

Establishment and Validation of Anticoagulant Rodenticides in Animal Samples by HPLC-MS/MS, Focusing on Evaluating the Effect of Modified QuEChERS Protocol on Matrix Effect Reduction

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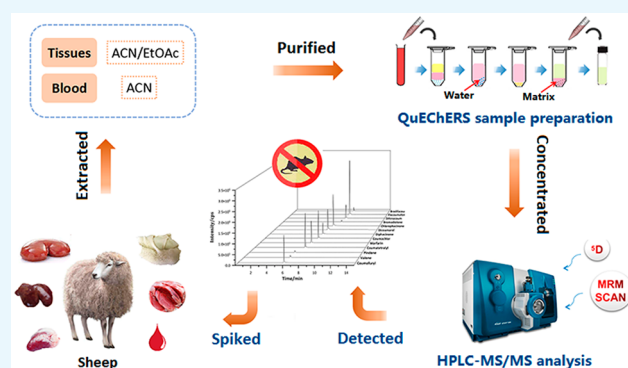


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ABSTRACT: A rapid, accurate, and selective analytical method to simultaneously quantify 13 anticoagulant rodenticides in animal biological samples was developed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) coupled with electrospray ionization (ESI) in negative mode. Samples were extracted and purified based on a modified QuEChERS (quick, easy, cheap, effective, rugged, safe) sample preparation technique. The sample pH and the type of extraction solvent and cleanup sorbent used to estimate the procedure's effectiveness were optimized. To improve the matrix effects and obtain acceptable recoveries for 13 rodenticides, 0.1 mL/g biological sample and 1 mL acetonitrile (or acetonitrile: EtOAc = 1:1/(v:v)) extraction followed by Florisil/HC-C18/anhidrous Na₂SO₄ (NaCl) cleanup under alkaline conditions was fully validated and shown to be selective, precise, accurate, and linear in the range from 1 to 100 ng/mL (g). The mean recoveries were between 52.78 and 110.69%, while the limits of detection and quantification ranged from 0.05 to 0.5 and 0.1–1 ng/mL (μ g/kg), respectively. Ideal soft matrix effects ($\leq 20\%$) were observed for the vast majority of rodenticides ($>95\%$) showing either suppression or enhancement. This method meets international criteria and is capable of simultaneously identifying and quantifying anticoagulant rodenticides in animal blood and tissues and can be suitable for the detection of poisoning cases in the field of forensic or public health.



1. INTRODUCTION

In recent years, incidents of livestock poisoning caused by accidental or deliberate poisoning with anticoagulant rodenticides (ARs) have occurred frequently, and illegal poaching using rodent poisoning for profit has also been reported from time to time.^{1–3} ARs can accumulate in cattle, sheep, and other livestock after ingestion of the drug, and when such livestock are processed into animal-derived foods such as meat products and dairy products, the resulting products may pose risks to human health.^{4,5} In addition, wildlife protection survey results also show that poisoning caused by this type of rodenticide is also an important cause of wildlife mortality. Target animals taking poisonous rodenticide bait and nontarget wildlife scavenging on carcasses containing high concentrations of poisons can both cause poisoning or death.^{6,7} Because of the wide variety of ARs, most of them are permitted for use in farmland, pasture, and even residential areas to eliminate the damage of voles and other rodents to cash crops and foods. ARs are commonplace chemicals that can be found in many homes, hardware stores, and big box stores and can be purchased in many forms, including blocks, pellets, and powders. Therefore, safety accidents involving such poisons

have the characteristics of concealment, suddenness, and complexity, and realizing timely and effective management and handling of AR poisoning is a great challenge. Currently, the presence of these chemicals in food and other wildlife samples has prompted warnings from the Centers for Disease Control (CDC) and Wildlife Conservation Associations of various countries.^{8–10}

There are two classes of ARs, hydroxycoumarins and indandiones, whose active constituents are 4-hydroxycoumarins and indandione derivatives, respectively (see structures in Supporting Information, Figure S1).^{11,12} These kinds of drugs can block the vitamin K epoxide reductase required to reduce vitamin K epoxide, an essential factor in the biosynthesis of clotting factors.^{10,12} In voles and other rodents, the synthesis of prothrombin is inhibited in the body, making the capillary wall

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brittle after taking the drugs, resulting in visceral coagulation disorders, hemorrhage, and even death after a few days.^{11,13,14} The representative types of commonly used indandione rodenticides include diphacinone, valone, and pindone.^{15,16} Hydroxycoumarin rodenticides are usually divided into two generations. The first generation is known as chronic drugs, mainly coumatetralyl, warfarin, coumachlor, and so forth, whose rodent-killing effect is slow and requires accumulation of several doses within a few days. The second generation, including bromadiolone, brodifacoum, and difenacoum, is a class of high-efficiency compounds that can kill rodents in a single use.¹⁴ ARs are characterized by low toxicity to humans while simultaneously having good rodent-killing effects.¹⁵ The highest concentration in human blood can be reached within a few minutes to 1 h after ingestion, and the clinical onset time is longer, usually more than 36 h.¹⁴ In animal experiments, the half-life period of warfarin in the blood of dogs is approximately 14 h, and those of diphacinone and brodifacoum are approximately 4.5 and 6 days, respectively.¹⁷ Therefore, the long half-life makes blood the first choice for AR poisoning detection.^{12,14}

At present, a number of analytical methods are used to detect ARs, including thin-layer chromatography,¹⁸ spectrophotometry,¹⁹ high-performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence detectors (FLDs),^{12,20} gas chromatography–mass spectrometry,^{21,22} and liquid chromatography–mass spectrometry (LC-MS).²³ However, these methods usually suffer from a lack of sensitivity and selectivity and complicated sample preparation at the same time, and the qualitative and quantitative accuracy is easily affected by instrument errors, which make it difficult to meet the rapid screening and trace detection requirements of ARs. In recent years, liquid chromatography coupled with electrospray ionization tandem mass spectrometry based on a triple quadrupole configuration (LC-MS/MS) has been widely developed and used in poisoning cases with the advantages of high instrument sensitivity, excellent specificity, speed and reliable detection results and ability to screen multiple ARs in complex samples at the same time.¹¹ Generally, cases involving AR poisoning mainly use the blood of freshly poisoned persons/animals as biological samples.^{24,25} However, the field survey results show that the poisoning or death of wildlife or livestock after being exposed to poisonous bait or animal carcasses containing ARs is often not detected in time.⁷ The symptoms of such drug poisoning are easily confused with some medical diseases.^{14,26} Therefore, mastering the detection methods of AR residues and specifically determining their content in animal tissues is of great significance for scientific investigation of the cause of animal poisoning/death and for biosafety and human health.

Previous studies have shown that it is necessary to consider the interference of impurities in the samples on the detection process of target compounds when using animal tissue as a biomaterial.²⁷ It is known that the water content of animal samples is approximately 70–80% in addition to a variety of proteins, carbohydrates, pigments, fats, and sterols. These components are the main interferences of the analyte in the sample preparation process and at the same time the main source of the matrix effect.²⁸ Their existence not only affects the detection results but also causes pollution to instruments with high sensitivity. Therefore, choosing an appropriate sample pretreatment method is a crucial step for the detection of target compounds in complex biological samples. Since the

QuEChERS (quick, easy, cheap, effective, rugged, safe) sample preparation technique was first proposed by Anastassiades et al. in 2003,²⁹ this method has been widely used in the field of multiresidue detection of foods and medicines because of its rapid, simple, environmentally friendly, and inexpensive qualities. With the progress and development of this method, researchers continue to improve the technology and apply it to the detection of ARs in food and animal plasma.^{11,30} At present, limited research reports on the extraction and purification of ARs from animal tissues by QuEChERS methodology have been published.³¹ There has been no systematic comparison of the extraction efficiency and matrix effect of common extraction solvents and cleanup sorbents.

This study aims to use sheep blood and tissue as experimental samples to improve the cleanup step to effectively improve matrix effects while maintaining ideal recoveries in the analysis of 13 ARs in sheep whole blood, heart, liver, kidney, muscle, and stomach wall based on a modified QuEChERS sample preparation method using high-performance liquid chromatography–triple quadrupole/linear ion trap tandem mass spectrometry (HPLC-QTrap-MS/MS). Purification steps based on different extraction solvents, dehydrants, d-SPE sorbent combinations and a step without cleanup were compared. By comparison, the extraction and purification steps for 13 ARs in blood and various tissues of sheep were obtained. In addition, this study minimized the matrix effect interference by optimizing instrument parameters, using isotope internal standards and matrix-matched calibration for AR analyses. The developed method was successfully applied to the determination of ARs and the forensic judgment of a case of goat poisoning in a pasture.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Methanol and acetonitrile (HPLC grade) were both purchased from Merck (Darmstadt, Germany). Ethyl acetate (EtOAc, HPLC grade) and diethyl ether (Et₂O, analytical grade) were both provided by Kemiou Chemical Reagent Co. (Tianjin, China). All other chemicals were obtained at the highest quality grade from commercial sources. Ammonium acetate (HPLC grade) and formic acid (analytical grade, 98% purity) were supplied by Fluka Chemical Co. (Bruchs, Switzerland). Analytical reagent-grade hydrochloric acid, sodium hydroxide, and inorganic salts of sodium chloride (NaCl), anhydrous sodium sulfate (Na₂SO₄), magnesium sulfate (MgSO₄), and sodium carbonate (Na₂CO₃) were all obtained from Kemiou Chemical Reagent Co. (Tianjin, China). Ultrapurified water was obtained from a Milli-Q Advantage Elix Essential 3.5.10.15 system (Merck KGaA, Darmstadt, Germany).

The QuEChERS sorbents (1) alumina-N (Al-N, 100–200 mesh), (2) HC-C18 (40–63 mesh), (3) graphitized carbon black (GCB, 120–400 mesh), (4) primary-secondary amine (PSA, 40–63 mesh), (5) polystyrene-divinylbenzene (PS-DVB, 80–160 mesh), and (6) classic Florisil diatomite (100–200 mesh) were supplied by CNW (Shanghai, China) and Aladdin Reagent Co. (Shanghai, China).

2.2. Standard and Working Solutions. The AR standards (coumafuryl, valone, pindone, coumatetralyl, warfarin, coumachlor, diphacinone, dicoumarol, chlorophacinone, bromadiolone, difenacoum, flocoumafen, brodifacoum) examined in our work were purchased from Cerilliant (Round Rock, AK, U.S.A.). Warfarin-D5 (100 μg/mL) used as an internal standard (IS) was provided by Cato (Guangzhou, China). The

Table 1. QuEChERS Steps for Sheep Biological Samples

serial number	step	blood	tissue
1	homogenization	directly homogenized using vortex	the tissue sample was placed in a tube containing porcelain bumping beads before adding 100 μ L of 0.1% formic acid water, and homogenized with a high-efficiency sample breaker at low temperature (4 $^{\circ}$ C)
2	add solvent	1 mL acetonitrile	0.5 mL acetonitrile + 0.5 mL EtOAc
3	pH regulation		≥ 9
4	extraction	cryogenic ultrasound 10 min	cryogenic ultrasound 5 min, and 1800 r/min oscillation for 5 min
5	centrifugation	centrifuged at 8000 r/min for 5 min, and transferred the supernatant to a new 2 mL centrifuge tube containing dehydrants and d-SPE sorbents	
6	dehydration	20 mg Na_2SO_4	20 mg Na_2SO_4 + 50 mg NaCl
7	purification	20 mg Florisil + 10 mg HC-C18	20 mg Florisil + 20 mg HC-C18
8	oscillation	2000 r/min for 10 min	
9	centrifugation	centrifuged at 8000 r/min for 5 min, and the supernatant was transferred to a new 5 mL glass tube	

Table 2. MRM Parameters and Rts of 13 ARs and IS

peak number	analytes	precursor ion (m/z)	product ion (m/z)	DP (V)	CE (eV)	Rt (min)
1	coumafuryl	297.1	161.1 ^a	-90.000	-24.000	5.84
			240.1	-90.000	-25.000	
2	valone	229.0	144.9 ^a	-90.000	-32.000	6.03
			116.0	-90.000	-50.000	
3	pindone	229.1	116.2 ^a	-90.000	-45.000	6.03
			172.0	-90.000	-28.000	
4	coumatetralyl	291.0	140.9 ^a	-90.000	-35.000	6.80
			274.1	-90.000	-30.000	
5	warfarin	307.2	160.8 ^a	-90.000	-26.000	7.06
			250.0	-90.000	-30.000	
6	coumachlor	341.0	161.0 ^a	-90.000	-28.000	7.49
			283.8	-90.000	-31.000	
7	diphacinone	339.1	167.1 ^a	-90.000	-30.000	7.59
			145.2	-90.000	-27.000	
8	dicoumarol	335.0	160.8 ^a	-40.000	-18.000	7.77
9	chlorophacinone	373.0	200.9 ^a	-90.000	-30.000	8.07
			145.2	-90.000	-35.000	
10	bromadiolone	525.1	273.1 ^a	-90.000	-48.000	8.84
			250.0	-90.000	-48.000	
11	difenacoum	443.3	135.0 ^a	-90.000	-43.000	9.01
			293.2	-90.000	-44.000	
12	flocoumafen	541.3	382.300 ^a	-90.000	-33.000	9.10
			161.100	-90.000	-45.000	
13	brodifacoum	523.1	80.9 ^a	-90.000	-94.000	9.32
			93.0	-90.000	-94.000	
IS	warfarin-D5	312.2	161.0 ^a	-90.000	-38.000	7.07
			255.0	-90.000	-38.000	

^aUsed as the quantitative ion.

purities of the standards were >99% (w/w), and they were used within established reanalysis dates. Standard stock solutions (100 μ g/mL) of 13 kinds of rodenticides were prepared separately in methanol or acetonitrile and stored in the dark at a temperature below -20 $^{\circ}$ C for at least 6 months. Mixed standard working solutions at a level of 1.0 μ g/mL were prepared by dilution of stock solutions in methanol. The IS working solution (1.0 μ g/mL) was prepared by dilution of warfarin-D5 (100 μ g/mL) in methanol. All of the standard working solutions were stored in the dark below 4 $^{\circ}$ C and prepared monthly when used.

2.3. Sample Preparation. In this study, we analyzed the extraction and detection methods of 13 ARs in sheep whole blood, heart, liver, kidney, muscle and stomach wall. Fresh sheep tissues/organs (heart, liver, kidney, muscle, stomach

wall) for the experiment were purchased from the Dongfa Farmers Market (Huoju Road), Rengcheng District, Jining City, Shandong Province from Jan 16 to Apr 20, 2021. Sheep whole blood was purchased from Solarbio (Beijing, China). All biological samples were stored at -20 $^{\circ}$ C until the time of testing.

All samples were extracted and purified with a modified QuEChERS method. First, the samples were defrosted, and 0.1 mL of blood or 0.1 g of tissue samples was accurately placed in a 5 mL centrifuge tube. The samples were spiked with 5 μ L of IS working solution (1 μ g/mL) to achieve an IS concentration of 5 ng/mL (spiked at 5 μ g/kg). Subsequently, the QuEChERS method was used to extract and purify sheep blood/tissue samples. The specific steps are shown in Table 1. After centrifugation, the supernatant was transferred into a 5

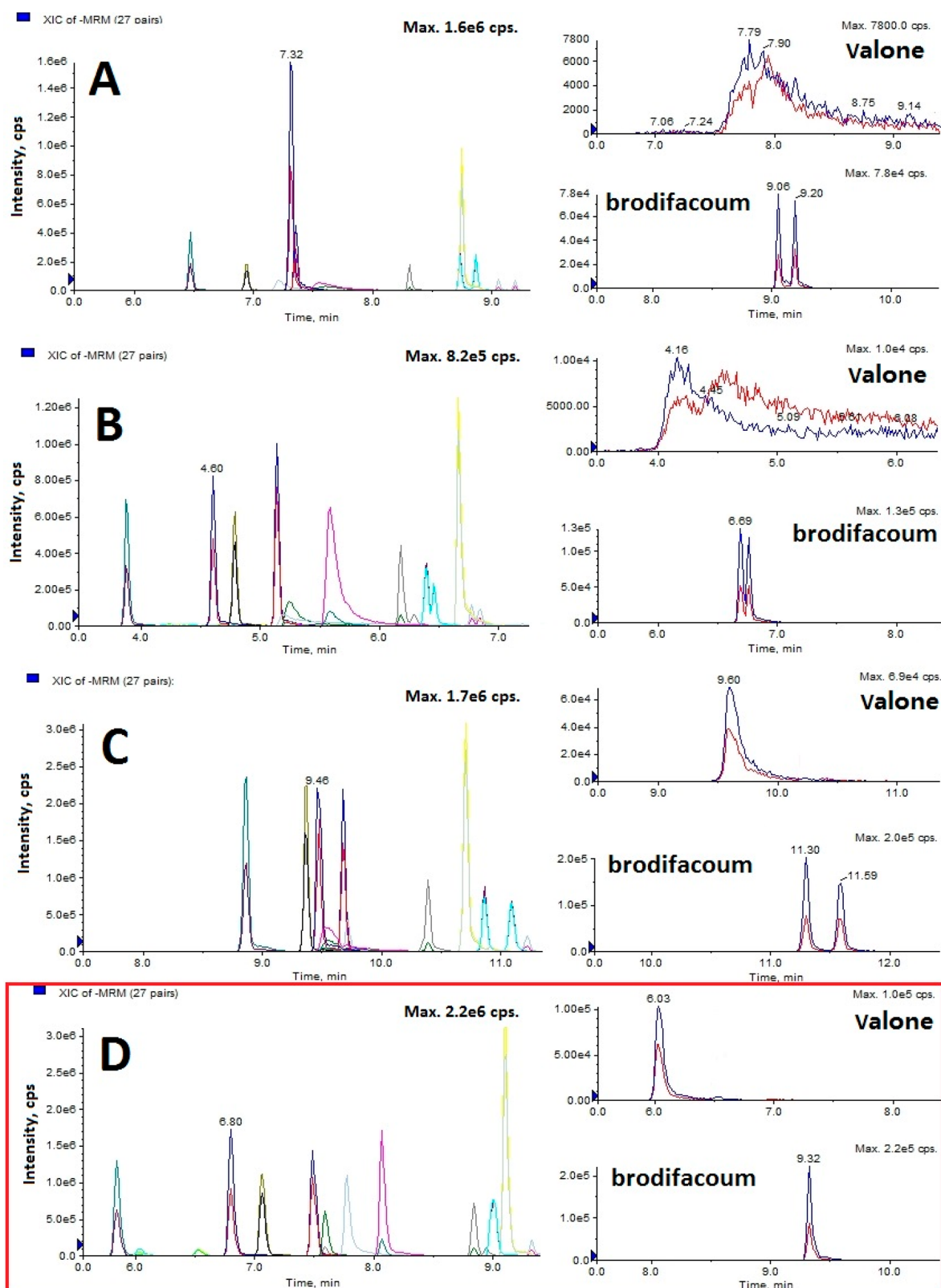


Figure 1. Chromatograms of 13 ARs using gradient elution with different mobile phases: (A) 5 mM ammonium acetate (0.1% formic acid)-acetonitrile, (B) 5 mM ammonium acetate-acetonitrile, (C) 5 mM ammonium acetate (0.1% formic acid)-methanol, and (D) 5 mM ammonium acetate-methanol. The injection concentration was 50 ng/mL.

mL glass tube, placed in a nitrogen stream at 60 °C and blown to near dryness. The residue was redissolved in 0.1 mL of methanol and filtered through a 0.22 μ m nylon filter into an autosampler vial for LC-MS/MS analysis.

2.4. LC-MS/MS Conditions. Chromatographic separation was carried out on a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) with a Kinetex Biphenyl 100 Å column (100 \times 3.0 mm, 2.6 μ m, Phenomenex, Torrance, CA, U.S.A.)

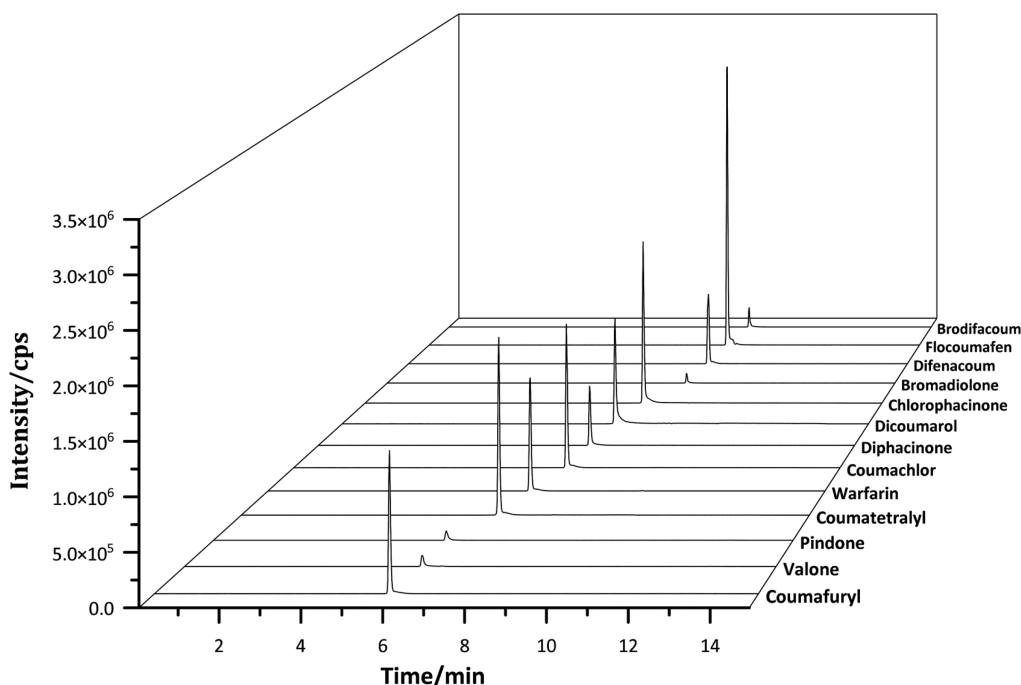


Figure 2. Extracted ion (quantitative) chromatograms of blank blood spiked with the 13 ARs mixed standard solutions at 25 ng/mL.

at 35 °C. On the basis of the findings of the optimization study, experiments used 5 mmol/L ammonium acetate buffer mixed with eluent A and methanol as eluent B. The gradient elution was as follows: 10% B at 0–0.5 min, 10–90% B at 0.5–9.0 min, 90% B at 9.0–12.5 min, 90–10% B at 12.5–12.6 min, and re-equilibration at 10% B for 1.4 min. The flow rate was 0.45 mL/min, and the sample injection volume was set at 2 μ L. The total run time for one injection is 14 min.

The effluent from the HPLC system was introduced into an API 5500 Qtrap-MS/MS (Applied Biosystems Sciex, Foster City, CA, U.S.A.) equipped with an electrospray Turbo spray interface operating in negative ion mode (ESI⁻). The detection conditions were optimized previously to afford the highest relative intensity: an ion spray (IS) voltage of -4500 V and a source temperature of 550 °C were applied. The curtain gas (CUR) was 30 psi, the ion source gas 1 (GS1) was 45 psi and the ion source gas 2 (GS2) was 30 psi. To optimize the declustering potential (DP) and collision energies (CE), a multiple reaction monitoring (MRM) scan mode was used with a dwell time of 25 ms. Two precursor/product ion transitions for each target compound were monitored in MRM mode, and both transitions were used for quantification and confirmation purposes (SANCO, 2011). Masshunter workstation software data Analyst 1.6.3 and MultiQuant 3.0.2 were used for data acquisition and processing. Precursor ions, product ions, retention times (Rt), and the optimum turning parameters (DP, CE) for target compounds are shown in Table 2.

2.5. Quality Assurance/Quality Control. Quality assurance and quality control of the analytical process were carried out using duplicates, method blanks, and matrix spikes. A procedural blank (one laboratory blank, one control blank spiked with IS only, and one standard-spiked matrix sample) was run before samples to assess for potential interference and cross-contamination from the procedure. During the injection time, the 13 target ARs were not detected in the procedural blanks. Three duplicate samples were set in each station, and

the Rts of the target compounds in parallel samples were within the tolerance range. The concentrations of 13 kinds of ARs in sheep blood and tissue samples were quantitatively determined by the isotope IS method using peak area, which was kept consistent in parallel samples.

2.6. Statistical Analyses. Data is presented as the mean \pm standard error (SE). The differences between the groups were analyzed by one-way analysis of variance (one-way ANOVA). If significant ($P < 0.05$) differences were found by the ANOVA test, the *t* test was used to determine pairwise differences between means. All statistical analyses were carried out using SPSS version 16.0.

3. RESULTS AND DISCUSSION

3.1. Optimization of LC–MS/MS Condition. To effectively separate the 13 target ARs with different structures and molecular weights, the mobile phase parameters were first experimentally optimized. In addition to affecting the retention time and peak shape of the target compound, the selection of the mobile phase also influenced the ionization of the analyte in the mass spectrum. Usually, adding an appropriate amount of alkaline substance to the mobile phase can increase the response value of the target in negative ion mode. Previous studies have shown that compared to ammonia, adding ammonium acetate to the aqueous phase (A) was more conducive to obtaining a good symmetrical peak shape in the detection of ARs.³⁰ On this basis, our study compared the difference between methanol and acetonitrile as the organic phase and the effect of adding formic acid (0.1%) to the mobile phase. More satisfactory sensitivity and peak shape for the target compounds could be obtained when methanol was used as the organic phase (especially for valone), and some compounds showed double peaks after adding formic acid (such as brodifacoum), as shown in Figure 1. Therefore, the A/5 mM ammonium acetate–B/methanol system was selected as the mobile phase in the experiment.

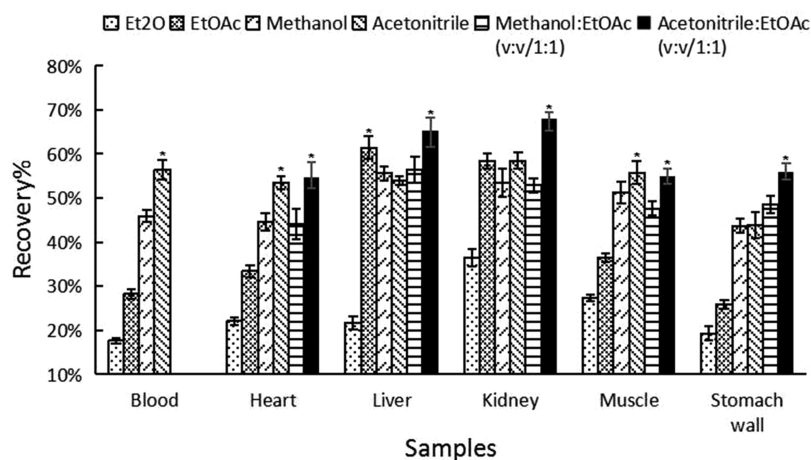


Figure 3. Average recoveries of 13 ARs in spiked biological samples with different solvents ($P < 0.05$). * Indicates a significant difference in observations between groups. Data is mean \pm SE ($n = 3$).

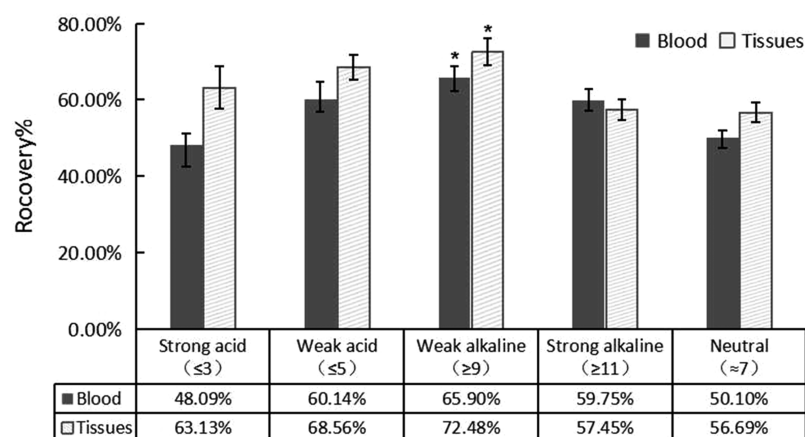


Figure 4. Average recoveries of 13 ARs in spiked samples under different pH conditions ($P < 0.05$). * Indicates a significant difference in observations between groups. Data is mean \pm SE ($n = 3$).

To optimize the mass spectrometric parameters of the 13 target ARs, a single standard solution (50 ng/mL) was injected into the ESI ion source continuously at a flow rate of 7 μ L/min with a flow syringe pump. Thirteen target compounds were analyzed by first-order mass spectrometry to obtain precursor ions (Q1) after optimizing the extraction voltage in ESI-detection mode. Then, the precursor ion was analyzed by secondary mass spectrometry (product ion scan) to obtain product ion information, and the DP and CE were optimized by using the multireaction monitor to maximize the response signal of the parent/product ion pairs. The optimized RT, precursor ions, and product ions as well as used DP and CE are listed in Table 2. The extracted ion (quantitative) chromatograms of blank blood spiked with the 13 ARs mixed standard solutions at 25 ng/mL are shown in Figure 2.

3.2. Optimization of the Sample Preparation Process.

On the basis of the classic pretreatment methodology QuEChERS, this study took sheep blood and tissues as the object by optimizing the sample pH, selecting extraction methods and solvents, and determining the effects and dosages of different dehydrating and purifying agents through single-factor experiments to explore the extraction and purification effect of this method and its influence on the matrix effect. The study established a set of rapid, low cost, and easy to use experimental parameters with good performance characteristics

suitable for a variety of biological matrices, which provides a reference for the rapid detection of multiple AR residues in organisms.

First, to weaken the interactions between analytes and the matrix, 0.1 mL of deionized water was added to the grinding flask containing 0.1 g of sheep tissue sample before extraction, and the tissue sample was thoroughly ground to ensure adequate partitioning. Previous research on pesticide determination methods in herbs has shown that adding an appropriate amount of distilled water to low-moisture samples before the extraction step can help increase the extraction efficiency,³² and this conclusion was also applied in this study.

The selection of an extracting solvent in the sample pretreatment process with a proper polarity to match the analyte was beneficial to improve recovery. The experiment compared the extraction efficiency of 13 ARs in sheep biological samples with four different organic solvents. One milliliter of Et₂O, EtOAc, acetonitrile, and methanol were selected in turn as solvents for 0.1 mL of spiked blood or 0.1 g of tissue samples, and the comparison results are shown in Figure 3. For blood samples, the extraction efficiency using either Et₂O or EtOAc was significantly lower than that of the other two solvents ($P < 0.05$). The average extraction efficiencies of Et₂O, EtOAc, acetonitrile and methanol for the 13 target compounds in spiked blood were 17.65%,

28.23%, 56.47%, and 45.93%, respectively. Therefore, acetonitrile was selected as the extraction solvent for blood samples. However, the extractability of the four organic solvents for the analytes in the five biological tissues was quite different. Among them, EtOAc had a higher ability for the liver and kidneys, and an ideal extraction effect was obtained when methanol or acetonitrile was used to extract the heart, muscle, and stomach wall samples. Therefore, the experiment then compared the extraction effect of the combination of an equal volume of EtOAc and acetonitrile or methanol and finally selected EtOAc/acetonitrile (v/v: 1:1) as the unified extraction solvent for the five biological tissues. The results showed that the combined effect of two solvents can generally improve the extraction efficiency of 13 ARs in the spiked tissue samples with a recovery rate ranging from 54.75% to 67.98% (Figure 3). In a previous study, Jia et al. studied the LC-MS/MS detection method of ARs in whole blood samples of poisoned patients and stated that compared with Et₂O, EtOAc, and acetone, when acetonitrile was used to extract blood samples it could effectively precipitate the proteins in the samples and reduce matrix interference.¹⁴ This conclusion was also confirmed in the present study. In addition, Cao et al. analyzed the screening methods of ARs in animal-derived foods and pointed out that pigments and other impurities in foods were easily extracted and absorbed by acetone and EtOAc, thereby enhancing the matrix effect, which could be effectively improved by adding acetonitrile or methanol.¹¹

Generally, relative to ionic compounds, molecular-type compounds are easier to extract effectively, and the higher the ionization degree of the compound is the more difficult the extraction is.³³ The pH of the solution affects the degree of hydrolysis and ionization of the compounds in the sample, which has a certain impact on the extraction efficiency. This study compared the recoveries of 13 ARs under neutral, acidic and alkaline pH conditions, and the results showed that the pH of the sample had a significant impact on the extraction efficiency, as shown in Figure 4. When the sample pH was neutral, the spiked recovery rate of most ARs was significantly lower than that under acidic and alkaline conditions, and the average recovery rate under strong acid–base conditions (pH ≤ 3 or pH ≥ 11) was generally lower than that under weakly acidic or basic conditions (pH ≤ 5 or pH ≥ 9) ($P < 0.05$). The extraction efficiencies of coumachlor, flocoumafen, and bromadiolone under weakly alkaline conditions (pH ≥ 9) were slightly lower than those under weakly acidic conditions (pH ≤ 5), except the recoveries of other target compounds were generally higher. As a class of weakly acidic compounds,³⁰ ARs under acidic conditions mainly exist in their neutral molecular form, which is more conducive to organic solvent extraction and separation. However, when the pH of the sample was adjusted in this experiment to be acidic, the organic solution of the sample extraction layer became more turbid, and the color changed from light yellow to brownish red. Previous studies by Zhong et al. have shown that when using the acetonitrile protein precipitation method to extract pharmaceutical components from liver tissue, acidic solvent pH could cause liver tissue to be dissolved by acid.³⁴ Under acidic conditions, membranes and organelles are destroyed by acid, and a large amount of broken organelles, pigments, proteins, and other substances flow into the matrix fluid, which makes the matrix more complicated and it even becomes turbid, which is not conducive to purification. Hemoglobin in the liver was also released into the matrix after the cells were

destroyed, which discolored the extraction solvent and affected the subsequent extraction. Therefore, the molecular form of the analyte in this study was maintained as much as possible without damaging the cell structure of the biological matrix, and the pH of the sample solution was adjusted to weakly alkaline (≥9) before extraction.

While the solvent can extract the target substance, it also absorbs the moisture in the sample. The presence of water makes the analyte partly dissolve in it and forms a competitive relationship with the extraction solvent. Therefore, it is necessary to select suitable dehydrants to remove water from biological samples. Studies have shown that adding appropriate inorganic salts can precipitate a small amount of protein in the system while absorbing water, which can not only enhance the protein precipitation effect of the extractant but also improve the matrix effect to a certain extent. NaCl, anhydrous Na₂SO₄, MgSO₄, and so forth are commonly used water absorbents, which can be used alone or in combination. Among them, NaCl can promote phase separation and make the target compounds undergo liquid–liquid distribution in the sample; anhydrous Na₂SO₄ or MgSO₄ can combine with the water in the system to distribute and transfer the analyte from the biological sample to the organic phase.^{35,36} This study analyzed the water absorption and salting-out effects of NaCl, anhydrous Na₂CO₃, Na₂SO₄, and MgSO₄. Figure 5 shows

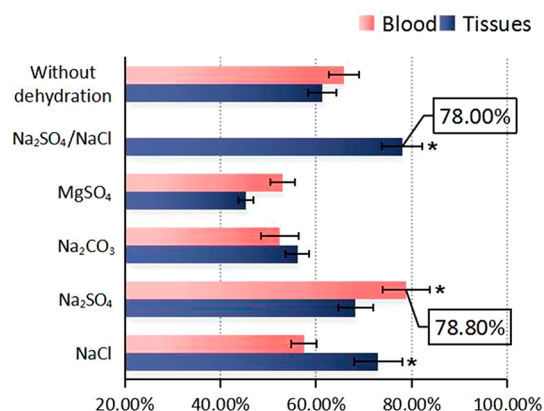


Figure 5. Average recoveries of 13 ARs for the modified QuEChERS procedures with and without dehydration ($P < 0.05$). * Indicates a significant difference in observations between treatments and controls. Data is mean \pm SE ($n = 3$).

that when anhydrous Na₂CO₃ or MgSO₄ was used as a dehydrant, the recoveries of the 13 target ARs in the samples were significantly lower than those of anhydrous Na₂SO₄ and NaCl. Previous studies have confirmed that although anhydrous MgSO₄ absorbs water more thoroughly, if Mg²⁺ fails to quickly absorb water after addition, it will form a chelate with the target compound, thereby increasing the difficulty of extraction.³⁷ Anhydrous Na₂CO₃ can generate NaOH after hydrolysis, which will change the pH of the system and reduce the extraction efficiency. In contrast, the recoveries of the analytes after adding anhydrous Na₂SO₄ to spiked blood samples were significantly higher than that of NaCl at the same dosage ($P < 0.05$). By comparing the extraction effects of different dosages, 20 mg of anhydrous Na₂SO₄ was selected as the dehydrant for blood samples in the experiment. Meanwhile, due to the different dehydration effects of anhydrous Na₂SO₄ and NaCl on the five tissue samples, to simplify and

unify the extraction steps the experiment then compared the extraction efficiency of the two agents in combination, and finally selected the combination of 20 mg of anhydrous Na_2SO_4 and 50 mg of NaCl as the common dehydrants for the five tissue samples. Compared with the samples analyzed without dehydration, the average recoveries of the target compounds in the blood and tissue samples after adding the above-mentioned inorganic salts increased by 13.02% and 16.71%, respectively ($P < 0.05$).

Biological samples contain a variety of complex matrices, including carbohydrates, macromolecular proteins, lipids, natural pigments, and sterols, which make analysis more complicated. The step without cleanup showed that over 10% of the analytes exhibited a significant ME (above 50%). Therefore, cleanup of the different sheep biological extracts was needed to minimize the matrix effect.

During the experiments, it was found that various d-SPE sorbents and their combinations had a significant influence on the purification and recoveries of target analytes. As shown in Figure 6, Al-N and GCB were not suitable as adsorbents for

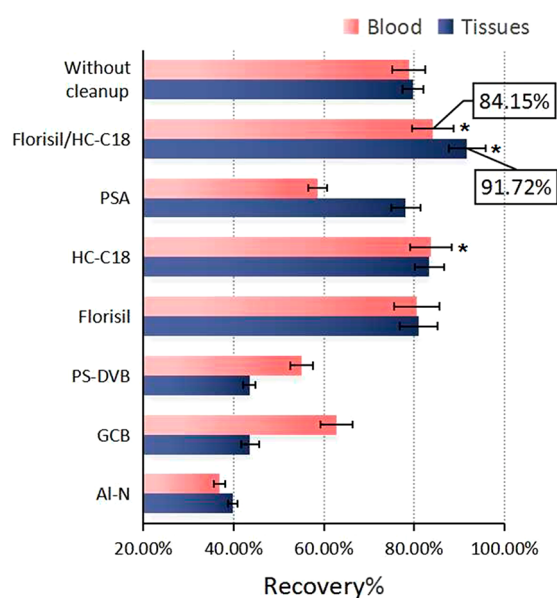


Figure 6. Average recoveries of 13 ARs for the modified QuEChERS procedures with and without cleanup ($P < 0.05$). * Indicates a significant difference in observations between treatments and controls. Data is mean \pm SE ($n = 3$).

either sheep blood or tissue samples. The recoveries of 13 ARs in sheep biological samples were not significantly ameliorative or even lower than those of unpurified samples. Studies have shown that Al-N can adsorb polar substances containing amino ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) groups, such as organic acids and alcohols.³⁸ As an electrophile with Lewis acid/base properties, Al-N can inhibit the ionization of the basic group and maintain its free state, so it has a better purification effect on alkaline substances; in contrast, Al-N reacts with compounds containing acidic groups (such as ARs) to form a salt, thereby causing tailing and difficult separation.^{39,40} As a kind of nonporous reversed-phase sorbent, GCB has been reported to be a highly effective sorbent for sample cleanup and can remove planar molecules such as natural pigments (e.g., chlorophyll, hemoglobin, and carotenoids), sterols, and nonpolar interferences.^{29,41,42} However, compounds such as

ARs with a planar structure will also be adsorbed.⁴³ Previous research by Rutkowska et al. showed that the use of high amounts of GCB (>10 mg per 1 mL of acetonitrile extracts) may lead to unacceptable losses of some planar pesticides.⁴⁴

In this experiment, when Florisil and HC-C18 were used as sorbents, the matrix effect of the spiked samples was significantly improved. Florisil is a highly polar magnesium silicate sorbent that can be used to extract polar compounds from nonpolar solutions.³⁰ It can effectively remove fat and has a significant purification effect on biological samples with high lipid content.³² Octadecyl-like HC-C18 is the sorbent most commonly used to remove coextractives from biological samples.²⁷ Its octadecyl functional group can adsorb fat and other nonpolar interfering substances, which can be used to remove oil, sterols, vitamins, and so forth in blood or tissues.⁴⁵ Previous studies have confirmed that compared to PSA with a similar structure, HC-C18 has a better purification ability for biological samples.²⁷

In addition, PS-DVB was considered for use in this study. As a new type of functional material, there is a lack of reports on the subject of compound determination in biological samples using this sorbent. With a main functional group of microporous polymer microspheres, PS-DVB is reported to be able to efficiently separate drug molecules such as natural products, antibiotics, organic compounds, peptides, proteins, and oligonucleotides.⁴⁶ In this study, when PS-DVB was used as a sorbent (10 mg per 0.1 mL/g sample) for blood or tissue samples, only 30% of ARs had acceptable recoveries ($\geq 50\%$). Thus, it was not applicable to this research.

To avoid the reduction of recovery and minimize additional cost caused by excessive adsorption, the experiment subsequently compared the purification effects of two sorbents (HC-C18 and Florisil) at 10, 20, and 50 mg (per 0.1 mL/g sample). The results showed that 10 mg or 20 mg of the two sorbents used alone can achieve acceptable matrix removal effects on blood and tissue samples, respectively. This advantage was replaced by excessive adsorption when the dosage was ≥ 50 mg, and the recoveries of more than 34% of the target analytes in spiked samples were less than 50%. The next experiment involved mixing the d-SPE sorbents in combination, and the results showed that the purification effect of the sample was better than that of using either sorbent alone, thereby significantly improving the matrix effect. This study confirmed that excellent results were achieved using the 20 mg Florisil/10 mg HC-C18/20 mg Na_2SO_4 combination in sheep blood samples, which provided satisfactory recoveries (63.5–104.27%) in the blood matrix ($P < 0.05$). For the tissue samples, the dosage of sorbents was slightly increased, which was a combination of 20 mg Florisil/20 mg HC-C18/20 mg Na_2SO_4 /50 mg NaCl. The purification efficiency of this combination brought about an 11% improvement in recoveries for 5 tissue matrices ($P < 0.05$), and the recoveries of the 13 target compounds reached between 64.16 and 110.65%.

3.3. Method Validation. The optimized analytical method for the determination of 13 ARs in sheep blood and tissue samples using HPLC-MS/MS was evaluated according to Peters et al., Matuszewski et al., and Meng et al.^{47–49} A series of parameters, including selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, recovery, matrix effect, stability, and carryover, were performed to validate the modified QuEChERS method under optimized conditions.

To evaluate the selectivity of the method, the interference effects of complex components in biological samples such as the matrix, metabolites, impurities, and so forth on target compounds were investigated. The selectivity of the method was tested in 10 different blank samples by purchasing sheep blood products of different batch numbers and collecting different healthy sheep tissues to analyze whether the endogenous substances in the whole blood or tissues interfered with the detection of the target compounds. Results showed that there were no compounds determined in the selectivity experiment.

For the biological samples with complex matrices, calibration curves were obtained from matrix-matching calibration solutions to assess the linearity of the method. The linearity was studied in the range of 1–100 ng/mL (g) with seven calibration points (1, 2, 5, 10, 20, 50, and 100 ng/mL (g)), performing three replicates at each level. The calibration curves were derived by plotting the peak areas of analytes to IS versus the spiked concentration using a $1/x$ weighted linear least-squares regression model. The results of the calibration curves are shown in [Supporting Information](#), Table S1. A good linearity coefficient of determination (R^2) value was obtained for each target compound in this study, ranging from 0.99 to 0.9999.

The LOQ was regarded as the lowest spiking level or detectable concentration of the analyte that could be quantified with acceptable accuracy and precision. The LOD was defined as the lowest concentration of the analyte that could only be detected but was unable to be quantified accurately. Both are important validation parameters and were used to evaluate the sensitivity of the analytical methods. In this study, the minimal values with signal-to-noise ratios of 3 ($S/N \geq 3$) and 10 ($S/N \geq 10$) in spiked samples were determined as the LOD and LOQ, respectively. Furthermore, an accuracy and precision within $\pm 20\%$ was required for the LOQ. The data are shown in [Table 3](#), and the values were low enough to sensitively determine the concentrations of the 13 target ARs in animal blood or tissue samples.

The accuracy and precision of the method were evaluated at three concentrations (5, 20, 80 ng/mL (g)) in spiked samples within the linear range. Each level was replicated six times. The accuracy was defined as the percent deviation between the mean calculated value and the corresponding spiked concentration, and the precision was expressed by the relative

standard deviation (RSD%) during the same day (intraday precision) and for three consecutive days (interday precision). The criteria of accuracy should be in the range of 85% to 115% (80–120% near the LOQ), and precision should be within 15% RSD (20% near the LOQ). In this study, parallel sample results obtained for the 13 target ARs indicated good method accuracy and overall precision, ranging from 88.28 to 114.21% and 0.29–14.85%, respectively, as shown in [Supporting Information](#), Tables S2 and S3.

Extraction recovery (ER) and matrix effect (ME) experiments were also performed in six blank spiked samples at three levels (5, 20, 80 ng/mL (g)). Each level was replicated five times. By comparing the difference in the instrument response value of each analyte between the spiked samples, the standard in the matrix solution, and the standard in methanol at the same concentrations, the ER and ME of different types of samples were validated. Their formulas are as follows

$$ER = \text{set 1} / \text{set 2} \times 100$$

$$ME = (\text{set 2} / \text{set 3} - 1) \times 100$$

where set 1 is the concentration of each analyte in extracted blank blood/tissue samples spiked with the compounds and treated as described in [Section 2.3](#); set 2 is estimated by the extracted blank blood/tissue samples treated as described in [Section 2.3](#) and then spiked with the same amounts of rodenticides and IS when reconstituting the dry residues; and set 3 is the standard working solution diluted by methanol to the designated concentration.

In this study, ERs obtained for 13 ARs were satisfactory and ranged from 52.78 to 110.69% ([Supporting Information](#), Table S4). The average ERs of the target analytes in the six biological spiked samples (blood, heart, liver, kidney, muscle, and stomach wall) were 81.22%, 68.66%, 76.83%, 80.12%, 86.50%, and 79.00%, respectively, which all met the method verification requirements ($\geq 50\%$).

Studies have shown that the matrix effect has become a very serious and common phenomenon in LC-MS/MS analysis, affected by sample type, chromatographic separation, the mobile phase, ionization and other factors. In this study, six biological samples showed different degrees of matrix enhancement or inhibition effects. During the chromatographic analysis of pesticide residues, Hajšlová et al. stated that coextracted matrix components can compete with target analytes for access to the active site during the injection process, which may result in enhancement of the detector's signal. Meanwhile, (semi)-polar or thermal-sensitive analytes may decompose at active sites in the liners, column, and detector, giving losses and distorted peak shapes, which may result in suppression of the chromatographic signal. Previous studies by Cao et al. showed that matrix effects can severely compromise qualitative and quantitative analysis of the target compounds at trace levels as well as method reproducibility, especially when electrospray ionization is used.¹¹ In this study, the analysis without cleanup showed that more than 50% of the analytes had MEs higher than 20%, and nearly 10% exhibited significant MEs ($\geq 50\%$), as shown in [Figure 7](#). The average ME of blood samples was the lowest, and the heart and stomach wall were more severe. This was not only related to the complex matrix composition but also involved the group structure, polarity, and molecular mass of the target compounds. Research by Rutkowska et al. showed that compounds with amino ($-R-NH-$), azole ($-N=$), benzimidazole, carbamate ($-O-CO-NH-$), car-

Table 3. LOD and LOQ Obtained from Blank Sheep Blood and Tissues Spiked with 13 ARs

compound	LOD ($S/N \geq 3$) (ng/mL (g))	LOQ ($S/N \geq 10$) (ng/mL (g))
coumafuryl	0.1	0.2
valone	0.5	1
pindone	0.5	1
coumatetralyl	0.1	0.2
warfarin	0.1	0.2
coumachlor	0.1	0.2
diphacinone	0.2	0.5
dicoumarol	0.1	0.2
chlorophacinone	0.1	0.2
bromadiolone	0.1	0.2
difenacoum	0.1	0.2
flocoumafen	0.05	0.1
brodifacoum	0.5	1

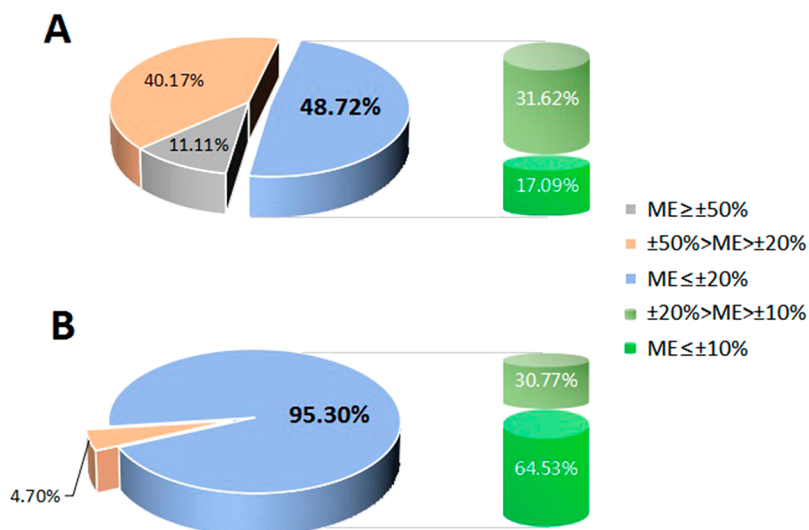


Figure 7. Matrix effects of the 13 target ARs for the modified QuEChERS procedures: (A) extraction using organic solvents only; (B) extraction after the optimized method (pH adjusted, dehydrating and purifying agents added).

boxyl (–COOH), hydroxyl (–OH), imidazole, and urea (–NH–CO–NH–) groups are most susceptible to matrix effects.³⁶ In addition, compounds with high polarity and high molecular mass (over 400 g/mol), such as bromadiolone and difenacoum, were also susceptible to ME. Therefore, to reduce the influence of complex matrices and obtain more reliable results, in addition to optimizing the instrument parameters and using a matrix-matched internal standard calibration curve for quantification, QuEChERS technology was used in this work to systematically optimize the sample pretreatment steps to minimize the interference of matrix components on the analyte ion detection process. Excellent results were achieved using the Florisil/HC-C18/anhydrous Na₂SO₄ (NaCl) combination, which brought an approximately 47% improvement in ME for six biological matrices, and over 90% exhibited acceptable soft ME (≤20%) (Supporting Information, Table S5).

Examining stability is also necessary for the effectiveness of the entire method. This study first verified the freeze/thaw stability of 13 ARs through three spiked points (5, 20, 80 ng/mL (g)) at low, medium, and high concentrations. Following an initial freezing period of 24 h (–20 °C), the samples were thawed for 12 h (to 25 °C). The period was performed for three freeze–thaw cycles before preparation. When comparing the repeated freeze–thaw samples with the control samples analyzed immediately, the analyte peaks and the average measured concentration of the processed samples were unchanged by these test conditions. Additionally, other stability experiments (room-temperature stability, long-term stability and autosampler stability) were also verified in turn. By comparing the measured concentration of the spiked samples stored at room temperature (25 °C) for 24 h or –20 °C for 1 month with the average value of the quality control samples analyzed immediately after preparation, the results of room temperature stability and long-term stability were obtained. The autosampler stability experiment analyzed the changes of samples after pretreatment and storage in the autosampler for 24 h. The results showed that all of the compounds were stable in the various matrices assayed under three different experimental conditions, and there were no significant differences when comparing values obtained from

the samples with those of control samples. In all tests, the absolute %RSD values were less than 15% (method requirement was within ±20%).

Carryover was assessed by placing one control blank (without analyte and I.S) immediately after every maximum concentration sample (80 ng/mL (mg)) in each accuracy and precision run. It was considered acceptable if the carryover blank had analyte peaks that were <20.0% of the lowest peak area of LOQ and had IS peaks that were <5.00% of the mean peak area of IS in the run. In this study, the target compounds and IS residues in the blank sample met the requirements of the method at the corresponding retention time. By calculating the peak area of the compounds in the blank sample at the corresponding retention time, the average peak area of 13 ARs in the blank sample was less than 15% LOQ, and the residual IS was less than 3%.

To the best of our knowledge, this is the first study that uses a QuEChERS method for the analysis of 13 kinds of ARs in various biological materials. The method is easy and fast to implement in a forensic or food analysis laboratory. The main advantages over most published methods are the extensive practical values with 13 common ARs that are widely used and its application in six biological matrices. Compared with the previous QuEChERS method used in the field of pesticide residue detection, this modified protocol reduced the sample size, thereby saving the consumption of extraction reagents and sorbents.^{27,32} Furthermore, while simplifying the experimental steps, accuracy, precision and matrix purification capacity obtained was comparable or better than those methods that used liquid–liquid extraction (LLE) or solid-phase extraction (SPE).^{24,50} Next, the application and verification of this method in actual poisoning cases can be used as research content of important scientific merit in future work.

Method Application. Our laboratory received samples from a public security bureau in Genhe City, Inner Mongolia, where a farmer in its jurisdiction reported that several goats died on their ranch. The dead goats had bleeding from the nose and mouth, and it was suspected of being poisoned. After processing and analysis by the above method established in this study, warfarin and bromadiolone were detected in the heart blood and stomach wall of the three goats submitted for

inspection, and the detected contents were all >1 ng/mL (g). At the same time, sampling and testing of soil samples from mountain slopes and herdsman's residences were carried out. It was confirmed that bromadiolone was detected on the mountain slopes within the range of goats' activities, and warfarin was extracted from the sheepfold at the herder's home. As a type of rodenticide with low acute toxicity, the single oral LD₅₀ of warfarin and bromadiolone in rats is 3.0 and 1.1 mg/kg, respectively. In animal poisoning cases involving such drugs, multiple administrations are often required to exert their efficacy, which can also be enhanced by combined use. In this case, although warfarin and bromadiolone in the heart blood and stomach wall of the dead goats did not reach the rat's single oral LD₅₀, it was inferred that they may have ingested the two drugs for multiple days during grazing and accumulated poisoning. In summary, combining the symptoms of the dead animals and determination results, it was concluded that the three goats submitted in this case died from ingesting the ARs warfarin and bromadiolone.

CONCLUSIONS

A multiresidue analysis method based on a modified QuEChERS pretreatment methodology for the simultaneous screening and detection of 13 ARs in biological samples (sheep whole blood, heart, liver, kidney, muscle, stomach wall) was demonstrated, and the matrix effect of the detection method was evaluated and compensated. In this work, a tandem mass spectrometry library was established for the 13 target compounds, and simultaneous qualitative and quantitative analysis was carried out using MRM-EPI scan mode. The mobile phase and mass spectrometric parameters were optimized first, and the isotope IS and matrix matching standard curve were applied to this method to effectively compensate for the interference of matrix effects on the quantitative results. The study discussed the impact of pretreatment parameters, such as sample pH and type of extraction solvent and cleanup sorbent, on recoveries and matrix effects, with the aim of choosing suitable methods for multiresidue analyses. Finally, the modified QuEChERS method based on acetonitrile (or acetonitrile: EtOAc = 1:1/ (v:v)) extraction followed by Florisil/HC-C18/anhydrous Na₂SO₄ (NaCl) cleanup under alkaline conditions was validated in terms of selectivity, linearity, LOD, LOQ, accuracy, precision, ER, ME, stability, and carryover. The correlation coefficients of the 13 target ARs in spiked biological matrices were between 0.9900–0.9999 in the range of 1–100 ng/mL (g), and the LODs were less than 0.05–0.5 ng/mL (g). The ERs of the target compounds were between 52.78 and 110.69%, and the RSDs were consistently <12.8%. It is worth noting that this method ensured an ideal ME. Over 46% of the obvious matrix enhancement or suppression effects (ME ≥ 20%) of the target compounds were effectively improved. The developed method meets the requirements of international guidelines with the advantages of being robust, inexpensive, and straightforward, and it is convenient for practical application. It has been successfully applied to a series of livestock death cases caused by suspected AR poisoning, and its application can be useful to improve the detection capabilities of dealing with rodenticide poisoning cases for forensic authentication and biosafety assessment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c01863>.

Chemical information for all compounds (Figure S1) and detailed methodological validation results (Tables S1–S5) (PDF)

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Notes

The authors declare no competing financial interest.

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