A vortex-assisted liquid-liquid extraction followed by dispersive-solid phase extraction (VA-LLE/d-SPE) for the determination of eight benzoylphenylureas insecticides in tomatoes and cucumbers

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ABSTRACT

Monitoring benzoylphenylureas (BPUs) residues in ready-to-eat vegetables is of great interest for an adequate assessment of human pesticide exposure. A rapid, inexpensive, simple, and effective method for determining 8 BPUs insecticides in tomatoes and cucumbers was developed and validated. Vortex-assisted liquid-liquid extraction (VA-LLE) followed by dispersive solid-phase extraction (d-SPE) using graphitized carbon black (GCB) for cleanup was used before LC-MS/MS analysis. Different parameters were optimized, including the type and volume of extractants, vortex time, and the type and amount of adsorbents used for cleanup. The evaluation showed that the method has excellent linearity ($R^2 \geq 0.994$). The recovered 8 BPUs insecticides from spiked tomato and cucumber samples at 0.01, 0.05, and 0.25 mg kg$^{-1}$ ranged from 83.2 to 105.2%, with RSD of 4.9–14.6%. The limits of quantitation (LOQs) were 0.0025 mg kg$^{-1}$ (0.005 mg kg$^{-1}$ for lufenuron). Within-day repeatability ranged from 3.9 to 13.9%, while between-day repeatability ranged from 8.9% to 17.7%. The optimized method was used to analyze 100 samples of tomatoes and cucumbers marketed in Saudi Arabia.

KEYWORDS

benzoylphenylureas, LC-MS/MS, method validation, monitoring, VA-LLE

INTRODUCTION

Farmers use pesticides to protect and increase their agricultural yields. Despite the positive aspects of pesticide use, irresponsible use can result in high levels of pesticide residues that can cause serious harm to human health and the environment [1–4]. Vegetables are among the most essential human nutrient supplements but are also one of the foods most contaminated with pesticide residues [5, 6].
Due to a lack of knowledge about pesticide safety and judicious application, farmers spray pesticides too close to harvest [5, 6]. Some organizations and countries have set maximum residue limits (MRLs) for pesticide residues in food to protect consumers. Due to the widespread use of pesticides in Saudi Arabia, comprehensive testing is required to monitor pesticide residues in agricultural products. Therefore, methods for detecting residues of various pesticides need to be developed.

Benzoylphenylureas insecticides (BPDs) were introduced in 1978 by Bayer in Germany [7] as effective and promising third-generation insecticides for the control of a wide range of insect pests, such as the cotton leafworm (Spodoptera littoralis) in many crops [8]. They have some exciting properties such as efficacy at low doses, low persistence in the environment, easy biodegradability, and low toxicity to mammals with low uptake or translocation into the plant system (non-systemic), and their ability to act as insect growth regulators [9–16], making them the most critical group among urea pesticides commonly used by farmers in integrated pest management programs for vegetables and fruits.

Several analytical approaches have been published for determining BPDs insecticides in different matrices using different extraction, cleaning up, and chromatographic devices. Due to the high polarity and low volatility of BPDs insecticides, it is generally difficult to determine them by GC or GC/MS [17–21]. Therefore, most BPDs insecticides have been determined mainly by LC using UV [13, 22, 23], fluorescence [24–27], chemiluminescence [12], MS [28–31], MS/MS [7, 21, 29, 30, 32–34], and MS/IT [34]. In previous studies, the determination of BPDs insecticide residues mainly focused on juice [35], tea [29], environmental water [27, 36, 37], and vegetables and fruits [7, 13, 21, 28–30, 32, 34, 38, 39].

Routine procedures for evaluating pesticide residues in the environment and food often include sample preparation with the primary objective of extracting, purifying and concentrating the analytes of interest for the analytical system. The main sample preparation techniques include liquid-liquid extraction (LLE), solid-phase extraction (SPE), and dispersive solid-phase extraction (d-SPE) [13, 39–41]. However, LLE is laborious and time-consuming and requires enormous amounts of organic solvents, some of which can be hazardous and costly due to the high purity required. While SPE drastically reduces the amount of solvents used compared to LLE, significant amounts can still be consumed throughout the process due to column conditioning, elution with organic solvents, and another step to concentrate the extract [42].

Despite their widespread use in agriculture, only a few data are available on BPDs residues in crops. Our study aimed to develop a methodology that would allow a routine analysis of 8 BPDs (chlorfluanid, chlorfluazuron, diflubenzuron, flufenoxuron, hexafluorouron, teflubenzuron, trifluralin, and lufenuron) (Fig. S1) insecticides in high water content ready to eat vegetables (tomatoes and cucumbers). The developed method involved a one-shot liquid-liquid extraction using a minute amount of a high-density solvent, clean-up with a dispersing sorbent, and subsequent LC-MS/MS for quantitation of the 8 BPDs at concentrations below their respective minimum residue limits (MRLs). One hundred natural tomato and cucumber samples from local markets in Saudi Arabia were analyzed using this approach to evaluate the pesticide residue levels of the 8 BPDs, which can serve as a reference for future regulation on pesticide use patterns.

**EXPERIMENTAL**

**Chemicals and reagents**

Chlorfluazuron (98.2%, purity), diflubenzuron (99.5%, purity), flufenoxuron (98.9%, purity), hexafluorouron (99.5%, purity), lufenuron (99.5%, purity), teflubenzuron (99.9%, purity), trifluralin (98.3%, purity), and lufenuron (98.7%, purity) certified reference materials were purchased from Chem Service Inc. (West Chester, PA, USA). LC-MS acetonitrile, methanol, ammonium formate, formic acid, HPLC-grade dichloromethane (CH2Cl2, density 1.32 g L−1, % solubility in water = 1.6, log kow = 0.84), chloroform (CHCl3, density = 1.49 g L−1, % solubility in water = 0.815, log kow = 0.84), and carbon tetrachloride (CCl4, density 1.49 g L−1, % solubility in water = 0.08, log kow = 3) [43] were obtained from Fisher Scientific (Loughborough, UK). Agilent Technologies Inc. (Wilmington, DE, USA) supplied the primary secondary amine (PSA), graphite carbon black (GCB), and ceramic homogenizer. The ultrapure water (resistivity 18.2 MΩ cm, TOC <10 ppb and bacterial count <10 CFU mL−1) was prepared using the Ultra Clear™ system (Evoqua Water Technologies LLC, Guenzburg, Germany).

**Standard and reagent solutions**

Appropriate weights of each standard active ingredient were individually weighed into a dark-calibrated volumetric flask to obtain a standard stock solution of 1,000 mg kg−1 in acetonitrile, then diluted ten times in acetonitrile to prepare the intermediate standard solution of 100 mg kg−1.

A working standard solution mixture of 10 mg L−1 of the 8 BPDs was serially diluted to 0.0001, 0.00025, 0.005, 0.005, 0.001, 0.025, 0.05, 0.1, and 0.2 mg L−1 in acetonitrile. The same series was prepared in the final sample extract and then used to generate matrix-matched calibration curves. The standard solutions were stored at −20 °C.

**Sample preparation**

In a 50-mL polypropylene centrifuge tube, 10 ± 0.2 g of the homogenized frozen sample of tomato and cucumber was weighed. After allowing it to melt at room temperature, 2 mL of dichloromethane (CH2Cl2) was added for extraction after adding a piece of ceramic homogenizer for complete interaction between solvent and sample tissues. The tube was vortexed for 4 min and then centrifuged at 5,000 rpm.
for 5 min 200 μL (equivalent to 1 g of sample) of the lower separated organic phase was transferred to a 2 mL tube using a 250-μL glass Hamilton syringe and evaporated under light nitrogen steam. Another 1 mL of acetonitrile was used to dissolve the dried residue and vortex for 1 min. For the cleanup step, 5 mg GCB was added to the tube, vortexed for 1 min, centrifuged for 3 min at 5,000 rpm, and then was filtered with a 0.22-μm nylon syringe filter (Whatman, USA) before being analyzed with LC-MS/MS.

**LC-MS/MS**

The analysis was carried out using a Dionex Ultimate 3000 RS UHPLC and a TSQ Altis triple quadrupole mass spectrometer (Thermo Fisher Scientific, Austin, TX, USA). Selected Reaction Monitoring (SRM) was performed by switching between positive and negative modes using an electrospray ionization (ESI) source. The ESI source was optimized in positive (ESI+) mode for the determination of diflubenzuron, triflumuron, flufenoxuron, novaluron, lufenuron, chlorfluazuron, and in negative (ESI-) mode for the determination of hexafluoruron and tebufluzuron. Sheath and auxiliary gasses were 40 and 10 Arb, respectively; argon was used as collision gas; capillary voltage was 4.0 kV in positive mode and −3.5 kV in negative mode; source temperature was 325 °C; and desolvation temperature was 350 °C. The mass transitions, cone voltage settings, and collision energies for the 8 BPUs are listed in Table 1. Chromatographic separation was performed using an Accucore RP-MS C18 column (100 × 2.1 mm, 2.6 μm film thickness, Thermo Fisher Scientific) at 40 °C. The flow rate was 0.3 mL min⁻¹, and the injection volume was 2 μL. The mobile phases consisted of two elements: Water (A) and methanol (B). The gradient elution program was as follows: 25% B for the first minute (0–1 min), then gradually increasing to 90% of B for 3 min (1–4 min), and holding for 4 min (4–8 min), then 25% B for the last 8 min for column equilibration (8.1–16 min). Data were acquired and processed using Trace Finder software (version 4.1).

**Method validation**

Selectivity, linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), precision, and matrix effects were tested for method validity based on SANTE guidelines [44]. The selectivity was investigated by monitoring each analytes characteristic transition in the corresponding retention time of the final extract of the analyzed blank sample (pre-analyzed for the absence of the tested pesticide residues). Linearity was assessed by evaluating the $R^2$ of the constructed calibration curves of the target analytes at concentrations ranging from 0.001 to 0.5 mg kg⁻¹. The recovery rates and RSD of the method were determined by analyzing spiked samples at three concentration levels of 0.01, 0.05, and 0.25 mg kg⁻¹. The LOD is the concentration that gives a peak-to-peak signal-to-noise ratio of 3. In practice, LOQ is the lowest spike concentration, with average recoveries between 70% and 120% with an RSD of less than 20%. Repeatability and reproducibility were estimated at the LOQ level and expressed as RSD. The matrix effect was calculated by comparing the slopes of calibration curves generated in pure acetonitrile to those generated using the blank sample’s extract. The matrix effect (ME) was calculated using Equation (1) [45, 46]:

$$\text{ME}\% = \frac{\text{slope(matrix)} - \text{slope(solvent)}}{\text{slope(solvent)}} \times 100 \quad (1)$$

**Natural samples**

Tomatoes and cucumbers are two of the most critical greenhouse crops for a healthy diet in Saudi Arabia; they are eaten raw or processed [47]. One hundred samples, 50 tomatoes, and 50 cucumbers were collected following Directive 2002/63/CE [48] from local markets in Saudi Arabia.

### Table 1. Optimization conditions of MS/MS for the analysis of 8 BPUs insecticides

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Polarity</th>
<th>Precursor (m/z)</th>
<th>Product (m/z)</th>
<th>Collision energy (V)</th>
<th>RF lens (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diflu benzuron</td>
<td>7.63</td>
<td>+</td>
<td>311.1</td>
<td>141</td>
<td>31</td>
<td>51</td>
</tr>
<tr>
<td>Triflumuron</td>
<td>7.90</td>
<td>+</td>
<td>359.3</td>
<td>139</td>
<td>31</td>
<td>51</td>
</tr>
<tr>
<td>Hexafluoruron</td>
<td>8.08</td>
<td>–</td>
<td>460.8</td>
<td>403.8</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>Novaluron</td>
<td>8.14</td>
<td>+</td>
<td>493</td>
<td>141</td>
<td>41</td>
<td>70</td>
</tr>
<tr>
<td>Lufenuron</td>
<td>8.44</td>
<td>+</td>
<td>511.1</td>
<td>141</td>
<td>39</td>
<td>80</td>
</tr>
<tr>
<td>Teflubenzuron</td>
<td>8.52</td>
<td>–</td>
<td>380.5</td>
<td>296.2</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Flufenoxuron</td>
<td>8.70</td>
<td>+</td>
<td>489.2</td>
<td>141</td>
<td>43</td>
<td>65</td>
</tr>
<tr>
<td>Chlorfluazuron</td>
<td>9.94</td>
<td>+</td>
<td>540</td>
<td>158</td>
<td>19</td>
<td>76</td>
</tr>
</tbody>
</table>

The bold ions were used as quantifiers.
Arabia between February and June 2023. Two kilograms of each sample were packed in sterile polyethylene bags, labeled, immediately taken to the laboratory, cut into 2–3 cm³ pieces, frozen, and then homogenized. For each sample type, three replicates (10 ± 0.2 g each) were weighed into 50-mL centrifuge tubes and frozen at −20 °C until analysis.

Statistical analysis
A one-way analysis of variance (ANOVA) (Microsoft® Excel 2021) was used to test the hypothesis that multiple means are equal. ANOVA compares the mean values of detected pesticides as a function of the optimized parameters. A significant difference was inferred if the probability criterion (p) was less than 0.05.

RESULTS AND DISCUSSION

Mass evaluation
One of the main objectives of pesticide research is to identify pesticide residues in food to assess the quality of products as well as possible and reduce potential health risks for humans. The MS/MS parameters of the analytes studied were tuned using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA). A mixture of methanol and water (50:50 v/v) was used for infusion with a 300 μL min⁻¹ flow rate during scanning and fragmentation. The positive and negative ion spray voltages were automatically optimized. It was found that the optimal values for the ESI⁺ voltages were 4 kV, while the ESI⁻-voltages were 3.5 kV. The parent ions of the 8 BPUs were recorded in full scan mode (Table 1). Most of the parent ions showed higher signal intensities in positive mode, except hexaflumuron and teflubenzuron, which showed higher intensities in negative mode. The mass spectra obtained in positive or negative ESI mode contained several ions with high relative intensities, which gave good measurement selectivity. According to the SANTE guideline [44], the quantifier ion with the most incredible abundance and the qualifier with the second greatest abundance were selected (Table 1). Figure S2 shows a representative chromatogram of the separated analytes.

Mobile phase optimization
Multiple injections showed good peak shape and high sensitivity of the 6 BPUs without adding formic acid (0.1%), whereas adding formic acid suppressed the sensitivity of the 6 BPUs. This could be due to the suppression of the signal due to the formation of formic acid adducts [M+FA]⁺ or the proton adduct [M+H]⁺, which suppressed the intensity responses of the ions. Kokkonen (2011) has reported that adding formic acid as a solvent modifier can lead to signal suppression in some toxins [49]. In contrast, novaluron and lufenuron showed signal enhancement when 0.1% formic acid was added to the mobile phase (Fig. S3). Therefore, we used pure water and MeOH as the mobile phase to separate the 8 BPUs in this study.

Procedure optimization
The effective parameters to optimize the VA-LLE method for determining 8 BPUs insecticides were studied in tomato samples (as a representative of the high water content of ready-to-eat vegetables). The optimization parameters included the vortex time, extraction solvent type and volume, and the adsorbent type and amount used in the cleaning step.

A. Extraction
High-density extraction solvent selection. The efficiency of different high-density solvents, including CH₂Cl₂, CHCl₃, and CCl₄, for extracting the 8 BPUs residues were studied, keeping the other factors unchanged (sample weight 10 ± 0.2 g, volume of extraction solvent 2 mL, vortex time 5 min, and no cleaning up step). The type of extraction solvent was selected based on the solubility of the BPUs, as they are much more lipophilic, so partitioning these compounds into a layer of lipophilic solvent should be relatively easy [50]. Figure 1 shows that dichloromethane has a significantly higher recovery than chloroform and carbon tetrachloride for lufenuron (P ≤ 0.041), chlorfluanuron (P ≤ 0.047), and teflubenzuron (P ≤ 0.048). Also, the co-eluting matrix was evaluated in blank samples after collecting the separated organic phase, and UV–Vis
spectrophotometry (Uvmini-1240, Shimadzu, Japan) was performed at 520 nm to determine the intensity of anthocyanin, which is mainly present in bright-colored fruits and vegetables [51]. Maximum absorbance was obtained for chloroform extract (0.593 absorbance units after 1000-fold dilution). On the other hand, dichloromethane had a lower absorbance (0.080 absorbance units). In comparison, carbon tetrachloride had an even lower absorbance (0.032 absorbance units), which indicates the lower co-eluting compounds in carbon tetrachloride < dichloromethane < chloroform. Consequently, dichloromethane was selected as the extraction solvent for further studies, considering the importance of cleaning up the extract.

**Volume of extraction solvent.** The performance of the presented VA-LLE procedure was tested using different volumes of CH$_2$Cl$_2$ (1.5–4 mL). As shown in Fig. 2, the extraction efficiency increased with the increase of the extraction solvent from 1.5 to 3 mL. The recovery decreases with more than 3 mL of dichloromethane, probably due to increased miscibility with the sample water during extraction. Based on these findings, the ideal amount of dichloromethane as an extraction solvent for the VA-LLE method was 2 mL.

**Vortex time.** In the VA-LLE process, “extraction time” is the time between adding organic solvents and the centrifugation process. The effect of extraction time on extraction efficiency was tested in a time range of 1–5 min, as shown in Fig. S4. It has been shown that the extraction time has no significant effect ($P \geq 0.21$) on the VA-LLE process if it is more than 4 min. Overall, the optimized factors for extraction were 2 mL of CH$_2$Cl$_2$ as an extraction solvent and a vortex time of 4 min. The BPUs have neither acidic nor basic properties [32], so pH optimization is not required during extraction.

**B. Cleanup.** Matrix interference from co-extracted tomatoes makes the identification of target compounds doubtful. The previous method used tandem adsorbents of graphitized carbon black (GCB) and primary secondary amine (PSA) to eliminate co-extracts and color-interfering chemicals. In our study, the use of PSA did not result in a sufficiently clean extract (Fig. 3). In contrast, the use of GCB resulted in a more transparent and colorless solution because it adsorbed the pigments and other co-eluting materials effectively and provided nearly pure extract. The percentage matrix effect (ME%) was used to evaluate the efficacy of using GCB for cleaning up. The extract of the blank sample was spiked with the tested analytes at a concentration level of 0.01 mg kg$^{-1}$, then treated with different weights of GCB, and finally determined by LC-MS/MS based on the calibration curves of the tested analytes generated in pure solvent. Additional weights were taken: 5 mg, 10 mg, 15 mg, and 20 mg per 1 g sample (200 μL of extract was evaporated and re-dissolved in acetonitrile). The results shown in Figs 3 and 4 indicated that with increasing the weight of GCB, the extract transparency increased while the determined analyte concentration significantly decreased; lufenuron and chlorfluanuron were most affected by increasing GCB weight. So, 5 mg of GCB is sufficient for reducing the co-eluting compounds and improving the matrix effect (<20%). Therefore, 5 mg GCB/1 mL extract (equivalent to 200 μL CH$_2$Cl$_2$ extract and 1 g sample) was selected as an adsorbent for further validation studies for the cleanup step (μd-SPE).

**Method validation**

**Linearity and matrix effects.** Linearity was assessed by constructing nine-level matrix-matched calibration curves using tomatoes and cucumbers’ final extracts over a 0.0005–0.25 mg kg$^{-1}$ concentration range. Correlation coefficients ($R^2$) were ≥0.9958 for diflubenzuron, flufenoxuron, and hexaflumuron over the range of 0.001–0.1 mg kg$^{-1}$, and

![Fig. 2. Effect of different volumes of CH$_2$Cl$_2$ on percent recovery for 8 BPUs at 10 μg kg$^{-1}$ using the VA-LLE method (sample weight = 10 g, $n = 6$, vortex time = 5 min, without clean-up)](image-url)

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for triflumuron, novaluron, chlorfluazuron, and teflubenzuron over the range of 0.002–0.1 mg kg\(^{-1}\), and 0.005–0.1 mg kg\(^{-1}\) for lufenuron. The deviation of the individual residuals was less than 20%, indicating an excellent linear relation (Table 2).

The slopes of the constructed curves in matrix-matched and pure acetonitrile were used to evaluate the percentage matrix effect (% ME) (Table 2). The co-extracted compounds caused signal enhancement for diflubenzuron, triflumuron, teflubenzuron, and hexafluorobenzuron. In contrast, signal suppression was observed for flufenoxuron, novaluron, lufenuron, and chlorfluazuron. The co-eluted compounds in the final extract of the tomato and cucumber samples caused insignificant enhancement and suppression of the signals of the eight benzoylphenylureas in the range of \(-12.5\) to \(9.3\%\) and \(-8.9\) to \(7.7\%\), respectively. This variability is close to the repeatability standard deviation (RSD) value of 20%. The residues of the actual samples were calculated using the matrix-matched calibration curves to obtain more accurate results.

**LOD and LOQ.** The high sensitivity of this approach is reflected in the LODs of the 8 BPs calculated at a signal-to-noise ratio of 3, ranging from \(0.238 \times 10^{-3}\) to \(0.931 \times 10^{-3}\) mg kg\(^{-1}\) and \(0.291 \times 10^{-3}\) to \(0.774 \times 10^{-3}\) mg kg\(^{-1}\) for tomato and cucumber, respectively. The LOQs, the lowest spiking level, achieved sufficient recovery (70–120%) and precision (<20%) at 0.0025 mg kg\(^{-1}\) (0.005 mg kg\(^{-1}\) for lufenuron) (Table 3) [44]. LOQs were well below the MRLs established by the European Union and FAO/WHO for tomatoes and cucumbers.

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**Table 2.** Linearity range, correlation coefficient (\(R^2\)), and Matrix effect (%ME) of the 8 BPs in tomato and cucumber

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Range (mg kg(^{-1}))</th>
<th>Tomatoes</th>
<th>Cucumber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R^2)</td>
<td>ME%</td>
<td>(R^2)</td>
</tr>
<tr>
<td>Diflubenzuron</td>
<td>0.001–0.1</td>
<td>0.9982</td>
<td>+9.3</td>
</tr>
<tr>
<td>Triflumuron</td>
<td>0.002–0.1</td>
<td>0.9981</td>
<td>+5.4</td>
</tr>
<tr>
<td>Flufenoxuron</td>
<td>0.001–0.1</td>
<td>0.9982</td>
<td>-7.1</td>
</tr>
<tr>
<td>Novaluron</td>
<td>0.002–0.1</td>
<td>0.9979</td>
<td>-12.5</td>
</tr>
<tr>
<td>Lufenuron</td>
<td>0.005–0.1</td>
<td>0.9945</td>
<td>-8.8</td>
</tr>
<tr>
<td>Chlorfluazuron</td>
<td>0.002–0.1</td>
<td>0.9963</td>
<td>-4.8</td>
</tr>
<tr>
<td>Teflubenzuron</td>
<td>0.002–0.1</td>
<td>0.9965</td>
<td>+6.5</td>
</tr>
<tr>
<td>Hexafluorobenzuron</td>
<td>0.001–0.1</td>
<td>0.9968</td>
<td>+4.1</td>
</tr>
</tbody>
</table>
Table 3. Limits of detection (LOD), limits of quantitation (LOQ), and precision (RSD, and RSDR) of the 8 BPUs in tomatoes and cucumbers

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Tomatoes (mg kg(^{-1}))</th>
<th>Cucumber (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOD</td>
<td>LOQ</td>
</tr>
<tr>
<td>Diffubenzuron</td>
<td>0.238 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>0.388 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td>Triflumuron</td>
<td>0.424 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>0.313 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td>Flufenoxuron</td>
<td>0.327 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>0.411 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td>Novaluron</td>
<td>0.482 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>0.291 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td>Lufenuron</td>
<td>0.931 × 10(^{-3})</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>0.774 × 10(^{-3})</td>
<td>0.005</td>
</tr>
<tr>
<td>Chlorfluazuron</td>
<td>0.713 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>0.551 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td>Tefubenzuron</td>
<td>0.651 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td></td>
<td>0.418 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
<tr>
<td>Hexafluoruron</td>
<td>0.521 × 10(^{-3})</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

\(^a\)Intra-days repeatability (n = 6) set at the LOQ levels.

\(^b\)Inter-days repeatability (n = 18, at three different days, 6 days' intervals) set at the LOQ levels.

**Precision.** At the LOQ level of 0.0025 mg kg\(^{-1}\) (0.005 mg kg\(^{-1}\) for lufenuron), the precision of the method was determined on the same day (within-day repeatability, RSD, n = 6) and different days (between-day repeatability, RSDR, n = 18). RSD ranged from 5.8 to 13.2% and 3.9–13.7%, while RSDR ranged from 9.1 to 16.2% and from 9.1 to 16.2% and 8.9–17.7% for tomatoes and cucumbers, respectively (Table 3). Precision results were within the allowable limit of 20% of the European Commission Guidance Document for the Validation and Quality Control of Pesticide Residue Analysis [44].

**Accuracy.** The recovery of the analytes studied was measured in tomato and cucumber samples spiked with three different concentrations (0.01, 0.05, and 0.25 mg kg\(^{-1}\)), with six replicates for each concentration. The average recoveries and relative standard deviations (RSD, %) of the 8 BPUs in tomatoes ranged from 83.2 to 105.2% and 4.3–14.4%, respectively, and from 84.8 to 98.1% and 4.9–13.7% for cucumber, respectively, as shown in Table 4.

**Comparison of the optimized method with the previous studies**

The presented method VA-LLE-LC/MS/MS is superior to the previous method regarding recoveries, LOQs, time required for complete extraction, solvent consumption, and operating efficiency (Table 5). The LOQs of the targeted 8 BPUs are significantly lower in this method than those in the previously reported methods, and the percent recoveries are sufficiently high. Regarding organic solvent consumption, only 2 mL of CH\(_2\)Cl\(_2\) is required for extraction and 1 mL of acetonitrile for solvent exchange, compared with the high solvent consumption of the previous methods [8, 38, 39, 52–56]. In addition, the reported methods used an SPE cartridge for the cleanup step, while our proposed method required only 5 mg of GCB for the cleanup step. Reducing the analysis time was a significant focus of our research. It was found that the time needed for extraction in this study was about 16 min, so this method is more environmentally friendly, economical, and faster quantify.

To evaluate the greenness of the proposed methodology, Pena-Pereira et al. developed the AGREE metric approach [57]. The metric approach compares analytical methods according to the 12 principles of green chemistry. Each principle is labeled with a score from 0 to 1 in a pictogram. The methods with the same chromatographic instrument and a nearly similar sample weight to the proposed method were considered to allow a fair comparison. Two reported methods, Method A [38] and Method B [28], were selected for comparison with the method proposed in this study (Method C). The exact weight of 2 was set for all 12 evaluated principles (assuming all evaluation criteria are equally important). The AGREE software [58] was also used to
create the pictogram centered on the overall evaluation score of the method (Fig. 5).

For principle 1, sample preparation, the methods compared were offline analyses, and a score of 0.48 was achieved. Regarding sample weight (principle 2), a sample weight of 10 g (0.32 score) was used, while 15 g was used for methods A (0.29 score) and B (0.27 score). Since the location of the analytical equipment (principle 3) must be in the

![Fig. 5. Comparison of the Greenness principles of method A, method B, and the method proposed in this study (method C)](image)
laboratory, the method steps were performed manually and not miniaturized (principle 5), the chromatographic and lab
equipment used for the determination is energy intensive (principle 9), none of the reagents and solvents are from bio-
based sources (principle 10), and the threats to aquatic life, bioaccumulation, persistence, high flammability, explosivity,
and corrosively of the solvents used in all the methods compared cannot be avoided (principle 12). Therefore,
principles 3, 5, 9, 10, and 12 did not comply with analytical
greenness and received the same score of zero. The compared methods do not include derivatization steps
(Principle 6) and achieve similar scores of 1. Also, the analytes analyzed per run are 4, 5, and 8 for Method A,
Method B, and the proposed method in this study (Method C) (Principle 8) and achieve scores of 1, 0.81, and 1,
respectively. The method proposed in this study (Method C) outperforms Methods A and B in the number of significant
steps for sample preparation (Principle 4), as it includes only five major steps: sample homogenization, vortex-assisted
liquid-liquid extraction, evaporation and solvent exchange, clean-up, and LC-MS/MS analysis, which were lower than
Method A (11 steps) and Method B (8 steps), scoring 0.6.
In contrast, the other methods scored zero (Principle 4). A significant advantage of the proposed method (method c)
over methods A and B is the amount of analytical waste it generates (principle 7). It scored 0.35 due to the high con-
sumption of solvents, salts, and adsorbents for cleaning up the extract in methods A and B. The compared methods
used acetonitrile and dichloromethane considered toxic solvents, for sample preparation (Principle 12). In contrast,
the proposed method (Method C) consumed a small amount of acetonitrile (1 mL) and dichloromethane (2 mL) in
addition to the solvents used in the mobile phase for chromatographic separation (Principle 12) and thus scored 0.
The overall analysis of the AGREE results showed that the proposed method had an overall value of 0.34 compared to
methods A and B, which scored 0.23 and 0.22, respectively, which means that the proposed method is greener than the
reported methods (Fig. 5).

Natural samples

Table 6 provides an overview of the occurrence of the 8 BPUs insecticides in tomatoes and cucumbers after
analyzing 100 samples. In 50 tomato samples, 24 (48%) were contaminated with pesticide residues, and 16 (32%) were
contaminated at a higher concentration than the CODEX and EU-MRL. In 50 cucumber samples, 10 samples (20%) were
contaminated with diflubenzuron, flufenoxuron, and lufenuron residues, of which 8 samples (16%) had higher
contamination levels of diflubenzuron and flufenoxuron residues than the CODEX and EU-MRL.

In tomatoes, diflubenzuron and flufenoxuron residues were above the EU-MRL of 0.01 mg kg$^{-1}$ in 22% and 10%,
with a mean residue of 0.091 mg kg$^{-1}$ and 0.038 mg kg$^{-1}$, respectively. On the other hand, Novaluron and lufenuron
were detected at concentrations below the CODEX MRL of 0.7 mg kg$^{-1}$ and 0.4 mg kg$^{-1}$ and EU-MRL of 0.01 and 0.4
mg kg$^{-1}$, respectively. Diflubenzuron residues were found most frequently in cucumbers, followed by flufenox-
uron and lufenuron, with a mean residue of 0.031, 0.027, and 0.034 mg kg$^{-1}$. Of the 50 cucumber samples tested, residues of diflubenzuron and flufenoxuron exceeded the
MRL of 0.01 mg kg$^{-1}$ (EU-MRL) in 5 (10%) and 3 (6%), respectively. Of the 100 samples, a higher percentage (16%)
of samples exceeded the MRLs detected in the tomato samples than in the cucumber samples (8%). The most
frequently detected insecticides in tomato and cucumber samples were diflubenzuron, flufenoxuron, and lufenuron.
The results showed that the percentage of contaminated tomato and cucumber samples with diflubenzuron residues exceeded the EU MRL of 0.01 mg kg$^{-1}$ in 22% and 10%,
respectively. Diflubenzuron was the first commercially available insecticide from the benzoylphenyleurea group [59],
classified as chronic risk level III [60]. It was approved for legal use on various crops in Saudi Arabia till now [59], which
explains the detection of its residues in the analyzed samples; therefore, farmers must be encouraged to comply with Good
Agricultural Practices (GAP) to minimize the contamination levels. Also, Alhamami et al. (2023) mentioned that the res-
ides of diflubenzuron in tomatoes did not pose significant potential health risks to consumers after the application of
24% SC at the authorized and high doses during a field study conducted in Najran City, Saudi Arabia [61].

On the other hand, flufenoxuron residues exceeded the maximum residue limit of 0.01 mg kg$^{-1}$ in 10% and 3% of
tomato and cucumber samples, respectively. Flufenoxuron is one of the pesticides banned in the Kingdom of Saudi Arabia
(Ministry of Environment Water and Agriculture, KSA, 2022) [62]. So, controls on banned pesticides must be tightened by Concerned parties, and regular inspections of
crops, especially those intended for direct consumption, must be intensified.

![Table 6. Residues of 8 BPUs in tomatoes and cucumbers collected from the local markets](image-url)
CONCLUSION

A rapid, cheap, effective, and environmentally friendly method was optimized for analyzing 8 BPUs insecticides in tomato and cucumber samples. The vortex-assisted liquid-liquid extraction (VA-LLE) method involved using 2 mL of dichloromethane followed by d-SPE for cleanup using 5 mg of GCB adsorbent and LC-MS/MS for quantitation. Several parameters were optimized, including the type of extraction solvent, its volume, vortex time, and the efficiency of PSA and GCB adsorbent in the cleanup step. The verified LOQs achieved satisfactory accuracy and precision for the tested 8 BPUs, and the LOQs were well below the MRLs. According to the SANTE guidelines, the validation parameters of the proposed method exhibited acceptable linearity, recovery, and precision. The advantages of the proposed method include high extraction efficiency, low matrix effects, low solvent volume for extraction, and time-saving. The extraction method can be considered environmentally friendly, safe, and straightforward. It could be an excellent alternative to the techniques currently used to analyze the 8 BPUs from vegetables and fruits with high water content at sufficient sensitivity and low concentrations below the EU MRLs. The optimized method was used for the analysis of 100 tomato and cucumber samples collected from Saudi markets. Of the 100 samples analyzed, 34 samples were positive for diflubenzuron, flufenoxuron, novalurin, and lufenuron residues, with diflubenzuron and flufenoxuron found in 16 samples at concentration levels above the MRLs set by the EU regulations. Further studies may be needed to determine whether the established VA-LLE procedure is suitable for analyzing the 8 BPUs in leafy vegetables and dried fruits.

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SUPPLEMENTARY DATA

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