Analysis ofazole fungicides in fish muscle tissues: Multi-factor optimization and application to environmental samples

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GRAPHICAL ABSTRACT

HIGHLIGHTS
• A sensitive method for analysis of azole fungicides in fish muscle was developed.
• Hierarchical clustering heatmap was used for multi-factor optimization.
• Accumulation of azole fungicides in wild fish muscle was demonstrated.

ABSTRACT
Azole fungicides have been reported to be accumulated in fish tissue. In this study, a sensitive and robust method using high-performance liquid chromatography-tandem mass spectrometry combined with ultrasonic extraction, solid-liquid clean-up, liquid–liquid extraction and solid–phase extraction (SPE) for enrichment and purification have been proposed for determination of azole fungicides in fish muscle samples. According to the results of non-statistical analysis and statistical analysis, ethyl acetate,

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1. Introduction

Azole fungicides are extensively used as antifungal active ingredients in various products including pesticides, pharmaceuticals and personal care products. As one of the azole fungicides, the consumption of climbazole was estimated up to 3800 tons per annum in China [1]. These azole fungicides have been found in surface water, wastewater, sediment, dewatered sludge and soil [2–9]. The presence of azoles in the aquatic environment may not only cause adverse effects in non-target organisms [9–18], but also lead to accumulation in tissues of aquatic biota [10,11,19]. Lazartigues et al. [19] reported that carbendazim was the most frequently detected compounds in muscle tissues of cultured fish from dam ponds. Through the exposure experiments on crucian carp (Carassius auratus), ketoconazole has been proved to be accumulated in muscle tissues [11]. In addition, long exposure duration and low exposure concentration of these azoles could result in a higher bioaccumulation in fish tissues [10,11]. These results implied the potential risk of azole fungicides to humans through edible wild fish. Zarn et al. [20] concluded that many azole fungicides could inhibit mammalian sterol 14α-demethylase (CYP51) and mammalian aromatase with unknown potencies. Rather than fungal CYP51, miconazole prefers to suppress human CYP51 [21]. About the occurrence of azole fungicides in wild fish tissue, however, is still very limited. Therefore, a multi-residue screening method is crucial for quantification of azole fungicides in environmental captured fish.

Analytical protocols for analysis of azole fungicides in surface water, wastewater, sediment, sludge, and soil have been reported in recent year [22–25]. Solid-phase extraction, ultrasonic extraction and matrix solid-phase dispersion were employed for sample enrichment and purification, and high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) for chromatographic separation and mass spectrometry detection. Breakthrough experiments by Casado et al. [24] demonstrated that mixed-mode solid-phase extraction (MCX cartridge) was more efficient to improve the selectively of azole fungicides extraction from water samples and reduce the matrix interference than conventional reversed-phase extraction (HLB cartridge). To date, there are few reports on the analytical method of azole fungicides in fish muscle samples. A previous multi-residue method for fish muscle samples displayed the serious matrix effect and unsatisfied method detection limit of miconazole was attained by simple sample preparation without clean-up step [26]. Although prolonging the operation time, purification procedures, e.g., addition of clean-up sorbent [25,27–29] and liquid–liquid extraction with nonpolar solvent [30], were necessary to perform in the method for elimination of interruption of lipids and other impurities from muscle tissue matrices. Besides, the presence of corresponding isotopic labeled internal standard for each analyte was highly recommended to be the most desired compensating effects on the precision and reliability of quantification [31]. With the multi-factor optimization, we developed a sensitive and robust monitoring method for azole fungicides in muscle tissues of wild fish.

The aim of this study was to establish a multi-residue analytical method for determination of six commonly used azole fungicides in fish muscle using ultrasonic extraction, solid-liquid clean-up, liquid–liquid extraction and solid-phase extraction as preconcentration and purification step, followed by a HPLC–MS/MS analysis. The developed method was successfully applied for biomonitoring of different fish species collected from Beijiang River and its tributaries, and a preliminary study was carried out. These results indicated the bioaccumulation of azole fungicides in environmental fish species, and provided a practical tool for further investigation.

2. Materials and methods

2.1. Chemicals and reagents

Six target azole fungicides, including climbazole, clotrimazole, ketoconazole, miconazole, fluconazole and carbendazim, were investigated in this study. Their basic information can be referred to Table S1. Supplier sources of the chemicals and reagents are given in the text of Supplementary material (Text S1).

2.2. Sample collection

Different varieties of fish samples were used for method development, validation and application tests. Fish samples were collected by fishing and netting from one fish pond (S0) and five monitoring sites (S1–S5) in the Qingyuan section of Beijiang River and its tributaries including Dayan River and Pajiang River. Detailed information of the sampling sites and collected fish is listed in Table S2. The source water of fish pond S0 was not affected by effluent discharge. Target analytes were not detected in F1 muscle sample so that this tissue could be applied for method development (Fig. S1). Common fish in southern China, e.g., silver carp (F1), common carp (F2), mrigal carp (F3), nile tilapia (F4) and grass carp (F5), were used for method validation. All collected wild fish species in this study were employed for method application (Table S2). The captured wild fish were immediately anaesthetized and sacrificed by dissection in the field. Fish muscle was cut into pieces after removing the skin. Each muscle tissue was then put in polyethylene bags and stored at −20 °C prior to extraction.

2.3. Sample extraction and purification

For sample extraction, an ultrasonic extraction method was chosen because of its convenience and good performance [32]. For sample purification, a solid-liquid clean-up [25,27–29], liquid–liquid extraction [30] and solid-phase extraction [23,24] were consecutively used to remove the lipid and other impurities before HPLC–MS/MS analysis. Therefore, multi-factor for extraction and purification, including extraction solvent, sorbent, dehydrant,
Table 1

Optimized condition in the extraction and purification procedure*.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Extraction solvent†</th>
<th>Sorbent‡</th>
<th>Dehydrant§</th>
<th>SPE cartridge</th>
<th>Rinse solution∥</th>
<th>Eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>30 mL MN</td>
<td>–</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C2</td>
<td>30 mL ACN</td>
<td>–</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C3</td>
<td>30 mL EA</td>
<td>1 g Al₂O₃-N</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C4</td>
<td>30 mL EA</td>
<td>1 g Florisil</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C5</td>
<td>30 mL EA</td>
<td>1 g C₁₂</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C6</td>
<td>30 mL EA</td>
<td>1 g Florisil</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C7</td>
<td>30 mL EA</td>
<td>1 g PSA</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C8</td>
<td>30 mL EA</td>
<td>1 g Florisil +0.5 g PSA</td>
<td>With sorbent</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C9</td>
<td>30 mL EA</td>
<td>1 g Florisil +0.5 g PSA</td>
<td>With sorbent</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C10</td>
<td>30 mL EA</td>
<td>0.5 g Florisil +0.5 g PSA</td>
<td>With sorbent</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C11</td>
<td>30 mL EA</td>
<td>0.5 g Florisil +0.5 g PSA</td>
<td>With tissue</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C12</td>
<td>30 mL EA</td>
<td>0.5 g Florisil +0.5 g PSA</td>
<td>–</td>
<td>HLB</td>
<td>6 mL 5%MN in H₂O</td>
<td>5 mL EA</td>
</tr>
<tr>
<td>C13</td>
<td>30 mL EA</td>
<td>0.5 g Florisil +0.5 g PSA</td>
<td>With sorbent</td>
<td>MCX</td>
<td>6 mL 5%MN in H₂O + 2.5 mL</td>
<td>2 mL MN + 2 mL 0.5%NH₃ in MN</td>
</tr>
<tr>
<td>C14</td>
<td>30 mL EA</td>
<td>0.5 g Florisil +0.5 g PSA</td>
<td>With sorbent</td>
<td>MCX</td>
<td>6 mL 5%MN in H₂O + 2.5 mL</td>
<td>4 mL 0.5%NH₃ in MN</td>
</tr>
<tr>
<td>C15</td>
<td>30 mL EA</td>
<td>0.5 g Florisil +0.5 g PSA</td>
<td>With sorbent</td>
<td>MCX</td>
<td>0.15%FA in MN</td>
<td>0.15%FA in MN</td>
</tr>
<tr>
<td>C16</td>
<td>30 mL EA</td>
<td>1 g Florisil</td>
<td>–</td>
<td>MCX</td>
<td>6 mL 5%MN in H₂O + 2.5 mL</td>
<td>4 mL 0.5%NH₃ in MN</td>
</tr>
<tr>
<td>C17</td>
<td>30 mL EA</td>
<td>1 g PSA</td>
<td>–</td>
<td>MCX</td>
<td>6 mL 5%MN in H₂O + 2.5 mL</td>
<td>4 mL 0.5%NH₃ in MN</td>
</tr>
<tr>
<td>C18</td>
<td>30 mL EA</td>
<td>1 g Florisil +0.5 g PSA</td>
<td>–</td>
<td>MCX</td>
<td>6 mL 5%MN in H₂O + 2.5 mL</td>
<td>4 mL 0.5%NH₃ in MN</td>
</tr>
<tr>
<td>C19</td>
<td>30 mL EA</td>
<td>0.5 g Florisil</td>
<td>–</td>
<td>MCX</td>
<td>6 mL 5%MN in H₂O + 2.5 mL</td>
<td>4 mL 0.5%NH₃ in MN</td>
</tr>
<tr>
<td>C20</td>
<td>30 mL EA</td>
<td>0.5 g PSA</td>
<td>–</td>
<td>MCX</td>
<td>0.15%FA in MN</td>
<td>0.15%FA in MN</td>
</tr>
</tbody>
</table>

* All optimized condition were carried out through liquid–liquid extraction with 16 mL n-hexane (saturation with methanol).
† MN, methanol; ACN, acetonitrile; EA, ethyl acetate.
‡ Al₂O₃-N, neutral alumina; PSA, primary secondary amine.
§ 1 g anhydrous MgSO₄.
∥ FA, formic acid.

SPE cartridge. SPE washing solution and SPE elution solution, were optimized by spiking of silver carp (F1) with all target azoles and corresponding deuterated analogues (internal standards) (Table 1). The most effective condition is those performing maximum recovery, minimum matrix effect and minimum standard deviation. Under optimized conditions, approximately 2.0 g (wet weight, ww) of fish muscle sample was weighed into a 50 mL polypropylene centrifuge tube. Thereafter, 100 ng of mixed internal standard solution was spiked accurately. The tube was shaken, left at fume cupboard for the solvent evaporation and stored overnight at 4 °C. The aged tissue combined with 15 mL of ethyl acetate was homogenized using an ultra-turrax homogenizer (IKA, Germany) at 30,000 rpm. Then the tube was vortex mixed for 30 s, extracted with ultrasonic for 5 min and centrifuged at 4500 rpm for 5 min. The supernatant was decanted into another 50 mL polypropylene centrifuge tube, followed another 15 mL of ethyl acetate rinsed the ultra-turrax and the cleaning fluid combined with the muscle tissue. The extracted procedure was repeated without homogenization.

All supernatants were combined and added 0.5 g of PSA. The tube was shaken vigorously for 1 min and centrifuged at 4500 rpm for 5 min. Then the supernatant was transferred into a 100 mL glass pear-shaped flask and the solvent was evaporated to dryness. The residue was dissolved in 10 mL of 5% methanol aqueous solution (v/v) and adjusted pH to 3.

Eight milliliter of n-hexane, which was saturated with methanol, was added to the above extract. The flask was shaken fiercely for 2 min, then the mixture was stood over for 15 min. The supernatant was removed. This degreasing step was repeated.

Oasis MCX cartridge (60 mg, 3 mL) was preconditioned successively with 5 mL of methanol and 5 mL of acidic Milli-Q water (pH = 3) prior to use. The above extract sample was loaded into the MCX cartridge at a flow rate of 5–10 mL/min. Then the sorbent bed was consecutively washed with 6 mL aliquots of 5% methanol aqueous solution (v/v) and 2.5 mL of 0.1% formic acid in methanol (v/v). After drying under vacuum for 30 min, the cartridge was eluted with 4 mL of 0.5% NH₃ in methanol (v/v). The eluate was dried with a gentle stream of nitrogen, reconstructed in 0.5 mL of methanol, filtered through a 0.22 μm membrane filter (Anpled, China) into a 2 mL amber glass vial (Agilent, USA), and then stored in −20 °C before HPLC–MS/MS analysis. Additional SPE procedure using Oasis HLB cartridge (60 mg, 3 mL) was conducted with previous described steps in Chen et al. [23].

2.4. Instrumental analysis

Liquid chromatography separation was performed by a Waters Alliance 2695 HPLC system (Waters, USA) coupled with an Agilent Zorbax SB-C18 column (3.0 × 100 mm, 3.5 μm) and its corresponding guard column (4.6 × 15 mm, 5.0 μm) from Agilent Technologies (Agilent, USA). The HPLC instrument was coupled to a Waters Quatro Micro triple quadrupole mass spectrometer (Waters, USA) with an electrospray ionization (ESI) source operating in positive ionization mode. Quantitative analysis was conducted on multiple reaction monitoring (MRM) mode. Table 2 showed the optimized MRM transitions of azole fungicides with the retention times, the relative abundances between confirmation ion and quantification ion, and the permitted tolerances [33]. The corresponding deuterated analogues were used as internal standard to compensate for matrix effects. These internal standards only monitored one ion transition for quantitation (Table 2). The optimized HPLC and MS parameters are listed in Table S3. The total ion chromatograms for all analytes in a 20 ng/mL standard solution are displayed in Fig. S2.
Table 2
Optimized MRM mode parameters and performance of the analysis ofazole fungicides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Ion transition (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (V)</th>
<th>Corresponding IS</th>
<th>Ion ration (%)</th>
<th>Permitted tolerances</th>
<th>Linearity (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Climbazole</td>
<td>9.16</td>
<td>293.1&lt;-&gt;69.0</td>
<td>30</td>
<td>20</td>
<td>Climbazole-D₄</td>
<td>26.5±2.20</td>
<td>±25</td>
<td>0.9999</td>
</tr>
<tr>
<td>(CBZ)</td>
<td>293.1&lt;-&gt;225.0</td>
<td>30</td>
<td>15</td>
<td>Clotrimazole-D₅</td>
<td>30.4±2.61</td>
<td>±25</td>
<td>0.9998</td>
<td></td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>10.76</td>
<td>277.1&lt;-&gt;165.0</td>
<td>40</td>
<td>30</td>
<td>(CTZ-D₅)</td>
<td>56.2±8.01</td>
<td>±20</td>
<td>0.9987</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>8.57</td>
<td>531.2&lt;-&gt;82.1</td>
<td>55</td>
<td>55</td>
<td>(KTZ-D₅)</td>
<td>18.8±2.21</td>
<td>±30</td>
<td>0.9996</td>
</tr>
<tr>
<td>Miconazole</td>
<td>11.88</td>
<td>471.0&lt;-&gt;123.0</td>
<td>45</td>
<td>30</td>
<td>Miconazole-D₅</td>
<td>8.47</td>
<td>±20</td>
<td>0.9990</td>
</tr>
<tr>
<td>Clotrimazole-D₃</td>
<td>531.2&lt;-&gt;489.1</td>
<td>55</td>
<td>32</td>
<td>Clotrimazole-D₅</td>
<td>18.8±2.21</td>
<td>±30</td>
<td>0.9996</td>
<td></td>
</tr>
<tr>
<td>Miconazole-D₃</td>
<td>11.81</td>
<td>422.0&lt;-&gt;164.0</td>
<td>45</td>
<td>70</td>
<td>(MCZ-D₅)</td>
<td>8.47</td>
<td>±20</td>
<td>0.9990</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>2.88</td>
<td>307.1&lt;-&gt;220.0</td>
<td>30</td>
<td>20</td>
<td>Fluconazole-D₄</td>
<td>90.2±9.98</td>
<td>±20</td>
<td>0.9990</td>
</tr>
<tr>
<td>Fluconazole-D₃</td>
<td>307.1&lt;-&gt;238.1</td>
<td>30</td>
<td>15</td>
<td>Fluconazole-D₄</td>
<td>90.2±9.98</td>
<td>±20</td>
<td>0.9990</td>
<td></td>
</tr>
<tr>
<td>Carbendazim</td>
<td>3.18</td>
<td>192.1&lt;-&gt;132.0</td>
<td>30</td>
<td>30</td>
<td>Carbendazim-D₄</td>
<td>17.3±1.10</td>
<td>±30</td>
<td>0.9995</td>
</tr>
<tr>
<td>(CBD)</td>
<td>192.1&lt;-&gt;160.0</td>
<td>30</td>
<td>20</td>
<td>CBD-D₄</td>
<td>17.3±1.10</td>
<td>±30</td>
<td>0.9995</td>
<td></td>
</tr>
</tbody>
</table>

Isotope-labeled internal standard (IS)

- Climbazole-D₄ 11.81 297.1<->229.0 30 15
- Clotrimazole-D₅ 10.71 282.1<->170.0 40 25
- Ketoconazole-D₅ 8.47 539.2<->497.1 55 32
- Miconazole-D₃ 11.81 422.0<->164.0 45 70
- Fluconazole-D₄ 2.86 311.1<->242.1 30 15
- Carbendazim-D₄ 3.13 196.1<->146.0 30 20

A Quantitation ion (underline).
B Relative intensity between confirmation ion and quantification ion.
C Permitted tolerances in the light of relative intensity to achieve with EU guidelines [31].

2.5. Method validation

All fish muscle samples underwent the extraction and purification procedure described above. For sample blank (background value) tests, samples were unspiked with mixed standard solution, but spiked with mixed internal standards solution.

For recovery tests, fish muscle samples were spiked with mixed standard solution (5–50 ng/g of each target analyte) and mixed internal standards solution (50 ng/g of each isotope analogue). Absolute recovery (Rabs) and relative recovery (Rrel) were calculated by Eqs. (1) and (2):

\[ R_{abs}(\%) = \frac{[A_S - A_B]}{A_S} \times 100 \]  

(1)

where \( A_S \) and \( A_B \) (background value) are the measured response (peak area without internal standard correction) of an analyte in the spiked and corresponding unspiked fish samples, and \( A_S \) is the response of a standard solution containing the same concentration of each analyte. Absolute recovery can be defined as actual recovery, which was used to assess the efficiency of extraction without any correction. The higher the value of \( R_{abs} \) was close to 100%, the greater sensitive it made to an analyte.

\[ R_{rel}(\%) = \frac{[C_S - C_B]}{C_S} \times 100 \]  

(2)

where \( C_S \) and \( C_B \) (background value) are the measured concentration (with internal standard correction) of an analyte in the spiked and corresponding unspiked fish samples, and \( C_S \) is the spiking concentration of each analyte. Relative recovery can be defined as apparent recovery, which was used to evaluate the trueness of extraction with internal standard correction.

Matrix effect (ME) was determined according to the method proposed in our previous study [23] with moderate modifications. Samples were not spiked neither standard solution nor internal standard solution before sample extraction and purification. Final extract was dried under a gentle nitrogen stream, and then added with spiked solution including each analyte standard and internal standard. This solution can be defined as the spiked final extract. Absolute matrix effect (MEabs) and relative matrix effect (MErel) were calculated by Eqs. (3) and (4):

\[ ME_{abs} = \frac{[A_S - A_B - A_S]}{A_S} \times 100 \]  

(3)

\[ ME_{rel} = \frac{[C_S - C_B]}{C_S} \times 100 \]  

(4)

where \( A_S \) and \( A_B \) (background value) are the measured response (peak area without internal standard correction) of an analyte in the spiked final extract and corresponding unspiked fish samples, and \( A_S \) is the response of a standard solution containing the same concentration of each analyte. Absolute matrix effect can be defined as actual matrix effect, which was used to assess the influence of matrix interference on actual recovery without any correction. 

\[ ME_{rel} = \frac{[C_S - C_B - C_S]}{C_S} \times 100 \]  

(4) 

where \( C_S \) and \( C_B \) (background value) are the measured concentration (with internal standard correction) of an analyte in the spiked final extract and corresponding unspiked fish samples, and \( C_S \) is the spiking concentration of each analyte. ME = 0 means no matrix interference, while ME > 0 or ME < 0 represent signal enhancement or suppression, respectively. The higher absolute value of the ME means the greater matrix interference. Relative matrix effect can be defined as apparent matrix effect, which was used to assess the influence of matrix interference on apparent recovery with internal standard correction.

Method detection limit (MDL) and method quantification limit (MQL) of each analyte were defined as 3 and 10 times of the signal-to-noise ratio (S/N) under the lowest spiked concentration of different fish muscle samples. For intra-day and inter-day tests, five standard solutions with the same concentration of each analyte were determined in the same day and in five different days over the course of one month, respectively. Intra-day and inter-day precision were expressed as relative standard deviation (RSD) of five measured concentrations.

For method application tests, samples were only spiked with mixed internal standards solution. The levels of target azoles in wild fish muscle samples were quantified using calibration standard curves with internal standard correction, which were established by analyzing the standard solution at the concentration range of 1–200 ng/mL. To monitor potential background contamination and instrument performance, a procedure blank, a reagent blank and a standard solution were executed during detection of each sample batch.

MassLynx 4.1 software (Waters, USA) and R project 3.2.2 software (Vienna, Austria) [34] were used for raw data acquisition and hierarchically clustered heatmap analysis (Text S2). According to our rules for optimization of analytical method, Rabs ≥ 60%,
Fig. 1. Comparison of the absolute recoveries (a), relative recoveries (b), absolute matrix effects (c) and relative matrix effects (d) for azole fungicides in different optimized conditions by hierarchical clustering heatmap.

90% ≤ R_{rel} ≤ 110%, ME_{abs} ≤ 10% or ME_{rel} ≤ 5% for the average, or R_{abs} ≤ 30%, R_{rel} ≤ 10%, ME_{abs} ≤ 10% or ME_{rel} ≤ 5% for the standard deviation can be defined as good performance of an optimized condition, otherwise they are defined as bad performance.

3. Results and discussion

3.1. Optimization of instrumental conditions

Instrumental conditions including liquid chromatography parameters and mass spectrometry parameters were optimized to
Fig. 2. MRM chromatograms of quantitative ions for azole fungicides in a 20 ng/mL standard solution (a) and five species of fish muscle sample, including silver carp (b), common carp (c), mirgal carp (d), nile tilapia (e) and grass carp (f), with spiked concentrations of 20 ng/mL. Retention time and intensity of each azole fungicide are presented in the chromatograms.

Table 3
Measured concentrations (ng/g wet weight) of azole fungicides in fish muscle samples.

<table>
<thead>
<tr>
<th>Sampling sites</th>
<th>Fish</th>
<th>Fish species</th>
<th>Climbazole</th>
<th>Clotrimazole</th>
<th>Ketoconazole</th>
<th>Miconazole</th>
<th>Fluconazole</th>
<th>Carbendazim</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>F1</td>
<td>Silver carp (Hypophthalmichthys molitrix)</td>
<td>ND a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F2</td>
<td>Common carp (Cyprinus carpio)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F3</td>
<td>Mirgal carp (Cirrhinus mirgala)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F4</td>
<td>Nile tilapia (Tilapia nilotica)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S2</td>
<td>F5</td>
<td>Grass carp (Ctenopharyngodon idella)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F6</td>
<td>Mirgal carp (Cirrhinus mirgala)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F7</td>
<td>Common carp (Cyprinus carpio)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F8</td>
<td>Mud carp (Labeo rohita)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F9</td>
<td>Nile tilapia (Tilapia nilotica)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S5</td>
<td>F10</td>
<td>Common carp (Cyprinus carpio)</td>
<td>ND</td>
<td>0.78</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F11</td>
<td>Crucian carp (Carassius asumts)</td>
<td>ND</td>
<td>1.45</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ND, not detected.

b <MQL, lower than method quantification limit.

improve the separation of azole fungicides, to sharpen the target peak, to shorten the analytical time, and to enhance their signal response. Liquid chromatography parameters (e.g., mobile phase, flow rate, column temperature and injection volume) were referred to our previous study [23] with a little modification of gradient program (Table S3). Mass spectrometry parameters (e.g., ion transition, cone voltage and collision energy) were optimized by direct infusion of individual target compound into the electrospray ionization source (Table 2). Two MRM transitions are required for each target azole [33]. The most prominent ion transition was selected for quantitation while the other one was used for confirmation. To elevate the compensation performance of the internal standards, the most intense ion transition may not be applied as the quantitative ion of an analyte. For instance, the selective quantitation ion transition for climbazole and climbazole-D4 was 293.1 > 225.0 and 297.1 > 229.0, which were not the most abundant ion transitions. The difference value between daughter ion of climbazole and daughter ion of climbazole-D4 was equal to the difference value of parent ion. It displayed that mass spectrometric behavior of this azole was involved in deuterated portion (Fig. S3) so that the stable isotope-labeled internal standard could play an important role for compensation of matrix effect [31].

According to the ion transition of each analyte, proposed dissociation pathway is provided in Fig. S3. With the exception of
clotrimazole, selected parent ions represent the molecular ion \([M+H]^+\) for each analyte. The most intense parent ion for clotrimazole was found to be the \([M+H-C_3H_7N_2]^+\) at 277.1 m/z, which lost the imidazole moiety in the ESI source. This was in accordance with previous observation [22,24]. Presumably, imidazole ring of clotrimazole is prone to remove due to the potential steric hindrance effect of other three benzene rings attached to the same carbon atom. For the daughter ion of each analyte, nitrogen heterocyclic ring is also easier to depart from parent compound except for carbendazim. Mass transition analysis of theseazole fungicides in mass spectrometer is help to predict and identify the transformation by-products of their environmental fate.

3.2. Optimization of sample extraction and purification

For sample extraction of this study, all optimized conditions were carried out through ultrasonic extraction, liquid–liquid extraction and solid-phase extraction. To our knowledge, ultrasonic extraction and solid-phase extraction are the key procedures for the extraction ofazole fungicides from solid medium [23], and liquid–liquid extraction with nonpolar or low polar solvent is effective to remove the lipid from tissue sample [30], to minimize the loss of the polar target compounds (including moderate polar and high polar), n-hexane as clean-up solvent was pretreated with methanol to reach saturation. In this study, target compounds are expected to be protonated at \(pH<3\) (Table S1), without different \(pH\) value evaluations because of the little effect on recovery of pharmaceuticals by \(pH\) variation [26].

Three extraction solvents (methanol, acetonitrile and ethyl acetate) (C1–C3) with different polarities were tested in a side-by-side comparison. Ethyl acetate (C3) resulted in quite good absolute recovery (14.7–67.7%) and relative recovery (86.1–97.2%) (Tables S4 and S5). The lower absolute recovery of miconazole (14.7%) with corresponding higher absolute matrix effect (−67.3%) revealed the serious matrix suppression, which led to the low absolute recovery of miconazole. As shown in Table S6, absolute matrix effects of other analytes were in the range from −33.4% to −51.3%, indicated that the higher matrix interference was not deducted in C3. Therefore, the elimination of matrix effect should be conducted in the purification process.

Addition of sorbent in the extract seems to be an effective means to remove complex impurities from agricultural products (e.g., vegetable, meat and milk) based on the quick, easy, cheap, effective, rugged and safe method (QuEChERS) [19,27]. Following ultrasonic extraction process, four different kinds of sorbents (MgSO4, C18, Florisil and PSA) (C4–C7) were added into the extracts to investigate the effects of removing the impurities, prior to liquid–liquid extraction. The results showed that absolute matrix effect was obviously reduced with sorbent clean-up (5.23–19.7%) compared to without sorbent clean-up (C3, 48.3%) (Table S6). Florisil and PSA showed favorable efficiencies on removal of impurities on the basis of the absolute recoveries of C6 and C7 for each analyte. However, the absolute recoveries of C6 and C7 for fluniconazole were 137% and 128%. This demonstrated the presence of extreme matrix enhancement for fluniconazole.

In order to lower such strong matrices, mixture of sorbents and addition of dehydrant were investigated. Different ratios of florisor and PSA (C8–C11) were evaluated on the conditions of simultaneous addition of 1 g anhydrous MgSO4 in the extracts. Absolute matrix effects of fluniconazole were improved in the range between −1.47% and 7.79%, but the absolute recoveries of some analytes (e.g., ketoconazole and miconazole) were declined (Tables S4 and S6). The effectiveness of dehydrant was also tested. One gram of anhydrous MgSO4 was added in the extract with sorbent (C11) and in the muscle tissue before ultrasonic extraction (C12), and not added (C13). Absolute recoveries and absolute matrix effects of C13 were slightly superior to C11 and C12. These two results implied that mixture of sorbents and addition of dehydrant seemed to have scarcely any effects on extraction and purification.

Due to the common use and low cost, HLB cartridges were employed in solid-phase extraction for the pretreatment of target azoles on the conditions of C1–C13. Relative recoveries and relative matrix effects of all analytes were mostly satisfied in the range of 80–120% and 5–5% (Tables S5 and S7). But the absolute recoveries, especially for ketoconazole (≤42.8%) and miconazole (≤40.2%), were unfavorable (Table S4) even if optimization of other factors. This can be attributed to the incomplete removal of impurities and the loss of target analytes during the extraction and purification process. As mentioned by Casado et al. [24,25] that MCX cartridge, with a mixed-mode cation exchange sorbent, had a significant advantage to selectively fractionate basic compounds such as azole fungicides. Thus, clean-up procedure was optimized using MCX cartridge instead of HLB cartridge. Optimization of rinse solution and eluent was also conducted. Absolute matrix effects of all analytes were more serious in C14 (−44.9–0.23%) than in C15 (−16.1–6.65%), suggested that neutral interferences are effectively eliminated by acidified methanol (0.1% formic acid in methanol) and target analytes can be selectively eluted by basic methanol (0.5% NH3 in methanol) (Table S6). However, absolute recoveries of ketoconazole and miconazole were still poor. Further, florisil and PSA were assessed as single sorbent (C16–C17, C19–C20) or mixture sorbent (C18) without dehydrant using MCX cartridge based on the results of C1–C15. The results showed that absolute recoveries of all analytes were much better under the conditions of C16–C20 than C1–C15. For ketoconazole, similar absolute matrix effects were found among last five optimized conditions, whereas absolute recoveries of C17 and C20 were significantly superior to the others (Table S6). It meant that less absorption of ketoconazole and more elimination of matrix interference in clean-up process were performed by PSA alone. As we known, PSA was an effective clean-up sorbent for removing many polar matrix components (e.g., organic acids, certain polar pigments and sugars) to some extent from the food extracts [27]. In addition, no obvious variations of recoveries and matrix effects for all analytes were observed between C17 and C20. In consideration of the cost of experiment, the volume of PSA was selected as 0.5 g. Therefore, the optimized condition of C20 was used in the final extraction and purification procedure.

Optimization of different conditions in the above discussion was appraised by comparing the recoveries and matrix effects without statistical relevance. To further identify the differences among different optimized conditions with statistics, a heatmap system with hierarchical cluster analysis was plotted in this study (Fig. 1). The clustering was based on the absolute recovery, relative recovery, absolute matrix effect or relative matrix effect of each azole fungicide in various conditions. In general, better relative recoveries and relative matrix effects were found for most optimized conditions, suggested that individual isolate-labeled internal standard correction method is conductive to obtain better apparent recovery and matrix effect, as well as better precision and reliability of quantification [31]. The results of hierarchical clustering clearly depicted that both the conditions of C17 and C20 could be easily separated from the rest of conditions (Fig. 1a), while the other three profiles revealed that the condition of C20 was more suitable to be the final selected condition than C17 (Fig. 1b–d).

3.3. Performance of analytical method

In the linearity study, the correlation coefficients \(R^2\) of calibration curves were greater than 0.998 for all analytes in the concentration range of 1–200 ng/mL (Table 2), indicated the good linearity. The analytical method was validated through quantita-
tion of each targetazole fungicide in five common species of fish muscle tissues (Tables S8–S12). The MRM chromatograms of quantitative ions for azole fungicides in various fish muscle samples, including silver carp, common carp, mirror carp, nile tilapia and grass carp, with spiked concentrations of 20 ng/mL are depicted in Fig. 2. The absolute recoveries and absolute matrix effects of all analytes in different fish muscle tissues were 22.7–106% and –8.76–9.14%, suggested the satisfactory extraction efficiency and matrix interference of this method. Based on the individual isotope-labeled internal standard compensation, the relative recoveries and the relative matrix effects were outstanding in the range of 81.7–104% and –6.34–7.16%, with the associated standard derivations within 10%, manifested that one-to-one correspondence between analyte and its deuterated analogue was effective for compensation of matrix effect [31]. The method detection limit (MDL) and method quantification limit (MQL) in different fish muscle tissues were 0.02–0.85 ng/g and 0.07–2.83 ng/g for all analytes. These results proved that the analytical method was more sensitive for azole fungicides monitoring in common fish muscle samples than those without any clean-up process [26]. The repeatability was less than 2.66% for intra-day and 4.71% for inter-day analysis (Table S13), implied that the analytical method was robust. Therefore, a sensitive and robust analytical method has been developed to determine commonly azole fungicides in fish muscle samples.

4. Conclusions

This work proposed a sensitive and robust multi-residue method to extract and quantify six commonly used azole fungicides in the matrix of fish muscle tissues. Numerous factors (including extraction solvents, sorbents, dehydrants and SPE cartridges) were investigated for the optimized conditions. According to the results of non-statistical analysis (side-by-side comparison) and statistical analysis (hierarchical clustering heatmap), finally, the analytical method involved ultrasonic extraction, solid-liquid clean-up, liquid-liquid extraction and solid-phase extraction for extraction and purification, and HPLC–MS/MS for determination. Recoveries, matrix effects, method detection limits and method quantification limits were favorable for method validation tests with different species of fish muscle samples. The developed method was successfully applied in wild populations of fish from Beijiang River basin. The obtained results suggested that the analytical method was practical and effective for the determination of azole fungicides in field fish muscle tissues. In addition, the presence of azole-contaminated fish in the environment deserves more urgent attentions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.11.024.

References


