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Simultaneous Quantification of Avermectins in Six Aquatic Foods by UHPLC/FLD with Precolumn Derivatization

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Abstract: In this study, a fast, concise and reliable ultra-high performance liquid chromatography-fluorescence (UHPLC/FLD) detection method for simultaneous quantification of avermectins (AVMs), including avermectin (AVM), ivermectin (IVM), emamectin (EMM), moxidectin (MOX) and doramectin (DOR) in six aquatic foods was established. Based on the QuEChERS pretreatment method, the samples were extracted with 0.2% (*v*/*v*) ammonia acetonitrile. N-methyl imidazole mixed with acetonitrile (1:1, *v*/*v*) and trifluoroacetic anhydride with acetonitrile (1:2, *v*/*v*) were used as derivatization reagents. The mobile phase consists of acetonitrile and water with a flow rate of 1.0 mL/min. An Infinity Lab Poroshell 120 EC-C18 column was used for optimum chromatographic separation of target analytes at 40 °C; the excitation and emission wavelengths were set at 365 nm and 465 nm, respectively. In six kinds of aquatic foods, the limits of detection (LODs) of AVM, IVM, EMM, MOX, and DOR were 2.7 μg/kg, 1.8 μg/kg, 2.1 μg/kg, 1.2 μg/kg, and 2.7 μg/kg, respectively, and the limits of quantification (LOQs) of AVM, IVM, EMM, MOX, and DOR were 5 μg/kg, 4.5 μg/kg, 4.5 μg/kg, 3.5 μg/kg and 5.0 μg/kg, respectively. The recoveries were all above 85.38% when the samples were spiked with the target compounds at the concentration level of 5, 10, 50, and 100 μg/kg. The intra-day and inter-day relative standard deviations (RSDs) were all less than 15%. This method considers the requirements of sensitivity, accuracy, and economics of the instrument.

Keywords: analytes; aquaculture; UHPLC-FLD; multiclass; veterinary drug residues

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1. Introduction

The avermectins (AVMs), a class of macrocyclic lactones, AVMs are produced by the soil-dwelling actinomycetes *Streptomyces avermitilis*, including avermectin (AVM), emamectin (EMM), ivermectin (IVM), eprinomectin (EPR), doramectin (DOR) and moxidectin (MOX). The AVM consists of a mixture of AVM B1a (at least 80%) and AVM B1b (not more than 20%). In addition, EMM consists of 90% or more of the B1a component and not more than 10% of the B1b component. Group a and b components belong to secbutyl and isopropyl homologs, respectively, which possess similar toxicological and functional activities, and, therefore, it is unnecessary to separate group a and b components [1]. The mechanism of action involves the stimulation of high-affinity GABA receptors and a consequent increase in membrane chloride ion permeability. Animal studies indicate a wide margin of safety because mammalian species are much less sensitive due to lower GABA receptor affinities and relative impermeability of the blood-brain barrier [2]. With increasing large-scale breeding, various parasitic diseases are seriously hindering economic output in the aquaculture industry. Avermectins (AVMs) in aquaculture are widely used as

antiparasitic drugs against internal and external parasites in rainbow trout, crucian carp, sea bass, and so on [3–6]. The widespread use of these drugs may lead to drug residues in various aquatic products, causing potential risks to human health. For this reason, maximum residue limits (MRLs) on AVMs have been established strictly. Currently, the European Medicines Agency (EMEA) has set a maximum residue limit of EMM in salmon meat and skin of 100 μg/kg [7], the Codex Aliment Arius Commission (CAC) has set a maximum residue limit of 100 μg/kg for EMM in salmon, trout muscle, and fillet (natural ratio of muscle plus skin) [8], the Health Canada has been set maximum residue limits for EMM in muscle and skin of salmonids of 100 μg/kg and 1000 μg/kg, respectively [9], Japan has been stipulated a maximum residue limit of 100 μg/kg for EMM in salmonids such as salmon and trout [10], the World Health Organization (WHO) limits MOX at 20 μg/kg in cattle muscle [11], and the European Union (EU) has been set the maximum residue limit of AVM and EMM in amphibians at 100 μg/kg [12].

Recently, the QuEChERS method has been the preferred method in the field of pesticide multi-residue analysis [13–17]. It involves miniaturized extraction with acetonitrile, liquid–liquid partition by using sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) as salts. QuEChERS pretreatment method is a common and effective pretreatment method, which is commonly used for pretreatment of residue detection in fruits, vegetables [14,15], and animal-derived food [13,16,17]. AVMs were mostly extracted with acetonitrile; modified QuEChERS methods were used for preparation. For animal samples, NaCl was used [18]. EDTA-treated sand was used for meat sample preparation [19]. Homogenized fish samples were extracted with acetonitrile, magnesium sulfate anhydrous, and sodium chloride [20].

AVMs have been determined mainly in vegetables [21–24], milk [25–28], and meat products [19,29–31]. Fewer studies have been reported on the determination of fish [13,32,33]. So far, pieces of literature have reported different detection methods for AVMs. Among them, AVM was detected by UPLC- UV in cabbage, where the LOD was 10.9 μg/kg [21], and the LOD of AVM detected by HPLC-UV in rape was 10 μg/kg [22]. Obviously, the LODs of those methods are not excellent enough. Apart from this, the AVM and IVM were detected simultaneously by HPLC-FLD in fish muscle samples, perch, and aquatic products. The LOQ was 3 μg/kg in fish muscle samples [34], the recovery values of AVM and IVM was 78.8–88% in perch [35], and the LOQs of AVM and IVM in aquatic products detected by the same method were both 3 μg/kg within 23 min (mins) [36]. The residues of AVM, IVM, DOR, and MOX were determined simultaneously by HPLC-FLD, and the detection time of each sample was 25 min [37]. The detection method is time-consuming and inefficient. Precision and recovery rates need to be improved. Over and above, detected by UPLC-MS, the LODs of AVM and IVM in milk were 1.0 μg/kg [38], and the LODs of AVM, DOR, and IVM in eel were 0.43~0.32 μg/kg [39]. However, the MS detector is always equipped with electrospray ionization (ESI) sources and is not suitable for mass detection because of its complicated pretreatment and fancy price.

Briefly, UHPLC-FLD combined the advantages of these methods. Not only is the equipment cheaper, but also the precision and the detection time are better, which is suitable for the detection of large quantities of multiple residues. In addition, few existing detection methods can simultaneously detect AVMs, which are widely used in a variety of aquatic products. Therefore, this study intended to establish a rapid, easy, and reliable UHPLC-FLD method to detect five AVMs (AVM, IVM, EMM, DOR, and MOX) simultaneously in aquatic products.

2. Materials and Methods

2.1. Chemicals and Reagents

EMM and IVM (purity $\geq$ 97.0%) were procured from ChemService (West Chester, PA, USA). AVM, MOX, and DOR (purity $\geq$ 97.0%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). HPLC grade acetonitrile, ethyl acetate, and n-hexane used for analysis were all obtained from J. T. Baker (Deventer, Holland). LC-MS grade water was bought from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was attained

from Sigma-Aldrich (Shanghai, China). Concentrated ammonium water (AR, 25–28%) was ordered from Macklin (Shanghai, China). N-methyl imidazole was purchased from Hwrk Chem (Beijing, China). $MgSO_4$, NaCl, heparin sodium, and trifluoroacetic anhydride were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). The centrifugal tubes and 0.22 μm nylon syringe filters were acquired from CNW Technologies (Shanghai, China).

0.2% (v/v) ammonia acetonitrile solution as an extractant was made by the addition of 0.2 mL ammonia water in 100 mL acetonitrile and mixed well.

2.2. Equipment

An Agilent 1260 Infinity II SFC/UHPLC system (Milford, MA, USA) equipped with a quart solvent manager with a quart solvent pump, a sampler manager with an autosampler, and a fluorescence detector (FLD) was used. Data acquisition was performed by openlab CDS 2 software.

Other equipment, such as KQ 2200 DE ultrasonic cleaner (Ultrasonic Instruments, Kunshan, China), Vortex-HQ-60-IV (TZ-Biotech Develop, Beijing, China), AOSHENG nitrogen blower (Allsheng instrument, Hangzhou, China), Hitachi 20PR-520 automatic high-speed frozen centrifuge (Hitachi, Tokyo, Japan) and AX205 analytical balance (Mettler-Toledo, Zurich, Switzerland) were also applied in this study.

2.3. Standard Solution Preparation

Standard stock solutions were prepared by dissolving 10 mg AVM, IVM, EMM, MOX, and DOR standards in acetonitrile and diluted to a final concentration of 100 mg/L, respectively.

Then, 1 mL AVM, IVM, EMM, MOX, and DOR standard stock solutions were taken, diluted to 10 mL with acetonitrile, respectively, and a single standard solution with a mass concentration of 10 mg/L was prepared.

Finally, 1 mL of AVM, IVM, EMM, MOX, and DOR single standard solutions were placed in the same 100 mL volumetric flasks, diluted and fixed with acetonitrile, containing AVM, IVM, EMM, MOX, and DOR 1 mg/L. All standard solutions were contained in screw thread amber glass bottles and stored at −20 °C.

2.4. Sample Preparation

Six kinds of aquatic samples (green pond frog, eel, grass carp, crayfish, white shrimp, and soft-shelled turtle) were collected from Baishazhou agricultural and sideline products market (Wuhan, China). The eatable tissues were taken and homogenized. The samples were stored at −20 °C.

Before analysis, the samples were thawed at room temperature. Two-gram samples were weighed into a 10 mL centrifuge tube. Briefly, 5 mL of 0.2% ammonia acetonitrile solution was added, then vortexed 30 s and ultrasonically extracted for 2 min. Next, 1.2 g $MgSO_4$ and 0.3 g NaCl were added. After vortexing, the sample was centrifuged at 5000 r/min for 5 min, and then the supernatant was transferred into a new 15 mL centrifuge tube. The residues were re-extracted with 5 mL of 0.2% ammonia acetonitrile solution, and the obtained upper layer was combined, and then 2 mL n-hexane saturated with acetonitrile was added into the tubes and then vortexed 30 s. After centrifugation at 5000 r/min for 5 min, the hexane layer was removed. The tubes filled with extract solution were condensed to dryness by a gentle nitrogen stream at 50 °C. Afterward, 100 μL of derivatization reagent A (N-methyl imidazole and acetonitrile (1:1, v/v)) and 150 μL of derivatization reagent B (trifluoroacetic anhydride and acetonitrile (1:2, v/v)) was added into the tubes. After vortex-mixing evenly, derivatization reaction lasted for 20 min at room temperature. Subsequently, the derivative was reconstituted by 1 mL acetonitrile. Finally, the obtained solution was filtered by 0.22 μm nylon syringe filter for UHPLC-FLD analysis.

2.5. Chromatographic Conditions

The analytical compounds were separated on an Infinity Lab Poroshell 120 EC-C18 column (2.7 μm, 100 mm × 4.6 mm) (Agilent, PaloAlto, CA, USA) at 40 °C. The injection volume was 20 μL. The detector was operated at an excitation wavelength of 245 nm and emission wavelength of 365 nm, respectively. The mobile phase was consisted of water (A) and acetonitrile (B) and applied at a flow rate of 1 mL/min. The chromatographic separation was carried out using gradient elution, the gradient elution program for AVMS separation was shown in Table 1, starting from 94% of eluent B, increased at 99% in 6 min and then kept stable for further 2 min, subsequently, decreased at 94% and re-equilibration time was 5 min, giving a total run time of 13 min.

Table 1. Gradient elution program for AVMS separation.

Time (min)	Water (%)	Acetonitrile (%)
0.0	6	94
6.0	6	94
6.1	1	99
8.0	1	99
8.1	6	94
13.0	6	94

2.6. Method Validation

Validation characteristics, including linearity, recovery, and precision were performed according to FDA guidelines for the chromatographic method [40].

Standard calibration curves for AVMs were constructed by plotting AVM's peak areas versus seven concentrations (5, 10, 50, 100, 200, 500, and 1000 μg/kg) of AVMs in acetonitrile, respectively.

Recovery was determined by analyzing samples spiked at four levels (5, 10, 50, and 200 μg/kg) in six blank replicates, and recoveries were determined by comparing the peak areas of the analytes extracted from the samples with those of the target compounds in the standard solutions.

Precision, including intra-day precision and inter-day precision, was evaluated by relative standard deviation (RSD). As for intra-day precision, samples tested three times in a single day; for inter-day precision, samples were evaluated three times within three days.

When the signal-to-noise ratio (S/N) $\geq$ 3, the corresponding additive concentration was the LOD of the analytical method. When S/N $\geq$ 10, the corresponding additive concentration was the LOQ of the analytical method.

3. Results and Discussion

3.1. Optimization of Detection Wavelengths

Detection wavelengths (excitation wavelengths and emission wavelengths) are necessary parameters for fluorescence detection and can directly affect the sensitivity of detection. According to national standards [41–43] and previous studies [25–27,29,44], we make adjustments to the excitation wavelength and emission wavelength. Ultimately, according to the sensitivities and responses of target compounds, the excitation wavelength and emission wavelength of the target compounds were chosen as 245 and 365 nm, respectively.

3.2. Optimization of the Mobile Phase

The study used a gradient elution procedure, which started with 94% of acetonitrile, increased to 99% in 6 min, and was then kept stable for a further 2 min; it subsequently decreased at 94%, and re-equilibration time was 5 min.

Several mobile phases were tested: (1) MeOH:ACN (1:1, *v*/*v*) with 0.1% formic acid [45], (2): acetonitrile and aquatic 0.1 mM ammonium format containing 0.1% formic acid [30], (3): 10 mM TEA aqueous solution and acetonitrile [14], and (4): acetonitrile and water.

Because of the complex composition of these mobile phases and the appropriate peak shapes and peak areas, (4): acetonitrile and water were selected. The influence of different mobile phase additives on the peak area of 100 μg/kg standard AVMs was illustrated in Figure 1.

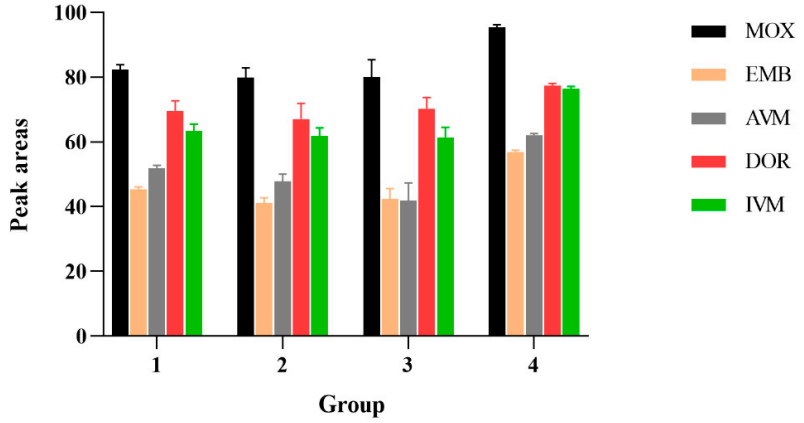

Figure 1. The influence of different mobile phase additives on the peak area of 100 μg/kg standard AVMs. 1: MeOH: ACN (1:1, *v*/*v*) with 0.1% formic acid; 2: acetonitrile and aquatic 0.1 mM ammonium format containing 0.1% formic acid; 3: 10 mM TEA aqueous solution and acetonitrile; 4: acetonitrile and water.

When acetonitrile accounted for 90% of the mobile phase, the peak was unstable; when the mobile phase ratio was 99:1, the baseline chromatogram was stable, the analysis time was shortened, and the separation effects of each target were optimal. Compared to the isocratic elution procedure, high responses and sharp peak shapes were obtained from gradient elution procedures.

3.3. Optimization of Column Temperature

The analytes were commonly separated on a C_{18} column [39,45–47]. In our study, the same stationary column was used. Proper column temperature can reduce the separation time of the target analytes and optimize the peak shapes. In this study, the chromatographic peak shapes of five target compounds were tested at different chromatographic column temperatures (30 °C, 35 °C, and 40 °C), the result is shown in Figure 2, the retention times are listed in Table 2 and the tailing factors are listed in Table A1 (Appendix A). When the column temperature was 30 °C, the retention times of the target compounds were delayed. When the column temperature was set to 35 °C or 40 °C, the peak shape of the target analytes was improved, and the separation of the peaks was affected. When the temperature increased to 40 °C, earlier retention time, complete separation, and no interaction between compounds were obtained. Ultimately, considering excessive temperature would cause irreversible damage to the column, a column temperature of 40 °C was selected in this study.

3.4. Optimization of Sample Preparation

AVMs were mainly extracted by acetonitrile [28,30,39,48–50]; based on the previous research, four different solutions were tested in order to optimize the extracts of the selected compounds: 1: acetonitrile; 2: 0.2% ammonia acetonitrile solution; 3: acetyl acetate; 4: acetonitrile: ethyl acetate (1:1, *v*/*v*). The result is shown in Figure 3; the drug recovery rates were 47.11% to 116.43% by extracted with acetonitrile, 75.65% to 114.39% through using 0.2% ammonia acetonitrile solution, 16.84% to 44.08% by extracting with acetonitrile: ethyl acetate (1:1, *v*/*v*) and when ethyl acetate was used as the extracting agent, the recovery rate of AVM, IVM, MOX, and DOR reached 32.34% to 44.49%, and the EMM recovery rate was only 3.94% to 5.47%. In conclusion, 0.2% ammonia acetonitrile solution as extractant has a higher recovery rate than other extractants; therefore, 0.2% ammonia acetonitrile solution was finally selected as the extractant.

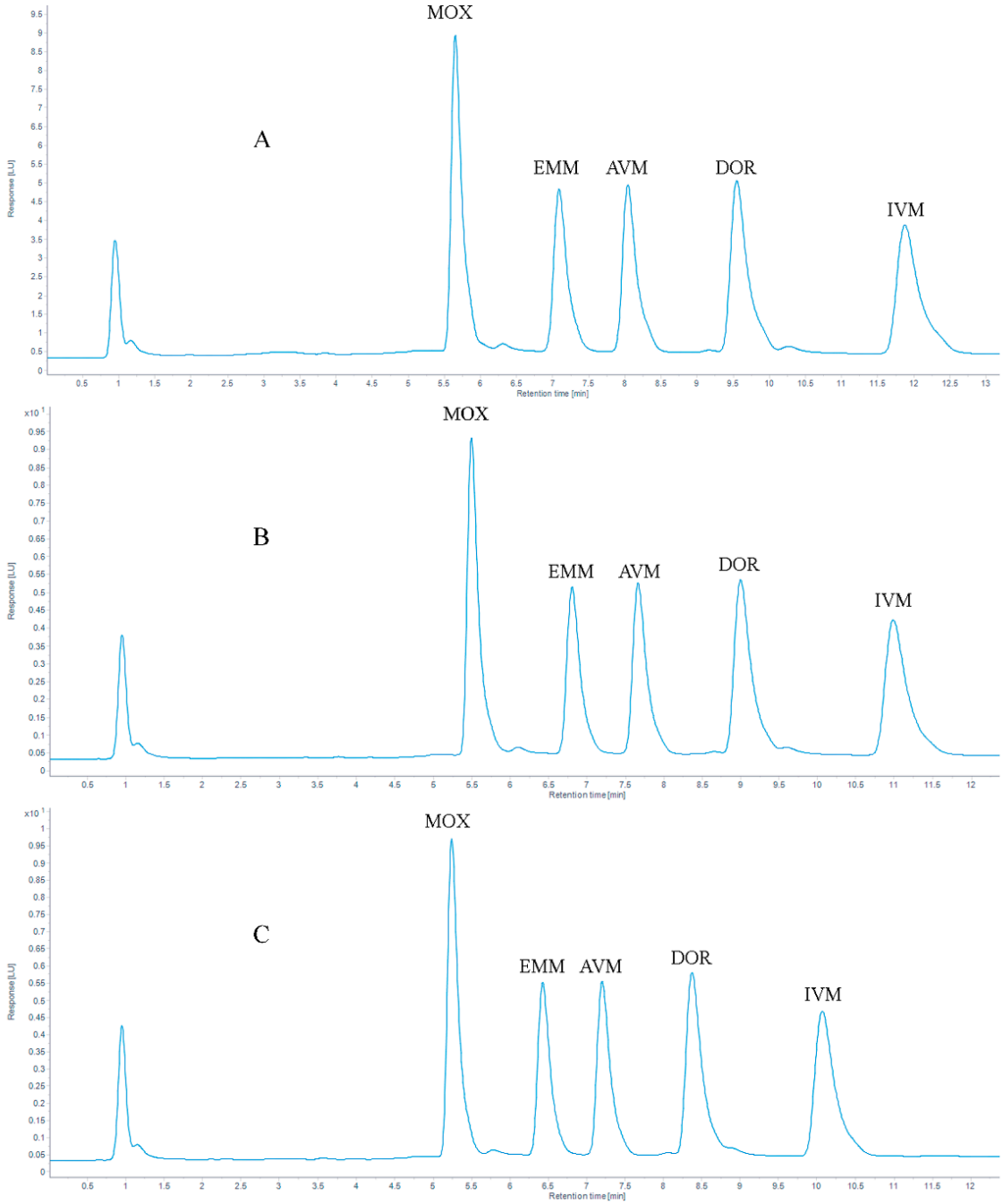

Figure 2. Chromatogram of 100 μg/kg AVMs standards at (**A**) 30 °C, (**B**) 35 °C and (**C**) 40 °C.

For the simultaneous determination of organic pollutants in aquatic products, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, and 0.5 g disodium citrate sesquihydrate were added to samples [51]. A total of 2 g $MgSO_4$ and 2 g anhydrous sodium sulfate were used for the simultaneous determination of AVMs in aquatic products [13]. For animal samples, 3 g NaCl was used for the determination of AVMs [18].

Refereed to the relevant literature, we added 3 g $MgSO_4$ with 1 g NaCl; however, the extractant was significantly reduced. Subsequently, we adjusted the amount of $MgSO_4$ and NaCl, 1.2 g $MgSO_4$ with 0.3 g NaCl was added. The results showed no significant reduction in extractant and good drug recoveries; therefore, the combination of 1.2 g $MgSO_4$ with 0.3 g NaCl was selected as a purifying agent.

Regarding derivatization reaction time, some studies have shown that the best derivatization reaction time was 15 min [34,35,52]. In addition, AVMs in milk samples were taken

30 min at 70 °C [25], and announcement No. 1025-5-2008 of the Ministry of Agriculture and Rural Affairs (MARA) mentioned that AVMs in the bovine liver, muscle, and porcine liver were determined and their derivatization reactions were carried out at 96 °C for 100 min [43]. In another study, AVMs in cattle, sheep, and swine muscle were derived for 60 min at 67 ± 2 °C [53].

Table 2. The retention time of 100 μg/kg AVMs standard at 30 °C, 35 °C and 40 °C.

Compound	Retention Time (min)		
	30 °C	35 °C	40 °C
MOX	5.64	5.49	5.20
EMM	7.10	6.82	6.49
AVM	8.05	7.68	7.21
IVM	9.56	9.01	8.48
DOR	11.88	10.99	10.17

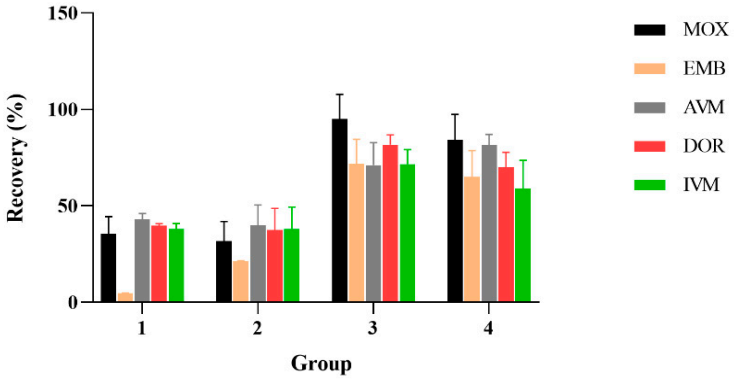

Figure 3. Effect of different extract reagents on the recoveries of the five AVMs (n = 6). 1: extraction agent is ethyl acetate; 2: extraction agent is ethyl acetate mixed with acetonitrile (1:1); 3: extraction agent is 0.2% ammonia acetonitrile; 4: extraction agent is acetonitrile.

In order to screen out the optimal derivatization reaction time, the effect of derivatization reaction time on drug detection was examined using standard working solution samples configured at a concentration of 1 μg/mL for 10, 15, 10, 20, 30, 40, 50, and 60 min. The result was shown in Figure 4; the peak areas of all five drug-derived products were maximum at 20 min and remained constant for 1 h after the derivatization products. Therefore, the derivatization reaction was performed for 20 min at room temperature. Considering the stability of AVMs, the drug-derived reaction was performed in a brown inlet sample vial.

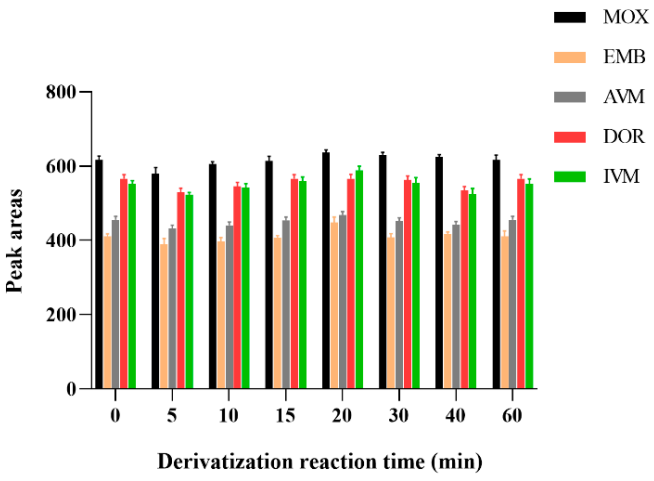

Figure 4. Effect of different derivatization reaction time on the recoveries of the five AVMs (n = 6).

3.5. Validation Results of the Method

The chromatograms of 100 μg/kg AVMs standards are shown in Figure 5. With mass concentration as the abscissa and peak area as the ordinate, the linear regression equations and coefficients of determination (R^2) of the standard working fluids are shown in Table 3. The results showed that the correlation coefficient (R^2) ≥ 0.999 in the concentration range of 5 to 1000 μg/kg.

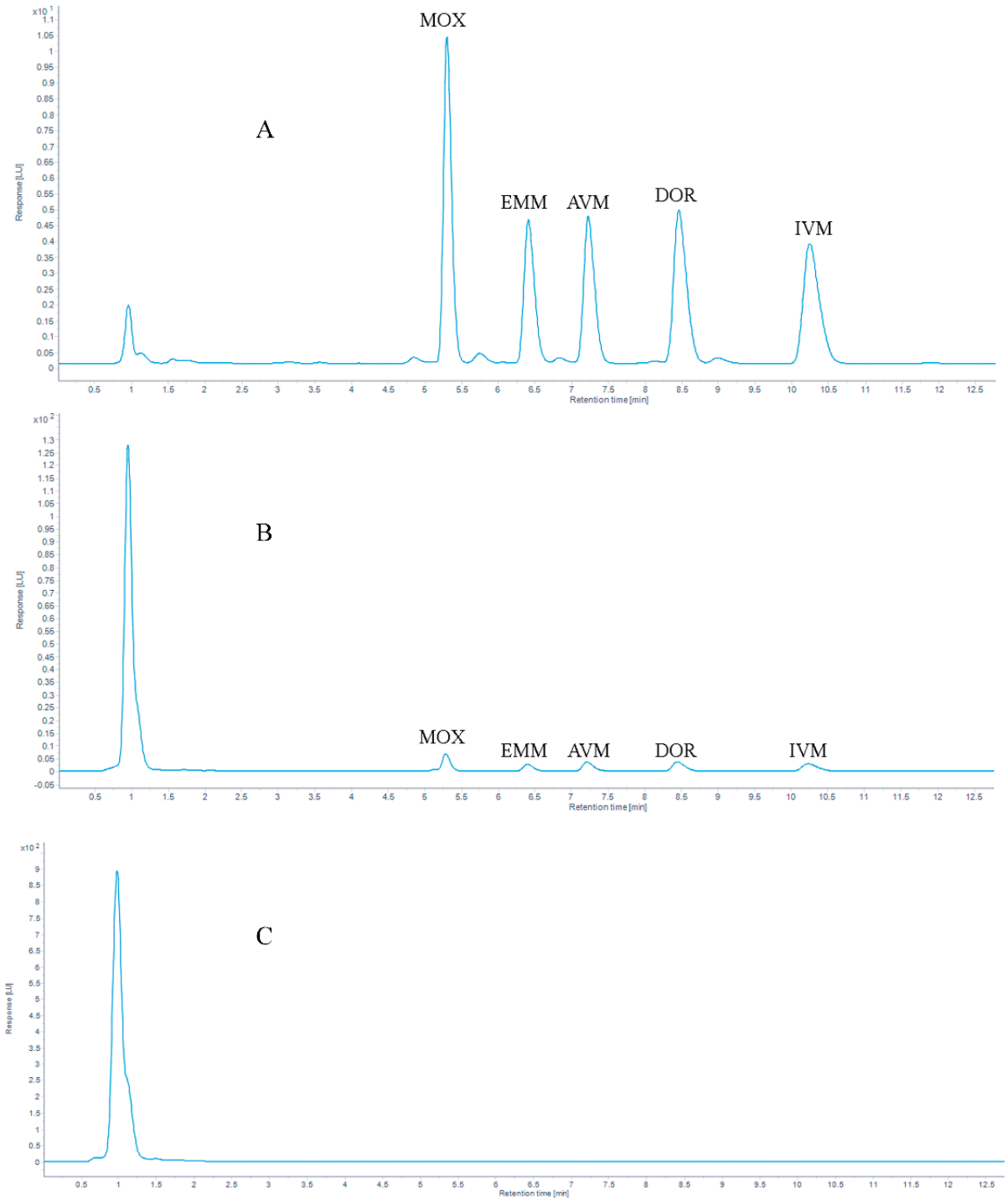

Figure 5. Chromatograms of (**A**) 100 μg/kg five AVMs standards, (**B**) 10 μg/kg five AVMs standards spiked in a grass carp muscle sample and (**C**) a blank grass carp muscle sample.

Table 3. Regression equations and correlation coefficients of five AVMs in matrix matching standard solutions.

Compound	Regression Equation	R^2	RSD (Intercept)	RSD (Slope)
MOX	A = 0.9544C + 3.0318	0.9995	3.3	0.008
EMM	A = 0.5618C + 1.037	0.9994	2.2	0.005
AVM	A = 0.6168C + 0.9645	0.9996	2.0	0.004
IVM	A = 0.7535C + 1.3696	0.9996	2.4	0.006
DOR	A = 0.7568C + 1.0593	0.9996	2.4	0.006

The LOQs (S/N $\geq$ 10) and LODs (S/N $\geq$ 3) of AVMs were examined. The results are listed in Table 4. These values are lower than the LOQ values obtained in previous studies [54–57]; the summary of date validation obtained by others authors is listed in Table 5.

Table 4. LODs and LOQs of five AVMs in matrix matching standard solutions.

Compound	LOD (μg/kg)	LOQ (μg/kg)
MOX	1.2	3.5
EMM	2.1	4.5
AVM	2.7	5.0
IVM	1.8	4.5
DOR	2.7	5.0

Table 5. Summary of dates validation obtained by others authors.

Compound	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%)	Reference
AVM, IVM and DOR	1.7–2.5	5.6–6.5	84.9–114.6	[54]
AVM, IVM and DOR	1.1–4.5	5.0	76.0–105.0	[55]
AVM, IVM, DOR and EPR	5.0	10.0	87.2–101.4	[56]
IVM	2.5	5.0	37.0–79.0	[57]
AVM, IVM, DOR, MOX, EPR and EMM	–	5.0	65.0–89.0	[44]
AVM, IVM, DOR and MOX	1.2–2.7	3.5–5.0	85.38–118.4	Our study

The results of the recovery and precision are listed in Tables 6–8. When the concentrations of AVMs were added to blank samples at 5, 10, 50, and 100 μg/kg levels, the recovery rates of the AVMs were $\geq$ 85.38%, and the RSDs were $\leq$13.62%.

Table 6. Recoveries of five AVMs in aquatic products (n = 6).

Aquatic Products	Concentration (μg/kg)	Recovery (%)				
		Moxidectin	Emamectin	Avermectin	Doramectin	Ivermectin
Pond green frog	5	85.4	97.2	89.0	90.2	90.4
	10	87.1	89.9	90.0	89.1	86.0
	50	90.1	87.8	86.3	88.2	91.2
	100	88.9	88.8	86.4	95.0	90.8
Eel	5	88.9	92.1	86.8	88.4	91.5
	10	87.2	87.1	89.2	96.2	94.8
	50	86.9	90.8	92.5	93.1	89.3
	100	118.4	105.0	86.9	87.4	101.9
Grass carp	5	89.6	93.7	92.7	98.5	92.9
	10	86.3	89.7	91.1	88.8	97.9
	50	90.4	93.4	88.4	92.0	91.5
	100	85.5	88.3	89.8	87.5	86.8

Table 6. *Cont.*

Aquatic Products	Concentration (μg/kg)	Recovery (%)				
		Moxidectin	Emamectin	Avermectin	Doramectin	Ivermectin
White shrimp	5	87.9	93.8	92.3	88.5	92.4
	10	87.2	91.6	93.3	90.6	93.6
	50	87.9	85.8	93.0	88.6	89.4
	100	87.3	86.7	90.8	95.0	93.8
Crayfish	5	90.4	94.7	90.0	98.5	94.0
	10	87.2	90.3	93.8	86.8	92.2
	50	93.0	85.8	93.1	87.5	89.5
	100	89.6	86.2	88.9	92.0	90.4
Soft-shelled turtle	5	90.6	89.5	90.4	94.5	85.6
	10	106.5	107.5	100.3	89.5	106.5
	50	90.0	93.2	94.9	91.4	90.0
	100	90.8	88.8	97.3	85.7	90.8

Table 7. Intra-day precision of five AVMs in aquatic products (n = 6).

Aquatic Products	Concentration (μg/kg)	Intra-Day Precision (CV, %)				
		MOX	EMB	AVM	DOR	IVM
Pond green frog	5	3.0	4.3	7.9	5.0	4.9
	10	2.7	1.4	2.8	2.3	2.0
	50	1.4	2.6	3.9	2.0	3.2
	100	1.8	2.5	1.1	1.6	2.5
Eel	5	1.1	2.1	3.2	2.9	1.3
	10	3.5	3.1	3.0	1.3	4.7
	50	1.5	1.1	4.3	4.7	2.4
	100	3.5	6.4	6.0	3.6	2.9
Grass carp	5	1.5	2.9	3.0	1.7	2.6
	10	4.1	6.9	5.6	5.3	3.0
	50	2.4	8.2	6.1	6.6	6.7
	100	4.1	5.3	1.3	5.2	5.5
White shrimp	5	1.0	1.5	0.2	1.5	1.2
	10	9.8	2.3	4.1	1.4	1.0
	50	3.8	4.6	4.7	3.7	6.7
	100	4.1	2.6	3.1	5.8	1.9
Crayfish	5	1.2	4.4	1.5	1.3	2.1
	10	2.0	2.6	5.0	5.1	3.6
	50	3.6	1.4	6.1	1.6	4.6
	100	8.3	2.1	1.7	8.8	4.4
Soft-shelled turtle	5	1.6	3.5	2.2	1.9	2.2
	10	1.3	2.9	3.4	3.3	3.7
	50	5.4	4.6	3.2	6.1	3.2
	100	3.4	4.1	3.4	2.9	3.6

Table 8. Inter-day precisions of five AVMs in aquatic products (n = 6).

Aquatic Products	Concentration (μg/kg)	Inter-Day Precision (CV, %)				
		MOX	EMB	AVM	DOR	IVM
Pond green frog	5	8.2	11.8	12.4	5.1	7.4
	10	7.5	6.1	4.2	5.0	4.0
	50	12.7	7.7	7.0	7.6	4.4
	100	6.8	8.3	2.3	5.3	3.8

Table 8. *Cont.*

Aquatic Products	Concentration (μg/kg)	Inter-Day Precision (CV, %)				
		MOX	EMB	AVM	DOR	IVM
Eel	5	3.5	2.7	6.1	4.8	4.9
	10	10.8	7.6	6.8	8.3	6.6
	50	2.8	4.3	1.1	10.9	11.4
	100	6.6	4.1	8.6	5.3	6.6
Grass carp	5	4.3	6.3	5.4	4.1	5.7
	10	7.1	11.3	12.5	7.9	8.6
	50	7.4	6.0	10.4	8.7	12.0
	100	9.0	13.2	11.4	10.0	12.1
White shrimp	5	6.1	13.9	7.4	6.8	6.6
	10	8.6	4.9	9.9	11.4	10.3
	50	10.2	12.8	10.7	13.2	10.5
	100	7.2	10.9	5.0	6.9	9.0
Crayfish	5	5.4	13.6	10.1	5.0	8.2
	10	8.4	3.4	7.9	9.2	9.3
	50	10.2	12.9	10.8	12.5	10.6
	100	6.1	10.8	4.7	7.5	8.9
Soft-shelled turtle	5	4.0	5.1	3.3	2.9	3.4
	10	4.4	13.2	8.7	6.8	9.5
	50	5.6	12.3	8.6	13.0	3.8
	100	4.4	11.9	10.1	7.2	12.2

4. Real Sample Analysis

To evaluate the feasibility and applicability of the present method, 72 samples, including common frogs (pond green frog and bullfrog), crustaceans (proto-shrimp, white shrimp), and freshwater farmed fish (crucian carp, grass carp, yellow jawed fish, silver carp, carp, bighead carp, bluefish, and loach) sold in Baishazhou agricultural and sideline products market (Wuhan, China). AVMs residues were not detected in most of the samples; however, AVM was detected in grass carp, ranging from 5.59 to 18.04 μg/kg, which is listed in Table 9. AVMs residues were not detected in the actual samples in other studies [13,34]. The main reason may be that AVMs are highly toxic to fish, and the concentration should be kept at a low level in use.

Table 9. Data of real sample analysis (n = 6).

Grass Carp	Avermectin (μg/kg)	Intra-Day Precision (CV, %)
1	5.59	3.1
2	16.25	1.0
3	10.40	3.2
4	18.04	1.3
5	7.85	2.5
6	9.39	1.4

5. Conclusions

A fast and effective sample preparation method coupled with the UHPLC-FLD method was established for the detection of multiple residues of AVMs in multi-kinds of aquatic products. The extraction method exhibited a good linear relationship ($R^2 \geq 0.999$), the obtained recoveries were all greater than 85.38%, and the RSDs were ≤13.62%. This method was found to have relatively high sensitivity, great precision, and a short detection time. Moreover, the newly developed method was successfully applied to the analysis of real samples, which proved the applicability of this method.

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Institutional Review Board Statement: The animal study protocol and care of animals were approved by the Fish Ethics Committee of Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China (ID 2021-Liu Yongtao-03).

Informed Consent Statement: The study did not involve humans.

Data Availability Statement: Not applicable.

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

Appendix A

Table A1. The tailing factor of 100 µg/kg AVMs standard at 30 °C, 35 °C and 40 °C.

Compound	Tailing Factor		
	30 °C	**35 °C**	**40 °C**
MOX	1.25	1.17	1.14
EMM	1.14	1.15	1.09
AVM	1.17	1.12	1.07
IVM	1.24	1.22	1.14
DOR	1.25	1.21	1.15

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