Air-Assisted Liquid Liquid–Microextraction for the Analysis of Fungicides from Environmental Water and Juice Samples

Shiji Wu1, Tingting Jin1, Jing Cheng1*, Hongbin Zhou1 and Min Cheng2

1Key Laboratory of Pesticide and Chemical Biology, Ministry of Education, Institute of Environmental Chemistry, College of Chemistry, Central China Normal University, Wuhan 430079, China, and 2School of Mechanical Science and Engineering, Hua Zhong University of Science and Technology, Wuhan 430074, China

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In this work, a rapid method based on air-assisted liquid liquid microextraction (AALLME) was developed for the determination of three fungicides (azoxystrobin, diethofencarb and pyrimethanil) in water and juice samples. A narrow-neck glass tube was made to facilitate collection of the low-density extractant. The mixture of extractant and sample solution is rapidly sucked into a 5-mL glass syringe and then is injected into the narrow-neck glass tube and the procedure is repeated six times. A homogeneous solution was formed and then with the continuous injection of air by a 20-mL glass syringe, phase separation happened and the extractant was collected on the top of the sample solution. No centrifugation separation step was involved. It took only 90 s to complete the pretreatment process. The influence of main factors on the extraction efficiency is studied. Under optimal conditions, enrichment factors for the three fungicides varied from 145 to 178. The limits of detection for azoxystrobin, diethofencarb and pyrimethanil were 0.08, 0.16 and 0.25 μg L−1, respectively. Reasonable relative recoveries were varied from 72.3 to 108.0%. And satisfactory intra-assay (5.3–6.2%, n = 6) and inter-assay (6.8–9.3%, n = 6) precision illustrated good performance of the analytical procedure.

Introduction

Azoxystrobin, diethofencarb and pyrimethanil which have different mechanisms of action, have recently been mixed to improve control over grey mould on fruits and vegetables. However, these fungicides may enter into the environment through various routes, such as spraying, the discharge of waste water and soil seepage, resulting in the possible pollution of water, fruits and vegetables, thus causing a threat to a person’s physical health and the environmental safety.

Sample preparation displays an essential part in the determination of trace analytes in complex matrices. The most common extraction techniques used in environmental analysis are liquid–liquid extraction (LLE) and solid-phase extraction (SPE). But these techniques require large amounts of poisonous organic solvent which are often hazardous. Moreover, the operation is time consuming and tedious (3–4–6). Therefore, analytical researchers have been searching for approaches with lower consumption of toxic organic solvents to minimize the damage to operators. Solid-phase microextraction (SPME) introduced in 1990 by Pawliszyn (7) is based on an equilibrium of analytes concentration between the sample matrix and a fused silica fiber coated with a stationary phase. SPME satisfies most of the requirements of a good sample preparation technique, including simplicity of use, automation (8). But the extraction fiber is expensive and fragile, and sample carry-over is also a problem.

In recent years, efforts have been directed towards miniaturization of the LLE procedure by greatly reducing the amount of organic solvent, leading to the development of solvent microextraction methods. Liquid-phase microextraction (LPME) is now becoming one of the most common methods of microextraction particularly for organic compounds from aqueous matrices. In 1996, it was introduced as one version of LPME that is single drop microextraction (SDME) (9–12). It performed by exposing a single drop of solvent to the headspace or directly into the matrix of the sample. SDME also has some disadvantages, such as unstable organic drop, long extraction time. In 2006, a novel LPME method named dispersive liquid–liquid microextraction (DLLME) was introduced by Assadi and co-workers (13). In DLLME, extraction solvent is dispersed into aqueous sample solution with the aid of a disperser (13–15). The advantages of this microextraction technique are simplicity of operation, low cost, rapidity of extraction and high enrichment factor. However, just like a coin has two faces, the disadvantages of DLLME may lie in two points: One is that the extraction solvent must be denser than water; thus, toxic solvents such as chlorobenzene, chloroform and carbon tetrachloride are employed. To overcome the disadvantages of the aforementioned techniques, DLLME based on solidification of floating drop (DLLME-SFO) was introduced and applied widely (16–20). The other disadvantage is the presence of a disperser in DLLME reduces the polarity of aqueous phase which leads to increase the solubility of analytes into aqueous phase and decreases extraction efficiency. In order to resolve the above-mentioned problem, some dispersive solvent-free techniques such as ultrasound-assisted emulsification microextraction (USEME) (21, 22), vortex-assisted liquid–liquid microextraction (VALLME) (23), and air-assisted liquid–liquid microextraction (AALLME) (24) have been developed. In AALLME, a few microliters of extraction solvent is transferred into aqueous sample solution and then the mixture is repeatedly sucked into a glass syringe and then injected into the tube. After centrifugation of cloudy solution, the extractant is settled down on the bottom of the centrifuge tube and used for further analysis. The main disadvantage of AALLME is that the separation of two phases must use centrifugation procedure. Furthermore, neither the traditional DLLME nor AALLME can avoid the centrifugation step, which inevitably prolong the pretreatment time.
In this work, a novel AALLME method was proposed for the determination of azoxystrobin, diethofencarb and pyrimethanil in environmental water and juice samples. In this method, the solvent with lower density than water was employed as the extraction solvent, and a narrow-neck glass tube was used to facilitate the collection of low-density extraction solvent. Both the extraction and phase separation process were performed with the air-assistant. No centrifugation was required in this procedure and the sample pretreatment time is very short (90 s). Particularly, the absence of a disperser enhanced the extraction efficiency. Hence, the proposed method is rapid, simple and environmentally friendly.

Experimental

Chemicals and supplies
Azoxystrobin (98.0%), diethofencarb (98.0%) and pyrimethanil (98.0%) were obtained from Sigma-Aldrich (St Louis, MO, USA). 1-undecanol (>99%), 1-octanol (>99%), 1-dodecanol (>99%) and n-tridecane (>99%) of analytical reagent grade were purchased from Tokyo Chemical Industry (Tokyo, Japan). HPLC-grade methanol and acetonitrile were obtained from Tedia Company (Fair Lawn, NJ, USA). Sodium chloride was procured from Tianjin Kermel chemical reagent development center (Tianjin, China). Deionized water was purified by a Milli-Q water purification system (Millipore Corporation, Billerica, MA, USA).

The stock solutions containing 20 μg mL⁻¹ of azoxystrobin, diethofencarb and pyrimethanil were prepared in HPLC-grade methanol. A series of working solution were obtained by dilution of the stock solution, and all of which were stored at 4°C in a refrigerator.

South lake water samples were collected from South lake (Wuhan city, Hubei province, China). Farmland water samples were collected from vegetable fields (Wuhan city, Hubei province, China). Lemonade samples were purchased from a local supermarket (Wuhan, Hubei province, China), and all of them were kept at 4°C. These real samples were filtered through a 0.45 μm membrane (the material of 0.45 μm filtration membrane is polyether sulfone), and it was purchased from Wuhan Shenshi Chemical Industry Co. Ltd (Wuhan, Hubei province, China) prior to analysis.

Instrument and apparatus
Chromatographic separations were performed on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto injector and a diode array detector (DAD). An Agilent HC-C18 column (250 mm × 4.6 mm, i.d. 5 μm) was used for the separation of analytes. The mobile phase was methanol–water (67:33, volume ratio) and the flow rate was 1.0 mL min⁻¹. The detection wavelength was set at 254 nm, and the column temperature was 30°C. The injection volume was 5 μL. A magnetic stirrer (Heidolph MR3001, Hong Kong, China) was used for the extraction.

Extraction procedure
The experimental setup is illustrated in Figure 1. A 6-mL aqueous sample containing 15% NaCl was placed in a narrow-neck glass tube; 30 μL 1-octanol (extractive solvent) was added to the tube. The mixture of extractant and sample solution is rapidly sucked into a 5-mL glass syringe and then injected into the tube and the procedure is repeated six times. In this step, a homogeneous solution was formed and then with the continuation injection of air by 10-mL glass syringe, phase separation occurred and the organic extraction phase floated on the upper layer, and then added water along the wall to make it up to the neck of the tube. Subsequently, 10 μL extractant was taken into a microsyringe and injected into the HPLC system.

Results and discussion
To validate the applicability of the proposed method, a series of extraction parameters were investigated using the spiked ultrapure water. In order to get high extraction efficiency, various
parameters affecting the performance of the proposed method, such as the kind and volume of extraction solvent, number of extraction cycles, salt concentration were optimized. All optimizations were performed in triplicate.

**Effect of the kind and volume of extraction solvent**

The selection of an appropriate organic extractant can play a vital role in the proposed procedure. It should have certain characteristics: high affinity to the target analytes, a lower density than water, low solubility in water. In addition, it should have good chromatographic behavior. According to these considerations, 1-undecanol, 1-dodecanol, 1-octanol, n-tridecane were investigated. As shown in Figure 2, 1-octanol got the highest extraction efficiency for the three fungicides, so 1-octanol was selected as the suitable solvent in the subsequent experiment.

The effect of the volume of extractant was investigated in the range of 20–40 μL. As shown in Figure 3, the peak areas increased with the increase of the volume from 20 to 30 μL, and then decreased with the increase of volume from 30 to 40 μL. On one hand, the reason may be that larger volume of extractant could extract more amounts of analytes, and therefore enhancing the extraction efficiency. On the other hand, the increasing volume of extractant could dilute the concentration of the analytes, hence decrease the extraction efficiency. Consequently, 30 μL extractant was selected for the following work.

**Effect of the ionic strength**

Salt addition often improves the extraction efficiency, and this could reduce the amount of the analytes in water. In this work, the concentration of NaCl was added into the water sample in the range of 0–25% (w/v). The increase of the salt concentration from 0 to 15% caused the extraction efficiency to be increased. However, further increase of salt concentration caused the extraction efficiency to be decreased. This phenomenon may be attributed to the following two points. On one hand, the addition of salt to the aqueous solution decreased the solubility of the analytes in the aqueous samples and enhanced the allocation into the organic solvent. The salting-out agent attracts a number of free water molecules and reduces the amount of free water.

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**Figure 2.** Effect of different types of the extractants on extraction efficiency. Conditions: 6 mL aqueous sample at room temperature (25 ± 0.5°C); extractant volume: 30 μL; extraction cycle 10 times. No salt addition.

**Figure 3.** Effect of extractant volume on extraction efficiency. Conditions: 6 mL aqueous sample at room temperature (25 ± 0.5°C); extractant: 1-octanol; extraction cycle 10 times. No salt addition.
molecules in the aqueous solution, so the concentration of the target molecules in the water increases and the extraction efficiency increases as well. On the other hand, the electrostatic interaction between the analyte and the salt ions blocks the analyte being extracted into the organic phase. When the concentration of salt is low, the former is predominant; when the concentration of salt becomes higher, the latter occupied the major part. Therefore, the best extraction efficiency was acquired when 15% NaCl (w/v) was added into the sample solution.

Optimization of number of extraction cycles

In this study, the mixture of extractive solvent and sample solution was rapidly sucked into a 10-mL glass syringe and then was injected into the tube. The number of suction/injection cycles is considered as the 'number of extraction cycles'. It was predictable that extraction efficiency would increased by the increasing number of extraction cycles. Therefore to reach the equilibrium status, the number of extraction cycles was studied in the range of 2–10 times. The results in Figure 4 showed that by increasing the number of extraction cycles, analytical signals also increased until the sixth extraction circle achieved the maximal signals. So, the number of extraction cycles for the subsequent experiments was fixed at 6. It is noted that this step was performed in <1 min.

Evaluation of the method performance

The performance of the method under the optimal conditions is shown in Table I. Good linearity of each analyte was obtained in the range of 0.5–1000 µg L⁻¹ for azoxystrobin and diethofencarb, 1–1000 µg L⁻¹ for pyrimethanil, with correlation coefficients (R) from 0.9991–0.9995. Six extractions of a mixture

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of number of extraction cycles on extraction efficiency. Conditions: 6 mL aqueous sample at room temperature (25 ± 0.5 °C); extractant: 1-octanol; extractant volume: 30 µL. No salt addition.

![Table I](https://example.com/table1.png)

**Table I**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear range (µg L⁻¹)</th>
<th>R</th>
<th>LOD (µg L⁻¹)</th>
<th>RSD (%) (n = 6)</th>
<th>EF Intra-day</th>
<th>EF Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azoxystrobin</td>
<td>0.5–1000</td>
<td>0.9995</td>
<td>0.08</td>
<td>5.3</td>
<td>6.8</td>
<td>156</td>
</tr>
<tr>
<td>Diethofencarb</td>
<td>0.5–1000</td>
<td>0.9984</td>
<td>0.16</td>
<td>6.2</td>
<td>9.3</td>
<td>178</td>
</tr>
<tr>
<td>Pyrimethanil</td>
<td>1–1000</td>
<td>0.9991</td>
<td>0.25</td>
<td>5.7</td>
<td>7.9</td>
<td>145</td>
</tr>
</tbody>
</table>

LOD, limit of detection (S/N = 3); RSD, relative standard deviation; EF, enrichment factor.
sample solution over a day gave the intra-day RSDs, and the inter-day RSDs were determined by extracting a mixture sample solution that had been independently prepared for continuous 3 days. The results are summarized in Table I. Acceptable precision was obtained with RSD values <9.3%, showing good repeatability of the method. Based on a signal-to-noise ratio (S/N) of 3, good limits of detection (LODs) in the range of 0.08–0.25 μg L⁻¹ were obtained. For the EIs calculation, three replicate extractions were performed from the aqueous solution containing 0.2 μg mL⁻¹ of the analytes, and good EIs of each analyte were also obtained in the range of 145–178.

Application of the proposed method to the real samples
The proposed method under optimal conditions was applied to determine the residue levels of azoxystrobin, diethofencarb and pyrimethanil in two real water samples including South Lake water, ponding water and two real juice samples including grape juice and lemon juice. The real samples were directly extracted without any pretreatment. In order to evaluate the accuracy of the developed method in real samples, they were spiked with three concentration levels of the analytes, including 5, 50 and 200 μg L⁻¹. The subsequent results are shown in Table II. No analytes were found in South Lake water, farmland water and grape juice, lemon juice. The result demonstrated good recoveries ranging from 72.3 to 108.0%. The chromatograms of the blank and spiked South Lake water with extraction by the proposed method are shown in Figure 5.

Comparison with other reported methods
A comparison between the proposed method with other reported methods for the analysis of trace fungicides (azoxystrobin, diethofencarb and pyrimethanil) is summarized in Table III. It is clear that the proposed method had better EIs, and a shorter pretreatment time than that of the other methods. Detection limits of the presented method are lower than most of the methods, even with mass spectrometry as the detector. Therefore, the results indicated that the proposed method is a rapid, simple and sensitive method which can be used for the determination and preconcentration of fungicides in complicated matrix.

Conclusions
In the present study, a rapid, simple and efficient simultaneous extraction and phase separation based on AALLME method has been successfully developed for the analysis of three fungicides (azoxyystrobin, diethofencarb and pyrimethanil) in water and juice samples. The whole pretreatment time is only 90 s. The use of low-density extractive solvent is more environment friendly and widens the application range of AALLME due to more low-density solvents than high-density ones. Meanwhile, the experiment operation is simpler and the pretreatment time fairly shorter due to phase separation with the continuous injection of air. Neither a disperser solvent or centrifugation step was involved. Compared with the other reported method, the novel technique can achieve excellent EIs (145–178), low LODs (0.08–0.25 μg L⁻¹), good linear ranges (r > 0.999) and fairly shorter pretreatment time (90 s).

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