, regression coefficients with 95% confidence intervals that included zero (based on the residual mean square) were considered statistically non-significant and excluded. The resulting reduced quadratic model, shown in Equation 4, retained only statistically meaningful terms, with the estimated coefficients and their confidence intervals (±) presented in parentheses:

$$\hat{y} = 72.99(\pm 3.33) + 5.64(\pm 2.44)x_1 + 6.25(\pm 2.44)x_2 - 7.53(\pm 2.44)x_3 - 7.33(\pm 2.42)x_2^2 + 7.61(\pm 2.42)x_3^2 - 3.91(\pm 3.19)x_1x_2 \quad \text{Equation 4}$$

where ŷ represents the predicted mean absolute recovery of pesticides (%), and x_1, x_2, and x_3 correspond to the coded levels of H_2O, ACN, and plasma volumes, respectively.

The significance and quality of this reduced model were evaluated through analysis of variance (ANOVA), as summarized in Table 5. ANOVA is a fundamental statistical method for assessing how much of the total variability in the response can be explained by the tested factors and interactions [38]. The model showed a strong correlation, with an R^2 value of 0.95 and an adjusted R^2 of 0.92, indicating that the selected variables explained 95% of the total variability in pesticide recovery. The F-value for the regression ($F_{cal} = 36.15$) was significantly greater than the critical value ($F_{tab} = 3.00$), with a p-value of 5.3×10^{-7}, confirming the model's statistical significance. The lack-of-fit test was not significant ($F_{cal} = 5.11 < F_{tab} = 6.04$, p = 0.07), indicating that the model adequately describes the experimental data and that the variation not explained by the model can be attributed to random error rather than systematic inadequacy.

Table 5. Analysis of variance (ANOVA) for the second-order polynomial model fitted to the CCD data.

	Sum of Squares	Degrees of Freedom	Mean Square	F_{cal}	F_{tab}	F_{cal}/F_{tab}	p-value
Regression	3652	6	609	36.15	3.00	12.07	5.3×10^{-7}
Residual	202	12	17				
Total	3854	18	214				
Pure Error	18	4	5	5.11	6.04	0.85	0.07
Lack of Fit	184	8	23				
R^2	0.95						
Adjusted-R^2	0.92						

Following the validation of the reduced quadratic response surface model, a numerical optimization procedure was used to identify the experimental conditions that maximize pesticide recovery. The traditional strategy for this task involves solving the system of partial derivatives of the fitted model to locate the stationary point—i.e., the point at which the gradient of the response surface is zero, corresponding to a potential maximum, minimum, or saddle point. However, in this study, the stationary point was found at $x_1 = -3.80$, which lies well outside the experimental design space (coded range: –1.68 to +1.68 for all variables). This value corresponds to a negative water volume (approximately –156 µL), which is physically infeasible and highlights a common limitation of unconstrained optimization in response surface methodology: mathematically valid solutions may fall outside the practical experimental domain, rendering them unusable.

To overcome this limitation and ensure that the optimization was conducted within the validated region of the design space, a constrained numerical optimization approach was employed. The coded values of the independent variables were restricted to the interval [–1.682, +1.682], thus ensuring that all predicted conditions remained within the experimentally supported range. Under these constraints, the optimal extraction condition was identified as $x_1 = 1.68$, $x_2 = 0.0$ and $x_3 = -1.68$, corresponding to 500 µL of ultrapure water, 1000 µL of ACN, and 250 µL of plasma, respectively. According to the model, this combination yields a predicted mean absolute recovery of 117 ± 10%.

To visualize the response distribution across the experimental domain and support the optimization outcome, a three-dimensional scatter plot with color-coded recovery values was generated (Figure 3). The highest predicted recoveries are shown in red and clearly delineate the optimal region, near the boundary of the design space, further reinforcing the importance of applying constrained optimization to avoid extrapolation errors.

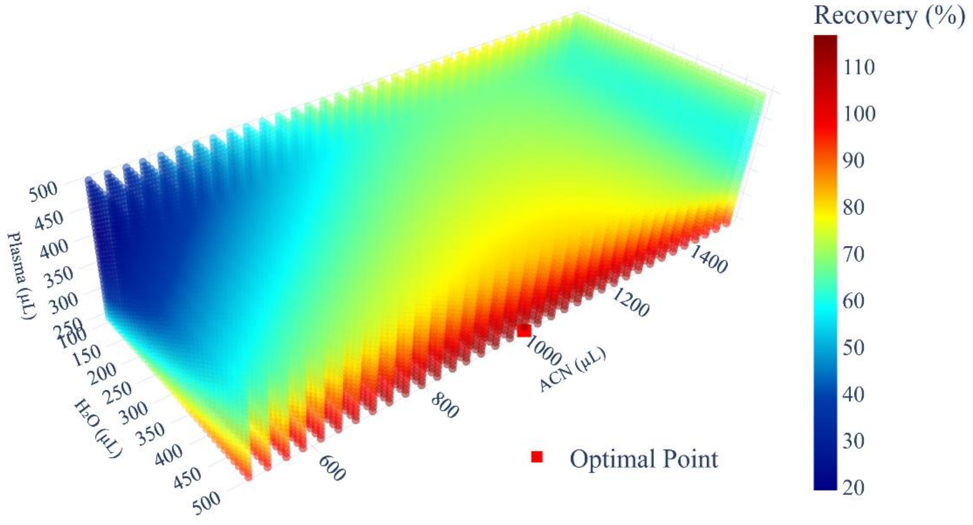


Figure 3. Three-dimensional scatter plot representing the experimental design space. Each axis corresponds to one experimental factor (H_2O, ACN, plasma), and color represents recovery. The red region indicates the area with the highest predicted recovery.

In addition, two-dimensional response surface plots were constructed to evaluate the interaction effects among variables near the predicted optimum. Rather than fixing the third variable at its central (zero) level, it was held at its predicted optimal value to provide a more realistic and targeted visualization of system behavior near the optimal conditions. Figure 4 presents the response surface for H_2O volume versus ACN volume, with plasma volume fixed at 250 µL. The plot shows a pronounced interaction effect and a clearly defined optimal region at the design boundary, indicating that small deviations in water or ACN volume can significantly influence extraction efficiency. Finally, the predicted optimal condition (500 µL water, 1000 µL ACN, 250 µL plasma) was experimentally tested and confirmed by the model, confirming the reliability of the optimized model. The results of this experimental confirmation, along with the evaluation of miniaturized extraction protocols, are presented and discussed in the next section.

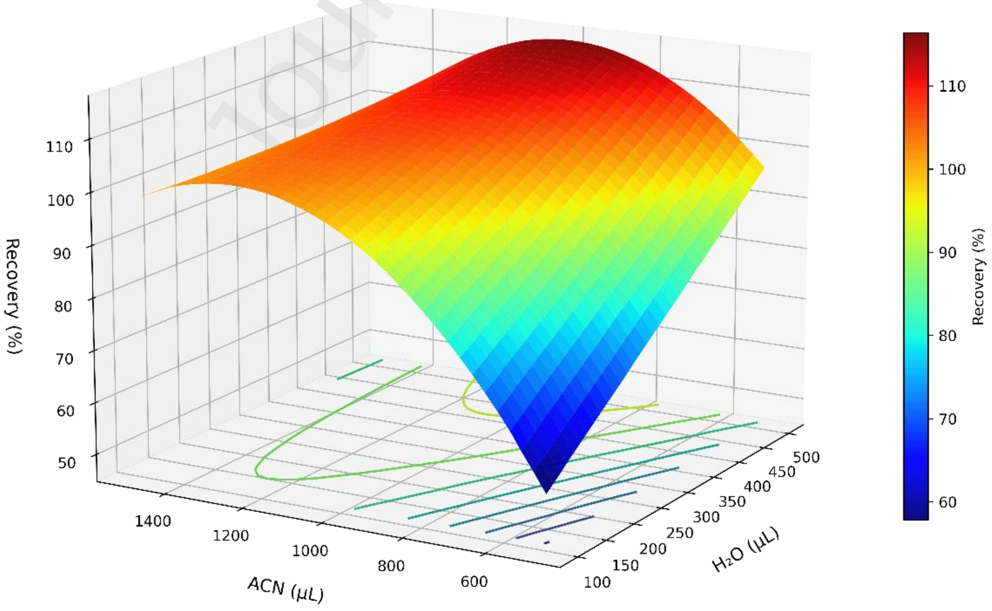


Figure 4. Response surface showing the interaction between water and ACN volumes on pesticide recovery, with plasma volume fixed at its optimal level (250 µL).

3.3. Miniaturization of the Optimized LLE–LTP Procedure.

Based on the optimized extraction conditions established in the previous section, a separate miniaturization study was performed to evaluate whether reducing sample and solvent volumes would preserve extraction performance while improving operational sustainability. Three miniaturized protocols were therefore tested and compared to the optimized full-scale condition. The mean absolute recoveries and associated standard deviations for each condition are summarized in Table 6. All protocols maintained the same extraction ratio and were evaluated under identical experimental conditions. A one-way ANOVA was conducted to determine whether the observed differences in mean absolute recoveries were statistically significant. As shown in Table 7, no significant difference was found among the conditions ($F_{cal} = 0.95 < F_{tab} = 4.07$; $p = 0.46$), confirming that miniaturization did not adversely affect the extraction performance. These findings support the robustness of the LLE–LTP method when scaled down in terms of extraction accuracy. The effect of miniaturization on method precision (repeatability and intermediate precision) was subsequently evaluated during the validation stage, as discussed in the next section.

Table 6. Mean Absolute Recoveries Under Different Miniaturization Conditions.

	Plasma (µL)	**ACN (µL)**	**H_2O (µL)**	**Mean Absolute Recovery (%) ± SD**
Optimized **(reference)**	250	1000	500	111 ± 5
Miniaturized Condition 1	50	200	100	112 ± 1
Miniaturized Condition 2 **(selected)**	100	400	200	107 ± 5
Miniaturized Condition 3	125	500	250	109 ± 3

Table 7. One-Way ANOVA for Miniaturization Comparison.

Source of Variation	**Sum of Squares**	**Degrees of Freedom**	**Mean Square**	**F_{cal}**	**F_{tab}**	**F_{cal}/F_{tab}**	**p-value**
Between Groups	45.21	3	15.07	0.95	4.07	0.23	0.46

Within Groups	127.15	8	15.89
Total	172.36	11	

Despite the equivalent analytical performance, practical handling during extraction influenced the selection of the final protocol. The condition using 50 µL of plasma, although efficient, posed challenges because rapid thawing of the aqueous phase complicated phase separation and compromised reproducibility. Therefore, the protocol using 100 µL of plasma, 400 µL of ACN, and 200 µL of water was selected for subsequent validation. This condition provided consistently high selectivity, a mean absolute recovery of 112 ± 1%, and an operationally convenient procedure for routine application.

In quantitative terms, the optimized full-scale condition used 250 µL of plasma and a total solvent volume of 1500 µL (1000 µL ACN + 500 µL water), yielding a solvent-to-sample ratio of 6:1. The final miniaturized protocol maintained the same solvent-to-sample ratio (600 µL solvent for 100 µL plasma, 6:1) while reducing both sample and solvent volumes by 60%. This represents a substantial reduction in absolute solvent consumption and biological sample requirements without compromising analytical performance, thereby improving the method's sustainability and practicality.

Moreover, the sample volume used here represents a substantial reduction compared with several human plasma pesticide-analysis methods in the literature, which typically use larger aliquots (typically 500 µL–1 mL). For example, Srivastava et al. (2017) and Farajzadeh et al. (2020) both reported protocols based on 1 mL plasma, whereas Godage et al. (2023) used 500 µL; conversely, Gross et al. (2024) demonstrated extraction from 100 µL plasma in an environmental monitoring context [25–28].

3.4. Analytical Method Validation.

The evaluation of matrix effects is a critical step in method validation, particularly when dealing with complex biological matrices such as human plasma. In this study, a statistical comparison of the analytical curves prepared in solvent (pesticide-spiked ultrapure water) and in plasma matrix (pesticide-spiked human plasma) showed no significant matrix effect for any of the 30 pesticides analyzed. This was supported by p-values above the 0.05 threshold in all cases (Table 3), as determined by a Student's t-test applied to the slope coefficients, indicating no statistically significant difference in analytical sensitivity between the two curves. Given these findings, all the quantification assays were performed using solvent-based calibration curves prepared from pesticide-spiked ultrapure water and subjected to the complete LLE–LTP extraction procedure, following the recommendations outlined in the INMETRO validation guide [30]. Furthermore, the calculated ME% values ranged from –14.76% to +3.37%, remaining within the commonly accepted range of ±20%, indicating that matrix effects were present but not significant enough to compromise quantitative performance [29].

All analytes demonstrated excellent linearity across their respective concentration ranges, with correlation coefficients (r) exceeding 0.997 for all compounds (Table 3). These results confirm that the method provides a consistent, proportional response across a wide range of concentrations, ensuring reliable quantification of pesticide residues in plasma, even at trace levels.

The method exhibited high sensitivity, with limits of quantification (LOQ) ranging from 0.01 to 30 ng·mL^{-1} and limits of detection (LOD) from 0.004 to 10 ng·mL^{-1} (Table 3). These limits are comparable to or better than several reported bioanalytical workflows: for instance, Srivastava et al. (2017) reported LOQs of 0.12–13.53 ng·mL^{-1} and LODs of 0.04–4.10 ng·mL^{-1}, Farajzadeh et al. (2020) reported LODs of 1.4–2.3 ng·mL^{-1} for selected pesticides, and Godage et al. (2023) achieved LOQs between 0.01 and 5 ng·mL^{-1} for a large panel of analytes.[25,26,28] Thus, the present LLE–LTP–LC–MS/MS workflow attains comparable or better sensitivity using only 100 µL of plasma, while conventional procedures often require 500–1000 µL, representing a 5-10 times reduction in sample consumption without compromising analytical performance.

Table 3. Matrix effect (ME%), p-values, correlation coefficients (r), linear ranges, and calculated LODs and LOQs for the target pesticides.

Analyte	p-value	ME (%)	r	Linear range*	LOQ*	LOD*
2,4,5-T	0.95	0.49	0.9990	1.2-40	0.95	0.32
2,4-D	0.59	-10.46	0.9998	50-200	20	6.8
Acephate	0.94	-1.29	0.9992	1.2-40	1.0	0.35
Alachlor	0.17	-7.52	0.9986	0.08-40	0.02	0.01
Aldicarb	0.16	-8.43	0.9987	0.8-200	0.47	0.16
Ametryn	0.09	-5.85	0.9992	1.6-200	0.02	0.01
Atrazine	0.26	-7.97	0.9999	25-40	1.4	0.45
Benzidine	0.11	-6.64	0.9995	2.5-40	0.20	0.07
Carbendazim	0.40	3.37	0.9988	1.6-200	0.49	0.16
Carbofuran	0.06	0.65	0.9998	0.4-200	0.27	0.09
Chlorpyrifos	0.06	-0.99	0.9971	6-200	5.2	1.7
Cyproconazole	0.11	-6.27	0.9998	0.4-200	0.05	0.02
Deisopropylatrazine	0.21	-5.50	0.9992	0.08-40	0.06	0.02
Demeton	0.40	-3.91	0.9998	0.08-40	0.03	0.01
Desethylatrazine	0.30	-2.98	0.9983	0.08-40	0.09	0.03
Desthio-Prothioconazole	0.22	-7.13	0.9990	0.08-40	0.38	0.13
Dimethoate	0.61	-1.96	0.9994	0.8-200	0.48	0.16
Diuron	0.40	-5.15	0.9995	1.6-800	0.64	0.21
Epoxiconazole	0.25	-5.66	0.9996	1.6-800	0.30	0.10
Fipronil	0.45	-14.62	0.9974	0.8-50	0.05	0.02
Flutriafol	0.63	-4.20	0.9975	25-200	21	7.1
Methamidophos	0.71	-7.08	0.9985	0.6-10	0.01	0.004
Metolachlor	0.21	-4.72	0.9987	0.08-40	0.05	0.02
Metribuzin	0.23	-6.36	0.9997	0.8-200	0.78	0.26
Molinate	0.19	-14.76	0.9995	5-40	2.8	0.94
Picloram	0.12	-7.70	0.9998	40-800	30	10
Simazine	0.16	-7.44	0.9996	2.5-40	1.60	0.53
Tebuconazole	0.22	-7.36	0.9995	1.6-800	0.10	0.03
Thiamethoxam	0.68	-7.50	0.9996	1.6-800	0.01	0.004
Thiodicarb	0.57	-13.55	0.9988	1.6-800	0.06	0.02

*ng mL^{-1} (in plasma)

Table 4. Accuracy and Precision Results for Quality Control Levels.

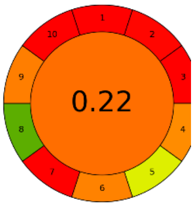
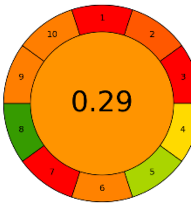
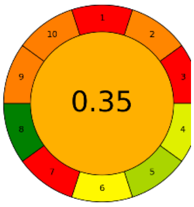
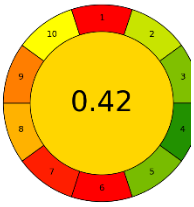
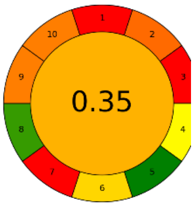
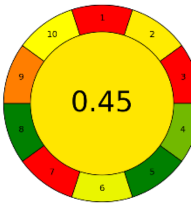
	Accuracy - Recovery (%)			**Repeatability - RSD (%)**			**Intermediate precision - RSD (%)**					
Analyte	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**		**MQC**		**HQC**	
	2 ng mL^{-1}	**4 ng mL^{-1}**	**10 ng mL^{-1}**	**2 ng mL^{-1}**	**4 ng mL^{-1}**	**10 ng mL^{-1}**	**2 ng mL^{-1}**	**p value**	**4 ng mL^{-1}**	**p value**	**10 ng mL^{-1}**	**p value**
Methamidophos	118	88	95	0.97	4.78	3.52	8.97	0.14	6.63	0.88	13.51	0.26
	4 ng mL^{-1}	**20 ng mL^{-1}**	**40 ng mL^{-1}**	**4 ng mL-1**	**20 ng mL^{-1}**	**40 ng mL^{-1}**	**4 ng mL^{-1}**	**p value**	**20 ng mL^{-1}**	**p value**	**40 ng mL^{-1}**	**p value**
2,4,5-T	87	102	100	4.24	6.83	3.08	12.05	0.47	10.24	0.38	5.78	0.11
Acephate	85	87	91	5.65	6.67	8.31	4.95	0.77	8.55	0.46	9.58	0.66
Benzidine	86	103	108	8.31	3.27	5.02	11.49	0.56	6.23	0.22	8.47	0.44
Deisopropylatrazine	100	103	106	4.51	3.36	2.21	4.84	0.12	4.57	0.33	4.24	0.45
Demeton	92	100	108	8.55	3.69	2.96	10.32	0.50	2.29	0.39	3.40	0.85
Desethylatrazine	95	104	105	5.73	2.52	2.22	3.97	0.29	3.19	0.78	3.23	0.76
Desthio-Prothioconazole	88	106	103	7.38	3.46	3.13	13.06	0.09	3.73	0.81	3.89	0.33
Metolachlor	97	98	101	6.64	3.49	4.36	11.75	0.91	3.88	0.71	5.35	0.65
Molinate	112	110	106	9.75	6.90	6.86	12.40	0.21	8.30	0.67	5.73	0.28
Simazine	99	99	104	3.14	2.18	4.55	9.76	0.62	2.00	0.96	4.99	0.97
	10 ng mL^{-1}	**20 ng mL^{-1}**	**40 ng mL^{-1}**	**10 ng mL^{-1}**	**20 ng mL^{-1}**	**40 ng mL^{-1}**	**10 ng mL^{-1}**	**p value**	**20 ng mL^{-1}**	**p value**	**40 ng mL^{-1}**	**p value**
Fipronil	115	101	103	2.74	4.11	3.98	5.34	0.41	9.16	0.73	4.87	0.57
	10 ng mL^{-1}	**100 ng mL^{-1}**	**200 ng mL^{-1}**	**10 ng mL^{-1}**	**100 ng mL^{-1}**	**200 ng mL^{-1}**	**10 ng mL^{-1}**	**p value**	**100 ng mL^{-1}**	**p value**	**200 ng mL^{-1}**	**p value**
Alachlor	93	106	109	10.06	5.98	3.95	6.72	0.77	13.10	0.09	3.97	0.86
Aldicarb	80	101	107	8.38	4.56	8.10	12.43	0.50	11.49	0.06	5.94	0.26
Carbofuran	108	99	104	4.82	2.59	3.80	3.98	0.79	12.69	0.44	5.59	0.53
Chlorpyrifos	120	94	104	6.07	5.41	8.69	6.29	0.16	11.78	0.15	9.40	0.50
Cyproconazole	90	103	108	6.22	3.75	3.98	3.69	0.72	3.62	0.98	4.02	0.79
Dimethoate	112	98	107	4.87	2.40	3.68	1.95	0.20	12.39	0.92	4.93	0.46
Metribuzin	99	99	102	6.88	2.96	3.29	1.78	0.25	2.70	0.94	4.64	0.40
	25 ng mL^{-1}	**32 ng mL^{-1}**	**40 ng mL^{-1}**	**25 ng mL^{-1}**	**32 ng mL^{-1}**	**40 ng mL^{-1}**	**25 ng mL^{-1}**	**p value**	**32 ng mL^{-1}**	**p value**	**40 ng mL^{-1}**	**p value**
Atrazine	92	91	103	2.94	5.95	4.02	3.27	0.75	5.98	0.79	4.10	0.07
	50 ng mL^{-1}	**100 ng mL^{-1}**	**200 ng mL^{-1}**	**50 ng mL^{-1}**	**100 ng mL^{-1}**	**200 ng mL^{-1}**	**50 ng mL^{-1}**	**p value**	**100 ng mL^{-1}**	**p value**	**200 ng mL^{-1}**	**p value**
2,4-D	84	91	92	8.27	14.11	11.86	8.27	0.95	12.96	0.79	8.13	0.24
Ametryn	100	96	97	5.79	7.07	10.43	7.81	0.75	3.41	0.37	10.43	0.45
Carbendazim	106	87	106	10.38	8.49	2.31	6.23	0.14	12.76	0.27	3.09	0.57
Flutriafol	103	102	106	12.45	1.80	3.76	3.68	0.19	2.59	0.67	4.06	0.81
	40 ng mL^{-1}	**400 ng mL^{-1}**	**800 ng mL^{-1}**	**40 ng mL^{-1}**	**400 ng mL^{-1}**	**800 ng mL^{-1}**	**40 ng mL^{-1}**	**p value**	**400 ng mL^{-1}**	**p value**	**800 ng mL^{-1}**	**p value**
Diuron	98	104	100	7.74	4.28	4.58	7.14	0.09	3.51	0.71	5.69	0.98
Epoxiconazole	88	100	102	4.63	3.20	5.62	12.27	0.81	3.29	0.79	4.66	0.28
Tebuconazole	101	100	104	8.47	1.73	3.40	5.90	0.74	2.22	0.37	4.85	0.41
Thiamethoxam	97	102	107	7.35	1.43	3.72	3.59	0.22	2.81	0.13	4.14	0.96
Thiodicarb	96	96	109	2.14	2.91	5.21	1.09	0.37	3.31	0.63	5.61	0.63
	80 ng mL^{-1}	**400 ng mL^{-1}**	**800 ng mL^{-1}**	**80 ng mL^{-1}**	**400 ng mL^{-1}**	**800 ng mL^{-1}**	**80 ng mL^{-1}**	**p value**	**400 ng mL^{-1}**	**p value**	**800 ng mL^{-1}**	**p value**
Picloram	97	95	85	7.24	10.91	8.48	2.87	0.64	10.08	0.20	12.60	0.20

Accuracy was evaluated through recovery studies at low (LQC), medium (MQC), and high (HQC) quality control levels (Table 4). Mean recoveries for all pesticides ranged between 80–120%, in agreement with national and international criteria. Comparable performance has been reported by Araoud et al. (2010) (65–106%, LLE), Srivastava et al. (2017) (74–109%, QuEChERS), and Farajzadeh et al. (2020) (59–80%, Dispersive Liquid-Liquid Microextraction –DLLME) [25,28,39]. Notably, the LLE–LTP procedure achieved equivalent or superior recovery values without the use of cleanup sorbents (e.g., PSA/C18), typically required in QuEChERS workflows, thereby reducing both consumables and sample manipulation.

Precision was assessed in terms of repeatability and intermediate precision (Table 4). Intra-day RSD values were below 15% for all analytes ($n = 5$), and no statistically significant differences ($p > 0.05$) were observed across days or analysts, confirming inter-day reproducibility, demonstrating that the LLE–LTP workflow provides reproducible quantification under routine laboratory conditions. These results align with reported values for similar workflows, such as Srivastava et al. (2017) (intra-day ≤ 6%, inter-day ≤ 11%) and Farajzadeh et al. (2020) (≤ 6%) [25,28].

To provide a broader perspective, Table 8 summarizes analytical performance and sustainability indicators (AGREEprep scores) of the present method in comparison with representative approaches from the literature [40]. The AGREEprep tool was applied to all methods with equal weighting of the ten greenness criteria. The resulting scores ranged from 0.22 to 0.45, with higher values indicating more sustainable workflows. The proposed LLE–LTP method achieved the highest AGREEprep score (0.45), surpassing QuEChERS and LLE-based protocols (0.22–0.35) and approaching the performance of more specialized green techniques such as bio-SPME (0.42). This improvement reflects the miniaturized extraction volume, elimination of sorbents, and reduced solvent consumption inherent to the LLE–LTP workflow.

Table 8. Comparative summary of analytical performance and sustainability assessment (AGREEprep) of plasma-based pesticide analysis methods.

Matrix (Reference)	**Sample volume (µL)**	**Extraction method (Cleanup step)**	**Analytical technique**	**Number of pesticides**	**Recovery %**	**Precision (RSD %)**	**LOQ (ng·mL^{-1})**	**LOD (ng·mL^{-1})**	**AGREEprep**[40]
Human serum [39]	2000	LLE (None)	LC–MS/MS	14	65 – 106	< 15%	5	2	
Human plasma [25]	1000	QuEChERS (PSA 50 mg)	GC–MS/MS	31	74 – 109	≤ 11%	0.12 – 13.53	0.04 – 4.10	
Human plasma [28]	1000	DLLME (None)	HPLC-DAD	9	59 – 80	≤ 6%	4.8 – 7.7	1.4 – 2.3	
Human plasma [26]	500	SPME (None)	LC–MS/MS	79	70 – 120	< 15%	0.01 – 5	-	
Fish plasma [27]	100	SPE (EMR 300 mg)	GC and LC-MS/MS	182	70 – 130	< 10%	-	0.4 – 13	
Human plasma (This work)	100	LLE–LTP (None)	LC–MS/MS	30	80 – 120	< 15%	0.01 – 30	0.04 – 10	

Legend: - : not related.

The comparative evaluation (Table 8) highlights the balance between analytical performance and environmental sustainability achieved by the proposed workflow. By integrating LTP, the method combines efficient phase separation, low sample and solvent volumes, and minimal consumable use—attributes that contribute to its improved AGREEprep score and operational simplicity. These features make the LLE–LTP–LC–MS/MS workflow a viable and sustainable alternative for large-scale biomonitoring studies of pesticide exposure in human plasma.

3.5. Comparison with the QuEChERS AOAC 2007.1 Method.

The performance of the micro-QuEChERS kit and the optimized LLE–LTP workflow was evaluated using recovery and precision for 30 pesticides at three QC levels (30 analytes × 3 levels = 90 recovery measurements per method), summarized in Table 9. LLE–LTP produced more consistent recoveries under the evaluated plasma conditions: all 90 recoveries fell within the 80–120% acceptance interval used in our validation [29,30], whereas only 77/90 (85.6%) of QuEChERS recoveries met this criterion (outliers in Table 9 are highlighted in red). Precision was also improved with LLE–LTP. The mean RSD across analytes decreased from 6.60% (QuEChERS) to 5.36% (LLE–LTP) (19% relative improvement), and 21 of 30 analytes exhibited lower mean RSDs with LLE–LTP. Representative examples include simazine (mean RSD reduced from 10.7% to 3.3%) and methamidophos (from 9.4% to 3.1%). Two factors account for the observed advantages of LLE–LTP in this plasma biomonitoring context. First, LLE–LTP minimizes physical manipulation (fewer transfers and no contact with PSA/C18 sorbents), reducing analyte losses from adsorption and limiting variability introduced by sorbent equilibration. Second, low-temperature partitioning in LLE–LTP promotes reproducible separation of ACN and the frozen aqueous fraction; the frozen aqueous "barrier" serves as an implicit clean-up step, yielding a more homogeneous organic extract and tighter analytical precision. Operationally, LLE–LTP also reduces recurring costs and consumable waste: it uses standard laboratory reagents (ACN, water). It employs routine freezer storage during extraction, avoiding the purchase of commercial salt kits and disposable sorbents [19,21,32,41].

Nevertheless, QuEChERS remains a well-established and highly robust approach for high-throughput applications, particularly in food and environmental analysis, where large sample batches, diverse matrices, and regulatory harmonization are critical [18,20,25]. The present results showed that LLE–LTP can be a competitive, context-specific alternative for small-volume biological matrices such as human plasma. Additionally, LLE-LTP required fewer steps than QuEChERS, improved method precision, and was a sustainable methodology because it eliminates the need for disposable sorbents and reduces residue generation. These features contribute to consistent analytical performance while reducing consumable use and operational costs for laboratories that prioritize resource efficiency.

Table 9. Comparison of recovery (%) and precision (RSD %, n = 5) for pesticides in plasma samples obtained using the micro-QuEChERS AOAC 2007.1 and the optimized LLE–LTP methods at three QC levels. The outliers are highlighted in red.

Analyte	**Accuracy - Recovery (%)**						**Repeatability - RSD (%)**					
	QuEChERS	**LTP**	**QuEChERS**	**LTP**	**QuEChERS**	**LTP**	**QuEChERS**	**LTP**	**QuEChERS**	**LTP**	**QuEChERS**	**LTP**
	LQC - 2 ng mL^{-1}		**MQC - 4 ng mL^{-1}**		**HQC - 10 ng mL^{-1}**		**LQC - 2 ng mL^{-1}**		**MQC - 4 ng mL^{-1}**		**HQC - 10 ng mL^{-1}**	
Methamidophos	110	118	82	88	124	95	7,61	0,97	11,2	4,78	9,33	3,52
	LQC - 4 ng mL^{-1}		**MQC - 20 ng mL^{-1}**		**HQC - 40 ng mL^{-1}**		**LQC - 4 ng mL^{-1}**		**MQC - 20 ng mL^{-1}**		**HQC - 40 ng mL^{-1}**	
2,4,5-T	98	87	93	102	97	100	12,17	4,24	6,99	6,83	7,19	3,08
Acephate	98	85	113	87	134	91	14,14	5,65	5,03	6,67	7,05	8,31
Benzidine	94	86	109	103	97	108	2,49	8,31	4,14	3,27	10,55	5,02
Deisopropylatrazine	97	100	105	103	106	106	2,06	4,51	3,8	3,36	6,29	2,21
Demeton	98	92	109	100	103	108	1,49	8,55	3,83	3,69	8,4	2,96
Desethylatrazine	112	95	145	104	127	105	4,51	5,73	9,46	2,52	9,75	2,22
Desthio-Prothioconazole	118	88	82	106	97	103	4,51	7,38	9,46	3,46	9,75	3,13
Metolachlor	127	97	102	98	82	101	3,95	6,64	5,61	3,49	4,43	4,36
Molinate	118	112	132	110	128	106	13,12	9,75	3,98	6,9	10,02	6,86
Simazine	91	99	99	99	89	104	10,86	3,14	8,89	2,18	12,38	4,55
	LQC - 10 ng mL^{-1}		**MQC - 20 ng mL^{-1}**		**HQC - 40 ng mL^{-1}**		**LQC - 10 ng mL^{-1}**		**MQC - 20 ng mL^{-1}**		**HQC - 40 ng mL^{-1}**	
Fipronil	121	115	120	101	122	103	6,45	2,74	7,74	4,11	3,05	3,98
	LQC - 10 ng mL^{-1}		**MQC – 100 ng mL^{-1}**		**HQC - 200 ng mL^{-1}**		**LQC - 10 ng mL^{-1}**		**MQC – 100 ng mL^{-1}**		**HQC - 200 ng mL^{-1}**	
Alachlor	89	93	104	106	99	109	7,15	10,06	3,92	5,98	5,83	3,95
Aldicarb	113	80	107	101	104	107	13,3	8,38	6,76	4,56	6,69	8,1
Carbofuran	103	108	107	99	106	104	3,86	4,82	7,29	2,59	5,6	3,8
Chlorpyrifos	85	120	97	94	96	104	8,23	6,07	3,28	5,41	6,84	8,69
Cyproconazole	84	90	89	103	85	108	6,74	6,22	4,07	3,75	4,48	3,98
Dimethoate	108	112	110	98	112	107	2,13	4,87	7,58	2,4	5,21	3,68
Metribuzin	99	99	97	99	93	102	6,78	6,88	4,96	2,96	6,9	3,29
	LQC - 25 ng mL^{-1}		**MQC - 32 ng mL^{-1}**		**HQC - 40 ng mL^{-1}**		**LQC - 25 ng mL^{-1}**		**MQC - 32 ng mL^{-1}**		**HQC - 40 ng mL^{-1}**	
Atrazine	97	92	87	91	100	103	2,7	2,94	7,29	5,95	6,99	4,02
	LQC - 50 ng mL^{-1}		**MQC - 100 ng mL^{-1}**		**HQC - 200 ng mL^{-1}**		**LQC - 50 ng mL^{-1}**		**MQC - 100 ng mL^{-1}**		**HQC - 200 ng mL^{-1}**	
2,4-D	78	84	86	91	89	92	6,86	8,27	11,88	14,11	8,72	11,86
Ametryn	92	100	109	96	104	97	10,75	5,79	7,2	7,07	4,35	10,43
Carbendazim	94	106	107	87	93	106	11,47	10,38	5,38	8,49	3,27	2,31
Flutriafol	82	103	92	102	91	106	13,41	12,45	7,59	1,8	7,71	3,76
	LQC - 40 ng mL^{-1}		**MQC - 400 ng mL^{-1}**		**HQC - 800 ng mL^{-1}**		**LQC - 40 ng mL^{-1}**		**MQC - 400 ng mL^{-1}**		**HQC - 800 ng mL^{-1}**	
Diuron	107	98	111	104	116	100	8,14	7,74	1,93	4,28	4,83	4,58
Epoxiconazole	90	88	102	100	104	102	6,43	4,63	6,33	3,2	3,92	5,62
Tebuconazole	90	101	90	100	120	104	8,71	8,47	1,93	1,73	5,35	3,4
Thiamethoxam	109	97	108	102	114	107	7,65	7,35	7,4	1,43	2,46	3,72
Thiodicarb	98	96	110	96	109	109	6,4	2,14	4,27	2,91	2,28	5,21
	LQC - 80 ng mL^{-1}		**MQC - 400 ng mL^{-1}**		**HQC - 800 ng mL^{-1}**		**LQC - 80 ng mL^{-1}**		**MQC - 400 ng mL^{-1}**		**HQC - 800 ng mL^{-1}**	
Picloram	75	97	126	95	136	85	1,86	7,24	3,08	10,91	6,61	8,48

3.6. Evaluation of Analytes in Farmers' Plasma Samples.

Pesticide residues were investigated in plasma samples from twenty-one agricultural workers. At least one compound was detected in eleven individuals (52%), indicating that more than half of the monitored population exhibited measurable exposure (Table 10). Although most detections were below the quantification limit (<LOQ) and therefore interpreted qualitatively, they still reveal contact with multiple pesticide classes. Only one sample (Plasma 3) showed a quantifiable concentration (chlorpyrifos at 14 ± 4.6 ng mL^{-1}), while other analytes, such as acephate, deisopropylatrazine, desethylatrazine, and desthio-prothioconazole, were detected at trace levels.

Table 10. Detection and quantification of pesticide residues in plasma samples from 21 agricultural workers with the LLE–LTP method.

Pesticide	**Plasma 1**	**Plasma 2**	**Plasma 3**	**Plasma 5**	**Plasma 9**	**Plasma 14**	**Plasma 16**	**Plasma 17**	**Plasma 18**	**Plasma 19**	**Plasma 20**
Acephate				< LOQ							
Chlorpyrifos			14.0 ng mL^{-1}								
Deisopropylatrazine	< LOQ					< LOQ		< LOQ	< LOQ	< LOQ	< LOQ
Desethylatrazine		< LOQ					< LOQ	< LOQ	< LOQ	< LOQ	
Desthio-Prothioconazole					< LOQ						

The detection of chlorpyrifos, even in a single quantifiable instance, is toxicologically relevant. From an analytical standpoint, this finding is noteworthy given the well-documented toxicological profile of this compound. Chlorpyrifos, an organophosphate widely used since the 1960s, acts as a potent acetylcholinesterase inhibitor and has been consistently associated with neurotoxic and endocrine-disrupting effects in both occupationally and environmentally exposed populations [42,43]. Chronic low-level exposure, even below conventional regulatory thresholds, has been linked to neurobehavioral alterations, hormonal imbalances, and developmental toxicity. These observations support the growing body of evidence suggesting that there is no truly safe exposure threshold for pesticide classes, particularly organophosphates [43–45]. Although the present study was not designed to assess toxicological risk or establish exposure limits, the detection of chlorpyrifos provides direct analytical evidence of recent exposure among the monitored individuals.

Importantly, the predominance of <LOQ detections should not be interpreted as an absence of risk. Plasma concentrations reflect only recent exposure windows and may not capture cumulative or bioaccumulative effects. Moreover, transient or sub-quantifiable levels may still produce biological alterations through chronic, low-dose exposure mechanisms. Complementary biomonitoring, such as measurement of urinary metabolites (e.g., TCPy for chlorpyrifos) or repeated plasma sampling campaigns, would provide a more accurate picture of long-term exposure patterns and individual variability [7–10,12].

Several methodological limitations that define the scope of these field data should also be acknowledged. First, the sample size (n = 21) was intended to demonstrate analytical feasibility and should not be considered statistically representative of broader occupational populations. Second, because samples were collected at a single time point, temporal variability and seasonal exposure patterns were not evaluated. Third, plasma is inherently a short-term exposure matrix; thus, measured concentrations primarily reflect recent contact rather than cumulative body burden. Finally, inter-individual exposure variability is intrinsic to agricultural settings, owing to differences in work tasks, application practices, personal protective equipment use, and environmental conditions—and these factors were not controlled in the present study. Consequently, robust exposure assessment requires larger cohorts, longitudinal sampling strategies, and integration with epidemiological frameworks [7–10].

Overall, these findings should be interpreted as a proof-of-concept application of the validated LLE–LTP–LC–MS/MS workflow to real biological samples, rather than as a comprehensive evaluation of occupational health risk.

From an occupational health perspective, these results highlight critical gaps in pesticide regulation and in preventing exposure. Despite the routine use of highly toxic compounds in agricultural settings.

4. Conclusion

A miniaturized LLE–LTP–LC–MS/MS workflow provides reliable multiresidue quantification of 30 pesticides from only 100 µL of human plasma. The method satisfied the applicable validation criteria and compared favourably with the AOAC 2007.01 micro-QuEChERS procedure, yielding more uniform recoveries and improved precision for most analytes while avoiding sorbent-based cleanup. Field application to 21 agricultural workers confirmed the method’s sensitivity and suitability for occupational biomonitoring. Given the documented risks associated with repeated low-level exposure to organophosphates and related compounds, these results reinforce the need for systematic surveillance. Adoption of the LLE–LTP workflow is therefore recommended as a pragmatic, resource-efficient analytical tool for occupational health programs, particularly in resource-constrained settings, as it supports exposure assessment and routine monitoring and provides high-quality analytical data to inform subsequent toxicological interpretation and risk evaluation. When combined with longitudinal sampling strategies and complementary biomarker or toxicological frameworks, this protocol enables a more comprehensive characterization of exposure patterns and dynamics, supporting the assessment of chronic exposure and the design of targeted public health interventions.

Compliance with ethics requirements

All procedures involving human biological material were conducted in accordance with ethical standards for research involving human participants and were approved by the Brazilian Research Ethics Committee (protocol number 7.081.319).]

Credit authorship contribution statement

João Victor Borges Assis: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Hugo G. Machado:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Yuri Arrates Rocha**: Methodology, Investigation, Writing - Original Draft, Visualization. **Almir Custodio Batista Júnior:** Methodology, Validation, Formal analysis, Investigation. **Simone Nascimento**: Methodology, Validation, Formal analysis, Investigation. **Miller Caldas Barradas:** Investigation, Resources. **Daniela de Melo e Silva**: Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Andrea Rodrigues Chaves**: Conceptualization, Resources, Data Curation, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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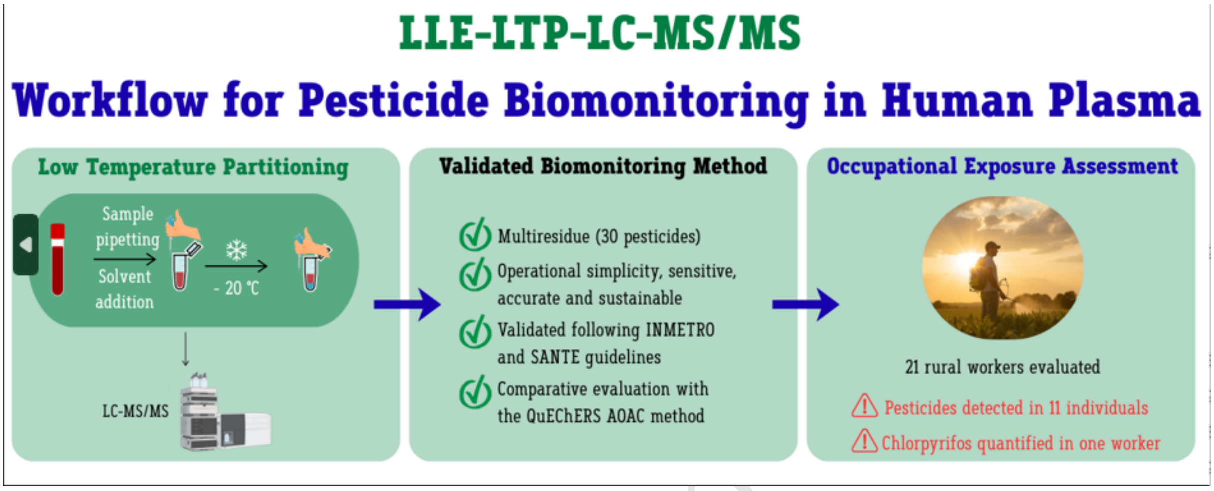
Declaration of Competing Interest

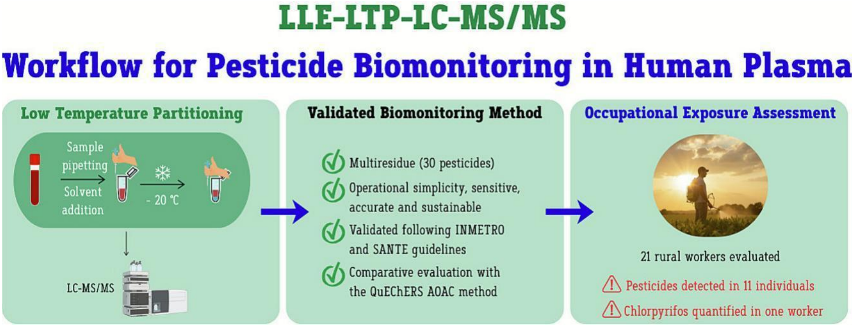
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical abstract



HIGHLIGHTS

- Development of a robust and environmentally sustainable LLE–LTP method for pesticide biomonitoring.
- Successful application of LLE–LTP for multiresidue extraction in human plasma.
- Use of multivariate experimental design for method optimization.
- Satisfactory analytical performance with recoveries of 80–120%, RSD ≤ 15%, and limits of quantification (LOQs) ranging from 0.01 to 30 ng mL^{-1}.
- Practical application of the method for occupational exposure assessment.



LLE-LTP-LC-MS/MS

Workflow for Pesticide Biomonitoring in Human Plasma

Low Temperature Partitioning

Validated Biomonitoring Method

- ✓ Multiresidue (30 pesticides)
- ✓ Operational simplicity, sensitive, accurate and sustainable
- ✓ Validated following INMETRO and SANTE guidelines
- ✓ Comparative evaluation with the QuEChERS AOAC method

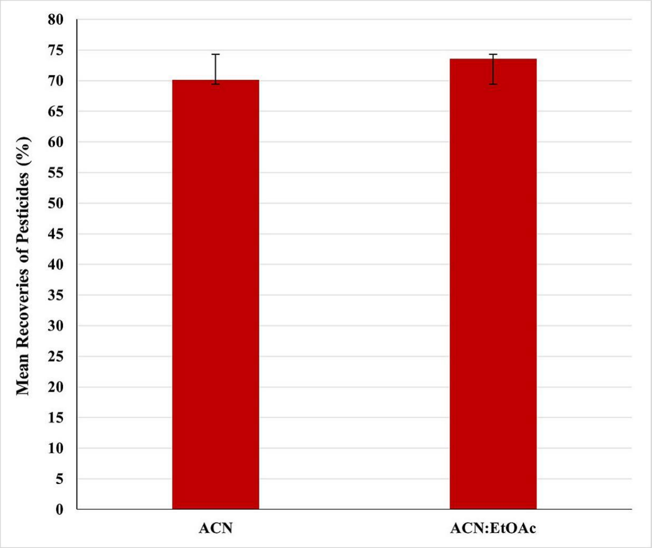
Occupational Exposure Assessment

21 rural workers evaluated

⚠ Pesticides detected in 11 individuals

⚠ Chlorpyrifos quantified in one worker

Graphics Abstract



Mean Recoveries of Pesticides (%)
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
ACN
ACN:EtOAc

Figure 1

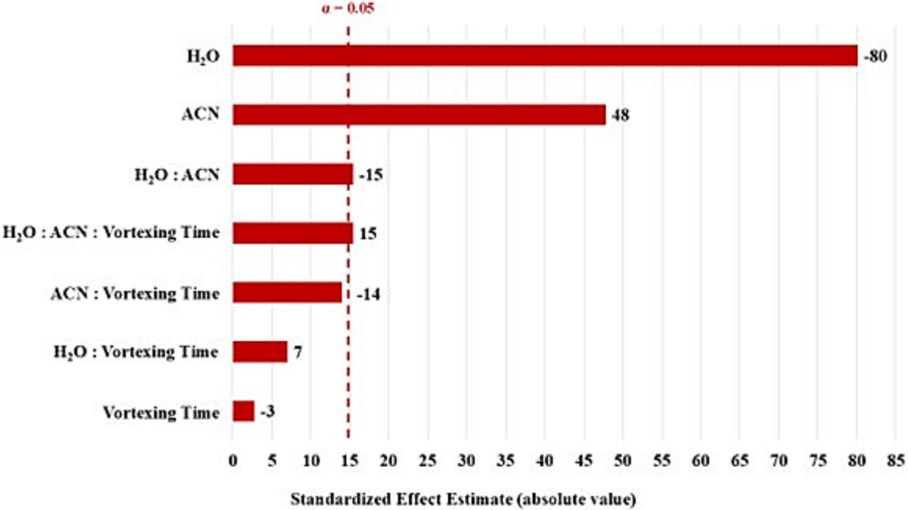

α = 0.05
H₂O
ACN
H₂O : ACN
H₂O : ACN : Vortexing Time
ACN : Vortexing Time
H₂O : Vortexing Time
Vortexing Time
-80
48
-15
15
-14
7
-3
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
Standardized Effect Estimate (absolute value)

Figure 2

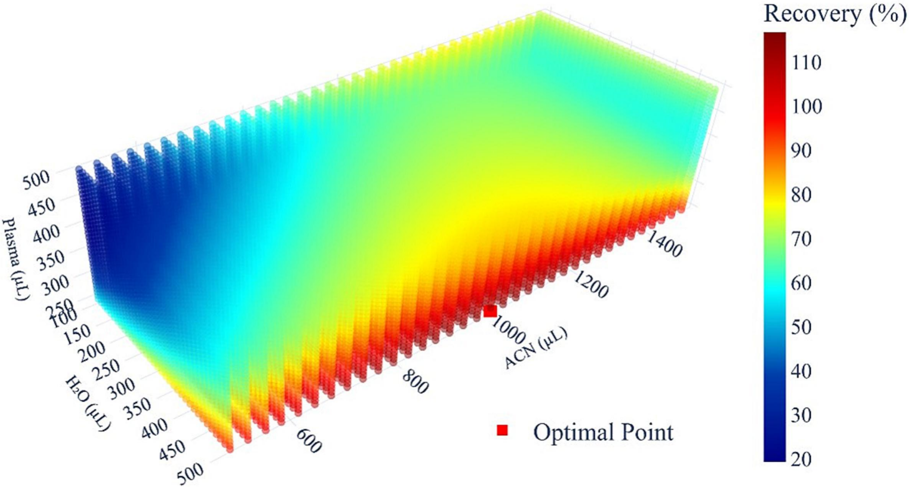

Recovery (%)
110
100
90
80
70
60
50
40
30
20
Plasma (μL)
500
450
400
350
300
250
H₂O (μL)
100
150
200
250
300
350
400
450
500
ACN (μL)
600
800
1000
1200
1400
Optimal Point

Figure 3

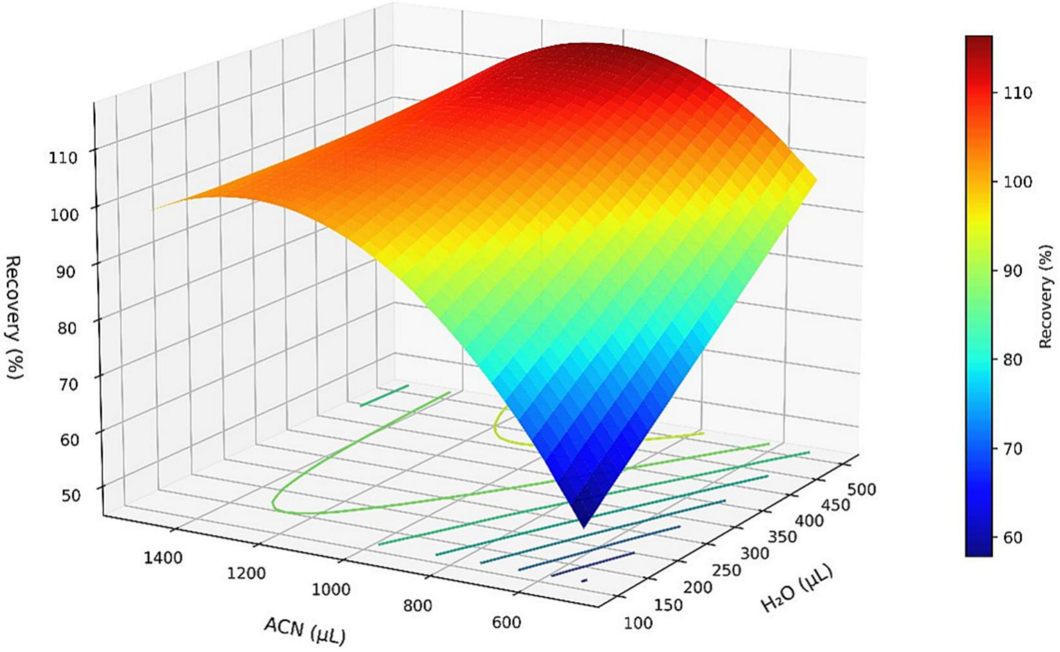

Recovery (%)
110
100
90
80
70
60
50
ACN (µL)
1400
1200
1000
800
600
H₂O (µL)
100
150
200
250
300
350
400
450
500
Recovery (%)
110
100
90
80
70
60

Figure 4