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Research Article

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Posted Date: November 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11485316/v1

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Additional Declarations: No competing interests reported.

Multiresidue Pesticide Determination in Honey by Liquid–Liquid Extraction with Low-Temperature Partitioning and UHPLC–MS/MS

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Abstract

A simplified, sensitive method combining liquid–liquid extraction with low-temperature partitioning (LLE-LTP) and ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) was developed for multiresidue pesticide determination in honey. Seven pesticides with different physicochemical properties were investigated: acephate, acetamiprid, carbendazim, diflubenzuron, imidacloprid, methomyl, and thiamethoxam. Extraction conditions were systematically optimized using a fractional factorial design followed by a central composite design. The optimized procedure used 3.0 g of honey, 4 mL of water, 4 mL of acetonitrile, 5 min of sonication, and low-temperature partitioning at −30 °C for 180 min. The organic extract was diluted tenfold before UHPLC-MS/MS analysis, without centrifugation, solvent evaporation, or additional clean-up. Matrix-matched calibration showed satisfactory linearity (R^2 = 0.9963–0.9992), with limits of quantification of 0.33–0.67 μg kg^{-1}. Six pesticides exhibited recoveries of 100.0–117.2% and precision below 7.5% (RSD), whereas acephate showed consistently low recoveries (41.6–46.2%), indicating limitations for highly hydrophilic analytes. Application to nine commercial honey samples from southeastern Brazil revealed pesticide residues, including quantifiable levels of imidacloprid (12.9 μg kg^{-1}) and methomyl (15.8–16.2 μg kg^{-1}). Overall, the proposed method combines low quantification limits, reduced solvent consumption, and simplified sample preparation, providing a practical approach for pesticide residue monitoring in honey.

Keywords: LLE-LTP, design of experiments, method validation, neonicotinoids.

1 Introduction

Honey is a natural food widely consumed worldwide and valued for its nutritional, sensory, and bioactive properties (Aga et al. 2023). However, its composition can also reflect environmental contamination in areas surrounding beehives. During foraging, honeybees interact with flowers, vegetation, water, soil particles, and other environmental compartments and may consequently be exposed to pesticides used in agricultural activities (Xiao et al. 2022). These compounds can be transported to the hive and eventually appear as residues in honey, making their determination relevant not only for food safety but also for assessing pesticide exposure in agricultural environments (Zhang et al. 2024). Therefore, reliable monitoring of pesticide residues in honey is important for evaluating consumer exposure, supporting regulatory compliance, and providing information on the contamination of environments visited by pollinators.

Determining pesticide residues in honey remains analytically challenging because of both the low concentrations at which these compounds may occur and the matrix's complexity (Orso et al. 2016; Choudhury et al. 2026). Honey consists predominantly of sugars and water but also contains organic acids, proteins, amino acids, minerals, pigments, phenolic compounds, and other minor constituents (Aga et al. 2023). During sample preparation, these components may be co-extracted with the target analytes and interfere with chromatographic analysis, particularly in liquid chromatography–tandem mass spectrometry (LC–MS/MS), where matrix components may cause ion suppression or enhancement (Suto et al. 2020; Thompson et al. 2025). Consequently, sample preparation plays a critical role in achieving adequate selectivity, sensitivity, accuracy, and robustness for pesticide residue determination in this matrix.

Different sample-preparation approaches have been used for pesticide analysis in honey, including dispersive solid-phase extraction (SPE) (Nemati et al. 2022; Rasoulizadeh et al. 2025), QuEChERS-based procedures (Silva and Faria 2020; Souza et al. 2021; Lewinski et al. 2023), and miniaturized extraction techniques (Elencovan et al. 2022; Khatami et al. 2024). QuEChERS combined with LC–MS/MS has been particularly useful for multiresidue analysis because of its broad applicability and compatibility with pesticides across chemical classes (Orso et al. 2016; Silva and Faria 2020; Lewinski et al. 2023). Nevertheless, depending on the analytical scope and matrix, these procedures may require relatively large solvent volumes, extraction salts, centrifugation, and additional dispersive clean-up using sorbents such as primary-secondary amine (PSA), C18, graphitized carbon materials, or combinations thereof (Oymen et al. 2022; Lamas et al. 2023). Although extensive clean-up can reduce matrix interferences, it may also increase sample-preparation complexity, solvent and material consumption, analysis costs, and analyte losses. Thus, simpler

approaches that provide sufficiently clean extracts while maintaining high extraction efficiency and low quantification limits remain desirable.

Liquid–liquid extraction with low-temperature partitioning (LLE-LTP) is an attractive alternative for this purpose. The technique combines analyte extraction into an organic phase with matrix separation promoted by cooling, during which the aqueous/matrix-rich phase solidifies or is retained. In contrast, the organic extract containing the analytes remains liquid (Goulart et al. 2024). This mechanism can provide extraction and cleanup within the same procedure, reducing the need for subsequent purification steps. LLE-LTP has previously been applied to pesticide determination in honey, demonstrating its feasibility for this matrix (Pinho et al. 2010; Freitas et al. 2021; Santos et al. 2022; Costa et al. 2024). For example, an early application used an acetonitrile–ethyl acetate mixture followed by an additional Florisil cleanup for the determination of four pesticides by gas chromatography, with quantification limits of approximately 0.032 μg g^{-1} (Pinho et al. 2010). More recently, LLE-LTP was also applied to the determination of pyroxasulfone in honey using an acetonitrile–ethyl acetate extraction mixture followed by GC–MS analysis (Costa et al. 2024). These studies demonstrate the potential of low-temperature partitioning for honey analysis but also indicate opportunities for further improvements in sensitivity, solvent consumption, procedural simplicity, and applicability to multiclass pesticide determination.

In this context, particular attention should be given to analytical strategies capable of simultaneously determining pesticides representative of current agricultural practices (ANVISA 2023). Selecting target compounds across pesticide classes and physicochemical properties increases the analytical challenge but also enhances the method's relevance for residue-monitoring applications. Although LLE-LTP has been used for pesticide determination in honey, to the best of our knowledge, its combination with LC–MS/MS has not yet been reported for contaminant analysis in this matrix. This combination is particularly promising because the high selectivity and sensitivity of tandem mass spectrometry may reduce the need for extensive extract purification, provided matrix effects are adequately controlled. Furthermore, reducing sample and solvent consumption and eliminating additional clean-up steps align with current efforts toward simpler and more environmentally sustainable sample-preparation procedures.

Therefore, this study aimed to develop a sensitive and simplified LLE-LTP–LC–MS/MS method for the simultaneous determination of acetamiprid, acephate, carbendazim, diflubenzuron, imidacloprid, methomyl, and thiamethoxam in honey. Extraction conditions were systematically investigated and optimized using experimental design, including an initial screening followed by a central composite design to evaluate the combined effects of the key extraction variables. The optimized method was then validated and applied to real honey samples. Compared with previously reported approaches for pesticide determination in honey, the proposed procedure was designed to achieve low limits of quantification using a reduced volume of organic solvent and without an additional sorbent-based clean-

up step, relying on low-temperature partitioning itself for matrix separation. Thus, the study seeks to combine analytical sensitivity, simplified sample preparation, reduced solvent consumption, and a pesticide panel relevant to agricultural applications within a single multiresidue procedure.

2 Materials and Methods

2.1 Chemicals and reference standards

Analytical standards of acephate (99.5%), acetamiprid (99.8%), carbendazim (99.2%), diflubenzuron (99.6%), imidacloprid (99.9%), methomyl (99.8%), and thiamethoxam (99.7%) were obtained from OuroFino Agrociência (Uberaba, Brazil). Physical and chemical information about the studied pesticides is presented in Table S1 (Supplementary Information). Acetonitrile and methanol (J.T. Baker, Radnor, PA, USA) were chromatographic grade, and ultrapure water (18.2 MΩ cm) was produced using a Milli-Q Direct 8 system (Merck Millipore, Darmstadt, Germany). Acetic acid was LC–MS grade (Merck, Darmstadt, Germany). Sodium chloride was analytical grade (Dinâmica, Indaiatuba, Brazil). All calibrated volumetric materials were used in an ISO/IEC 17025-accredited laboratory.

Individual standard solutions were prepared gravimetrically at approximately 100 mg L^{-1}. Acephate, methomyl, and carbendazim were dissolved in methanol, whereas the remaining compounds were dissolved in acetonitrile. A mixed working solution containing 10.0 µg L^{-1} of each analyte was prepared in ultrapure water. Calibration solutions at 0.50, 1.50, 2.50, 3.50, and 4.50 µg L^{-1} were prepared in both solvent and blank honey extract.

2.2 Honey samples

A pesticide-free wildflower honey was used for method development and validation. For method application, nine untreated commercial wildflower honey samples were obtained from apiaries in Uberlândia (samples A and H), Ituiutaba (B), Capinópolis (C), Patrocínio (D), Nova Ponte (E), and Uberaba (F, G, and I), cities located in Minas Gerais, Brazil. The samples were stored in their original glass containers at 4 ± 1 °C, coded to preserve manufacturer identity, and analyzed in sextuplicate.

2.3 UHPLC–ESI–MS/MS conditions

Chromatographic analyses were performed on a Waters UPLC system coupled to an Xevo TQ-S Micro triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Milford, MA, USA). Separation was achieved on an Acquity UPLC HSS T3 column (100 × 2.1 mm, 1.8 µm) maintained at 30 °C. Mobile phase A was ultrapure water with 0.1 % (v/v) acetic acid, and mobile phase B was acetonitrile with 0.1 % (v/v) acetic acid. The flow rate was 0.300 mL min^{-1}, and the injection volume was 2 µL. The gradient began at 10 % B, was held for 0.25 min, increased linearly to 98 % B at 7.75 min, was held until 8.50 min, returned to 10 % B at 8.51 min, and was re-equilibrated until 10.0 min.

The ESI source operated in positive-ion mode. The optimized parameters were a capillary voltage of 1.0 kV, a desolvation temperature of 450 °C, and a desolvation gas flow of 900 L h^{-1}. Compound-specific cone voltages, product ions, collision energies, and retention times are summarized in Table 1. One transition was used for quantification, and a second for confirmation.

Table 1. Pesticide-specific UHPLC–ESI–MS/MS conditions.

Pesticides	**Nominal molecular mass (g mol^{-1})**	**Cone voltage (V)**	**Quantifier m/z (CE, V)**	**Qualifier m/z (CE, V)**	**t_R (min)**
Acephate	184	10	49 (17)	143 (7)	1.55
Acetamiprid	223	10	126 (19)	56 (14)	4.38
Carbendazim	192	10	160 (16)	132 (29)	2.96
Diflubenzuron	311	20	158 (13)	141 (33)	7.23
Imidacloprid	256	10	209 (13)	175 (17)	4.18
Methomyl	163	10	88 (7)	106 (8)	3.30
Thiamethoxam	292	10	211 (11)	181 (22)	3.61

2.4 Liquid-liquid extraction with low-temperature partitioning of pesticides in honey samples

2.4.1 Selection of variables affecting LLE-LTP extraction efficiency

The variables affecting the extraction of pesticide residues from honey by LLE-LTP were initially screened using a 2^{6-2} fractional factorial design. Six factors were evaluated at two levels: sample mass (A), extraction solvent volume (B), extraction solvent (C), freezing time (D), extraction time (E), and NaCl concentration (F), as shown in Table 2. The design comprised 16 experimental runs, with factors E and F generated from the interactions E = A × B ×

C and F = B × C × D, respectively. The complete experimental matrix is provided in the Supplementary Information (Table S2). Pesticide recovery was used as an analytical response to evaluate the effect of each factor.

Table 2. Factors and levels evaluated in the 2^{6-2} fractional factorial design to screen variables affecting the LLE-LTP extraction of pesticides from honey.

Factor	Variable	Low level (−)	High level (+)
A	Sample mass (g)	2.0	3.0
B	Extraction solvent volume (mL)	2.0	6.0
C	Extraction solvent	Acetonitrile	Ethyl acetate
D	Freezing time (h)	2	4
E	Extraction time (min)	5	10
F	NaCl concentration (% w/v)	1	2

Each experimental condition was performed in triplicate. Honey samples were fortified with the pesticide mixture to achieve a final concentration of 3.3 μg kg^{-1}. The appropriate amount of honey (2.0 or 3.0 g) was weighed into a 50-mL polypropylene tube, followed by the addition of 4 mL of ultrapure water containing NaCl at the concentration specified in the experimental design (1 or 2 %, w/v). After vortexing, the extraction solvent (acetonitrile or ethyl acetate; 2 or 6 mL) was added, and the mixture was vortexed and then subjected to ultrasound-assisted extraction for 5 or 10 min. The tubes were then maintained at −30 ± 3 °C for 2 or 4 h, according to the experimental design.

After low-temperature partitioning, a 100-μL aliquot of the liquid organic phase was collected and diluted with 900 μL of ultrapure water prior to UHPLC-ESI-MS/MS analysis. Pesticide recovery was then calculated and used to estimate the effects of the variables under investigation.

2.4.2 Central composite design and response-surface methodology optimization

Following the screening study, the factors identified as relevant to LLE-LTP performance were further optimized using a central composite design (CCD). Three factors were investigated: sample mass (A), extraction solvent volume (B), and freezing time (D). The CCD consisted of a 23 factorial design, six axial points (α = ±1.73), and three replicates at the central point, resulting in 17 experimental runs. The coded and actual levels for each factor are presented in Table 3, and the complete experimental matrix is provided in the Supplementary Information (Table S3). Based on the results of the previous screening study, acetonitrile was used as the extraction solvent, extraction time was fixed at 5 min, and no NaCl was added.

Table 3. Factors and levels evaluated in the central composite design for optimizing the LLE-LTP extraction of pesticides from honey.

	Variable	Levels (coded and experimental)				
		−1.73	**−1**	**0**	**+1**	**+1.73**
A	Honey mass (g)	0.3	1.0	2.0	3.0	3.7
B	Acetonitrile volume (mL)	2.6	4.0	6.0	8.0	9.4
D	Freezing time (min)	60	90	135	180	210

Each experimental condition was performed in triplicate, and honey samples were fortified with the pesticide mixture to achieve a final concentration of 3.3 μg kg^{-1}. The appropriate amount of honey (0.3-3.7 g) was weighed into a 50-mL polypropylene tube, then 4 mL of ultrapure water was added, and the mixture was vortexed. Acetonitrile (2.6-9.4 mL) was then added, and the mixture was vortexed and subjected to ultrasonic extraction for 5 min. The tubes were then maintained at -30 °C for 60-210 min, according to the experimental design.

After the LTP approach, a 100-μL aliquot of the liquid organic phase was collected, diluted with 900 μL of ultrapure water, and analyzed by UHPLC-ESI-MS/MS. Pesticide recovery served as the response variable for optimizing the extraction conditions.

2.5 Method validation and quality assurance

The optimized LLE-LTP method was validated in accordance with the SANTE/11312/2021.v2 guidelines (European Commission 2026). The parameters evaluated included selectivity, linearity, matrix effect, limit of quantification (LOQ), accuracy, repeatability, within-laboratory reproducibility, and measurement uncertainty.

Selectivity was assessed by comparing extracts of blank honey samples with matrix extracts fortified with the target pesticides. Linearity and matrix effects were evaluated using five-level calibration curves prepared in solvent and in blank honey extracts, each in triplicate. Matrix effects were estimated by comparing the slopes of matrix-matched and solvent-based calibration curves. The LOQ was established as the lowest tested concentration that provided a signal-to-noise ratio ≥ 10 and met the identification criteria for the monitored MRM transitions.

Accuracy and precision were evaluated through recovery assays at three fortification levels (0.67, 3.33, and 6.00 μg kg^{-1}), with six replicates at each level. Repeatability was evaluated under the same analytical conditions, whereas within-laboratory reproducibility was assessed from experiments conducted on three different days. Acceptance criteria,

according to SANTE/11312/2021.v2, were recoveries of 70–120% and RSDs ≤ 20%. Potential outliers were evaluated using the Grubbs test at a 97.5% confidence level.

Measurement uncertainty was estimated using a top-down approach based on validation data, considering contributions from standard solution preparation, calibration, within-laboratory reproducibility, and recovery. The combined standard uncertainty was obtained by propagation of the individual uncertainty components, and the expanded uncertainty was calculated using a coverage factor of k = 2.

2.6 Method greenness assessment

The environmental performance of the optimized LLE-LTP procedure was evaluated using the Analytical Greenness metric for sample preparation (AGREEprep) (Wojnowski et al. 2022). The evaluation was based on the ten principles of green sample preparation and considered factors such as sample amount, solvent consumption, energy requirements, number of operational steps, sample throughput, waste generation, and operator safety. The experimental parameters of the optimized procedure were entered into the AGREEprep software according to the criteria established for each principle. The resulting pictogram and overall score, ranging from 0 to 1, were used to assess the environmental performance of the proposed sample preparation procedure and to identify its main strengths and limitations.

2.7 Analysis of real honey samples

The applicability of the optimized and validated LLE-LTP method before UHPLC-ESI-MS/MS was evaluated by analyzing nine wildflower honey samples (A to I) collected from different municipalities in the Triângulo Mineiro region, Minas Gerais, Brazil. Samples originated in Uberlândia (A and H), Ituiutaba (B), Capinópolis (C), Patrocínio (D), Nova Ponte (E), and Uberaba (F, G, and I). Samples were stored at 4 ± 1 °C until analysis. Each sample underwent the optimized LLE-LTP procedure and was analyzed for acephate, acetamiprid, carbendazim, diflubenzuron, imidacloprid, methomyl, and thiamethoxam. Residues below the validated LOQ were reported as <LOQ, whereas those at or above the LOQ were quantified using matrix-matched calibration.

3 Results and discussion

The development of multiresidue methods for pesticide determination in honey remains analytically challenging because efficient extraction must be paired with adequate removal of highly concentrated matrix

components while preserving analytes with markedly different physicochemical properties. In this context, LLE-LTP offers an attractive sample-preparation strategy by combining analyte extraction with low-temperature phase separation in a relatively simple workflow. The procedure requires only dilution of the honey matrix, extraction with a small volume of organic solvent, low-temperature partitioning, and dilution of the resulting extract before instrumental analysis, avoiding additional clean-up, centrifugation, evaporation, or solvent-exchange steps.

The present study therefore focused on establishing an LLE-LTP procedure suitable for the multiresidue determination of pesticides with different physicochemical characteristics in honey by UHPLC-ESI-MS/MS. Particular attention was given to elucidating the variables governing extraction efficiency and low-temperature partitioning, followed by optimization and analytical validation of the resulting procedure. The method's analytical performance, including its quantification capability and matrix effects, is discussed in relation to the characteristics of the LLE-LTP workflow and to previously reported approaches for pesticide residue analysis in honey.

3.1 Experimental design and multivariate optimization of the LLE-LTP procedure

3.1.1 Factor screening by fractional factorial design

The initial 2^{6-2} fractional factorial design revealed marked differences in pesticide recovery across the experimental conditions, demonstrating that the efficiency of LLE-LTP was governed by both extraction and low-temperature partitioning variables. Among the factors investigated, extraction solvent had the most pronounced overall effect, particularly for diflubenzuron, whereas solvent volume, honey mass, and freezing time also affected the analytical response. In contrast, sonication time and NaCl addition produced comparatively smaller effects within the experimental domain investigated (Fig. 1).

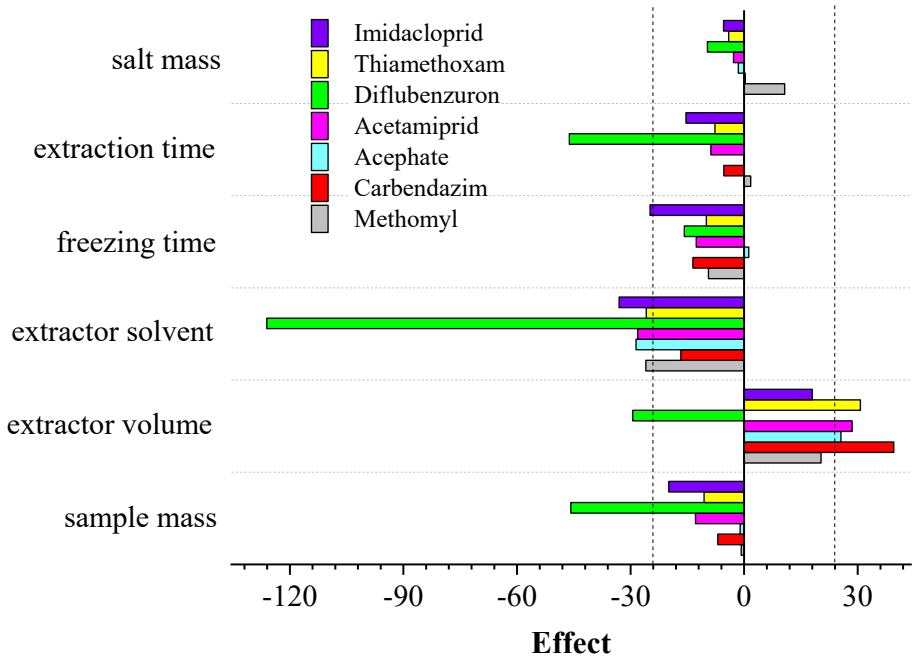


Fig. 1. Relative importance of the six variables evaluated in the fractional factorial design, expressed as the mean absolute main effect across seven target pesticides.

The pronounced influence of solvent type stems from the physicochemical diversity of the target pesticides and the distinct behavior of acetonitrile and ethyl acetate in the aqueous honey extract. Diluting honey with water reduces the viscosity of the sugar-rich matrix and improves contact between the analytes and the extraction solvent. Acetonitrile provided the best overall extraction performance for the multiclass analyte set and was therefore selected for subsequent optimization. Its miscibility with water before cooling promotes intimate contact with the diluted matrix, whereas low-temperature treatment promotes separation of the acetonitrile-rich liquid phase from the water-rich matrix (Silva and Faria 2026). This behavior is particularly advantageous for honey, since immobilization of the aqueous fraction at low temperature also retains a substantial proportion of the highly abundant sugars and other poorly soluble matrix constituents, allowing extraction and partial matrix removal to occur within the same workflow.

The effect of solvent volume was also significant and should not be interpreted solely as an increase in extraction capacity. Varying the amount of acetonitrile alters the solvent-to-sample ratio, analyte solvation, dilution of co-extracted matrix components, and the composition of the phases formed during low-temperature partitioning (Goulart et al. 2024). Similarly, the influence of honey mass reflects not only the amount of analyte available for extraction but also the increasing load of sugars and other matrix constituents introduced into the extraction system. Consequently, these two variables are intrinsically related through the sample-to-solvent ratio and were investigated simultaneously rather than optimized independently.

Freezing time also affected pesticide recovery, indicating that establishing the low-temperature partitioning equilibrium was relevant to extraction performance. At -30 °C, the water-rich fraction becomes immobilized while the acetonitrile-rich phase remains liquid, enabling its subsequent collection. Sufficient cooling time is therefore required to achieve effective phase separation and matrix immobilization (Silva and Faria 2026). On the other hand, extending sonication from 5 to 10 min did not provide a meaningful overall improvement within the studied range, indicating that prolonged ultrasound treatment was unnecessary once adequate dispersion of the sample and solvent had been achieved. Likewise, increasing NaCl concentration from 1 to 2 % (w/v) did not provide sufficient benefit to justify its inclusion in the subsequent optimization. Acetonitrile was therefore selected as the extraction solvent, sonication time was fixed at 5 min, and NaCl was omitted, reducing the number of operations and reagents required by the procedure.

3.1.2 Optimization by central composite design and response surface methodology

Based on the screening results, honey mass, acetonitrile volume, and freezing time were subsequently evaluated using a central composite design coupled with response surface methodology (RSM). Because the objective was to establish a single multiresidue extraction condition rather than analyte-specific procedures, the mean recovery across the seven pesticides was used as the response. Considerable variation was observed across the experimental domain, with mean recoveries ranging from 93.2 to 119.7%, reflecting the combined response of pesticides with different extraction behaviors (Table S4, Supplementary Information).

The quadratic model was statistically significant ($F = 18.02$, $p = 0.0005$), and the lack of fit was not significant ($F = 5.09$, $p = 0.1723$), indicating that the model adequately represented the experimental response within the investigated domain. As shown in the Analysis of Variance in Table 4, the model had a good coefficient of determination ($R^2 = 0.9586$). In contrast, the adjusted coefficient ($R^2_{adj} = 0.9054$) indicated that a large proportion of the response variability remained explained after accounting for the number of model terms.

Table 4. Analysis of variance (ANOVA) for the quadratic model describing the mean recovery of the seven target pesticides from honey samples obtained by LLE-LTP, using the central composite design.

Sources	Sum of squares	Degrees of freedom	Mean square	F-value	p-value	
Model	1074.79	9	119.42	18.02	0.0005	significant
Residual	46.39	7	6.63			
Lack of Fit	43.01	5	8.60	5.09	0.1723	not significant

Pure Error	3.38	2	1.69
$\mathbf{R^2}$	0.9586		
$\mathbf{R^2_{adj}}$	0,9054		

Among the individual model terms, acetonitrile volume was the dominant factor ($F = 145.82$, $p < 0.0001$), whereas honey mass ($p = 0.0710$) and freezing time ($p = 0.0502$) did not reach statistical significance at the 95% confidence level. None of the two-factor interactions or quadratic terms were significant ($p > 0.05$). These results indicate that, within the experimental region investigated, extraction performance was governed predominantly by the amount of acetonitrile rather than by strong interactions among the three factors.

This behavior is also evident in the response surfaces (Fig. 2). Decreasing the acetonitrile volume generally increased the mean pesticide recovery, whereas changes in honey mass and freezing time produced comparatively smaller effects on the response. The influence of acetonitrile volume on pesticide recovery stems from changes in the sample-to-solvent ratio and analyte partitioning during low-temperature extraction. Excessive solvent volumes can alter phase partitioning and dilute the extract without necessarily improving extraction efficiency (Rubensan et al. 2011), whereas insufficient volumes can limit analyte transfer and hinder extract collection. Thus, RSM enabled the identification of suitable extraction conditions that balance pesticide recovery, solvent consumption, and practical handling of the organic phase.

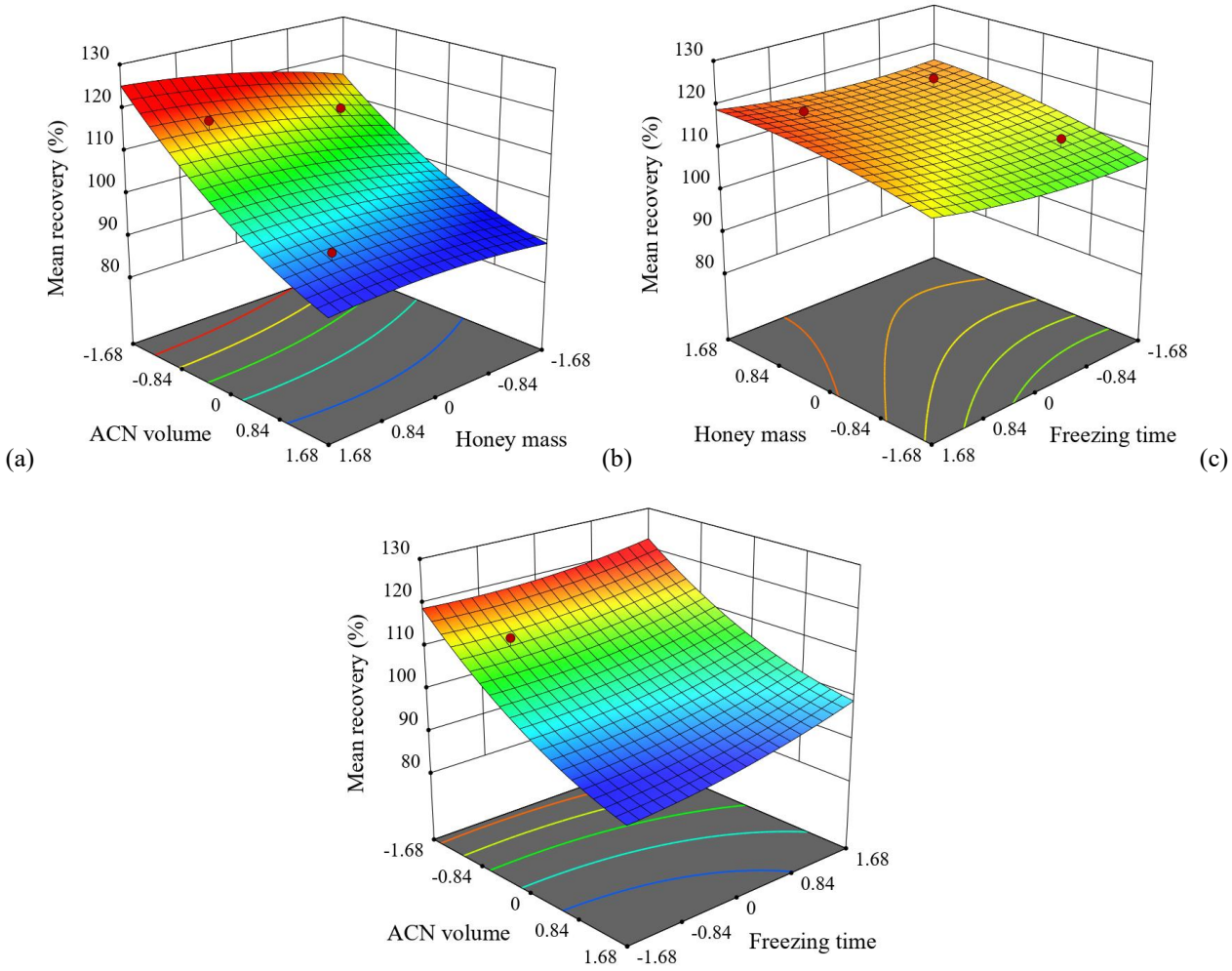


Fig. 2. Response surfaces from the central composite design showing the effects of (a) honey mass and acetonitrile volume, (b) honey mass and freezing time, and (c) acetonitrile volume and freezing time on the mean recovery of the seven target pesticides by LLE-LTP.

Freezing time plays a different role because it is primarily associated with establishing low-temperature partitioning rather than analyte solvation. At -30 °C, cooling immobilizes the water-rich fraction of the diluted honey matrix, while the acetonitrile-rich phase remains liquid, facilitating its collection. Once effective phase separation has been established, however, further extending the freezing period is not expected to proportionally increase extraction efficiency. This is consistent with the comparatively smaller statistical contribution of freezing time observed within the investigated range.

Numerical optimization of the response model identified 3.0 g of honey, 4.0 mL of acetonitrile, and 180 min at -30 °C as the optimal conditions, with a predicted mean recovery of 116.9 % for the seven pesticides. Under these conditions, a clear liquid extract was obtained and could be collected and diluted directly for UHPLC-ESI-MS/MS analysis. The resulting workflow required no centrifugation, evaporation, solvent exchange, or additional sorbent-based

clean-up, limiting active sample manipulation to dilution, extraction, phase collection, and final dilution prior to UHPLC-ESI-MS/MS analysis.

3.2 Analytical performance and method validation

The analytical performance of the optimized LLE-LTP with the UHPLC-ESI-MS/MS method was evaluated for selectivity, linearity, matrix effects, limit of quantification (LOQ), recovery, repeatability, within-laboratory reproducibility, and measurement uncertainty. Blank honey extracts showed no interfering signals at the retention times and MRM transitions used for the target pesticides, demonstrating adequate selectivity (Fig. 3). Identification criteria based on retention time and the relationship between quantification and confirmation transitions were also met (European Commission 2026).

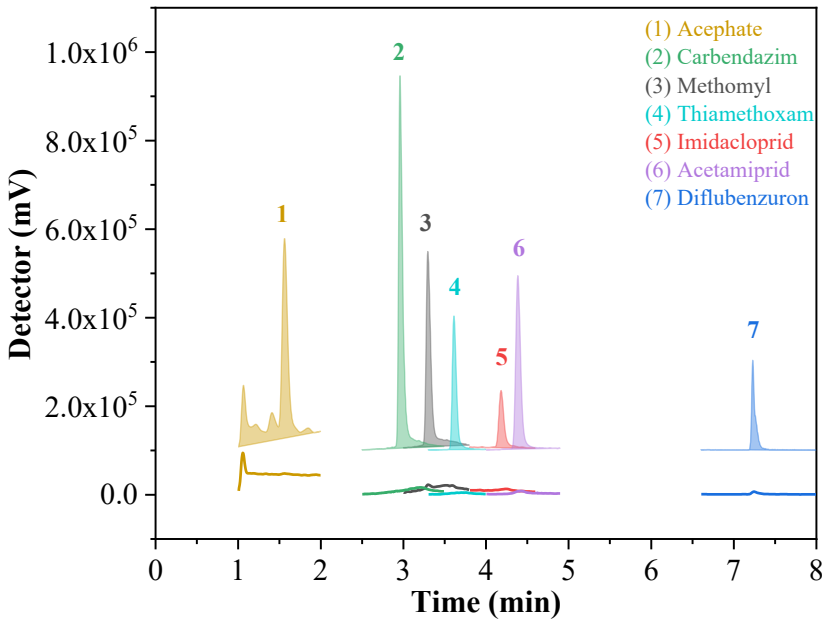


Fig. 3. Representative chromatograms of pesticide-free honey extracts (blank samples) and honey samples spiked with the target pesticides at 2.5 µg L^{-1}, obtained using the optimized LLE-LTP method. Chromatographic conditions are described in the Experimental section. Peak identification: (1) acephate, (2) carbendazim, (3) methomyl, (4) thiamethoxam, (5) imidacloprid, (6) acetamiprid, and (7) diflubenzuron.

Calibration curves prepared in solvent and in matrix extract showed satisfactory linearity over the investigated range. Coefficients of determination ranged from 0.9966 to 0.9994 for solvent-based calibration and from 0.9963 to 0.9992 for matrix-matched calibration (Table 5). The analytical response varied considerably among pesticides, with

carbendazim showing the highest calibration slope and methomyl the lowest. These differences mainly reflect compound-dependent MS/MS response and ionization efficiency and should therefore not be interpreted as differences in extraction performance.

Comparison of slopes from solvent and matrix-matched calibration revealed predominantly positive matrix effects. Signal enhancement was observed for most compounds, whereas acetamiprid showed a negligible matrix effect. The largest effect was observed for thiamethoxam (24.1 %). Although these results indicate that the relatively simple LLE-LTP workflow limits the magnitude of matrix-induced ionization effects, the occurrence of signal enhancement demonstrates that low-temperature partitioning does not eliminate co-extracted matrix components capable of affecting electrospray ionization. Matrix-matched calibration was therefore adopted for quantitative analysis. This approach is particularly appropriate for LC-ESI-MS/MS analysis, for which co-extracted matrix constituents may alter analyte ionization even in the absence of chromatographic interference (Prabhu et al. 2023).

Table 5. Analytical performance and validation parameters of the LLE-LTP-UHPLC-ESI-MS/MS method for pesticide determination in honey. Recovery and repeatability are expressed as recovery (%) / RSD (%), respectively (n = 6).

Pesticides	Matrix-matched R^2	ME (%)	LOQ (µg kg^{-1})	0.67 µg kg^{-1} Recovery (%) / RSD (%)	3.33 µg kg^{-1} Recovery (%) / RSD (%)	6.00 µg kg^{-1} Recovery (%) / RSD (%)	Within-lab. RSD (%)[a]	U (%)[b]
Acephate	0.9974	+6.6	0.67	41.6 / 5.2	43.0 / 2.7	46.2 / 4.0	≤ 4.4	22.3
Acetamiprid	0.9973	−0.1	0.67	112.1 / 1.9	117.2 / 5.7	116.9 / 2.3	≤ 1.5	27.6
Carbendazim	0.9992	+6.5	0.33	100.5 / 1.3	100.0 / 3.8	105.9 / 1.2	≤ 2.0	25.3
Diflubenzuron	0.9988	+9.5	0.67	105.2 / 1.8	110.8 / 2.1	112.8 / 2.1	≤ 1.7	37.6
Imidacloprid	0.9980	+1.9	0.67	115.3 / 3.5	115.4 / 2.2	116.8 / 2.4	≤ 4.8	19.9
Methomyl	0.9963	+11.6	0.67	107.7 / 5.0	114.5 / 2.8	114.5 / 1.7	≤ 4.9	25.5
Thiamethoxam	0.9990	+24.1	0.67	102.2 / 3.3	105.9 / 2.2	110.5 / 1.3	≤ 2.5	21.4

[a] Maximum RSD under within-laboratory reproducibility conditions across the three fortification levels. [b] Relative expanded measurement uncertainty, expressed as a percentage (k = 2). ME, matrix effect; LOQ, limit of quantification; RSD, relative standard deviation; U, relative expanded measurement uncertainty.

The method provided LOQs of 0.33 μg kg^{-1} for carbendazim and 0.67 μg kg^{-1} for the other target pesticides. These quantification limits are noteworthy, given that the acetonitrile extract was diluted tenfold with water before UHPLC-ESI-MS/MS analysis to reduce matrix loading into the chromatographic and ionization systems. Thus, adequate quantification capability was maintained despite the dilution step, without requiring additional concentration or sorbent-based clean-up.

Accuracy and precision were evaluated at three fortification levels of 0.67, 3.33, and 6.00 μg kg^{-1} in honey. For acetamiprid, carbendazim, diflubenzuron, imidacloprid, methomyl, and thiamethoxam, mean recoveries ranged from 100.0 to 117.2 %, with RSDs not exceeding 5.7 %, meeting the recovery and precision criteria established for pesticide residue analysis (European Commission 2026). The consistency of recovery across the three concentration levels also indicates that extraction performance remained essentially concentration-independent over the validated range.

Acephate exhibited markedly different behavior (Table 5). Recoveries were only 41.6, 43.0, and 46.2 % at the low, intermediate, and high fortification levels, respectively, although the corresponding RSDs remained low (2.7-5.2 %). The combination of consistently low recovery and good precision suggests a systematic extraction limitation rather than random analytical variability. This behavior can be attributed to acephate's markedly hydrophilic character and its preferential distribution toward the water-rich fraction of the extraction system (Barros et al. 2019). During low-temperature partitioning, a smaller proportion of this highly polar compound is therefore expected to remain in the liquid acetonitrile-rich phase collected for analysis. This observation is particularly relevant for a multiresidue method, since the extraction conditions represent a compromise among compounds with substantially different physicochemical properties.

Importantly, matrix-matched calibration compensates for matrix-induced changes in ESI response but cannot account for analyte loss during sample extraction when calibration standards are prepared in the final matrix extract (Barros et al. 2019). Consequently, the satisfactory precision observed for acephate does not compensate for its insufficient recovery. Under the selected LLE-LTP conditions, the method can therefore be considered quantitatively validated for acetamiprid, carbendazim, diflubenzuron, imidacloprid, methomyl, and thiamethoxam, whereas acephate remains outside the validated quantitative scope.

Precision was further confirmed under repeatability and within-laboratory reproducibility conditions. Overall RSDs ranged from 0.6 to 7.5 % for repeatability and from 0.6 to 4.9 % for within-laboratory reproducibility, remaining well below the 20 % acceptance criteria (European Commission 2026). The latter experiments were conducted on three different days, indicating that analytical performance was maintained despite normal within-laboratory temporal variation. No evidence of excessive dispersion was observed across the evaluated concentration levels.

Relative expanded measurement uncertainties (k = 2, approximately 95 % confidence) ranged from 19.9 to 37.6 % among the target pesticides. The lowest value was obtained for imidacloprid (19.9 %), whereas diflubenzuron showed the highest uncertainty (37.6 %). These values provide an estimate of the interval associated with quantitative results and complement the recovery and precision data by incorporating the major sources of variability involved in the analytical procedure.

The validation results demonstrate that the optimized LLE-LTP procedure provides a suitable multiresidue sample-preparation approach for the quantitative determination of six of the seven pesticides investigated in honey. The method combines LOQs of 0.33-0.67 μg kg^{-1} with recoveries of 100.0-117.2 % and low intra- and interday variability, while requiring only 4 mL of acetonitrile and no additional sorbent-based cleanup. However, the distinct behavior of acephate highlights the limitations of applying a single partitioning condition to analytes with markedly different affinities for the aqueous and organic phases.

3.3 Comparison with previously reported methods for pesticide determination in honey

The analytical performance and sample preparation characteristics of the proposed method were compared with those of previously reported approaches for pesticide determination in honey (Table 6). LLE-LTP has already been successfully applied to this matrix, particularly for determining individual pesticides or small groups of compounds using GC-MS and HPLC-DAD. However, the present method offers a favorable combination of analytical detectability, reduced solvent consumption, and operational simplicity.

A notable feature of the proposed method is the low LOQs achieved for the six pesticides (0.3–0.7 μg kg^{-1}). These values are lower than those reported for the other methods in Table 6, where LOQs ranged from 1 to 33 μg kg^{-1}. Even compared with the QuEChERS-UHPLC-MS/MS method reported by Pang et al. (2022), which provided LOQs of 1.0–4.0 μg kg^{-1}, the present method achieved lower quantification limits. Although direct comparison of LOQs should be interpreted with caution because analytical performance depends on the physicochemical properties of the target compounds, instrumental conditions, and validation criteria, these results demonstrate the high detectability of the proposed LLE-LTP-UHPLC-MS/MS approach.

Another advantage is the simplicity of sample preparation. The proposed procedure requires only 3.0 g of honey and 4.0 mL of acetonitrile, the lowest organic solvent volume among the methods compared. Previously reported LLE-LTP procedures generally employed 8 mL of extraction solvent, whereas QuEChERS-based methods required 5–10 mL of acetonitrile. Furthermore, no additional cleanup step is required after low-temperature partitioning. In contrast, some previously reported methods employed Florisil or dispersive SPE containing PSA, C18, $MgSO_4$, or graphene to reduce

matrix interferences (Vargas-Pérez 2019; Pang et al. 2022). Therefore, low-temperature partitioning serves as both an extraction and matrix-separation strategy, reducing the number of sample-handling operations and the consumption of additional sorbent materials.

In addition, the method targets a set of pesticides particularly relevant to current Brazilian agricultural practices, increasing its applicability to honey produced in regions potentially affected by intensive pesticide use (ANVISA 2023).

Table 6. Comparison of analytical performance and sample preparation characteristics of the proposed LLE-LTP–UHPLC-MS/MS method with previously reported methods for pesticide residue determination in honey.

Analites	Extraction technique	Analytical technique	Cleanup step	LOQ (µg kg^{-1})	Solvent	Honey mass (g)	Solvent volume (mL)	Recovery (%)	Precision (RSD)	Ref
Pyroxasulfone	LLE-LTP	GC-MS	-	3.0	ACN and Ethyl acetate	4	8.0	101-111	5-10	Costa et al. (2024)
Aldrin e Mirex	LLE-LTP	GC-MS	-	2.0-4.0	ACN	4	8.0	84-112	1-8	Santos et al. (2022)
DDT	LLE-LTP	HPLC-DAD	-	8.0	ACN	4	8.0	80-106	3-7	Freitas et al. (2021)
Chlorpyrifos, λ-Cyhalothrin, Cypermethrin, and Deltamethrin	LLE-LTP	GC-ECD and GC-MS	2 g Florisil	28-33	ACN and Ethyl acetate	4	8.0	85-101	1-10	Pinho et al. (2010)
Propoxycarbazone and 2-Hydroxy-propoxycarbazone	QuEChERS	LC-MS/MS and GC-MS/MS	dSPE: 50 mg PSA + 50 mg C18	25	ACN	5	10.0	101-110	< 19	Vargas-Pérez et al. (2019)
10 Insecticides	QuEChERS	UHPLC-MS/MS	dSPE: 50 mg PSA + 150 mg $MgSO_4$ +	1.0-4.0 [a]	ACN	2.5	5.0	69-116	1-16	Pang et al. (2022)

			2 mg graphene							
6 pesticides	LLE-LTP	UHPLC-MS/MS	-	0.3-0.7	ACN	3.0	4.0	100-117	< 5	This work

[a] LOD: Limit of detection; ACN: acetonitrile; DDT: dichlorodiphenyltrichloroethane;

Despite this simplified procedure, satisfactory analytical performance was maintained. Recoveries of 100–117% were obtained for the six pesticides, with RSDs below 5%, comparable to or better than those reported for the methods summarized in Table 6. In particular, the low RSDs indicate that reducing solvent consumption and eliminating an additional cleanup step did not compromise method precision.

The proposed method does not rely on LLE-LTP as a novel extraction principle, since this technique has previously been used for pesticide analysis in honey. Rather, its contribution is optimizing a streamlined LLE-LTP procedure coupled with UHPLC-MS/MS, which offers low solvent consumption, no additional cleanup, satisfactory accuracy and precision, and particularly low quantification limits. These characteristics make the method suitable for trace-level monitoring of pesticides in honey while reducing the complexity and material requirements of sample preparation.

3.4 Assessment of method greenness

The greenness of the proposed sample preparation procedure was evaluated using the AGREEprep metric, yielding an overall score of 0.54 (Fig. 4) (Wojnowski et al. 2022). This value reflects the combined contribution of the ten principles considered by AGREEprep and indicates a moderate overall greenness profile (Table S5 in Supplementary Information). The individual scores reveal both favorable aspects of the procedure and factors that constrain its overall environmental performance.

Among its favorable characteristics, the method requires only 4 mL of acetonitrile to extract 3.0 g of honey and does not require an additional cleanup step or sorbent materials. These features minimize the consumption of organic solvents and auxiliary materials while reducing the number of sample-preparation operations and associated waste generation. As discussed in the comparison with previously reported methods (Table 6), the solvent volume is lower than that used in other LLE-LTP and QuEChERS procedures, which typically require 5–10 mL of organic solvent. Compared with previous LLE-LTP methods that use 8 mL for 4 g of honey, the present procedure reduces absolute solvent consumption by 50% and the solvent-to-sample ratio from 2.0 to 1.33 mL g^{-1}.



Fig. 4. AGREEprep pictogram illustrating the greenness assessment of the LLE-LTP sample preparation procedure developed for pesticide residue determination in honey using UHPLC-MS/MS.

3.5 Application to commercial honey samples

The method was applied to nine honey samples from six municipalities in the Triângulo Mineiro region. Imidacloprid was detected in several samples and was quantified in sample F at 12.9 ± 2.6 μg kg^{-1}. Methomyl was detected in most samples and was quantified in samples H and I at 15.8 ± 4.0 and 16.2 ± 4.1 μg kg^{-1}, respectively. Carbendazim and thiamethoxam were also detected below their respective LOQs in some samples. Acetamiprid and diflubenzuron were not detected. Because acephate did not meet the recovery criteria in the validation study, any non-detection for this analyte should be interpreted with appropriate caution. The sample-by-sample results are summarized in Table S6 of the Supplementary Information.

Conclusions

The present study demonstrated the practicability of combining liquid–liquid extraction with low-temperature partitioning (LLE-LTP) and UHPLC-MS/MS to determine pesticide residues in honey, offering a simplified alternative to conventional multistep sample-preparation procedures. Multivariate optimization established extraction conditions suitable for pesticides with diverse physicochemical properties, using only 3.0 g of honey and 4 mL of acetonitrile, without centrifugation, solvent evaporation, or additional clean-up. The method achieved low limits of quantification (0.33–0.67 μg kg^{-1}), satisfactory recoveries (100.0–117.2%), and good precision (RSD < 7.5%) for six pesticides. However, the consistently low recovery of acephate highlighted the limitations of a single extraction for highly hydrophilic compounds. Compared with previously reported methods, the proposed approach offers a favorable combination of analytical sensitivity, reduced solvent consumption, and operational simplicity. Its application to

commercial honey samples confirmed its suitability for detecting and quantifying pesticide residues at trace levels. Overall, coupling LLE-LTP with UHPLC-MS/MS expands the analytical applicability of low-temperature partitioning and provides a practical strategy for routine pesticide residue monitoring in honey, with potential for adaptation to other multiclass contaminant groups.

Acknowledgements

The authors thank the Universidade Federal de Uberlândia for its support and infrastructure, and Ourofino Agrociência for providing the laboratory infrastructure and instrumentation used in this study.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) [grant number APQ-01901-22] and the Financiadora de Estudos e Pesquisa (FINEP) [grant 01.13.0371.00 and 01.11.0135.00].

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability statement

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Author contributions

RAGS: Conceptualization, Data curation, Methodology, Formal Analysis and Investigation, Validation; **ACBC**: Formal Analysis and Investigation, Writing - original draft preparation; **AMF**: Project administration, Supervision, Writing - original draft preparation, Writing - review and editing.

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Figure captions

Fig. 1. Relative importance of the six variables evaluated in the fractional factorial design, expressed as the mean absolute main effect across seven target pesticides.

Fig. 2. Response surfaces from the central composite design showing the effects of (a) honey mass and acetonitrile volume, (b) honey mass and freezing time, and (c) acetonitrile volume and freezing time on the mean recovery of the seven target pesticides by LLE-LTP.

Fig. 3. Representative chromatograms of pesticide-free honey extracts (blank samples) and honey samples spiked with the target pesticides at 2.5 μg L^{-1}, obtained using the optimized LLE-LTP method. Chromatographic conditions are described in the Experimental section. Peak identification: (1) acephate, (2) carbendazim, (3) methomyl, (4) thiamethoxam, (5) imidacloprid, (6) acetamiprid, and (7) diflubenzuron.

Fig. 4. AGREEprep pictogram illustrating the greenness assessment of the LLE-LTP sample preparation procedure developed for pesticide residue determination in honey using UHPLC-MS/MS.

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