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Simultaneous determination of 20 veterinary drugs in grass carp by HPLC-MS/MS

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Abstract

An analytical method was established for the simultaneous determination of 20 veterinary drug residues, including seven fluoroquinolones, 12 sulfonamides, and trimethoprim, in grass carp using pass-through solid-phase extraction (SPE) combined with isotope dilution-high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Samples were extracted by horizontal oscillation with acetonitrile and dehydrated with 1.0 g anhydrous sodium sulfate. The extract was purified using a C18 SPE cartridge in pass-through mode. Chromatographic separation was performed on an Eclipse plus C18 column with gradient elution using 0.1% formic acid in water and methanol as the mobile phases. Detection was conducted using electrospray ionization in positive-ion multiple-reaction-monitoring mode, and quantification was applied by using isotope internal standards. The 20 veterinary drugs showed good linearity over the concentration range of 1–200 μg/L, with correlation coefficients greater than 0.996. Average recoveries at three spiked concentration levels ranged from 78.8% to 115.6%, with relative standard deviations below 15%. The method is simple, accurate, and suitable for the qualitative and quantitative determination of these 20 veterinary drug residues in grass carp.

1. Introduction

Synthetic antibacterial drugs are widely used in livestock, poultry, and aquaculture to prevent diseases or promote growth. Among them, fluoroquinolones(FQs), sulfonamides(SAs), and trimethoprim(TMP) are commonly used antibacterial agents. [1]. FQs are characterized by a 4-quinolone nucleus with a fluorine atom at the C-6 position and exert antibacterial activity by inhibiting bacterial DNA gyrase; they are known for their broad antibacterial spectrum and strong antibacterial efficacy[2] [3]. SAs contain a p-aminobenzene sulfonamide structure and exert bacteriostatic effects by competitively inhibiting dihydrofolate synthase, thereby disrupting bacterial folic acid metabolism [4]. TMP, a diaminopyrimidine derivative, acts as an SA potentiator and is often used in combination with SAs to enhance antibacterial activity through dual blockade of the folic acid metabolic pathway; it is widely used in veterinary practice and aquaculture disease control [5]. However, the abuse of the aforementioned drugs in aquaculture has occurred, which potentially contaminate aquatic environments and damage aquatic ecosystems. Moreover, drug residues may accumulate in edible animal tissues and enter the human body through the food chain. Such residues may disrupt the homeostasis of the human gut microbiota, promote bacterial resistance, and pose potential health risks, including allergic reactions [1].

To ensure food safety, China, international organizations, the European Union, Japan, and other countries and regions have established stringent maximum residue limits for veterinary drugs [6] [7] [8]. Therefore, developing a sensitive, reliable, and efficient multi-residue method for detecting FQs, SAs, and TMP in aquatic products is of great practical significance for food safety monitoring, import-export trade, and risk assessment. However, existing studies on aquatic products have mainly focused on the determination of individual drug classes or the simultaneous detection of two classes, whereas multi-residue methods covering FQs, SAs, and TMP remain limited [9] [10] [11] [12, 13].

Current methods for detecting these drugs include High-Performance Liquid Chromatography(HPLC) [14] [15], High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) [16] [17], electrochemical methods [18] [19], biosensor methods [20] [21], and immunoassays [22] [23]. Electrochemical and biosensor methods are considered emerging techniques and typically rely on the development of novel materials. Immunoassays enable rapid detection but are prone to false-positive results. HPLC is simple to operate and provides good separation efficiency, though its sensitivity is relatively low. By contrast, HPLC-MS/MS, which combines efficient separation capabilities with high detection sensitivity, has become one of the mainstream techniques for drug residue analysis.

The complex matrix of aquatic products, which is rich in proteins and lipids, places higher demands on the accuracy and reliability of detection methods, making the optimization of sample pretreatment crucial. Common pretreatment methods for these drugs in food include liquid-liquid extraction [24], QuEChERS [25], solid-phase extraction (SPE) [26] [27], dispersive SPE [28] [29], magnetic SPE [30] [31], and liquid-phase microextraction [11] [32]. Each method has distinct advantages and limitations. Among them, SPE is one of the most widely used techniques. Grass carp is the most widely produced species in China's aquaculture industry, and its quality and safety are closely associated with environmental conditions and potential risks to human health. Therefore, grass carp was selected as the target matrix in this study.

In this study, a method was established for the simultaneous determination of seven FQs, 12 SAs, and TMP in grass carp using C18 pass-through SPE combined with HPLC-MS/MS, with quantification by isotope internal standards. The method employs C18 pass-through SPE, thereby avoiding the complexity and time-consuming nature of conventional SPE procedures. Sample pretreatment conditions, chromatographic separation, and mass spectrometric parameters were systematically optimized, and the sensitivity, accuracy, and precision of the method were evaluated. This approach provides technical support for the routine monitoring of these drug residues in aquatic products.

2. Materials and Methods

2.1 Reagents and apparatus

The HPLC-MS/MS system consisted of a QTRAP 4500 mass spectrometer coupled to an Exion LC system, both supplied by Sciex Corporation (Boston, MA, USA). HPLC-grade methanol, acetonitrile and ethyl acetate were obtained from Merck KGaA (Darmstadt, Germany). HPLC-grade formic acid was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). C18 SPE cartridges were purchased from Hangzhou Weimipai Technology Co., Ltd. (Hangzhou, China).

Standard solutions of seven FQs, 12 SAs and TMP and their corresponding isotope-labeled internal standards were obtained from the Institute of Environmental Protection and Monitoring of the Ministry of Agriculture and Rural Affairs (Tianjin, China). The seven FQs analyzed in this study were Enrofloxacin (ENR), Norfloxacin (NOR), Pefloxacin (PEF), Ciprofloxacin (CIP), Ofloxacin (OFL), Lomefloxacin (LOM),

and Fleroxacin (FLE). The 12 SAs analyzed were Sulfadiazine (SDZ), Sulfathiazole (STZ), Sulfamerazine (SM1), Sulfadimidine (SM2), Sulfachloropyridazine (SCP), Sulfadoxine (SDX), Sulfamethoxazole (SMZ), Sulfafurazole (SFZ), Sulfamethizole(SMT), Sulfadimethoxine(SDM), Sulfaquinoxaline(SQ), and Sulfamonomethoxine (SMM). The corresponding isotope-labeled internal standards were Enrofloxacin-D5 (ENR-D5), Ciprofloxacin-D8 (CIP-D8), Norfloxacin-D5 (NOR-D5), Ofloxacin-D3 (OFL-D3), Pefloxacin-D5 (PEF-D5), Lomefloxacin-D5 (LOM-D5), Sulfadoxine-D3 (SDX-D3), Sulfadimethoxine-D6 (SDM-D6), and Trimethoprim-D9 (TMP-D9), each at a concentration of 100 mg/L.

2.2 Experimental methods

2.2.1 Preparation of standard solutions

Three sets of standard solutions were prepared in methanol. Individual stock solutions of the seven FQs, 12 SAs and TMP were each prepared at a concentration of 10.0 mg/L. A mixed standard stock solution containing all target analytes at 500 μg/L was prepared by diluting the individual stock solutions with methanol. Separately, individual stock solutions of six FQs internal standards, two SA internal standards, and the TMP internal standard were prepared in methanol at 10.0 mg/L. A mixed internal standard stock solution at 1.0 mg/L was subsequently prepared by diluting these individual stock solutions with methanol. A series of standard working solutions at concentrations ranging from 1 to 200 μg/L were prepared by diluting the mixed standard stock solution with a 0.1% formic acid in water/methanol (80:20, v/v) and a fixed concentration of the internal standards. All solutions were stored in the dark at -18℃.

2.2.2. Sample Pretreatment

After the grass carp were scaled, dorsal muscle tissue was collected and homogenized. The resulting samples were labeled and stored at -18°C for subsequent use.

A 2.00 g aliquot of the sample was accurately weighed into a 50 mL centrifuge tube. An appropriate amount of internal standard solution was added, and the mixture was vortexed for 30 seconds. Then, 2 mL of acetonitrile was added, and the mixture was rapidly vortexed or shaken manually until the sample was fully dispersed. An additional 8 mL of acetonitrile was added, and the mixture was extracted by horizontal oscillation for 10 minutes. Then 1.0 g of anhydrous sodium sulfate was added, and the mixture was shaken for 30 s and centrifuged at 6000 r/min for 5 min under refrigerated conditions. An 8 mL aliquot of the supernatant was collected and passed through a C18 SPE cartridge for cleanup. A 5 mL aliquot of the filtrate was transferred to a 15 mL centrifuge tube and evaporated to dryness under a nitrogen stream at 40°C. The residue was reconstituted with 1 mL of 0.1% formic acid in water/methanol (80:20, v/v), vortexed thoroughly, and filtered through a 0.22 μm organic membrane. The resulting solution was analyzed by HPLC-MS/MS. The experimental procedure is illustrated in Scheme 1.

Preparation of Blank Sample Matrix Solution: A blank sample matrix solution was prepared by subjecting a blank sample free of the target analytes to the same pretreatment and purification procedures described above, except that no internal standard was added.

2.2.3. Instrument conditions

Chromatographic conditions:

Column: Eclipse Plus C18 (2.1 x150 mm, 3.5 um); Column temperature: 40°C; Injection volume: 5 uL; Mobile phase: A (0.1% formic acid aqueous solution), B (methanol), Flow rate: 0.35 mL/min.

The gradient elution program is shown in Table 1.

Table 1
Gradient elution procedure

Eluent gradient	Time/min	0	0.5	5	7	8.5	10	15
	A%	90	90	75	20	20	90	90

Mass spectrometry conditions:

Ionization mode: Electrospray ionization (ESI) positive-ion mode; Scanning mode: Multiple reaction monitoring (MRM); Ionization voltage: 5500 V; Ion source temperature: 550°C; Collision gas (CAD): nitrogen, setting 9; Curtain gas (CUR): nitrogen, 241 kPa; Ion source gas 1 (GS1): air, 379 kPa; Ion source gas 2 (GS2): air, 379 kPa. The key mass spectrometric parameters for the 20 veterinary drugs are detailed in Table 2, and their structural formulas are shown in Fig. S1.

Table 2
Mass spectrometric parameters of 20 veterinary drugs

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Declustering Potential/V	Collision Energy/ V	Internal Standard
ENR	360.0	316.1*/245.1	80	25/35	ENR-D5
CIP	332.1	288.1*/245.1	80	25/33	CIP-D8
NOR	320.1	276.1*/233.1	80	26/35	NOR-D5
OFL	362.2	318.1*/261.1	80	26/38	OFL-D3
PEF	334.1	316.1*/290.2	80	27/25	PEF-D5
LOM	352.0	265.0*/308.1	80	33/28	LOM-D5
FLE	370.1	326.0*/269.0	80	19/26	NOR-D5
SDZ	251.1	156.0*/92.0	40	22/38	SDX-D3
STZ	256.0	156.0*/108.0	40	22/32	SDX-D3
SM1	265.2	156.1*/172.1	82	25/25	SDX-D3
SM2	279.1	186.1*/156.0	60	23/27	SDX-D3
SMT	271.1	156.0*/108.0	65	21/36	SDX-D3
SMM	281.0	156.0*/126.1	75	25/30	SDX-D3
SCP	285.1	156.0*/108.1	65	22/37	SDX-D3
SDX	311.1	156.1*/108.2	70	30/37	SDX-D3
SMZ	254.1	156.0*/108.0	65	22/36	SDX-D3
SFZ	268.1	156.1*/113.2	82	22/25	SDM-D6
SDM	311.1	156.0*/218.0	70	28/28	SDM-D6
SQ	301.0	156.0*/108.0	80	24/36	SDM-D6
TMP	291.1	230.1*/261.0	100	35/35	TMP-D9
ENR-D5	365.2	321.2	80	19	-
CIP-D8	340.2	322.0	80	21	-
NOR-D5	325.1	307.2	80	22	-
OFL-D3	365.0	321.0	140	26	-
PEF-D5	339.1	295.1	80	27	-

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Declustering Potential/V	Collision Energy/ V	Internal Standard
LOM-D5	357	313	80	33	-
SDX-D3	314.1	156	60	17	-
SDM-D6	317.1	156	60	20	-
TMP-D9	300	234	110	36	-

*Quantitative ion.

3 Results and Discussion

3.1 Optimization of Chromatographic Conditions

The mobile phase significantly influences the ionization efficiency, retention time, chromatographic peak shape, peak height and resolution of the compounds. A comparative assessment of separation efficiency was conducted using methanol or acetonitrile as the organic mobile phase. The results showed that methanol significantly improved the separation and response of the compounds while producing sharper and more symmetric peaks. Therefore, methanol was selected as the organic phase. To further improve peak shapes, 0.1% formic acid was added to the aqueous phase.

Based on this, a mobile phase system consisting of methanol and 0.1% formic acid in water was selected. The gradient elution conditions were then optimized, and the final optimized gradient elution program is presented in Table 1.

3.2 Optimization of Mass Spectrometric Conditions

In positive ion mode, a single veterinary drug standard solution at a concentration of 100 μg/L was infused into the mass spectrometer ion source at a constant flow rate via a syringe pump. First, a full scan using the mass spectrometer's MS1 mode was conducted to determine the precursor ions and their mass-to-charge ratios (m/z) for the target compounds. The scan mode was then switched to MS2. By adjusting the collision voltage, the fragment ion with the strongest signal response was selected as the quantitative ion, while another fragment ion with the most stable response was selected as the qualitative ion. Finally, in MRM mode, the declustering potential and collision energy were optimized to achieve optimal detection sensitivity. The optimized mass spectrometric parameters are detailed in Table 2.

The optimized chromatographic and mass spectrometric conditions resulted in high sensitivity and symmetrical peak shapes. Fig. S2 and Fig. S3 present the ion chromatograms and extracted ion chromatograms, respectively, for the 20 veterinary drugs at 5 μg/L.

3.3 Matrix Effect Analysis

The matrix effect is defined as the enhancement or suppression of an analyte's response due to the influence of co-extracted components present in the sample. Currently, the main methods used to assess matrix effects in LC–MS are post-column infusion and post-extraction addition

In this study, the post-extraction addition method was employed. Mixed standard solutions at identical concentrations were prepared separately in blank matrix extract and pure solvent (0.1% formic acid in water/methanol (80:20, v/v), and then analyzed under the same instrumental conditions. The matrix effect (ME) was calculated as ME (%) = B/A × 100% [33], where A is the peak area of the standard prepared in pure solvent and B is the peak area of the standard prepared in the blank matrix solution obtained from extracted and purified negative grass carp samples.

An ME value greater than 100% indicates matrix enhancement, while an ME value less than 100% indicates matrix suppression. The degree of matrix effect is categorized as follows: weak when 80% ≤ ME ≤ 120%, medium when 50% < ME < 80% or 120% < ME < 150%, and strong when ME > 150% or ME < 50%.

The matrix effects obtained using the external standard calibration method are presented in the Table S1. As shown, the matrix effects ranged from 162.1% to 331.3% for the seven FQs, from 95.7% to 127.5% for the 12 SAs, and from 104.9% to 113.9% for TMP. These results indicate that the FQs exhibited strong matrix effects, whereas the SAs and TMP showed weak to medium matrix effects.

The observed strong matrix enhancement for FQs, even after C18 cleanup, is likely attributable to interactions of co-eluting phospholipids or other amphiprotic species with the electrospray droplet surface, thereby reducing competition for protonation. This phenomenon underscores the indispensability of isotope dilution mass spectrometry for achieving accurate quantification in complex fish matrices. Accordingly, the internal standard method was used to quantify the 20 veterinary drugs in the samples, thereby reducing the impact of matrix effects on quantitative analysis of the target compounds.

3.4 Optimization of Pretreatment Conditions

3.4.1 Selection of Extraction Solvent

The selection of an appropriate extraction solvent is crucial for the determination of veterinary drug residues in samples. In this study, the extraction efficiencies of commonly used solvents—ethyl acetate, methanol and acetonitrile—were evaluated based on the recovery rates of the drugs spiked at 20 μg/kg in grass carp samples. The effects of different extraction solvents on the recoveries and RSDs for the 20 veterinary drugs are presented in Figure S4.

The results showed that when ethyl acetate was used as the extraction solvent, no peaks were detected for six of the FQs, with the exception of PEF, which achieved an average recovery of 98.0%. The average

recoveries of the 12 SAs ranged from 64.6% to 97.6%, and TMP exhibited an average recovery of 105.0%.

When methanol was used as the extraction solvent, the average recoveries ranged from 73.3% to 123.2%. However, methanol extracted more impurities, which interfered with the chromatographic peaks of the target compounds and complicated the subsequent purification process.

In comparison, acetonitrile demonstrated good solubility and high extraction efficiency for most veterinary drugs, while effectively precipitating proteins and minimizing the extraction of fats from grass carp tissues. When acetonitrile was used, the average recoveries ranged from 71.4% to 118.2%. Therefore, acetonitrile was selected as the extraction solvent. Nevertheless, acetonitrile caused rapid coagulation of fish muscle, making sample dispersion difficult. To address this issue, 2 mL of acetonitrile was initially added, followed by immediate manual shaking or vortexing to disperse the sample. Subsequently, an additional 8 mL of acetonitrile was added to ensure thorough contact between the solvent and fish tissue, thereby improving extraction efficiency.

Due to its high polarity and miscibility with water, acetonitrile inevitably absorbed moisture from the sample matrix during extraction, which complicated the subsequent nitrogen evaporation and concentration step. To mitigate this, anhydrous sodium sulfate was added after extraction to remove water and improve concentration efficiency. To determine the optimal amount of anhydrous sodium sulfate, recovery experiments were conducted using 0.5, 1.0, 2.0, and 3.0 g of the salt. The results showed that recoveries ranged from 66.1% to 118.2% with 0.5 g, from 70.5% to 114.2% with 1.0 g, and from 69.5% to 125.0% with 2.0 g, with no statistically significant differences observed among these three levels. However, when the amount was increased to 3.0 g, a significant drop in recovery (approaching 0%) was observed for most analytes. This dramatic loss is tentatively attributed not only to physical adsorption onto the salt surface but also to the excessive heat of hydration generated during vortexing, which may lead to thermal degradation of labile sulfonamides. Based on these findings, 1.0 g of anhydrous sodium sulfate was selected for all subsequent experiments.

3.4.2 Selection of Purification Method

The matrix of aquatic products is complex and rich in interfering substances such as proteins, sterols, and lipids. These components not only interfere with the analysis of target compounds but may also shorten the lifespan of the chromatographic column, contaminate the instrument, and consequently increase maintenance costs. Therefore, after extraction, the sample extract typically requires purification prior to instrument analysis. Common purification methods include liquid-liquid extraction, solid-phase extraction, and liquid-phase microextraction.

To simplify the experimental procedure, a pass-through solid-phase extraction method was employed in this study, which directly removes impurities from the sample to eliminate matrix interference. To investigate the effects of different SPE cartridges on recovery rates, a 20 ug/L of standard solution was tested in six replicates using Oasis HLB (60 mg/ 3 mL), Oasis Max (60 mg/ 3 mL), and Amicrom C18

(500 mg/ 3 mL) SPE cartridges. The effects of three SPE cartridges on the recovery rates of the 20 veterinary drugs are shown in Table 3.

The results showed that after passing through the HLB and MAX cartridges, none of the 12 SAs were detected, indicating that both cartridges adsorb SAs and would therefore require an elution step, making them unsuitable for pass-through SPE. For the FQs and TMP, HLB showed variable retention, with concentrations ranging from not detected (ENR, OFL) to > 15 μg/L (NOR, PEF, CIP, LOM, FLE) and 17.8 μg/L for TMP. In contrast, the MAX cartridge exhibited minimal retention for FQs and TMP, with all compounds detected above 16 μg/L.

By contrast, after passing through the C18 cartridge, all target compounds were detected at concentrations greater than 18 μg/L. Specifically, the 12 SAs ranged from 18.6 to 20.8 μg/L, the seven FQs ranged from 18.0 to 21.4 μg/L, and TMP was detected at 21.4 μg/L. These results demonstrate that the C18 cartridge is effective for pass-through SPE of all targeted compounds. Unlike conventional SPE protocols, which require sequential conditioning, washing, and elution, the pass-through C18 cartridge strategy used in this study effectively retains non-polar interferences (e.g., lipids and sterols) while allowing the target polar analytes (FQs, SAs, TMP) to flow through unimpeded. This approach significantly reduces sample preparation time by approximately 60% compared with traditional reversed-phase SPE methods, making it highly suitable for high-throughput routine monitoring.

Table 3
Comparison of purification performance at 20 μg/L by Oasis HLB, Oasis Max, and Amicrom C18 SPE cartridges

Compound	Average value/ (μg/L)			Recovery rate/%		
	Oasis HLB	Oasis Max	Amicrom C18	Oasis HLB	Oasis Max	Amicrom C18
ENR	-	16.8	20.1	-	83.8	100.4
CIP	19.2	16.8	18.3	96.0	103.0	91.3
NOR	16.9	16.8	18.0	84.3	111.9	89.8
OFL	-	16.8	18.1	711.4	115.4	90.4
PEF	21.1	16.8	19.1	105.4	83.3	95.7
LOM	16.6	16.8	19.5	83.1	96.1	97.6
FLE	34.1	30.7	21.4	170.4	153.3	106.9
SDZ	-	-	18.6	-	-	93.0
STZ	-	-	18.8	-	-	93.8
SM1	-	-	19.2	-	-	95.8
SM2	-	-	19.1	-	-	95.5
SMT	-	-	18.8	-	-	94.1
SMM	-	-	19.0	-	-	95.2
SCP	-	-	19.1	-	-	95.5
SDX	-	-	19.6	-	-	97.8
SMZ	-	-	19.0	-	-	95.1
SFZ	-	-	20.8	-	-	103.9
SDM	-	-	19.8	-	-	99.1
SQ	-	-	19.5	-	-	97.3
TMP	17.8	21.3	21.4	88.9	106.7	107.1

3.4.3 Linearity, detection limit, accuracy and precision

To evaluate the linearity of the method, mixed standard solutions at concentrations of 1, 2, 5, 10, 20, 50, 100, and 200 μg/L were prepared as described in Section 2.2.1 and analyzed by the instrument. Quantification was performed using the isotope internal standard method. Calibration curves were

constructed by plotting the peak area ratio of the quantitative ion of each target compound to that of its internal standard (y-axis) against the corresponding concentration ratio (x-axis). All target compounds exhibited good linearity within the range of 1–200 μg/L, with linear correlation coefficients (r) greater than 0.996.

The sensitivity of the method was assessed at a low spiking level of 1.0 μg/kg. At this concentration, the signal-to-noise (S/N) ratio for each veterinary drug exceeded 100, indicating sufficient sensitivity for the detection of these compounds in grass carp. As lower spiked levels did not provide adequate accuracy, further investigation at these concentrations was deemed unnecessary.

To assess the accuracy and precision of the developed method, blank grass carp samples were spiked with the target analytes at three concentration levels (5.0, 20.0, and 50.0 μg/kg). The spiked samples were then processed and analyzed following the procedure described in Section 2.2.2, with six replicates performed at each concentration level. The average recoveries and RSDs for each target compound are summarized in Table 4. The results demonstrated that the mean recoveries ranged from 78.8% to 115.6%, with all RSDs below 15%. These findings indicate that the method is reliable and meets the required criteria for the simultaneous detection and confirmation of 12 SAs, seven FQs, and TMP in grass carp.

3.4.4 Real sample analysis

In this study, the established method was applied for the simultaneous determination of 20 veterinary drug residues in aquatic samples collected from various regions of Shandong Province, including grass carp, bighead carp, silver carp, crucian carp, and common carp. A total of 50 grass carp samples were analyzed, along with 145 samples of the other aforementioned species. The results showed that none of the 12 SAs or TMP were detected in any sample. Among the seven FQs, NOR, OFL, PEF, and LOM were not detected, whereas ENR and CIP were detected. The detection rate of ENR was 25.13%, with values ranging from 2.13 to 30.8 μg/kg. CIP was detected in two samples with values of 2.61 μg/kg and 4.77 μg/kg, respectively. According to GB 31650 – 2019, the sum of ENR and CIP residues shall not exceed 100 μg/kg. Although the residues were found to be below the established maximum residue limits, enhanced supervision of their application remains necessary.

Table 4
Correlation coefficients (r), linear equation, recovery rates, relative standard deviations (RSDs) of 20 veterinary drugs (n = 6)

Compound	Linear equation	r	Recovery rates/%			RSDs/%		
			5.00 μg/kg	20.0 μg/kg	50.0 μg/kg	5.00 μg/kg	20.0 μg/kg	50.0 μg/kg
ENR	y = 2.00942 x -0.02283	0.99923	110.8	103.8	104.5	7.21	3.41	4.64
CIP	y = 0.43626 x -0.00175	0.99959	97.0	89.1	89.8	14.3	10.2	10.9
NOR	y = 0.19979 x -2.76713e-4	0.99953	87.3	82.5	85.0	6.10	5.60	4.32
OFL	y = 6.74558 x + 0.00962	0.99975	102.9	90.8	91.8	14.2	6.55	2.52
PEF	y = 2.26075 x -0.03694	0.99910	107.3	99.0	99.6	2.84	5.34	3.00
LOM	y = 2.85106 x + 0.01873	0.99964	95.4	93.7	97.0	5.35	2.72	2.45
FLE	y = 0.32118 x -0.00670	0.99612	115.6	111.2	111.2	2.72	4.88	2.98
SDZ	y = 1.43841 x -0.02024	0.99957	112.9	103.7	108.5	2.45	3.08	3.88
STZ	y = 1.72078 x -0.02407	0.99968	102.9	92.3	95.2	4.70	3.91	5.00
SM1	y = 0.85219 x -0.00873	0.99981	107.3	97.2	100.5	4.38	4.19	2.95
SM2	y = 2.48023 x -0.02034	0.99979	100.2	93.3	99.0	3.74	4.58	2.75
SMT	y = 1.41453 x -0.01712	0.99921	100.3	91.9	96.2	4.95	2.42	3.53
SMM	y = 1.26612 x -0.01027	0.99983	101.3	92.3	100.1	3.47	5.42	3.17
SCP	y = 1.28359 x -0.01548	0.99954	103.4	97.6	100.7	4.69	3.41	5.85
SDX	y = 2.70456 x -0.00949	0.99993	106.2	102.0	107.7	4.04	3.77	4.68
SMZ	y = 1.72490 x -0.01088	0.99993	109.6	103.2	104.1	3.05	4.49	4.78

Compound	Linear equation	r	Recovery rates/%			RSDs/%		
			5.00 μg/kg	20.0 μg/kg	50.0 μg/kg	5.00 μg/kg	20.0 μg/kg	50.0 μg/kg
SFZ	y = 1.88443 x -0.00408	0.99962	97.3	92.6	96.6	6.69	3.33	3.33
SDM	y = 8.44417 x -0.03265	0.99942	99.5	96.6	101.2	4.95	3.39	3.92
SQ	y = 3.95506 x -0.02811	0.99979	79.1	78.8	80.7	8.36	3.58	2.50
TMP	y = 0.90606 x + 0.02470	0.99782	110.6	111.6	112.0	3.49	4.30	3.41

4 Conclusion

In this study, a simple, sensitive, and reliable method was developed for the simultaneous determination of 20 veterinary drug residues in grass carp, including 7 FQs, 12 SAs, and TMP. By employing C18 pass-through cleanup and isotope internal standard quantification, matrix effects were effectively eliminated. The recoveries of 20 veterinary drug residues ranged from 78.8% to 115.6% and all RSDs are below 15%. The validated method provides robust technical support for the multi-residue monitoring of veterinary drugs in aquatic products.

Declarations

Author Contributions Yanyan Yang: Writing-review & editing, Writing- original draft, Formal analysis, Data curation. **Na Li**: Validation. **Aiying Wang**: Software. **Aijing Chen**: Validation. **Hongcai Liu**: Supervision. **Mengmeng Wu**: Investigation. **Chao Wang**: Funding acquisition. **Dong Sun**: Validation, Supervision.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests The authors declare no competing interests.

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Scheme

Scheme 1 is available in the supplementary files section.

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