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Optimization of ASE and SPE conditions for the HPLC-FLD detection of piperazine in chicken tissues and pork

Chujun Liu[1,2] | Xing Xie[3] | Bo Wang[2,4] | Xia Zhao[1,2] | Yawen Guo[1,2] | Yangyang Zhang[1,2] | Xiaona Bu[1,2] | Kaizhou Xie[1,2] | Yajuan Wang[1,2] | Tao Zhang[1,2] | Genxi Zhang[1,2] | Xuezhong Liu[2,4] | Guojun Dai[1,2] | Jinyu Wang[1,2]

[1] College of Animal Science and Technology, Yangzhou University, Yangzhou, China

[2] Joint International Research Laboratory of Agriculture and Agri-Product Safety, Yangzhou University, Yangzhou, China

[3] Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, Institute of Veterinary Medicine, Nanjing, China

[4] College of Veterinary Medicine, Yangzhou University, Yangzhou, China

Correspondence
Kaizhou Xie, College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China.
Email: yzxkz168@163.com

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Abstract

Accelerated solvent extraction (ASE) and solid-phase extraction (SPE) conditions were optimized by a high-performance liquid chromatography-fluorescence detector (HPLC-FLD) method for the detection of piperazine in chicken tissues and pork. Piperazine residues were determined by precolumn derivatization with trimethylamine and dansyl chloride. Samples were extracted with 2% formic acid in acetonitrile using an ASE apparatus and purified using a Strata-X-C SPE column. The monosubstituted product of the reaction of piperazine with dansyl chloride was 1-dansyl piperazine (1-DNS-piperazine). Chromatographic separations were performed on an Athena C_{18} column (250 × 4.6 mm, id: 5 μm) with gradient elution using ultrapure water and acetonitrile (5:95, V/V) as the mobile phase. The calibration curves showed good linearity over a concentration range of LOQ-200.0 μg/kg with a coefficient of determination (R^2) ≥ .9992. The recoveries and relative standard deviations (RSD values) ranged from 78.49% to 97.56% and 1.19% to 5.32%, respectively, across the limit of quantification (LOQ) and 0.5, 1, and 2.0 times the maximum residue limit (MRL; μg/kg). The limits of detection (LODs) and LOQs were 0.96 to 1.85 μg/kg and 3.20 to 5.50 μg/kg, respectively. The method was successfully applied for the validation of animal products in the laboratory.

KEYWORDS

ASE, chicken tissues, HPLC-FLD, piperazine, pork, SPE

1 | INTRODUCTION

Piperazine and its salts are anthelmintics that are used extensively to treat specific nematode and roundworm parasites, and they have been applied widely in livestock animals.[1] Piperazine deworms by hyperpolarizing the neuromuscular junction to block the spread of the worm by inducing flaccid paralysis.[2,3] However, the excessive use of piperazines can result in drug residues in livestock animals, which can cause serious harm to animals and consumer health.[4] Therefore, to facilitate the safe management of veterinary drugs and ensure the safety of food

for consumers, the maximum residue limit (MRL) for piperazine in pork and chicken was set by the European Union (EU) and China Agriculture Department at 400 μg/kg,[5,6] while the Food and Drug Administration (FDA) set an MRL of 100 μg/kg for piperazine in pork and chicken, and in Japan, the MRL for piperazine in chicken is 100 μg/kg.[7,8]

Based on these standards, several methods, such as gas chromatography-tandem mass spectrometry (GC-MS/MS),[9] high-performance liquid chromatography (HPLC),[10-12] and ultra-performance liquid chromatography (UPLC),[13] have been used to detect piperazine in animal products or other creatures. However, sample preparation is different in these methods. Samples were derived with acetic anhydride, and sample preparation conditions were optimized via ASE and SPE in GC-MS/MS. This method has a higher sensitivity but a lower extraction efficiency.[9] Samples underwent liquid-liquid extraction (LLE) and were purified with a PCX cartridge in the HPLC-FLD method.[10] This analytical method was sensitive and accurate, but it was easy to produce human error in the sample preparation process, and the limit of quantification (LOQ) was higher. Samples were extracted with petroleum ether and ethyl acetate in a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI/MS/MS) method for the analysis of piperazine derivatives in fungus, and this method was relatively simpler but quantitatively inaccurate.[11] An internal standard method was used, and samples were extracted with ethyl acetate with recoveries of 86.68% to 91.29% in the HPLC-MS/MS method for the determination of piperazine phosphate in human plasma, which has important reference significance for animals.[12] A method using ultra-performance liquid chromatography-electrospray ionization-tandem triple quadrupole mass spectrometry (UPLC-ESI/MS/MS) for the identification of piperazine in animal products has been developed,[13] which is simple and has good sensitivity.

A method in which HPLC-FLD is applied after precolumn derivatization for the determination of piperazine in animal products has been previously reported.[10] The sample preparation method was optimized by using accelerated solvent extraction (ASE) instead of liquid-liquid extraction (LLE), extracting with 2% formic acid in acetonitrile, and purifying with Strata-X-C solid-phase extraction (SPE) columns, thereby improving the recovery, sensitivity, and accuracy. This experimental study is significant for ensuring the safety of animal-derived foods and human health, and the results show that the HPLC-FLD method can be considered suitable for the quantitative detection of piperazine residues in chicken tissues and pork.

2 | EXPERIMENTAL

2.1 | Standards and materials

A piperazine standard (≥99.0% purity) and dansyl chloride (DNS-Cl, ≥98.0% purity) were purchased from Sigma-Aldrich (St. Louis, Missouri) and the Yuanye Biological Technology Co (Shanghai, China), respectively. HPLC-grade acetonitrile and triethylamine (≥99.0% purity) were obtained from Fisher Scientific International Inc (Pittsburgh, Pennsylvania) and Thermo Fisher Scientific International Inc (Shanghai, China), respectively. Analytical-grade formic acid, n-hexane, and ammonia were provided by Sinopharm Chemical Reagent Co (Shanghai, China). The ultrapure water (18.2 MΩ cm^{-1}, 25°C) used in this study met the national laboratory water requirements (GB6682-1992). Strata-X-C SPE cartridges (3 mL, 60 mg) were obtained from Phenomenex (California). PCX-SPE cartridges (3 mL, 60 mg) were purchased from Agela Technologies (Tianjin, China). MCX-SPE cartridges (3 mL, 60 mg) were provided by Waters (Massachusetts). An Athena C_{18} (4.6 mm × 250 mm, id.: 5 μm) column and nylon needle filters (13 mm × 0.22 μm) were obtained from Anpu Technology Co, Ltd (Shanghai, China). DE and extraction cell filters were provided by Thermo Fisher Scientific Co, Ltd (Shanghai, China).

2.2 | Equipment

The HPLC system utilized in this study consisted of a Waters Alliance e2695 LC System and a Waters 2475 fluorescence detector (FLD) (Waters, Milford, Massachusetts). A model AE260S electronic analytical balance (Mettler Toledo, Zurich, Switzerland), a model P300H Ultrasonic cleaning machine (Elma, Hamburg, Germany), a model FD 115 oven (Binder, Tuttlingen, Germany), a model G560E vortex oscillator (Scientific Industries, New York), and a model ASE350 accelerated solvent extractor (Thermo Fisher, Massachusetts) were used. The Smart2-Pure water used herein was ultrapure (Thermo Fisher Scientific Co, Ltd, Shanghai, China), and an N-EVAP-112 nitrogen evaporator (Organomation, Massachusetts) was used.

2.3 | Standard stock solutions and working solutions

The standard stock solution of 1.0 mg/mL piperazine was prepared by weighing 10.11 mg of piperazine standard into a 10-mL brown volumetric flask and diluting to volume with acetonitrile. The working solutions of

piperazine with different concentrations (100.0 μg/mL, 10.0 μg/mL, 1.0 μg/mL, 100.0 ng/mL, and 10.0 ng/mL) were prepared by diluting the stock solution (1.0 mg/mL) with acetonitrile. The working solutions were stored at 4°C in the dark, and the stock standard solutions were stored at −70°C in an ultralow temperature refrigerator; the samples were stable for 4 months of storage.

2.4 | Standard curves

Standard curves of piperazine were constructed by diluting standard working solutions into blank chicken tissues (muscle, kidney, and liver) and pork to obtain the final concentrations of piperazine of the LOQ, 5.0, 10.0, 50.0, 100.0, 150.0, and 200.0 μg/kg, and then the samples were analyzed via HPLC-FLD. The external standard method was adopted, and the standard curves were prepared by plotting the peak areas of the piperazine derivatives against the piperazine concentration.

2.5 | HPLC analysis

HPLC separations were performed using an Athena C_{18} (250 × 4.6 mm, id: 5 μm) column. The column temperature was set at 25°C. The mobile phases consisted of ultrapure water (A) and acetonitrile (B) (5:95, V/V). The flow rate was 1.0 mL/min. The fluorescence detector was set at an excitation wavelength of 330 nm and an emission wavelength of 531 nm. The injection volume was 20 μL. Gradient elution was performed with solutions A and B at 1.0 mL/min: 0 minute, 5% A; 2 minutes, 15% A; 4.5 minutes, 12% A; and 7 to 10 minutes, 5% A.

2.6 | Sample preparation

Blank chicken tissue and pork samples were obtained from Jinghai Yellow chicken (Jinghai Poultry Company, Jiangsu) and Duroc-Landrace-Yorkshire swine (Kangle Farming Company, Jiangsu). Sample pretreatment included extraction, purification, concentration, redissolution, and derivatization. A homogenized blank sample (2 ± 0.02 g) was precisely weighed into a mortar and ground with 4.0 g of infusorial earth to achieve the best extraction efficiency. Then, the mixture was loaded into the ASE350 apparatus, and the sample was extracted three times. First, to remove fat with n-hexane, the static extraction process was continued for 5 minutes at 80°C and 1500 psi. The extracts were discarded, and the fat-removal process was repeated. Second, the fat-removed sample was extracted twice with 2% formic acid in acetonitrile. Finally, the extracts were collected and purified by a Strata-X-C SPE column, and the Strata-X-C SPE column was activated and balanced with 3.0 mL of methanol and 3.0 mL of 2.0% formic acid in water, respectively. Then, the loaded cartridge containing 20.0 mL of extraction solvent was washed with 2.0 mL of 0.1 mol/L hydrochloric acid in water and 2.0 mL of methanol. Finally, the analyte was eluted with 9.0 mL of 10% aqueous ammonia in methanol. The eluate was collected in a 15-mL centrifuge tube, evaporated to dryness in a speed vacuum concentrator, reconstituted with 1.0 mL of acetonitrile for derivatization, and sonicated for 10 minutes. Then, 100 μL of 0.12% triethylamine and 600 μL of dansyl chloride (1.0 mg/mL) were added, and the mixture was diluted to 2.0 mL with acetonitrile, reacted in darkness for 20 minutes at 50°C, and vortexed for 1 minutes. The mixture was passed through a 0.22-μm needle filter and analyzed by HPLC with FLD.

3 | RESULTS AND DISCUSSION

3.1 | Comparisons of extraction solvents and methods

Sample extraction was the key step in this method; thus, the selection of the most appropriate solvent system was important. Piperazine is an amine compound with high polarity, and its aqueous solution is slightly alkaline; this solution can generally be extracted with a solvent or acid of higher polarity.[14] In a previous study, 2% formic acid in water or a mixture of 2% formic acid in water and acetonitrile was selected as a solvent for the extraction of piperazine from animal products.[10] In this experiment, the ratios of 2% formic acid in water and in acetonitrile were optimized for the extraction process. The recoveries of piperazine using 2% formic acid in water and in acetonitrile (1:1, V/V) were 42.66% to 60.90%: Those using 2% formic acid in water and in acetonitrile (3:1, V/V) were 36.17% to 48.81%, and those using 2% formic acid in water and in acetonitrile (1:3, V/V) were 45.49% to 51.10%. Therefore, 2% formic acid in water and in acetonitrile (1:3, V/V) was selected as the best solution system for the experiment. The recovery of piperazine using 2% formic acid in water in LLE was higher than that using 2% formic acid in acetonitrile in ASE. However, the recovery of piperazine using 2% formic acid in acetonitrile in ASE was more than 80%, which was higher than that using 2% formic acid in water in LLE. Therefore, 2% formic acid in acetonitrile and ASE were selected as the extraction solvent and extraction method, respectively.

3.2 | Optimization of extraction conditions

The ASE method is superior to other traditional extraction methods, and high temperature and high pressure are important parameters for optimizing the ASE process. High temperature can reduce the viscosity of the solvent, increasing its ability to dissolve analytes,[15,16] and high pressure can maintain solvents in the liquid state, allowing them to pass quickly through the ASE system. However, pressure is typically maintained at 1500 psi and is not considered a key parameter as it has little effect on analyte recovery. Unlike pressure, temperature is considered a key parameter, and it has a large effect on extraction efficiency.[15,16] The extraction efficiency at 70°C is much higher than that at 60°C or 90°C.[17] Hence, temperature was evaluated in detail. In this experiment, the pressure in the ASE350 system was fixed at 1500 psi, and different temperatures (40°C, 60°C, 80°C, 100°C, and 120°C) were tested for piperazine extraction from different tissues to improve the recovery. A better purification effect and greater recovery of piperazine could be achieved at 80°C. Thus, 80°C was the best extraction temperature. In addition to the extraction temperature, the number of static cycles and extraction time will also affect the recovery of the samples. Increasing the static extraction time of ASE can provide sufficient time for the target compounds to diffuse into the extraction solvents, which can improve extraction efficiency. The extraction process was generally complete within 20 minutes; if the sample extraction required additional time, higher temperature, different solvents, or a greater number of static cycles was applied to reduce the total extraction time.[18,19] The sample was extracted for 5 minutes at 80°C and 1500 psi using two cycles, and these parameters provided the best extraction efficiency.

3.3 | Comparison of the SPE columns

Wang et al[9] selected a Strata-X-C SPE column to purify samples, and the recoveries were 77.46% to 96.26%. Park et al[10] selected the PCX SPE column (3 mL, 60 mg) for purification. Xie et al[13] used the Strata-X-C SPE column (3 mL, 60 mg) to purify samples. Kopciuch[20] selected the ODS column for purification. For the optimization of sample preparation, the Strata-X-C SPE column (3 mL, 60 mg) was selected. Meanwhile, the recoveries of piperazine on a PCX-SPE column (3 mL, 60 mg), an MCX-SPE column (3 mL, 60 mg), and a Strata-X-C SPE column (3 mL, 60 mg) were compared. The Strata-X-C SPE column (3 mL, 60 mg) not only provided a recovery greater than 80% but also yielded greater purification than did the other columns.

3.4 | Optimization of the reaction conditions for derivatization

To optimize the reaction conditions for the derivatization used in the analytical procedure, the effects of the reaction time, temperature, amount of triethylamine (100 μL of triethylamine at different concentrations), and amount of dansyl chloride added for the formation of the fluorescent derivatives of piperazine were investigated. When optimizing the reaction time, 100 μL of the working solution of piperazine (1.0 μg/mL) was transferred into a 10-mL centrifuge tube, and 100 μL of 0.10% triethylamine and 400 μL of dansyl chloride (1.0 mg/mL) were added. The reaction mixture was diluted to 2.0 mL with acetonitrile, and it was allowed to react in darkness for 10, 20, 30, 40, 50, and 60 minutes at 50°C. Each reaction time for the derivatization was tested with three parallel reactions. The fluorescent derivatives were vortexed for 1 minute after the reaction and then analyzed by HPLC-FLD. When optimizing the reaction temperature for the derivatization, the fluorescent derivatives were prepared in darkness for 30 minutes at 30, 40, 50, 60, 70, and 80°C. Other reaction conditions were evaluated using a system similar to that used to evaluate the reaction time. The concentration (0.02%, 0.04%, 0.06%, 0.08%, 0.10%, 0.12%, 0.14%, 0.16%, and 0.18%) of the 100 μL of triethylamine (with reaction time, 30 minutes; temperature, 50°C; 1.0 mg/mL dansyl chloride, 400 μL) and different amounts (100, 200, 300, 400, 500, 600, 700, and 800 μL) of dansyl chloride (with reaction time, 30 minutes; temperature, 50°C; 0.10% triethylamine, 100 μL) were evaluated in this manner. The optimum combination of reaction conditions consisted of 100 μL of piperazine (1.0 μg/mL) standard working solution mixed with 0.12% triethylamine and 600 μL of dansyl chloride (1 mg/mL) heated at 50°C for 20 minutes.

3.5 | Comparison of reagents for the derivatization reaction

Piperazine does not have ultraviolet-absorbing or fluorescent groups, so ultraviolet-absorbing or fluorescent groups need to be produced through derivative reactions to allow the use of ultraviolet or fluorescence detection. Piperazine is a six-membered heterocyclic amine that can be substituted with common derivatizing agents (such as ethyl chloroformate or isobutyl chloroformate,[21] benzaldehyde,[22] and dansyl chloride[10]). Piperazine in aqueous solutions and in human urine was derivatized with ethyl

chloroformate or isobutyl chloroformate, which was detected by capillary gas chromatographic determination using nitrogen- or mass-selective detection, which have lower recoveries.[21] The derivative formed by the reaction of piperazine with benzaldehyde only has UV activity.[22] In this experiment, dansyl chloride was chosen as the derivatization reagent because piperazine contains a secondary amine, and the product reacted with it has a fluorescent group and is structurally stable.[10-19] The reaction of piperazine with dansyl chloride can produce both monosubstituted and disubstituted products. Within 10 minutes in darkness at 30°C, piperazine can react with dansyl chloride to generate the monosubstituted piperazine (1-DNS-piperazine). The disubstituted piperazine (1,4-DNS-piperazine-DNS) is generated when the reaction is conducted in darkness at 50°C for 30 minutes.[23] The derivatization reactions are shown in Figure 1. The monosubstitution of piperazine with dansyl chloride produced 1-DNS-piperazine under the selected derivatization conditions in this experiment (temperature, 50°C; time, 20 minutes; 0.12% triethylamine solution), as determined by MS analysis. These results are consistent with previous reports.[12-23] The mass spectrum of 1-DNS-piperazine is shown in Figure 2. Additionally, to achieve a high fluorescence response, the derivatization reaction conditions were optimized, and the results of these experiments are shown in Figure 3.

3.6 | Optimization of the chromatographic conditions

In this experiment, an Athena C_{18} column (4.6 × 250 mm, id: 5 μm) and an XbridgeTM C_8 column (4.6 × 150 mm, id: 5 μm) were selected, and piperazine was not effectively retained on the C_8 column—the peak shape was severely tailed, it was prone to the overload phenomenon, the peak broadening was too large (more than 1.0 minute), the piperazine component and the interference component could not be completely separated, and the baseline separation peak was not reached—while piperazine on the C_{18} column had good retention, and the peak shape was sharp

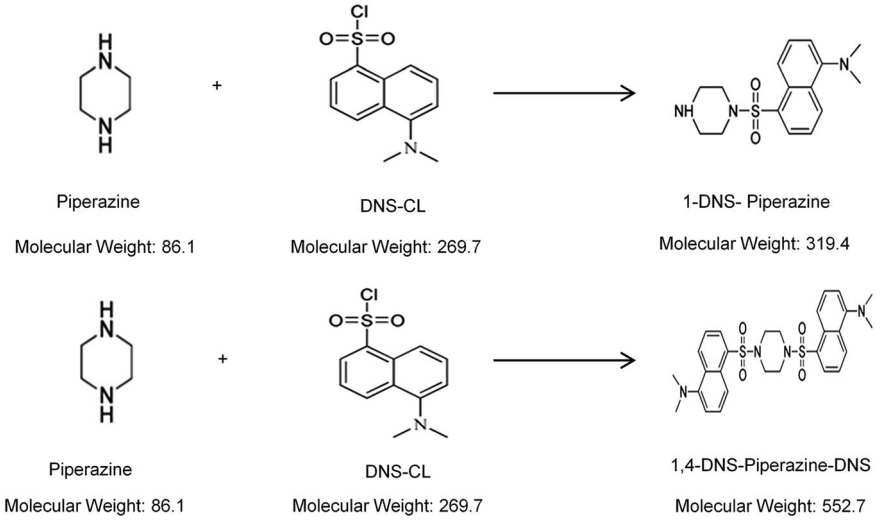

FIGURE 1 Reactions of piperazine with dansyl chloride

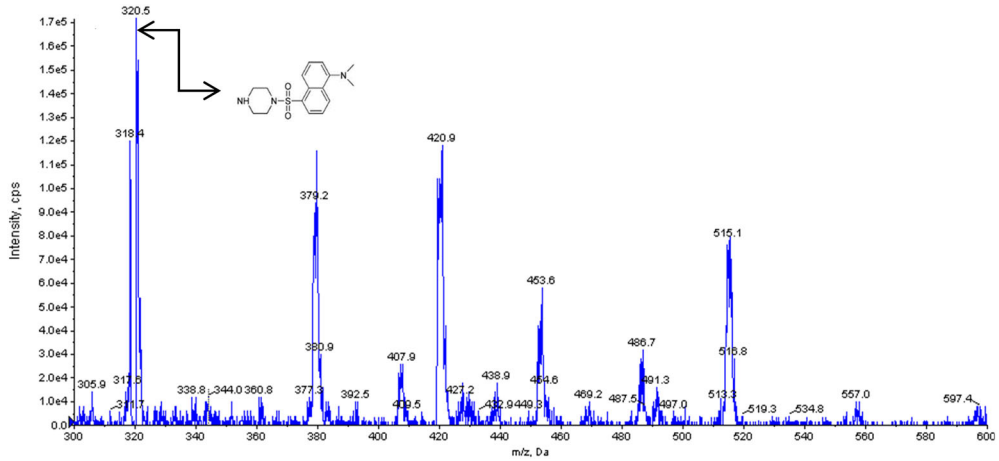

FIGURE 2 Mass spectrum of 1-DNS-piperazine

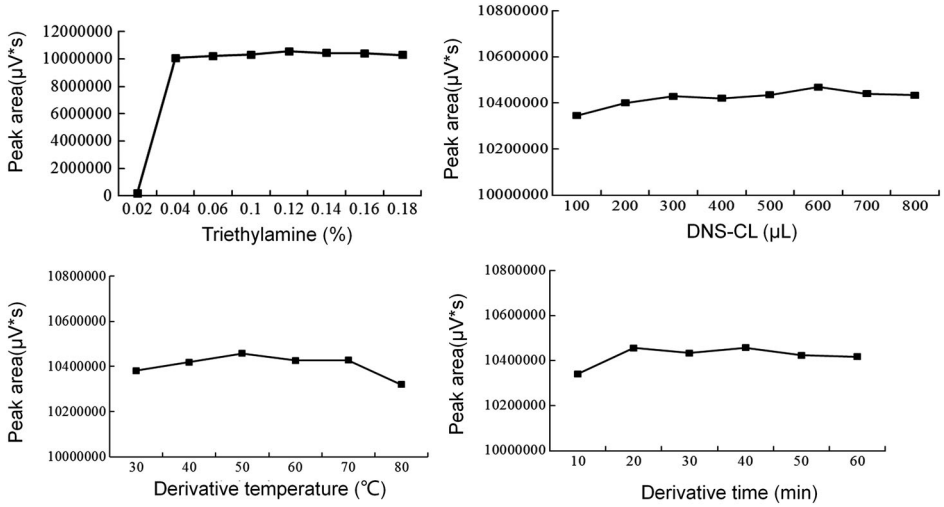

FIGURE 3 Effects of the concentration of triethylamine A, the amount of dansyl chloride B, the heating temperature C, and the heating time D, on the formation of flourescent of piperazine

and symmetrical, without trailing and overloading. Thus, an Athena C_{18} column (4.6 × 250 mm, id: 5 μm) was selected. Additionally, in order to shorten the retention time, different mobile phases were tested in detail. First, acetonitrile-water and methanol-water were investigated as mobile phases. However, the results showed that acetonitrile-water as the mobile phase provided better separation. When acetonitrile-water was used as the eluent, the peak width of the piperazine derivative was moderate, the response signal was good, and no impurity peaks were observed. When methanol-water was used as the eluent, the chromatographic peaks tailed, and there were interfering peaks from impurities. Therefore, acetonitrile-water was chosen as the mobile phase. Isocratic and gradient elution were also compared in the experiment. Compared with isocratic elution, gradient elution shortened the retention time, as shown in Table 1. Additionally, when acetonitrile-water (70:30, V/V) was selected as the mobile phase, the retention time of the derivatives in isocratic elution was 17.4 minutes.[10] While acetonitrile-water (95:5, V/V) was selected as the mobile phase, the retention time of the derivatives in gradient elution was 6.6 minutes, as shown in Table 1, which greatly improved the detection efficiency. Therefore, an Athena C_{18} column (4.6 × 250 mm, id: 5 μm) at a column temperature of 25°C with an injection volume of 20 μL, ultrapure water-acetonitrile (5:95) as the mobile phase, and a gradient elution program were used as the initial chromatographic conditions for the determination of piperazine. The piperazine derivatives and dansyl chloride standards were well separated from all impurities under optimized chromatographic conditions, and the retention time was 6.599 minutes. The retention times of the derivatives of piperazine in spiked samples (chicken muscle, chicken kidney, chicken liver, and pork) were 6.605, 6.602, 6.595, and 6.632 minutes, respectively, and these target peaks were not found in blank samples. The results are shown in Figure 4. The excitation and emission wavelengths of the HPLC-FLD method were 338 and 523 nm, respectively.[10] However, in this study, fluorescence scanning showed that when the excitation wavelength was 330 nm, the emission peak signal was strongest at the emission wavelength of 531 nm, and when the emission was 531 nm, the excitation peak signal was strongest at 330 nm. In this study, as the detectors were different from those in the previous study (see

TABLE 1 Comparison of different piperazine detection methods

Tissues	Mobile Phase, %	Detector	Retention Time, min	Recovery, %	LOD, μg/kg	LOQ, μg/kg
Chicken tissues	Ultrapure water: Acetonitrile (5:95, V/V)	FLD (Waters 2475)	6.6	78.49-97.56	0.96-1.85	3.20-5.50
Chicken tissues (Park et al[10])	Water: Acetonitrile (30:70, V/V)	FLD (G1321A)	17.4	96.40-97.30	6	20
Pork	Ultrapure water: Acetonitrile (5:95, V/V)	FLD (Waters 2475)	6.6	86.92-91.13	1.15	3.52
Pork (Park et al[10])	Water: Acetonitrile (30:70, V/V)	FLD (G1321A)	17.4	87.50-90.10	6	20

Abbreviations: LOD, limit of detection; LOQ, limit of quantification.

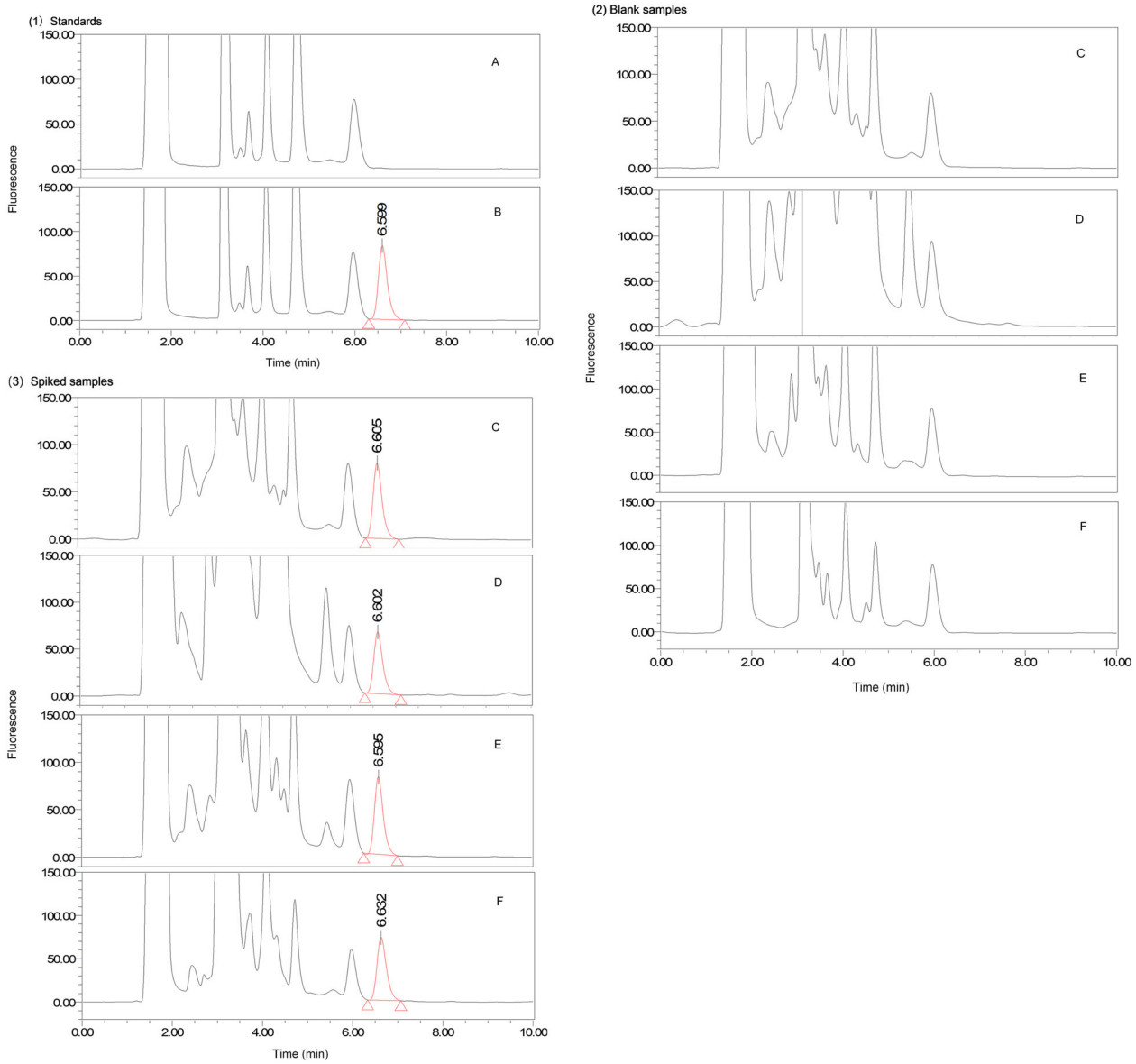

FIGURE 4 High-performance liquid chromatography-flourescence detection (HPLC-FLD) chromatograms of (1) standards, bank samples and spiked samples (50μg/kg). A, Dansyl chloride standard; B, piperazine standard (50μg/kg); C, chicken muscle; D, chicken kidney; E, chicken liver and F, prok

Table 1), an excitation wavelength of 330 nm and an emission wavelength of 531 nm were used.

3.7 | Method validation

3.7.1 | Linearity

The linearity of the method was evaluated for each matrix (chicken muscle, chicken kidney, chicken liver, and pork) using added concentrations of piperazine within the range of LOQ-200.0 μg/kg (four concentrations equal to the LOQ, 0.5 MRL, 1.0 MRL, and 2.0 MRL are reflected in the calibration curve). The linear ranges, the linear regression equations, and the determination coefficients ($R^2 \geq 0.9992$) are shown in Table 2. Compared with LOD and LOQ values of 6 and 20 μg/kg in previous studies,[10] respectively, LODs (0.96-1.85 μg/kg) and LOQs (3.20-5.50 μg/kg) were lower in this study, as shown in Table 1. This finding demonstrated that this method had higher sensitivity.

3.7.2 | Accuracy and precision

For methodological demonstrations, the European Union 2002/657/EC regulates[24] that the acceptable range for the recovery of analytes is 70% to 120%. In this study, the

TABLE 2 Linear ranges, linear regression equations, and determination coefficients of piperazine in chicken tissues and pork

Analyte	Tissues	Linear Range, μg/kg	Regression Lines	Relative Coefficient, R^2
Piperazine	Chicken muscle	3.20-200.0	y = 216097x − 47670	.9998
	Chicken kidney	5.50-200.0	y = 200108x − 123298	.9995
	Chicken liver	4.25-200.0	y = 206352x − 193268	.9993
	Pork	3.52-200.0	y = 207048x − 13936	.9992

recoveries were determined by spiked samples (chicken muscle, chicken kidney, chicken liver, and pork) with the LOQ, 0.5 MRL, 1.0 MRL, and 2.0 MRL piperazine (n = 6). The precision was determined by intraday RSD and interday RSD. The recoveries of piperazine from chicken tissues were 78.49% to 97.56% (RSD, 1.19%-5.32%; intraday RSD, 2.52%-5.42%; interday RSD, 3.32%-6.46%). The recoveries of piperazine from pork were 86.92% to 91.13% (RSD, 2.51% to 4.25%; intraday RSD, 3.71% to 4.65%; interday RSD, 4.75% to 7.06%). The results are shown in Table 3. The recoveries in this study were higher than those in a previous study[14] as shown in Table 1, thus demonstrating that this method is more accurate.

3.7.3 | Sensitivity and repeatability

The sensitivity was assessed based on the LOD and LOQ. The LODs in chicken muscle, kidney, and liver were 0.96, 1.85, and 1.60 μg/kg, respectively, and the LOQs were 3.20, 5.50, and 4.25 μg/kg, respectively. The LOD and LOQ in pork were 1.15 and 3.52 μg/kg, respectively, as shown in Table 4. The RSD of the retention time of the piperazine derivative among the six results was 0.12%, and the RSD of the peak area was 0.28%, which confirmed the repeatability of the determination of piperazine.

TABLE 3 Recovery and precision of piperazine added to blank chicken tissues and pork (n = 6)

Tissues	Addition Level, μg/kg	Recovery, %	RSD, %	Intraday RSD, %	Interday RSD, %
Chicken muscle	3.20	83.41 ± 2.71	3.25	3.70	4.29
	50.0	88.82 ± 1.06	1.19	3.08	5.24
	100.0[a]	97.56 ± 1.81	2.05	2.52	3.32
	200.0	96.23 ± 2.46	2.56	4.24	4.02
Chicken liver	5.50	78.49 ± 3.35	4.27	4.48	4.65
	50.0	84.96 ± 4.52	5.32	5.13	6.46
	100.0[a]	87.35 ± 2.15	2.46	3.10	3.77
	200.0	83.24 ± 3.53	4.24	5.41	5.31
Chicken kidney	4.25	79.72 ± 3.87	4.85	5.42	5.48
	50.0	87.08 ± 2.75	3.16	4.30	6.37
	100.0[a]	84.57 ± 2.49	2.95	3.26	3.84
	200.0	87.49 ± 1.04	1.19	2.91	3.51
Pork	3.52	87.44 ± 2.86	3.27	4.17	7.06
	50.0	86.92 ± 2.18	2.51	4.32	5.25
	100.0[a]	91.13 ± 3.54	3.88	3.71	5.44
	200.0	90.60 ± 3.85	4.25	4.65	4.75

Abbreviation: RSD, relative standard deviation.

[a]Maximum residue limit.

3.7.4 | Stability

The stability of derivative products has an important impact on detection results. The stability of the derivative is low, making the accuracy of the detection results poor, which is not suitable for quantitative analysis. Thus, HPLC-FLD analysis was conducted immediately after derivatization of the sample to ensure the accuracy of the results. The stability of the piperazine standard solution was investigated at −70°C, 4°C, and 25°C. The piperazine standard solution can be stored at −70°C protected from light for at least 2 months or at 4°C for 1 month without decomposition; however, piperazine gradually degrades after 15 hours at 25°C (room temperature). Therefore, −70°C was the best storage temperature. The stability of the derivatives at room temperature for 24 hours was assessed in this study; the stability was acceptable within 12 hours, but it degraded slowly after 16 hours, and the degradation was obvious at 24 hours.

3.7.5 | Real sample analysis

To evaluate the applicability and reliability of the validated method, we investigated 30 types of chicken tissues

TABLE 4 LODs and LOQs of piperazine in chicken tissues and pork

Analyte	Matrix	LOD, μg/kg	LOQ, μg/kg
Piperazine	Chicken muscle	0.96	3.20
	Chicken kidney	1.85	5.50
	Chicken liver	1.60	4.25
	Pork	1.15	3.52

Abbreviations: LOD, limit of detection; LOQ, limit of quantification.

(muscle, liver, and kidney) and pork from a local market. Among these tissue types, only chicken muscle (40, 50, and 60 μg/kg) and pork (30 and 40 μg/kg) were found to contain piperazine residues, although the residual amounts were below the MRL (less than 100 μg/kg) (FDA standard). The recoveries of these samples were 75.12% to 90.34% (RSD, 2.62%-5.32%; intraday RSD, 3.81%-5.51%; interday RSD, 4.85%-7.11%). Therefore, the HPLC-FLD method can be applied as a validated method to identify piperazine residues in chicken tissues and pork.

4 | CONCLUSION

In this study, ASE and SPE conditions and the derivatization and monosubstitution of piperazine with DNS-Cl were optimized, which further improved the detection of piperazine by the HPLC-FLD method in chicken tissues and pork. The optimization of the sample preparation procedure is of great significance for future research. The novelty of this test method is that ASE extraction and SPE purification of the sample have high sensitivity and can adequately trace the piperazine residues in animal-derived foods. Thus, this method can help deter livestock producers from using piperazine drugs in excess. The parameters for detecting piperazine were satisfactory and acceptable. Thus, this HPLC-FLD method, using precolumn derivatization, can be considered suitable for the detection of piperazine in both chicken tissues and pork.

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DISCLOSURE STATEMENT

We declare that there are no commercial relationships that might pose a conflict of interest in connection with the submitted manuscript.

ETHICAL APPROVAL

This article does not present any studies with human participants or animals performed by any of the authors.

ORCID

Kaizhou Xie https://orcid.org/0000-0002-2788-4323

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