Determination of Paraquat in Vegetables Using HPLC–MS-MS

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A simple, sensitive, reliable and economical method was developed for the determination of paraquat (a widely used herbicide) in four edible vegetables (cabbage, lettuce, spinach and Chinese cabbage) using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS-MS). The samples were extracted with water under sonication and cleaned up by weak cation exchange solid-phase extraction. Chromatographic separation of paraquat was achieved on a hydrophilic interaction liquid chromatography column (2.1 × 100 mm, 3 μm) with a gradient program using 10 mM ammonium acetate in 0.1% formic acid and acetonitrile as mobile phase. The low salt concentration used in the eluting buffer ensured extended LC–MS analysis of paraquat in different matrices without the necessity of frequent source cleaning. The validity of the developed method was evaluated by spiking paraquat in four edible vegetables at 50 and 500 ng g⁻¹. Recovery ranged from 43.6 to 73.5%. The limit of detection is 0.94 ng g⁻¹. With the developed method, the kinetic of paraquat entering plant tissue was also evaluated.

Introduction

Paraquat, 1,1'-dimethyl-4,4'-bipyridinium dichloride, is one of the most widely used herbicides for the control of weeds in crops. Its strong herbicidal activity combined with physical properties such as ease of handling, low vapor pressure, high water solubility and high binding potential with soil, which prevents leaching into aquifers, makes it suitable for widespread agricultural usage (1). Arabidopsis thaliana was used as a model system to study the mechanism of paraquat’s toxicity (2–4). It has been reported that paraquat exerts its herbicidal activity by interfering with the intracellular electron transfer system in plant, inhibiting reduction of NADP to NADPH during photosynthesis. This disruption leads to the formation of reactive oxygen species (ROS) that interact with unsaturated lipids of membranes and result in the destruction of plant organelles, inevitably lead to cell death (5, 6).

The widespread usage of paraquat in agriculture has drawn increasing concern regarding its residues in foods since paraquat has been demonstrated to be a highly toxic compound for human beings and animals. It can cause great damage to liver, lung and kidney (7–9). Chronic exposure to paraquat increases the risk of Parkinson’s disease (PD) so it is also considered as an environmental risk factor for PD (10, 11). In addition, paraquat has been involved in many cases of acute poisoning and suicides (12). Paraquat also brings risks to vegetation, wild life and soil microsystem and may cause long-term adverse effects in the aquatic environment (13). For these obvious adverse effects, paraquat is considered to be one of the most dangerous and controversial herbicides in the world. To protect the environment and human health, paraquat has been banned in many countries, including Sweden, Denmark, Austria and Finland (14). Chinese government also announced that paraquat will be banned from the market after 2016 (15). However, efficient and low toxic replacement of paraquat is difficult to find so illicit usage of paraquat will be a long-lasting problem.

At present, the maximum residue level of paraquat is 0.05 mg kg⁻¹ in most foodstuffs and the acceptable daily intake is 0.005 mg kg⁻¹ (15). Capillary electrophoresis (16), gas chromatography (17), liquid chromatography (18) and enzyme-linked immunosorbent assay (19) have been reported for the determination of paraquat in food crops such as potato, carrot and onion, those methods required complex extraction procedures and were generally low in sensitivity. Mass spectrometry based methods, such as gas chromatography–mass spectrometry and liquid chromatography tandem mass spectrometry (LC–MS-MS) showed better sensitivity. Such methods were used to determine paraquat in biological materials such as blood and urine (20–22) and in liquid matrices such as water (23–25). The high polarity of paraquat rendered poor retention on conventional C18 columns, ion-pairing agent can mitigate the situation, but lead to signal suppression and low sensitivity when coupled with mass spectrometric detectors (26). Hydrophilic interaction liquid chromatography (HILIC) columns showed promising retention of polar compounds like quaternary ammonium (27, 28), but a buffer with high salt concentration is required to maintain optimal performance of the column. When coupled with mass spectrometer, the ion-pairing agents and high salt concentration decrease sensitivity (29, 30) and cause clogging of the spray tip and the MS orifice. So, an LC protocol without ion-pairing agent and low buffer concentration is needed to achieve sensitive detection of paraquat with LC–MS.

Vegetable is an important category of human diet, it is also a paraquat carrier, because paraquat can enter into plant cell quickly, simple washing may not remove it, so it is an urgent need to develop a sensitive and accurate method for the determination of paraquat in such samples. Kolberg et al. (30) determined paraquat residues in potato and cereals with LC–MS-based methods. Aramendia determined paraquat in olive oil by ion-pair LC–ESI-MS coupled with an Xterra C8 column and got a detection limit at 4 μg kg⁻¹ (31); corn leaves as vegetation matrix was also tested by liquid chromatography–ESI–isotope-dilution mass spectrometry (LC–ESI–IDMS), 0.5 M HCl and dichloromethane were adopted for sample extraction (32). However, there was no report on the determination of paraquat in edible leafy vegetables by LC–MS-MS.

In this paper, we established a method for the determination of paraquat in four commonly edible leafy vegetables (cabbage,
lettuce, spinach and Chinese cabbage). Paraquat was extracted from vegetables simply by sonication in water, followed by solid-phase extraction (SPE) clean up. Chromatographic separation of paraquat was achieved on an HILIC column with 10 mM ammonium acetate in the elute buffer. The low salt concentration of the mobile phase ensured optimal performance of the mass spectrometer without the necessity of frequent source cleaning up. Sub-ppb level limit of detection (LOD) of paraquat could be achieved from vegetable samples.

Experimental

Instrumentation and reagents

LC–MS–MS was performed on an Agilent 1200 HPLC system coupled with a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Fremont, CA, USA); weak cation exchange (WCX) cartridges (60 mg, 3 mL) were used for sample purification (Waters Oasis, Dublin, Ireland). Ultrasonic cleaner (Kunshan, China) was used to promote sample extraction. Paraquat and high-performance liquid chromatography (HPLC) grade trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were bought from Fisher Scientific International (Hampton, NH, USA). HPLC-grade formic acid and ammonium acetate were purchased from Dikma Technology (Richmond Hill, ON, Canada). All other reagents were analytical grade. Ultrapure water with a resistance of 18.2 MΩ cm$^{-1}$ was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Paraquat standard solutions were prepared by dissolving paraquat with ultrapure water and diluting to proper concentrations.

Sample preparation

Vegetables (cabbage, spinach, lettuce, and Chinese cabbage) were purchased from local supermarket. Spiked samples were prepared by adding standard paraquat solution of different concentrations into vegetables. One gram of vegetable sample was weighed and grounded into powder in liquid nitrogen; all the powder was transferred into a 50-mL plastic centrifuge tube, weighed and added into solid matrix. After the extraction, the extract solution was centrifuged at 8,000 rpm for 20 min. The WCX cartridge was preconditioned with 3 mL of methanol and 3 mL of water. Four milliliters of the extract supernatant were loaded onto the column. The cartridge was washed with 1 mL methanol–water (1:1, v/v), and paraquat was eluted by 1 mL acetonitrile–TFA–water (80:2:18, v/v/v). The solution was evaporated to dryness in 50°C water bath under nitrogen. Finally, the residue was reconstituted with 0.4 mL starting mobile phase and injected into the LC–MS system for analysis. Blank samples were processed in the same manner.

For immersion experiments, 1 g of cabbage sample was immersed in 10 mL, 50 ng mL$^{-1}$ of paraquat solution for 30 min, 3 and 24 h. One gram of lettuce sample was immersed in 10 mL, 20 ng mL$^{-1}$ of paraquat solution for the same time period. After the immersion, the vegetable samples were lifted from the solution and analyzed by the developed method.

HPLC–MS–MS conditions

Chromatographic separation of paraquat was achieved on a Thermo Scientific Syncronis HILIC column (2.1 × 100 mm, 3 μm). The column temperature was set at 30°C. Mobile phase consists A: 10 mM ammonium acetate with 0.1% formic acid and B: acetonitrile. A gradient program was used for elution: 60% B (initial), 60–20% B (from 0 to 7 min) and 20% B (from 7 to 8 min). After 8 min, the ratio was increased to 60% B and held for 10 min for column equilibration before the next injection. The mobile phase was delivered at a flow rate of 0.2 mL min$^{-1}$, and the injection volume was 1 μL.

The triple quadrupole mass spectrometer operated under multiple reaction monitoring mode for quantitative and qualitative analysis. The optimized electrospray ionization condition was: gas temperature 350°C, gas flow 12 L min$^{-1}$, sheath gas temperature 350°C, sheath gas flow 10 L min$^{-1}$, capillary voltage 4,500 V and nozzle voltage 1,500 V. Positively charged molecular ion of paraquat at m/z 186 was selected as precursor ion. Two transitions: 186/171 and 186/155 were selected with 186/171 as the quantitative transition.

Results

Optimization of sample extraction

Extraction of pesticide residues from solid matrices generally involves sample homogenization and solvent extraction. In the case of paraquat, grinding the plant material in liquid nitrogen is required to release paraquat from cytoplasm and subcellular organelles. Literature reported extraction of paraquat from vegetable and cereal by refluxing or heating with strong sulfuric or hydrochloric acid to free herbicides from their adsorbed or bound state (33). In this experiment, 6 M HCl was tested and showed poor recovery. In comparison, water showed improved recovery, and consistency, water also had better compatibility with the following SPE cleanup and MS analysis procedure. Different extraction conditions were tested to improve the extraction efficiency. Sonication, water bath, shaking and combinations of these procedures were compared. The results showed that sonication in 50°C water bath obtained the best extract efficiency (Figure 1). Water bath extraction at elevated temperature, such as 80°C, induced decomposition of paraquat, which in turn led to significant variation in recovery between replicates. Sonication time (30 min, 1 and 2 h) was also tested, and 1 h was chosen as the final treatment time.

Figure 1. Efficiency of different extract methods: (A) sonication at 50°C, 1 h; (B) shaking at 250 rpm, 1 h; (C) water bath at 60°C, 1 h; (D) stirring, 1 h; (E) water bath at 60°C and sonication, 0.5 h, respectively and (F) water bath at 60°C and shaking at 250 rpm, 0.5 h, respectively (error bar represents SD; n = 4).
Optimization of HPLC–MS-MS condition
HILIC columns are commonly used for the separation of compounds with high polarity and hydrophilicity. Young et al. (26), Whitehead et al. (34) and Wang et al. (35) adopted an Atlantis HILIC column for the determination of paraquat in water, human urine, blood and tissue samples. In these reports, the mobile phase used consisting of 250 mM ammonium formate water, human urine, blood and tissue samples. In these reports, the mobile phase used consisting of 250 mM ammonium formate

Optimization of sample purification
The extract supernatant was injected directly into the HILIC column to assess the possibility of analyzing without SPE purification. But matrix in the supernatant induced poor chromatographic peak shape and low MS detection sensitivity so further SPE cleanup was adopted. SPE cartridges suitable for polar compounds were tested; these cartridges included WCX, Waters Oasis HLB (hydrophilic–lipophilic-balanced reversed-phase) and basic alumina column. HLB cartridge could not retain paraquat properly, >60% of the paraquat was lost in the loading step, WCX and basic alumina column showed better recoveries. By comparison, with WCX cartridge recovery up to 95% can be achieved, while with basic alumina column, the recovery was ~70%. So WCX cartridge was selected as the final cleanup cartridge.

Five different eluting solutions were also compared, which were acetonitrile–TFA–water (80 : 1 : 19, v/v/v), (80 : 2 : 18, v/v/v), (80 : 5 : 15, v/v/v), acetonitrile–formic acid–water (40 : 30 : 30, v/v/v) and acetonitrile–formic acid (90 : 10, v/v). Both acetonitrile–TFA–water (80 : 2 : 18, v/v/v) and acetonitrile–TFA–water (80 : 5 : 15, v/v/v) showed satisfactory recovery (Figure 2). TFA had significantly better eluting efficiency than formic acid due to its stronger acidity. Two percent of TFA was selected as the final eluting solution to reduce usage of the corrosive acid.

Method validation
Linearity, LOD, precision and recovery were determined to evaluate the validity of the method. Linearity was studied by diluting paraquat standard stock solution either with initial mobile phase or with blank vegetable sample extracts. Satisfactory linearity within the concentration range of 10–500 ng mL\(^{-1}\) \((R^2 ≥ 0.999)\) in different matrices was observed. LOD, which was defined as the concentration at three times the signal intensity of noise was in the range of 0.94–1.82 ng g\(^{-1}\) for different types of vegetables. Limit of quantitation (LOQ), which was defined as the concentration at 10 times the signal intensity of noise was in the range of 3.13–6.07 ng g\(^{-1}\) (Table I).

Recovery analysis was carried out by spiking paraquat into vegetable samples at 50 and 500 ng g\(^{-1}\), respectively. Four replicates were tested for each concentration. Extracted ion chromatograms for standard paraquat solution (500 ng mL\(^{-1}\), lettuce

Figure 2
Recoveries of paraquat in WCX cartridge using five different elute solutions: (A) acetonitrile–TFA–water (80 : 1 : 19, v/v/v), (B) acetonitrile–TFA–water (80 : 2 : 18, v/v/v), (C) acetonitrile–TFA–water (80 : 5 : 15, v/v/v), (D) acetonitrile–formic acid–water (30 : 30 : 40, v/v/v) and (E) acetonitrile–formic acid (90 : 10, v/v) (error bar represents SD, n = 4).

Figure 3
The influence of injection volumes on LC performance: (A) 1 μL, (B) 2 μL, (C) 5 μL and (D) 10 μL.
sample (spiked at 500 ng g\(^{-1}\)) and blank lettuce sample were shown in Figure 4. Calibration curves obtained from external standards diluted with blank sample extracts were used to calculate recovery of the developed method. The recoveries for spiked vegetable samples at 50 and 500 ng g\(^{-1}\) ranged from 43.6 to 49.8% and 63.8 to 73.5%, and RSDs were 14.1 and 8.9%, respectively (Table II).

**Analysis of real samples and immersion experiments**

Vegetable samples were collected from local supermarket in five consecutive days and analyzed with the developed method. Only in a few vegetable samples, small amounts of paraquat (no > 20 ng g\(^{-1}\)) were detected which were in the allowable level according to GB 26130-2010. The extracted ion chromatogram of one cabbage sample detected with paraquat (19.7 ng g\(^{-1}\)) was shown in Figure 4D.

Immersion experiments were performed to evaluate the effect of paraquat contaminated water on edible vegetable. It can be seen that paraquat enters plant cells quickly (Figure 5), the concentration in vegetable reaches plateau after 3 h of immersion. The concentration of paraquat inside plant tissue was much higher than the immersion solution, which also indicates paraquat was brought into the plant cell by active transportation mechanism.

**Discussion**

Paraquat is a hydrophilic compound, HILIC column is suitable for the separation of such compounds, but generally, considerable amount of salt (100–250 mM) is required in the eluting buffer to maintain optimal performance of the column (26; 34; 36; 39). Here, we reduced the amount of ammonium acetate to 10 mM and achieved good separation of the compound. The results indicated that it is possible to operate an HILIC column with low salt buffer by carefully control the experimental conditions and achieve satisfactory separation performance.

The previous reports on the determination of paraquat in leafy vegetable matrices using GC (17), ELISA (19) and LC–ESI–IDMS (32) showed LODs of 50 ng g\(^{-1}\) (for lettuce), 10 ng g\(^{-1}\) (for cabbage) and 20 ng g\(^{-1}\) (for corn leaves), respectively. LODs of the method we established were in the range of 0.94–1.82 ng g\(^{-1}\), which were much higher than these previous reports. In addition, leafy vegetable matrices were more complex than water. Grey et al. (32) determined paraquat in water and corn leaves by the same LC–ESI–IDMS method, the LOD for water was 0.2 ng mL\(^{-1}\), whereas the LOD for corn leaves was 20 ng g\(^{-1}\). Biological samples such as blood and urine were the common matrices for paraquat analysis, and the LODs of previously

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**Table I**

Linear Relationship, Sensitivity, LOD and LOQ for the Detection of Paraquat Using HPLC–MS-MS (n = 4)

<table>
<thead>
<tr>
<th>Parraquat</th>
<th>Linear range (ng mL(^{-1}))</th>
<th>Linear equation</th>
<th>(R^2)</th>
<th>LOD (ng g(^{-1}))</th>
<th>LOQ (ng g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting buffer</td>
<td>10–1,000</td>
<td>(y = 1307.9x - 1,661.8)</td>
<td>0.999</td>
<td>0.94</td>
<td>3.13</td>
</tr>
<tr>
<td>Cabbage</td>
<td>10–500</td>
<td>(y = 439.2x + 1,756.0)</td>
<td>0.999</td>
<td>1.69</td>
<td>5.63</td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>10–500</td>
<td>(y = 627.5x + 310.6)</td>
<td>0.999</td>
<td>1.19</td>
<td>3.97</td>
</tr>
<tr>
<td>Lettuce</td>
<td>10–500</td>
<td>(y = 411.3x - 1,668.8)</td>
<td>0.999</td>
<td>1.72</td>
<td>5.73</td>
</tr>
<tr>
<td>Spinach</td>
<td>10–500</td>
<td>(y = 346.8x + 862.7)</td>
<td>0.999</td>
<td>1.82</td>
<td>6.07</td>
</tr>
</tbody>
</table>

**Table II**

Percentage Recoveries (%) and RSD% of Paraquat in Different Matrices

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Recovery (n = 4)</th>
<th>50 (ng g(^{-1}))</th>
<th>500 (ng g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabbage</td>
<td>45.8 ± 5.9</td>
<td>70.1 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>49.8 ± 1.1</td>
<td>63.8 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>46.4 ± 7.9</td>
<td>73.5 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>43.6 ± 14.1</td>
<td>67.7 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.** Quantitative ion chromatograms (186/171) of paraquat: (A) paraquat standard solution diluted in blank lettuce extract solution (500 ng g\(^{-1}\)), (B) lettuce spiked with 500 ng g\(^{-1}\) paraquat, (C) blank lettuce sample and (D) cabbage sample detected with small amount of paraquat.

**Figure 5.** Paraquat concentration detected in selected vegetables after immersion treatment in paraquat solution: (A) lettuce immersed in paraquat solution (10 mL, 20 ng mL\(^{-1}\)) for 0.5, 3 and 24 h and (B) cabbage immersed in paraquat solution (10 mL, 50 ng mL\(^{-1}\)) for 0.5, 3 and 24 h.
reported LC–MS-MS methods ranged from 0.5 to 100 ng mL\(^{-1}\) (20, 22, 35, 39), which are comparable with the method reported here.

The result of immersion experiment showed that paraquat penetrates into plant cells quickly and resides inside the plant cell. There still might be minute amount of paraquat cannot be extracted, which resulted in the low recovery when spiking paraquat at lower concentration. The relatively low recovery of paraquat at low spike level can be alleviated by the addition of internal standards.

It was also found that paraquat had strong absorption to glass vessel. With 1 µg mL\(^{-1}\) standard solution stored in glass sample vial, the signal decreased distinctly over consecutive injections. After 4 days, the signal completely disappeared. Similarly, when glass homogenizer was used for sample extract, poor recovery was observed. Similar phenomenon has also been reported previously (30, 31). So plasticware was used throughout the experiment.

**Conclusion**

A method for determining paraquat in vegetables was developed in this paper, water extraction and WCX cleanup can reduce interference from sample matrix, LC–MS-MS can achieve high sensitivity and accuracy that realize the determination of paraquat with microinjection volume. This method was applied to the analysis of paraquat residues in vegetable samples collected from local market and also used to study the mechanism of paraquat entering plant cell.

**Acknowledgments**

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