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Multi-Residue Screening of Pesticides in Aquaculture Waters through Ultra-High-Performance Liquid Chromatography-Q/Orbitrap Mass Spectrometry

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Abstract: Pesticide residues in foodstuffs can lead to several undesirable effects. A simple and high-throughput targeted screening method analyzing multi-residue pesticide in aquaculture water based on ultra-high-performance liquid chromatography-Q/Orbitrap mass spectrometry (UHPLC-Q/Orbi MS) was developed and validated. In this technique, the peaks of the compound using precursor ions were recorded by the full scan, which was used for rough quantitative analysis with single point matrix matched calibration. The qualitative identification was performed following the stringent confirmation criteria with fragment ions, retention time, and an isotopic pattern. Additionally, solid-phase extraction with an HLB (Hydrophilic/Lipophilic Balanced) column was selected to enrich and separate target pesticides from water. The screening detection limit of 33 compounds are less than 2 ng·L^{-1}, while 26 compounds range from 2 ng·L^{-1} to 10 ng·L^{-1}, 19 compounds are at the range of 10–200 ng·L^{-1}, and the other two compounds are 200 ng·L^{-1} and 1000 ng·L^{-1}. Most of the recovery results were found to be between 60~130%. Finally, the method was successfully applied to the analysis of pesticide residues in 30 water samples from aquaculture environment in Shanghai, indicating its applicability in pesticide screening for environmental monitoring.

Keywords: environmental monitoring; solid phase extraction; trichlorfon; chlordimeform; fipronil; dimethoate

1. Introduction

The use of pesticides in agriculture plays an important role in preventing crop diseases, pests, rodents, etc., and also promoting high quality and high yield of agricultural products [1]. Due to the increases in demands of seafood, aquaculture has become one of the fastest growing food-production sectors [2]. In the past decade, the production and consumption of pesticides have been increasing in China, which inevitably causes an exposure of the environment to pesticides. Pesticide residues in foodstuffs could also have several undesirable effects, such as destructing the regulation of hormones and enzymes in organisms by leading to chronic diseases or cancer [3]. These residues distributed in the atmosphere, water, and soil can accumulate through natural circulation and eventually enter the human body via diet or ingestion, dermal contact, and inhalation of contaminated air [4,5]. Moreover, pesticide residues in water environments can damage the balance of ecosystems and biodiversity, which further negatively affects the growth of aquatic organisms [6]. Some pesticides have been specified in China's water quality standards for aquaculture and surface

water, in which the maximum concentration for individual pesticide ranges between 0.5 μg·L^{-1} and 1000 μg·L^{-1}. However, these specified pesticides are quite incomplete if used for comprehensive evaluation of the quality and potential pollution for aquaculture purposes. The analysis method for monitoring as many as possible pesticide residues in water is quite essential to ensure the high quality of aquaculture.

The analysis of pesticide contaminants in aquaculture environments mainly involves preconcentration and cleanup of the extracts before their instrumental analysis [7]. For the detection of pesticides in the environment, gas chromatography (GC) with mass spectrometry (MS) detection [8–11], nitrogen–phosphorus detection [12], flame photometric detection [1], electron–capture detection [13,14], and high performance liquid chromatography (HPLC), with diode-array detection [15], ultraviolet detection [16–19], and MS/MS [11,20], are the most common instrumental techniques. In the last few years, methods applied in pesticides based on GC or HPLC with high-resolution mass spectrometry (HRMS) have been increasingly explored [21–23]. Owing to the low concentration residues of contaminants in the environment and their complexity in the real samples, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are often used for extraction and purification of pesticides from water [1,24,25]. As the solid-phase extraction (SPE) allows the simultaneous enrichment and purification with less organic solvent, it is preferable for large volume samples pretreatment. However, the major concerns of the previous studies were organophosphorus, organochlorines, carbamates, or certain types of pollutants, respectively [26–29]. Studies focusing on the detection of pesticides in water or multi-residue detection of these pesticides in water with triple quadrupole (QqQ) have been extensively reported [30]. Less research has been reported on multi-residue detection of pesticides by HRMS with a standard database for comparison. To some extent, QqQ is inferior to HRMS in terms of anti-interference performance, accurate qualitative, and non-target screening without standards [31,32]. Moreover, the pesticide pollution in aquaculture water is of less concern, which is important for the quality and safety of aquatic products and water ecosystems.

In our work, a fast, simple, and reliable method based on a one-step SPE enrichment-cleanup-elution strategy, followed by ultra-high-performance liquid chromatography (UHPLC) quadrupole/Orbitrap mass spectrometry (Q/Orbi MS) for screening of pesticides in water was established for the adequate monitoring of the pesticide residues in environment. A total of 80 target analytes, including organophosphorus, organochlorines, carbamates, imidazoles, triazines, phenylpyrazoles, avermectins, pyrethroids, and other pesticides, were successfully screened. These compounds were frequently used as insecticides and fungicides in water and negatively impact human health [3,33]. Furthermore, 30 environmental water samples from different regions of rivers, lakes, and aquaculture environment in Shanghai were screened with this method.

2. Materials and Methods

2.1. Reagents and Chemicals

Acetonitrile (ACN), methanol (MeOH), ethyl acetate (EA), dichloromethane (DCM), acetone (ACE), and n-hexane of HPLC grade were purchased from J.T. Baker (USA). Formic acid (FA, LC-MS grade) was obtained from Fisher Scientific (USA). Ammonium formate was purchased from common domestic suppliers. Ultrapure water was prepared by Milli Q system (Millipore Molsheim, France). SPE cartridges, Oasis PRiME HLB (500 mg/6 mL), were obtained from Waters (Milford, MA, USA). A total of 80 standards of analytes (purity, >90%) are listed in Table S1. Carbofuran and dichlorvos were purchased from MANHAGE (Beijing, China) and thiofanox sulphone, aldicarb sulfone, phoratoxon sulfoxide, and sodium pentachlorophenol (PCP Na) were purchased from Accustandard (New Haven, CT, USA). The other 76 standards were acquired from Dr. Ehrenstofer GmbH (Augsburg, Germany). Qualitative filter paper (d = 11 cm, fast speed) was obtained from Sinopharm (Shanghai, China).

Individual stock solutions of all analytes with a concentration of around 0.1 mg·mL^{-1} were prepared in MeOH and stored at −40 °C in brown glassware. A small volume of FA (1.2 mg·mL^{-1})

was added in simazine, carbendazim, and indoxacarb to enhance the solubility. According to the chemical structure and properties, these compounds were categorized into organic phosphorus, carbamate, organic chlorine, imidazole, pyrethroid, triazine, phenylpyrazole, avermectin, and others. Mixed standard solutions were prepared at a concentration of 5 $\mu g \cdot mL^{-1}$, according to their category, and further stored at −40 °C in the dark.

2.2. Samples

The water samples for the development and evaluation of the methods were taken from tap water in Shanghai, China. In addition, 30 real water samples were collected from the rivers, lakes, and different aquaculture environments around Shanghai for screening purposes with this method. These water samples were stored at −20 °C in the dark prior to sample preparation procedures.

2.3. Sample Preparation

A total of 500 mL of each water sample was sat overnight and then filtrated with qualitative filter paper to remove sediment prior to sample preparation. The samples were then extracted by solid-phase extraction. The Oasis HLB cartridge (500 mg/6 mL) was firstly conditioned with 10 mL of ACN, followed by 10 mL of water before extraction. The water sample was passed through the HLB column at a flow rate of 3–5 $mL \cdot min^{-1}$. Subsequently, 10 mL of ultrapure water was added to remove the water-soluble interference, respectively, and the residual water in the column was dried under low pressure vacuum for 30 min. Columns were then eluted with 20 mL of ACN/EA/DCM (1:1:1, v/v/v) at a flow rate of 1 $mL \cdot min^{-1}$. The eluent was collected with a 20 mL glass tube. The solvents were blown under a gentle stream of nitrogen at 35 °C to a final volume of ca. 0.3 mL and avoid evaporation to dryness. Finally, 1 mL of methanol/water (1:1, v/v) was used to reconstitute the remaining solvent into the vial for subsequent analysis.

2.4. Instrument

Dionex Ultimate 3000 ultra-high-performance liquid chromatography coupled to a quadrupole/Orbitrap mass spectrometry (UHPLC-Q/Orbi MS) system operated in electrospray ionization (ESI) mode with Tracefinder analysis software (Q-Exactive, Thermo Fisher Scientific) was used to detect the pesticides. An Accucore aQ-MS column (100 mm × 2.1 mm, 2.6 μm, Thermo Fisher Scientific, Waltham, MA, USA) was used for the separation and elution of various pesticides at 30 °C. The binary mobile phases were prepared with water containing 0.1% formic acid and 5 mM ammonium formate (phase A) and methanol with 0.1% formic acid and 5 mM ammonium formate (phase B). The gradient elution was applied with 2% B to start, gradually increased to 20% B at 4 min, 40% B at 5.5 min, 98% B at 10.5 min, and kept to 12.9 min, further restored to 2% B at 15.0 min, and re-equilibrated for 5 min before the next injection. A flow rate of 0.3 $mL \cdot min^{-1}$ was performed for the instrumental analysis. A total of 10 μL of the samples was injected to the UHPLC-Q/Orbi MS system. All the parameters of the UHPLC-Q/Orbi MS system were controlled through the TraceFinder software.

A heated electrospray ion source (H-ESI) for simultaneous positive and negative ion acquisition was used at applied voltages: 3200 V (+) and 2800 V (−). The sheath gas with a flow rate of 40 $L \cdot min^{-1}$, auxiliary gas with a flow rate of 10 $L \cdot min^{-1}$ at a temperature of 350 °C, and sweep gas with a flow rate of 1.0 $L \cdot min^{-1}$ were performed. The capillary temperature was set at 325 °C. Data was acquired by the full-scan data dependent MS/MS (TopN) mode with an inclusion compound list. The Full Scan/ddMS2 acquisition mode with inclusion list could simultaneously record the precursor and the MS/MS (fragmentation) spectra for the selected precursors [34]. The mass spectral data were recorded at the scan range of m/z 100–1000, with full scan resolution at 70,000, and the MS/MS acquisition resolution at 17,500. The top five abundant precursors greater than 5×10^5 during each full scan were sequentially fragmented and transmitted to the Orbitrap mass analyzer. The stepped normalized collision energy (NCE) at 20, 50, and 80 were used for fragmentation at the high energy collision dissociation (HCD) cell.

2.5. Identification and Validation

The database established in the Tracefinder contained information of the m/z of the precursor ion, retention time (RT), and fragment ion, which were acquired through the UHPLC-Q/Orbi MS system with standards of the analytes at 100 ng·mL^{-1}. When the m/z deviation of the precursor ion was less than 5 ppm, the RT override was less than 0.25 min, at least one fragment ion matched with its abundance over 1×10^4 and a deviation of less than 20 ppm, and the precursor isotope pattern of fit threshold was higher than 75%, so the target compound could be identified.

The matrix effect for the one-step SPE enrichment-cleanup-elution strategy was examined by the ratio of abundance between the standards spiked matrix extract and standards matched reconstitution solvents. The matrix effect of all compounds was calculated using Equation (1):

$$matrix\ effect = \left(1 - \frac{A_b}{A_s}\right) \times 100\%, \quad (1)$$

where A_b is the signal area of the standards spiked matrix extract and as is the signal area of solvent-matched standard solution with equivalent concentration [35]. In order to evaluate the performance of the SPE enrichment, the blank aquaculture water samples spiked with all analytes at the concentration of 2 ng·L^{-1}, 10 ng·L^{-1}, 40 ng·L^{-1}, and 200 ng·L^{-1} were enriched with SPE, eluted through mixed solvent, and were finally analyzed by the UHPLC-Q/Orbi MS system to examine the number of targets that can be identified and confirmed under different spiked amounts. The ratio between the calculated amounts of the analytes added before and after pretreatment was calculated as the recoveries of the method. Recoveries at the spiked concentration of 10 ng·L^{-1}, 40 ng·L^{-1}, and 200 ng·L^{-1} were calculated with five duplicates for each spiked level. At the same time, the precision of the method was calculated by the relative standard deviation (RSD) of five replicates.

3. Results and Discussion

3.1. Data Acquisition with UHPLC-Q/Orbi-MS and the Ionization of Compounds

The data acquisition mode (full scan mode) can record all precursor ions generated through electrospray ionization at positive and negative modes. According to the data-dependent setup, the targets of interest in the inclusion list can fragment in the HCD cell with high efficiency and be recorded with accurate mass by Orbitrap. In the ion source, the precursor ions were formed through electrospray ionizations before being transferred to the mass analyzer. As shown in Table S1, the adduct of four phenylpyrazole pesticides (fipronil-desulfinyl, fipronil, fipronil-sulfide, and fipronil-sulfone) formed negative ions $[M–H]^-$, and the remaining $[M+H]^+$, $[M+Na]^+$, or $[M+NH_4]^+$ were all positive ions, respectively. In order to acquire high abundant fragments for the target compound, the NCE of some precursors was optimized as a typical compound fragmentation (Figure S1). The instrument detection limit (IDL) of 80 pesticides was validated to access the sensitivity of our instrument method (Table S1).

3.2. Selection of Extraction Cartridges

To enrich analytes from the water as much as possible, the loading efficiency of several different columns, such as HLB, Florisil, and C18, as well as their series use, was investigated. In total, 50 ng of the mixed standards was spiked in 20 mL of tap water and subsequently enriched, eluted, and reconstituted for UHPLC-Q/Orbi-MS analysis. The number of detected compounds after sample enrichment through the three columns was similar. However, three columns showed different recoveries for compounds of interest. As displayed in Figure 1a, avermectins, acephate, and methamidophos can be well recovered on HLB, while florisil performed excellently for pyrethroids. These results are consistent with previous papers [36,37], but no better results were obtained for other pesticides. Meanwhile, as shown in Figure 1b, the combined use of HLB with florisil or C18 was also examined. There was no improvement in terms of the number of detected compounds or their

recoveries obtained, which may be attributed to a less efficient elution during the combined use. As a result, HLB was finally selected for enrichment of pesticide analytes from environmental water.

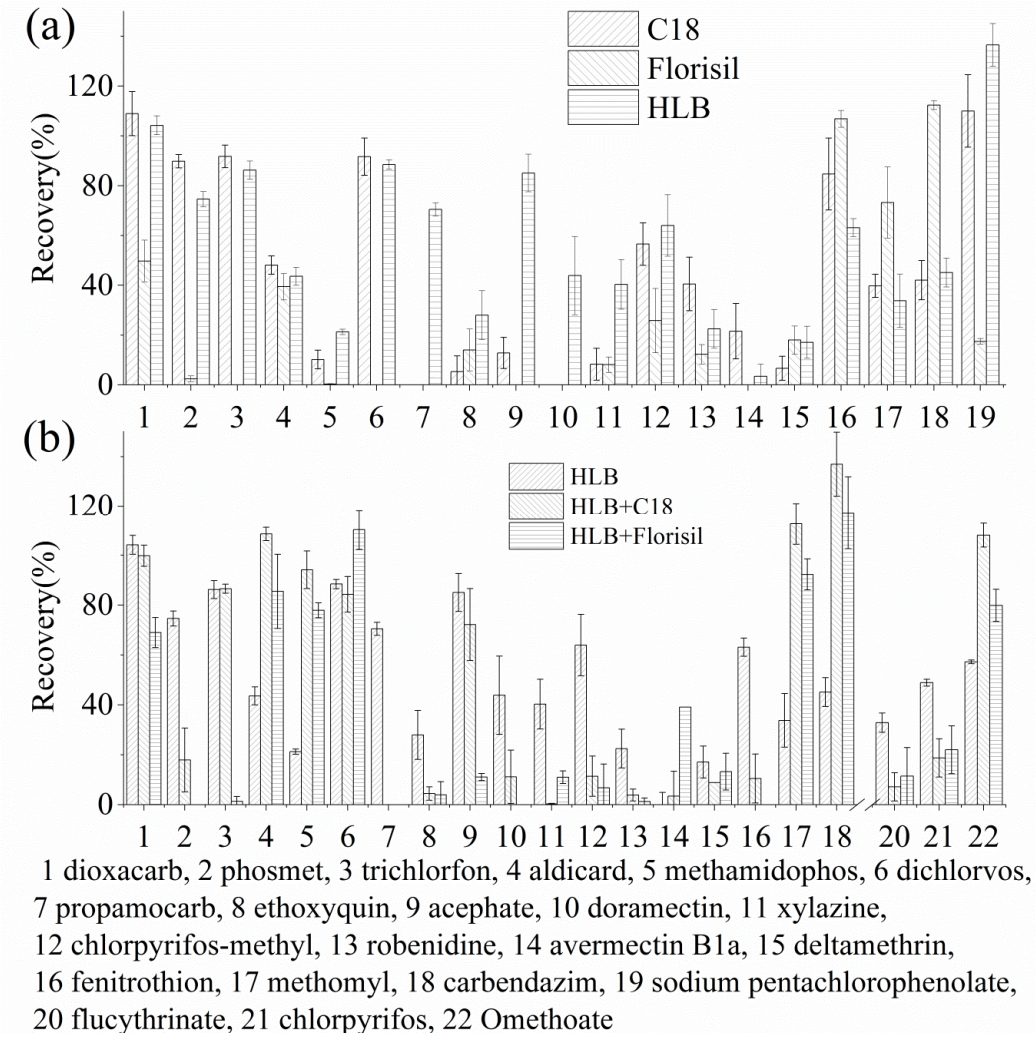

Figure 1. Differences in enrichment recovery between HLB, C18, Florisil (**a**), and their combinations (**b**).

3.3. Optimazation of Eluents

Eluent is critical for recovering compounds loaded on the HLB cartridge. In this research, compounds of interest were of wide polarity range, thus solvents of different polarity and ratios were examined to elute with high efficiency. In the beginning, single solvent, MeOH, ACN, and EA were investigated. Phorate and six pyrethroids, namely cyfluthrin, flumethrin, tau-fluvalinate, fenvalerate, deltamethrin, and bifenthrin, were only detected with ACN as eluent. Additionally, three pesticides (ethoxyquin, doramectin, and fenitrothion) were identified with ACN or EA as eluent, while these compounds were not recovered efficiently after MeOH elution, even if the recoveries of these compounds still had some drawbacks (Figure S2a). Furthermore, mixed solvents, including ACN/EA (1:1), ACN/EA (3:1), ACN/MeOH (1:1), MeOH/EA (1:1), ACE/ n-hexane (1:1), and ACE/n-hexane (3:1), were examined to get high elute efficiency. ACN/EA (1:1) had better eluting potential for most of the compounds, which were recollected with recoveries of over 60% among these mixed solvents; however, foracephate, phosmet, xylazine, aldicard. Nonetheless, thiophanate-methyl, methamidophos, thiophanate, propamocarb, and pyrethroid pesticides lost more than 85% in the result of these mixed eluents (Figure S2b).

Therefore, we considered one weaker polar solvent mixed with the two solvents to help increase recoveries of these pesticides with poor eluent efficiency. In Figure 2, three solvent mixtures, namely ACN/EA/n-hexane (1:1:1), ACN/EA/DCM (1:1:1), and ACN/ACE/n-hexane (2:2:1), were tested. The results show that ACN/EA/DCM (1:1:1) can perform better for most of the pesticides, with which the recoveries of acephate, PCP Na, methamidophos, fipronil, omethoate, and propamocarb were significantly higher than that of ACN/ACE/n-hexane (2:2:1). The elution abilities of ACN/EA/n-hexane (1:1:1) are similar to that of ACN/EA/DCM (1:1:1). However, xylazine, avermectin b1a, fenitrothion, and dodemorph show poor recoveries when ACN/EA/n-hexane (1:1:1) is used as an eluent. Finally, ACN/EA/DCM (1:1:1) was used as an efficient eluent for HLB loading.

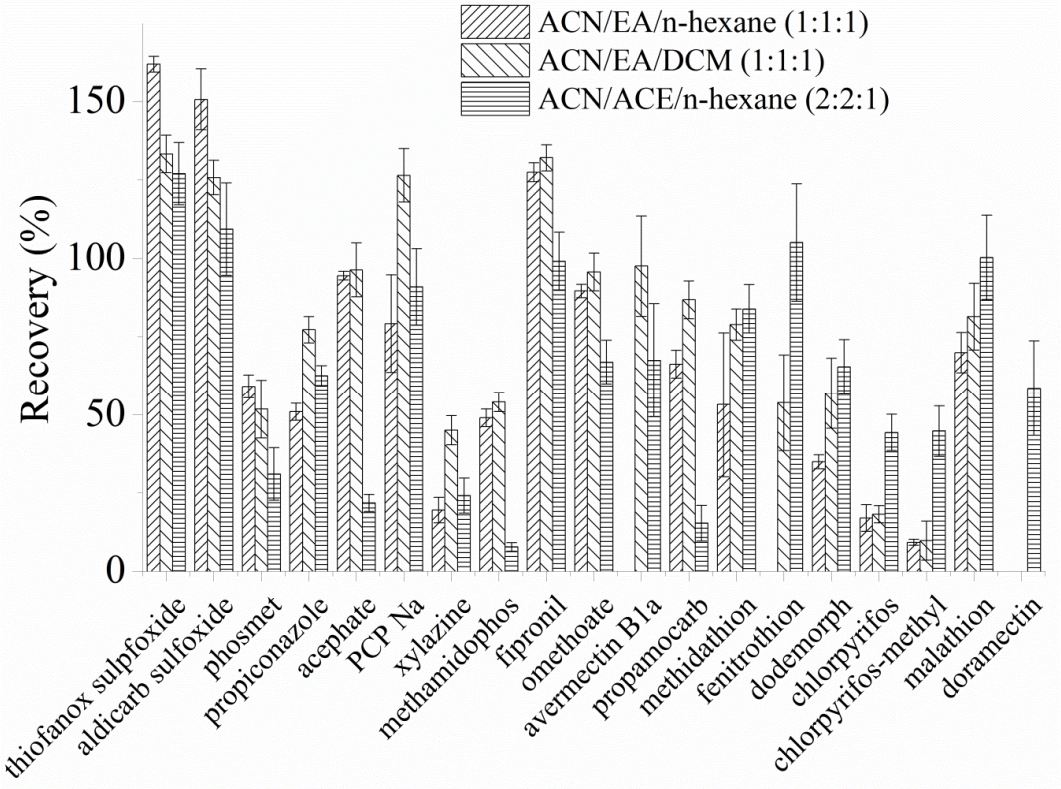

Figure 2. Compounds of significant recovery differences with three solvent mixtures as eluents after loading on the HLB column. Spiked level of 2.5 μg·L^{-1}.

3.4. Effect of the Cartridge Capacity on Pesticide Recovery

To avoid the saturation of the HLB column during enrichment, columns of different capacities (200 mg/6 mL, 500 mg/6 mL) were tested on their recovery ability for these pesticides. Results displayed no significant differences for the two columns. However, HLB (500 mg/6 mL) was applied in the pretreatment to avoid poor control of the flow rate of water samples when passing through the cartridge.

3.5. Method Performance and Matrix Effect

The performance of this screening method was validated with the sensitivity, precision, and accuracy. The numbers of analytes were identified at different concentrations of the standard mixture. The accuracy was evaluated based on the recovery calculated by adding standard compounds to blank aquaculture water at spiking levels of 10~200 ng·L^{-1} in five replicates. The blank aquaculture water was simply filtrated to remove precipitation and ensure similar physical states with tap water. The signal of positive components in the aquaculture water was subtracted from the spiked aquaculture

water to get an absolute signal response of the spiked pesticides. Precision was examined based on relative standard deviation (RSD).

According to SANTE/11813/2017 [38] with modification, five replicates of samples at the same level were used to get a validated screening detection limit (SDL). The lowest level of spiking concentration was regarded as the SDL for individual pesticides, if it was screened out in all five replicates. It showed that SDLs for 59 pesticides were between 2 and 10 ng·L^{-1}, the SDLs for 12 pesticides were between 10 and 40 ng·L^{-1}, and the SDLs for 9 pesticides were between 40 and 1000 ng·L^{-1} (Table S2). As shown in Figure S3, 78 compounds were screened out at 200 ng·L^{-1}, following the identification criteria set for the method, and 71, 59, and 33 of analytes were identified at 40 ng·L^{-1}, 10 ng·L^{-1}, and 2 ng·L^{-1}, respectively. The screening detection limit of our method can be used to evaluate whether the concentration of these specified pesticides in aquaculture water meets China's standards. Table S2 illustrates the recoveries and RSDs of the method at a spiked concentration of 10 ng·L^{-1}, 40 ng·L^{-1}, and 200 ng·L^{-1}. The RSDs for these compounds were found to be less than 20%, except for avermectinb1a and PCP Na. In total, 90% of these RSDs were below 15% in the water samples spiked at 200 ng·L^{-1}, 40 ng·L^{-1}, and 10 ng·L^{-1}. A total of 61, 55, and 48 compounds were found with recoveries between 70%–120%, when these compounds were spiked at 200, 40, and 10 ng·L^{-1}, respectively. Methiocarb, acephate, methamidophos, thiofanox, thiofanox sulfoxide, xylazine, isocarbophos, deltamethrin, avermectin B1a ethoxyquin, prothiofos, and cyfluthrin, were recovered out of 70%–120% at these spiked levels. These compounds were of relative strong polarity, resulting in easy loss during the enrichment process with SPE. It should be noted that ethoxyquin showed poor recoveries due to its transformation and decomposition during pretreatment. When these compounds were identified in real samples, their quantification results should be cautiously followed by this method.

As a result, this SPE method exhibits good reproducibility and high accuracy for screening pesticide residues in water, which is of great importance for monitoring the water environmental residues. Moreover, HLB was employed as enrichment and cleanup columns for pesticides in water. The matrix effect after pretreatment with this column was still tested. As shown in Table S2, 61 pesticides were detected with a weak matrix effect from −20% to 20%, 15 pesticides were with a medium inhibitive effect between 20%~40%, only 2 pesticides showed matrix enhancement effect of over 40%, and the other 2 pesticides showed a matrix inhibition effect of greater than 40%. These results displayed acceptable requirements for quantitative application for 61 pesticides with weak matrix effect. However, as for qualitative screening, these compounds with a matrix effect above 20% were also acceptable, even for semi-quantitative analysis in aquaculture water.

3.6. Application to Real Samples

After optimization and evaluation, this screening method was practically applied on 30 samples collected at different aquaculture areas of Shanghai in 2019. Results demonstrated that all positive components screened from these samples can be confirmed with stringent identification rules. For these screened pesticides, the deviation of precursor ions m/z was less than 3 ppm and their RT override was less than 0.1 min. In addition, the quantification of positive components was roughly calculated based on a single-point matrix-matched standard, where the proportion of an appropriate concentration of the components spiked was used. As shown in Table S3, among these 30 real samples, only one sample was not screened out with pesticides residues. Prometryn, carbendazim, propiconazole, fipronil-desulfinyl, fipronil-sulfone, simetryne, and fipronil-sulfide can be confirmed in more than one third of these samples. Drugs banned in agriculture, such as trichlorfon, chlordimeform, fipronil, and dimethoate, can be screened out in 16 samples. Typical extracted ion chromatography for pesticides in these samples are shown in Figure 3. It is noticed that, although most of the detected residues did not exceed the limit set by local authorities, carbendazim, trichlorfon, and other drugs, which exceeded 1 ppb. still need to be concerned, as they are prone to be accumulated in aquatic organisms [39,40].

Prometryn and carbendazims were found in 29 and 27 of these samples, indicating a potential threat to the safety of aquaculture as the environment can easily be exposed to these pesticides.

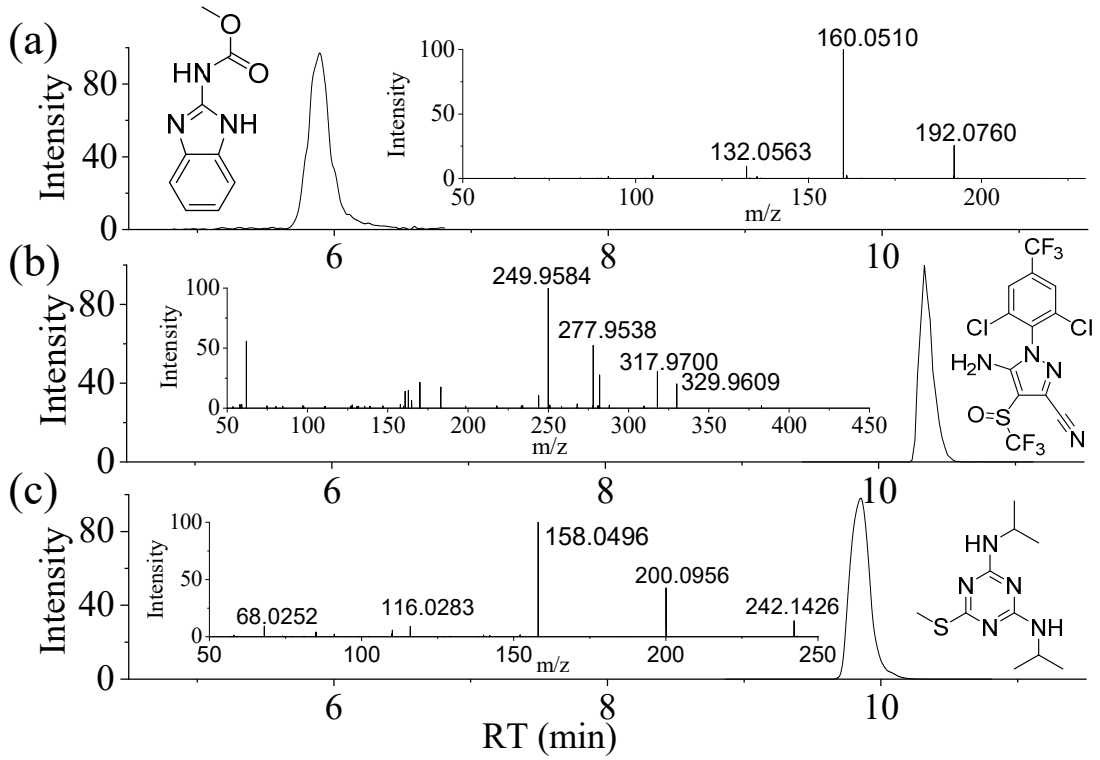

Figure 3. Typical compounds identified in real samples through ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS). (**a**) Extracted precursors chromatography for carbendazim, the inset of (**a**), the chemical structure and fragment ion spectrum acquired for carbendzaim; (**b**), extracted precursors chromatography for fipronil, the inset of (**b**), the chemical structure and fragment ion spectrum acquired for fipronil; (**c**), extracted precursors chromatography for prometryn, the inset of (**c**), the chemical structure and fragment ion spectrum acquired for prometryn.

4. Conclusions

In general, a screening method for multi-residue pesticides in environmental water based on SPE-UHPLC-Q-Orbi-HRMS has been established. These pesticides were screened based on their conformance with the database, which includes the high-resolution data of precursor ions and fragment ions, isotope fit, and RT. A stringent identification rule with a lower error tolerance was applied during the screening process. HLB demonstrated acceptable recoveries and the matrix effect. The screening detection limits of this method for 80 compounds were between 2~1000 ng·L^{-1}. Among them, 78 compounds could be screened out at <200 ng·L^{-1}, which is below the limits specified by local authorities, indicating the applicability of this method for China's regulations (NY5051-2001, NY5052-2001, GB 3838-2002). Meanwhile, the method was successfully validated at three spiking levels (200 ng·L^{-1}, 40 ng·L^{-1}, 10 ng·L^{-1}). Results suggest that most compounds can be detected with less than 20% of RSD and the desirable recovery is between 70%–120% for more than 90% of compounds. Practical application of this method demonstrated that 29 out of the 30 real samples were confirmed with positive pesticides analyzed in the database, suggesting that the method is feasible for monitoring control and surveillance.

Supplementary Materials: The following are available online, at http://www.mdpi.com/2073-4441/12/5/1238/s1, Figure S1: Comparison of MS/MS spectrum of thiabendazole generated by different NCE, (**a**) at NCE of 20, 50, and 80, (**b**) at NCE of 80; Figure S2: Compounds of significant recovery differences with single (**a**) or mixed

(**b**) solvents as eluents after loading on HLB columns. Spiked level of 2.5 μg·L^{-1}; Figure S3: Number of compounds identified at different spiked concentrations in blank aquaculture water samples; Table S1: Chromatographic mass spectrometry parameters and instrument detection limits (IDLs) of 80 pesticides; Table S2: The recovery, relative standard deviation (RSD) at three different levels of SDL and the matrix effect of 80 compounds; Table S3: Screened results for 30 real samples at different aquaculture environment of Shanghai.

Author Contributions: Conceptualization, C.K. and H.-J.Y.; methodology, H.-J.Y. and S.-Y.W.; software and validation, S.-Y.W.; formal analysis, C.K. and S.-Y.W.; investigation and resources, H.-J.Y. and S.-Y.W.; data curation, C.K. and S.-Y.W.; writing—original draft preparation, S.-Y.W.; writing—review and editing, C.K. and E.K.F.; visualization, C.K.; supervision, C.K.; project administration and funding acquisition, C.K. and H.-J.Y. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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