RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2025.014

ScienceAsia 51 (1): 2025: ID 2025014: **1–9**

Trace and qualitative evaluation of residual piperazine in chicken muscle by HPLC-MS/MS employing automatic ASE coupled to solid-phase preconcentration

Pengfei Gao[a,b], Yawen Guo[a,b], Zhaoyuan He[a,b], Mengxiao Xue[a], Yuhao Dong[c], Kaizhou Xie[a,b,*]

[a] Department of Animal Science and Technology, Yangzhou University, Yangzhou 225009 China
[b] Joint International Research Laboratory of Agriculture & Agri-Product Safety, Yangzhou University, Yangzhou 225009 China
[c] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095 China

[*]Corresponding author, e-mail: yzxkz168@163.com

Received 22 Sep 2023, Accepted 3 Jan 2025
Available online 17 Mar 2025

ABSTRACT: This paper described a clipping, reliable, and hypersensitive protocol for the trace measurement of piperazine residues in chicken muscle by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The sample matrices were defatted with n-hexane and extracted with 2% trichloroacetic acid/acetonitrile using accelerated solvent extraction (ASE) at 80 °C and 1500 psi. Before using the HPLC system for separation, the samples were purified utilizing solid-phase extraction (SPE) and filtered with a 0.22-µm syringe. The qualitative and quantitative analyses applied electrospray ionization positive ion (ESI+) mode and multiple reaction monitoring (MRM) mode combined with an external standard method. Under optimized sample pretreatment measures and analytical conditions, the test results exhibited good linearity over the added concentration range (1–200 µg/kg). When the target was added at concentrations of 50, 100, and 200 µg/kg, the recoveries were 82.22%–88.63%, and the relative standard deviations (RSDs) were 1.56%–4.55%. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.3 µg/kg and 1.0 µg/kg, respectively. This method met the requirements of the EU and was successfully applied for the determination of piperazine residues in chicken muscle samples.

KEYWORDS: piperazine, ASE, HPLC-MS/MS, antibiotic residues, chicken muscle

INTRODUCTION

Piperazine (PIP) has been extensively utilized in clinical veterinary and animal feeding because of its potent efficacy and low cost; and it is widely used in the primary treatment of certain nematode infections, such as *Ascaris lumbricoides* [1] and *Trichostrongylus colubriformis* [2]. The nonstandardized use of PIP causes excessive residue in animal-derived foods, which leads to the proliferation of PIP in the food chain and ultimately endangers human health. Some studies have confirmed the potential harm of PIP to the human body. PIP is toxic to marginal cells of the cerebrospinal fluid and to the basal layer of the brain, leading to cytoplasmic vacuoles and subsequently causing swelling of the choroidal epithelium [3]. PIP also induces absence seizure and catatonia and can cause ataxia or worm wobble [4, 5]. PIP and its derivatives are in great demand as synthetic intermediates in the pharmaceutical industry. In general, nitrogen-containing heterocyclic amines are extremely difficult to degrade in the environment, and PIP is considered the least biodegradable of all similar heterocyclic amines [6]. Using PIP analogs as potential antibiotic efflux pump inhibitors has been implicated as an important mechanism for causing antibiotic resistance [7]. To ensure the safety of animal-derived foods and the smooth progress of related product trade, veterinary drug residue detection methods must be established and optimized according to the maximum residue limit (MRL) standards set by various countries or international organizations. In this study, the US [8] and the Japanese [9] regulations for MRL in chicken muscle of 100 µg/kg was used as the data basis.

To date, numerous methods that utilize chromatography and mass spectrometry to measure PIP residues have been reported, including high-performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) [10], HPLC-ultraviolet detection (HPLC-UV) [11], HPLC-fluorescence detection (HPLC-FLD) [12–14], ultrahigh-performance liquid chromatography-FLD (UPLC-FLD) [15, 16], gas chromatography-tandem mass spectrometry (GC-MS/MS) [17], UPLC-MS/MS [18], and HPLC-MS/MS [19]. Most organic compounds with low relative molecular masses of nitrogenous aliphatic amines, such as PIP, have neither satisfactory absorption in the visible and UV regions nor fluorescence properties, so they need to be chemically derivatized for determination by LC methods. Mass spectrometry uses ionization of ion sources for target analytes to generate ions with different mass-to-charge ratios (m/z), which are then detected via their interaction with the electromagnetic field. Therefore, mass spectrometry has the qualitative ability that other detection methods do not possess.

The combination of liquid chromatography and mass spectrometry can integrate the separation ability of liquid chromatography and the qualitative and quantitative analysis capabilities of mass spectrometry.

The sample pretreatment process is a crucial bottleneck of the analysis and directly affects the reliability and accuracy of the detected results. The pretreatment process adopted by the abovementioned methods was manual extraction, which has low extraction efficiency and is greatly influenced by the experiment's operator. In the present study, the novel instrument ASE was used to extract the target analyte, and the impurities were removed by SPE. The purpose of this work was to establish a rapid, reliable, and sensitive ASE-SPE-HPLC-ESI/MS/MS method for quantitative and qualitative analyses of PIP residues in chicken muscle. The established method would be beneficial for measuring PIP and could be a methodological reference for determining PIP residues in animal-derived foods.

MATERIALS AND METHODS

Experimental apparatus

List of equipments used were as follows: disintegrator (FW800: Taisete Instrument Co., Ltd., Tianjin, China); Ultrasonic bath (KQ-300DE: Shumei, Kunshan, China); high-speed refrigerated centrifuge (5810R: Eppendorf Corp., Hamburg, Germany); ASE (ASE350: Thermo Fisher Scientific Corp., Massachusetts, USA); Strata-X-C SPE column (3 ml/60 mg: Phenomenex Inc., Torrance, USA); and nitrogen blower (No. N-EVAP-112: Organomation Corp., Maryland, USA).

For HPLC-ESI/MS/MS, the system consisted of an HPLC separation module (Alliance e2695, Waters Corp., Massachusetts, USA) integrated with a triple quadrupole mass spectrometer (Triple QuadTM 5500: AB Corp., Massachusetts, USA); and a Waters SunFireTM C18 column (4.6 mm × 150 mm; particle size, 5 µm) was used to separate target analytes and interferers.

Chemicals and reagents

The PIP standard (99% purity) was from Sigma-Aldrich Co., Ltd. (Missouri, USA). Methanol and acetonitrile (LC grade) were from Merck Co., Inc. (New Jersey, USA). Aqueous ammonia (25–28% purity), trichloroacetic acid, and n-hexane were of analytical grade from Sinopharm Co., Ltd. (Shanghai, China). Other reagents were also of analytical grade. Ultrapure water with a resistivity of 18.2 MΩ cm (25 °C), consistent with the national laboratory water regulations (GB6682-1992) [20], was generated by the Milli-Q integral treatment system (Merck Co., Inc).

Preparation of standard, stock, and working solutions

Ten mg of PIP standard was accurately weighed and dissolved in 10 ml of methanol to obtain PIP standard solution (1 mg/ml), subsequently stored at −34 °C in the dark. This standard solution was diluted with methanol/water (1:4, v/v) to obtain three concentrations (1, 2, and 4 µg/ml) of PIP stock solution. A series of PIP working solutions (1, 5, 10, 20, 50, 100, and 200 ng/ml) was prepared by stepwise thinning of the stock solution and stored in the dark at −34 °C. The working solutions were removed from the refrigerator only when standard analyses were performed, or when they were to be added to blank samples.

For stability analysis, temperature conditions should reflect the possible situations in the actual sample processing and analysis. The stability analysis measured the stability of the target analyte in each step during processing: storage, pretreatment, and detection. The PIP standard working solutions were determined to be stable at −34 °C (storage temperature), 25 °C (room temperature), and 4 °C (maintained temperature in the HPLC sample manager) for 10 h without obvious degradation. The PIP working solutions of different concentrations could be stabilized for 6 h at 40 °C (the temperature used for nitrogen purging and drying), 25 °C, 4 °C, and −34 °C. After 6 h at 40 °C, the PIP was degraded to a certain extent; therefore, the sample analysis needed to be completed within 10 h.

Preparation of samples

Breeding, slaughtering, and sampling processes of animals strictly followed the recommendations in the "Guide for the Care and Use of Laboratory Animals of Jiangsu Province" and relevant regulations issued by the Animal Care and Use Committee of the Ministry of Agriculture and Rural Affairs of the People's Republic of China. The slaughter of the chickens was conducted using a low voltage, high-frequency stun, and efforts were made to ensure that the experimental animals lost consciousness as quickly as possible and suffered minimal pain.

Muscle samples were taken from Jinghai yellow chickens (a new broiler breed with high quality and small size: Jinghai Poultry Company, Jiangsu Province, China). Sample chickens did not receive any drugs during the feeding process. The samples were comminuted using a pulverizer at ambient temperature (25 °C); and intermuscular fat, sebum, and broken bones were removed with tweezers and immediately transferred into a −34 °C freezer.

The muscle samples were homogenized by a knife grinder (HM100: Grinder Instrument Equipment Co., Ltd., Beijing), accurately weighed to 2 g (±0.02 g), and then transferred to a grinding bowl. They were then ground with 4 g (±0.02 g) of diatomaceous earth. To achieve optimized extraction efficiency, the samples

were ground into particles as small as possible and placed in a 22 ml stainless-steel extraction tank. If necessary, a suitable amount of diatomaceous earth could be supplemented to the extraction tank before capping.

Extractions were performed using an accelerated solvent extractor. The fat impurities were removed from the sample with n-hexane, and the target analyte was extracted using 2% trichloroacetic acid acetonitrile. The parameters of ASE were: extraction tank pressure, 1500 psi; extraction tank temperature, 80 °C; static extraction process time, 5 min; total account of extraction solvent, 40% of the extraction cell capacity; and purification interval between two extraction cycles (nitrogen), 60 s.

Purification and cleanup were performed using solid-phase extraction, and a Strata-X-C SPE column with a strong cation exchange mode was used. The column was activated with methanol (3 ml) and balanced with 2% formic acid in water (3 ml). Prior to elution, the column was rinsed with 2.0% formic acid in water, ultrapure water, and methanol in sequence; and their infusion volumes were 3 ml. The column was eluted with 10 ml of aqueous ammonia/acetonitrile (1:9, v/v).

The eluate was then completely collected into a centrifuge tube (15 ml) and placed in an aluminum sand bath at 40 °C. A blowing needle was drilled deeply until it reached the appropriate position above the liquid level of the eluent in the centrifuge tube. To minimize droplet splashing, the liquid surface was purged using a steady nitrogen flow. The powder residue attached to the bottom of the tube was redissolved with 20% methanol in water (2 ml). After vortexing, the tube was centrifuged at a speed of 12,000g for 10 min. Afterward, the collected supernatant was filtered using a 0.22-µm syringe, and 10 ml of the solution to be tested was placed in a sample tray. The solution (10 µl) was injected into the HPLC-ESI/MS/MS system by the injection needle for separation and detection.

HPLC-ESI/MS/MS analysis

The C18-HPLC column (as formerly described) and the analytical software used was Analyst 1.6.1 version (AB Corp., Massachusetts, USA). The mobile phase was composed of two sections: 0.1% formic acid water (set as A) and methanol (set as B). Gradient elution was performed at 0.4 ml/min as follows: 0–3 min, 92% A; 6.5–7.5 min, 40% A; 9.5 min, 80% A; and 10–12 min, 92% A.

A triple quadrupole mass spectrometer, ESI source, positive ion mode, combined with MRM scanning was used to analyze structures. The most abundant chemical precursor used for PIP monitoring corresponded to the fragment ion transition m/z 87.1 to 44.1. The spray voltage was adjusted to 5500 V, and the ion source temperature was set at 550 °C. The pressures of the spray gas, auxiliary gas (high purity nitrogen), curtain gas, and impact gas were 50, 50, 35, and 8 psi, respectively. The collision energy was 20.8 eV for the qualitative ion pair for the 87.1→70.0 mass transitions and 24.1 eV for the 87.1→44.1 mass transition. Both declustering potentials were 81.2 V.

Quality parameters

The linearity of this method was evaluated by plotting the calibration curve. The calibration curve was rendered by determining the peak area of the quantitative ions at the different addition concentrations of PIP and fitting these data based on the regression equation $Y = aX + b$ using least-squares linear regression. The linear assay was performed using six concentrations of PIP (1, 5, 10, 50, 100, and 200 µg/kg). The coefficients of determination (R^2) were determined, all of which should be $\geqslant 0.99$.

To evaluate the sensitivity of the method, the LOD and the LOQ values were assigned by the signal-to-noise ratio (SNR) of the product ions. With an SNR greater than or equal to 3 ($\geqslant 3$), the LOD was the lowest analyte concentration that could be detected; and the LOQ was the lowest analyte concentration that could be quantified when the SNR was greater than or equal to 10 ($\geqslant 10$).

One hundred microliters of the 1, 2, and 4 µg/ml PIP standard solutions were added to 2 g of homogenized blank sample matrices (the corresponding concentrations were 50, 100, and 200 µg/kg, respectively) to determine the recovery. The peak area was substituted into the regression equation ($Y = aX + b$) to calculate the concentration of PIP. The detected PIP concentration was compared with the actual concentration added to the sample to determine the recovery.

The intraday precision assessment was conducted by testing samples of different concentrations (0.5, 1.0, and 2.0 MRL) at six different times within a single day using the same standard curve and the same instrument. The interday precision assessment involved the performance of a six-consecutive day determination using the same instrument and different daily standard curves. The RSD (%) value was calculated using the Horwitz formula: $\mathrm{RSD}_{\max} = \mathrm{RSD} \times 2/3$, $\mathrm{RSD} = 2(1 - 0.5 \log c)$, where c representing the analyte concentration (g/g). A newly established method is perceived to be accurate and precise when its recovery ranges from 70–110%, and RSD does not exceed $\mathrm{RSD}_{\max}$ [21].

The standard deviation (SD) was determined by adding 2 µg/ml (100 µl) PIP working solution to 20 different chicken samples (2 g each), and the decision limit (CCα) value was calculated using equation CCα = MRL + 1.64 × SD (α = 5%). The detection capability (CCβ) was calculated using equation CCβ = CCα + 1.64 × SD (β = 5%).

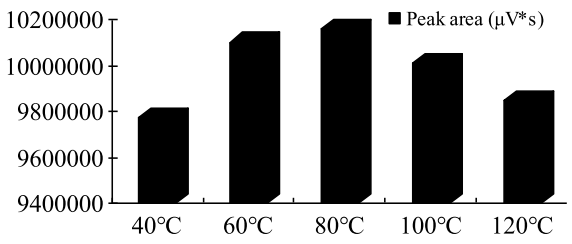

Fig. 1 Effects of temperature on ASE extraction efficiency.

Based on the regulations established by the EU [21], the established method was validated by determining the linearity, LOD, LOQ, recovery, precision, CCα, and CCβ values.

RESULTS AND DISCUSSION

Optimization of the pretreatment process

As solubility of interested components in the extracting agent is different from that of the impurities, liquid-liquid extraction (LLE) uses this principle to achieve separation. Emulsification often occurs during LLE, which greatly affects the reproducibility of the detection results [22]. Moreover, the extraction efficiency of the analyte in the LLE process largely depends on operational skills and parameters involved. Solvent extraction, ultrasonic-assisted liquid extraction. and supercritical fluid extraction were shown to be efficient in extracting bioactive compounds like flavonoids and glucosides from plant extracts [23].

ASE is a technique for extracting solid or semisolid samples using appropriate organic solvents. The goal is to obtain a higher extraction efficiency by increasing temperature and pressure [24]. In comparison to traditional manual extraction methods, e.g., LLE; ASE has many advantages, for instance, fewer organic solvents, speed, automation, simple operation, and multiple samples for one-time processing (a total of 24 extraction tanks).

SPE utilizes the ability of the mixture to be adsorbed within the solid filler to effectively separate the target analyte from the interfering components. Based on the chemical properties of PIP determined during the preexperiment [13], its recovery using three strong cation exchange columns (PCX, MCX, and Strata-X-C) was compared. The Strata-X-C SPE column had an obvious purification effect, no interfering peak existed, and the highest recovery rate. This result might be due to the type of filler, the packing technique, and the particle size of the filler that was used [25]. To ensure effective elution, choice and amount of eluent were compared, which resulted in the selection of 10 ml of 10% aqueous ammonia acetonitrile (aqueous ammonia:acetonitrile = 1:9, v/v) for elution.

The purpose of sample pretreatment was to extract the target analyte while removing impurities to the greatest extent possible. Hence, combining extraction and purification would be effective. The effects of ASE-SPE and LLE-SPE on recovery were also compared. The data in Table 1 clearly show that the recovery obtained using ASE-SPE was higher than that obtained using LLE-SPE. After considering the many advantages of ASE related to LLE, ASE-SPE was chosen for sample pretreatment in this study.

PIP is a relatively polar compound and weakly alkaline in an aqueous solution; therefore, it can generally be extracted with a polar solvent or acid solution [13]. The extraction effects of 2% trichloroacetic acid/ultrapure grade water, 2% trichloroacetic acid/methanol, and 2% trichloroacetic acid/acetonitrile were compared using ASE. When water or methanol was used, the extracts were more turbid due to high polarity, which is not conducive to the purification of SPE. In addition, the deproteinization effect of water or methanol is not as good as that of acetonitrile. When 2% trichloroacetic acid/acetonitrile was used as the extraction solvent, the recoveries of the target analyte were better (all above 80%; Table 1). Moreover, the extract was clear and free of visible impurities. Therefore, 2% trichloroacetic acid/acetonitrile was ultimately selected as the extraction reagent.

High temperature and high pressure are important parameters of ASE, in contrast to other traditional extraction methods. In this study, various extraction temperatures (40, 60, 80, 100, and 120 °C) were compared. The results showed that the impurity interference in the extract was the smallest and the recovery rate was the highest when the extraction temperature was 80 °C (Fig. 1). An increase in extraction pressure raises the boiling point of the liquid, allowing the extraction solvent to remain liquid at elevated temperatures. From our previous experiments, we found that extraction pressure had little effect on the recovery rate, so it was set to the instrument's default value of 1500 psi. In addition to the extraction temperature and pressure, the number of static cycles and the time of static extraction also affected the recovery. As the static extraction time increased, the extraction process gained more time to diffuse into the diatomaceous earth, contributing to improved extraction efficiency. Static extraction of the target analyte using two cycles was sufficient to ensure complete extraction. Moreover, a longer static extraction time or more static extraction cycles could reduce efficiency [26]. This study was carried out at 80 °C and 1500 psi for 5 min with 2 static cycles.

Optimization of HPLC-ESI/MS/MS analysis

The composition of the mobile phase, the gradient or isocratic elution procedures used, the column selection and temperature, and other chromatographic parameters could affect the detection of mass spectrometry [18, 19]. A good balance between chromato-

Table 1 Comparison of extraction methods and solvents used for the extraction efficiency of piperazine (%).

Spiked level	LLE-SPE			ASE-SPE		
(μg/kg)	2% trichloroacetic acid/ultrapure grade water	2% trichloroacetic acid/acetonitrile	2% trichloroacetic acid/methanol	2% trichloroacetic acid/ultrapure grade water	2% trichloroacetic acid/acetonitrile	2% trichloroacetic acid/methanol
50	57.43 ± 1.92	78.56 ± 1.06	61.24 ± 2.53	59.02 ± 1.92	82.82 ± 1.06	60.90 ± 2.53
100	60.12 ± 2.42	80.37 ± 2.52	61.59 ± 1.65	64.62 ± 6.42	84.59 ± 4.52	63.96 ± 1.65
200	67.35 ± 3.51	84.52 ± 2.18	62.50 ± 1.61	68.48 ± 3.51	89.59 ± 2.18	66.42 ± 1.61

graphic separation efficiency and MS/MS sensitivity was achieved by using a mobile phase consisting of 0.1% formic acid water and methanol. Because of the polarity of PIP, 0.1% formic acid was added to enrich PIP and ameliorate its peak shape. The polarity of the mobile phase was changed by altering the proportion of the solvents in the mobile phase, which allowed the PIP to be optimally separated within a short retention time, the peak shape to be improved, and the sensitivity to be increased. Since the mobile phase contained acid, a sufficient equilibrium time was set at the beginning and at the end of the elution procedure to achieve optimal separation on the column. Most studies, other than the one performed by Xie et al [18], used C18 columns [12–16, 19]. Therefore, this study also used C18 column to retain PIP residues; the column temperature was set at 25 °C.

The triple quadrupole mass spectrometer was equipped with an ESI source. The sample solution was rapidly atomized by high temperature and strong electric field to produce highly charged droplets. After the evaporation and shrinkage of the charged droplets, single-charge and multi-charge ions were produced. Three quadrupole mass analyzers were connected in series to form a mass analyzer, and the multi-charge ions were separated and allowed to enter the detector [27]. Fifty ng/ml PIP standard solutions were fully scanned in ESI (+) mode and ESI (−) mode, with the ESI (+) mode highly sensitive to PIP. In the Q1 scan, after data acquisition, the precursor ion was the abscissa molecular weight corresponding to the center of the target analyte peak in total ion chromatography. In the MS2 scan, the CE value was manually adjusted until the precursor ion signal was only approximately one-third of the strongest fragment ion signal, and the two strongest fragment ions were the product ions that quantify the target analyte. Different cone voltages and collision energies were tested and selected, and MRM mode was used to detect the target analyte.

Method validation

No interference peaks derived from endogenous compounds were found during the retention time of the target analyte, and the specificity of the method was proven (Fig. 2–Fig. 4). The abscissa (X) was the concentration of the target analyte added to the blank sample matrix, and the ordinate (Y) was the detected peak area so that the standard curve could be drawn. In the range of 1–200 μg/kg, the peak areas demonstrated a good linear relationship with the concentration of PIP ($y = 45237x + 33224$); and the coefficient of determination (R^2) was 0.9997.

The LOD determined for this method was 0.3 μg/kg, and the LOQ was 1 μg/kg. An additional test was performed at the LOQ concentration. The average recovery was 76.24%, and the intraday RSD and interday RSD were 7.08% and 9.16%, respectively. The LOQ was considered reliable when the recovery was greater than 70%, and the RSDs were less than 20% [21].

Three concentrations (50, 100, and 200 μg/kg) of PIP were tested to calculate recovery. Table 2 shows their recoveries of 82.22%–88.63%, and the RSDs of less than 4.95%. The differences between the precision (intraday precision and interday precision) and the RSD ranged from 0.2%–2.34%. The RSDs did not exceed the RSDmax of PIP, thus proving that the developed method met the requirements established by the European Medicines Agency [21].

Twenty blank samples containing 100 μg/kg PIP were tested; the CCα and CCβ values obtained were 106 μg/kg and 113 μg/kg, respectively, which were close to the MRL value.

Matrix effect assessment

Matrix effect was related to the characteristics of sample matrices, the interference from compounds contained in the matrix, the properties and concentration of the target analyte, the method used for sample pretreatment, the chromatographic conditions, and the structure of the ion source; and it could not be completely avoided [28]. Bonfiglio et al [29] analyzed three drugs with different polarities using reversed-phase chromatography. The results showed that the matrix effect was significantly correlated with the chemical structure and became more influential as the polarity increased. A matrix effect was generated because of the strong polarity of the amino group in the PIP structure. Additionally, PIP was more likely to be protonated within the background produced by the coeluting matrix components [18]. The PIP stock solution was diluted with a blank sample matrix to

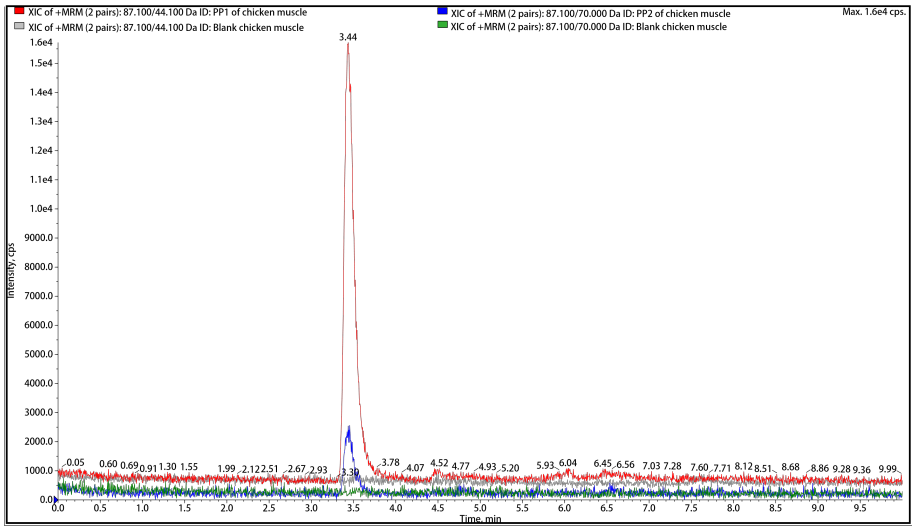

Fig. 2 Total ion chromatogram of blank chicken muscle samples and piperazine spiked in blank chicken muscle samples (5.0 µg/kg).

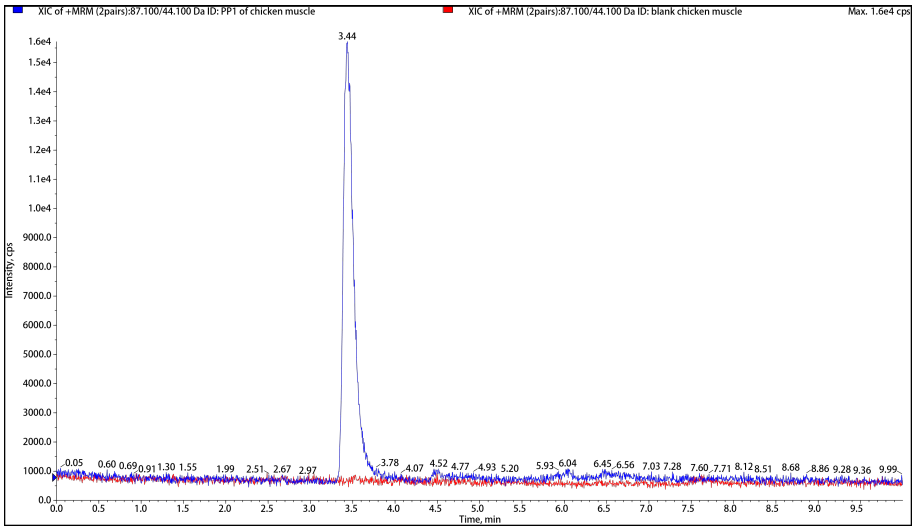

Fig. 3 Extracted ion chromatograms (87.100/44.100) of blank chicken muscle samples and piperazine spiked in blank chicken muscle samples (5.0 µg/kg).

compensate for the matrix effect.

Real sample analysis

To evaluate the applicability and reliability of the newly developed method, we analyzed muscle samples from 40 chickens obtained from local supermarkets. PIP residues were detected in only 5 samples, of which the concentrations in µg/kg were 6.84, 7.43, 11.86, 8.56, and 8.35. The SD values of each sample in which PIP was detected were 5.47%, 3.89%, 2.53%, 2.72%, and 5.08%, respectively. Therefore, this method could be used as a novel method for quantifying PIP in chicken

Table 2 Recovery and precision of the measurement of piperazine added to blank chicken muscle samples ($n = 6$).

Spiked level µg/kg	Recovery %	RSD %	Intraday RSD %	Interday RSD %	Intraday RSD_{max} %	Interday RSD_{max} %
50	86.43 ± 4.28	4.95	5.96	7.29	16.7	25.1
100	88.63 ± 3.51	3.97	2.19	3.77	15.1	22.7
200	82.22 ± 1.28	1.56	2.33	3.21	13.6	20.4

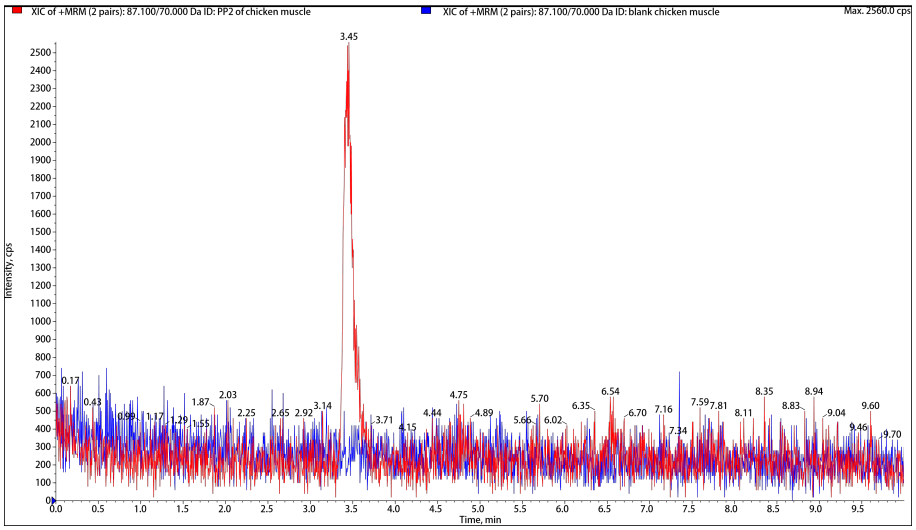

Fig. 4 Extracted ion chromatograms (87.100/70.000) of blank chicken muscle samples and piperazine spiked in blank chicken muscle samples (5.0 μg/kg).

muscle.

Comparison of different detection methods

PIP is difficult to determine using an ultraviolet or fluorescence detector because of its weak absorption in the ultraviolet-visible spectrum. Therefore, derivatization prior to liquid chromatography separation was used to bypass the determination restrictions of PIP in existing approaches. Different derivatization reagents have been used in different reports. 4-chloro-7-nitrobenzofuran was used as a derivatization reagent for determining PIP residues in pharmaceutical reagents with an ultraviolet detector by Navaneeswari and Reddy [11]. Skapring et al [30] used ethyl- and isobutyl-chloroformate as derivatization reagents and then determined the concentration with capillary gas chromatography. The detection limits for PIP in urine were approximately 20 ng/ml (nitrogen-selective detection) and 1 ng/ml (mass-selective detection). In studies on detecting PIP residues in animal products, derivatives with fluorescent groups were obtained by derivatizing PIP with dansyl chloride [12–16, 19, 31]. In the studies of Wang et al [17] and Pietsch et al [32], acetic anhydride and chloroformates were used as derivatization reagents, respectively. The present study did not require derivatization to circumvent the uncontrollable derivative reaction, possibly leading to a production of uncertain derivatives. Among the reported methods, only the study by Xie et al [18] did not perform derivatization. Our LC separation system and columns used were different from that reported by Xie et al [18]. While Lin et al [19] used an HPLC-MS/MS method with a sample matrix of human plasma, our study worked on a sample matrix of chicken muscle. In addition, automatic ASE technique was introduced to the sample pretreatment process in the present study to effectively avoid all kinds of errors caused by anthropic factors in the LLE process. Therefore, the present study could be considered as more meaningful advantage than the above two LC-MS/MS studies [18, 19]. Compared with other LC methods, the LC-MS/MS method had a higher selectivity and efficiently separated target analytes. We also compared the recovery, LOD, and LOQ with those of other studies (Table 3). The results showed that our newly developed method had high sensitivity and selectivity. The establishment of this method provided different experimental options and a scientific basis for detecting PIP. Because of automation and easy integration of ASE, it could also be widely used in drug screening, environmental monitoring, food safety, and other fields. Meanwhile, compared with the LLE method, the ASE technique had the advantages of high extraction efficiency, speed, low sample/reagent consumption for environmental protection, and less error compared with manual extraction, making it suitable for batch processing of samples.

CONCLUSION

This study established a pioneering, feasible, and sensitive HPLC-ESI/MS/MS protocol for determining piperazine residues in chicken muscle. Samples were extracted with 2% trichloroacetic acid/acetonitrile using the ASE technique and defatted with n-hexane saturated with acetonitrile. The manifold merits of ASE over manual operation ensured its effectiveness and applicability in sample pretreatment. The detection method used ESI (+) mode for ionization, MRM mode for detection, and the external standard method for quantitative analysis. Compared with other reported

Table 3 Recovery and precision of the measurement of piperazine added to blank chicken muscle samples ($n = 6$).

Method	Sample	Stationary phase	Mobile phase	Detector	Recovery, %	LOD	LOQ
HPLC-ELSD [10]	Naproxen piperazine, phenylbutazone piperazine	Alltech Alltima Cyano (250 mm × 4.6 mm)	95% acetonitrile, 4.85% deionized water, and 0.15% nitric acid	ELSD	59.8–89.2	10.0 ng/ml	–
HPLC-FLD [12]	Beef, pork, chicken, milk, eggs	Waters Xbridge C18 column (250 mm × 4.6 mm, 5 µm)	Water and HPLC-grade acetonitrile (30:70, v/v)	FLD	80.6–97.3	6.0 µg/kg	20.0 µg/kg
GC-MS/MS [17]	Chicken and pig tissues	Thermo Fisher TG-5 MS amine column (30 m × 0.25 mm, 0.25 µm)	Helium grade 5	Trace 1300 and TSQ 8000 selective MS/MS detector	77.5–96.3	1.4–1.6 µg/kg	4.8–5.2 µg/kg
UPLC-ME/MS [18]	Chicken muscle	Waters UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm)	0.1% formic acid-methanol (50:50, v/v)	Triple-quadrupole mass spectrometer	102.9–111.5	0.4 µg/kg	1.0 µg/kg
HPLC-MS/MS [19]	Human plasma	Agilent Zorbax SB-C18 column (150 mm × 2.1 mm, 3.5 µm)	Ammonium acetate solution (pH 3.0)-methanol (50:50, v/v)	Triple-quadrupole TSQ Quantum mass spectrometer	84.0–91.3	10.0 ng/ml	100.0 ng/ml
Method used in this work	Chicken muscle	Waters SunFireTM C18 (150 mm × 4.6 mm, 5 µm)	0.1% formic acid water and methanol	Triple-quadrupole TSQ Quantum mass spectrometer	82.2–88.6	0.3 µg/kg	1.0 µg/kg

methods, this optimized HPLC-ESI/MS/MS method met the requirements of the European Medicines Agency and provided a simple and precise alternative for measuring piperazine. This novel method was successfully applied to analyze 40 samples, demonstrating its applicability and reliability.

Acknowledgements: This work was supported by the National Key R&D Program of China (2019YFC1605400), the earmarked fund for the China Agriculture Research System (CARS-41), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Yangzhou University high-end talent support program, and the Postgraduate Research and Practice Innovation Program of Jiangsu Province (KYCX22_3526).

REFERENCES

1. Bueding E, Saz HJ, Farrow GW (1959) The effect of piperazine on succinate production by *Ascaris lumbricoides*. *Brit J Pharmacol* **14**, 497–500.
2. White WH, Gutierrez JA, Naylor SA, Cook CA, Gonzalez IC, Wisehart MA, Smith II CK, Thompson WA (2007) *In vitro* and *in vivo* characterization of p-aminophenethyl-m-trifluoromethylphenyl piperazine (PAPP), a novel serotonergic agonist with anthelmintic activity against *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. *Vet Parasitol* **146**, 58–65.
3. Conrad J, Edward S, Paul M, Daniel R, Juergen F, Georg K (2011) The distributional nexus of choroid plexus to cerebrospinal fluid, ependyma and brain. *Toxicol Pathol* **39**, 186–212.
4. Conners GP (1995) Piperazine neurotoxicity: worm wobble revisited. *J Emerg Med* **13**, 341–343.
5. Yohai D, Barnett SH (1989) Absence and atonic seizures induced by piperazine. *Pediatr Neurol* **5**, 393–394.
6. Charney DS, Woods SW, Goodman WK, Heninger GR (1987) Serotonin function in anxiety. II. Effects of the serotonin agonist, mCPP, in panic disorder patients and healthy subjects. *Psychopharmacology* **92**, 14–24.
7. Choquet M, Lohou E, Pair E, Sonnet P, Mullie C (2021) Efflux pump overexpression profiling in *Acinetobacter baumannii* and study of new 1-(1-naphthylmethyl)-piperazine analogs as potential efflux inhibitors. *Antimicrob Agents Chemother* **65**, aac.00710-21.
8. FDA (2014) *CFR – Code of Federal Regulations Title 21 Part 556 Tolerances for Residues of New Animal Drugs in Food*. Food and Drug Administration (FDA), USA.
9. JFCRF (2015) *Maximum Residue Limits (MRLs) List of Agricultural Chemicals in Foods*. The Japan Food Chemical Research Foundation (JFCRF), Japan.
10. McClintic C, Remick DM, Peterson JA, Risley DS (2003) Novel method for the determination of piperazine in pharmaceutical drug substances using hydrophilic interaction chromatography and evaporative light scattering detection. *J Liq Chromatogr R T* **26**, 3093–3104.
11. Patel J, Loeser E, Kircher R, Marrepalli HR, Fazio SD, Drinkwater DE, Drumm P (2010) Analytical method for 1-methyl-4-amino-piperazine in an active pharmaceutical ingredient using chemical derivatization and HPLC-UV. *J Liq Chromatogr R T* **33**, 712–719.
12. Park JA, Zhang D, Kim S, Kim SK, Cho S, Jeong D, Kim J, Shim J, et al (2015) Development of a high-performance liquid chromatography with fluorescence detection method for quantification of piperazine in animal products by using precolumn derivatization. *Food Chem* **196**, 1331–1337.
13. Liu C, Xie X, Wang B, Zhao X, Guo Y, Zhang Y, Bu X, Xie K, et al (2019) Optimization of ASE and SPE conditions for the HPLC-FLD detection of piperazine in chicken tissues

and pork. *Chirality* **31**, 845–854.
14. Bu X, Pang M, Wang B, Zhang Y, Xie K (2020) Determination of piperazine in eggs using accelerated solvent extraction (ASE) and solid phase extraction (SPE) with high-performance liquid chromatography-fluorescence detection (HPLC-FLD) and pre-column derivatization with dansyl chloride. *Anal Lett* **53**, 53–71.
15. Guo Y, Xie X, Wang B, Zhang Y, Xie K, Bu X (2020) The establishment of a practical method for the determination of piperazine residues using accelerated solvent extraction and UHPLC-FLD. *Qual Assur Saf Crop* **12**, 28–39.
16. Wang B, Zhang Y, Xie K, Bu X, Liu C (2020) Automated accelerated solvent extraction coupled with ultra-high-performance liquid chromatography for the analysis of piperazine in egg samples. *J Consum Prot Food S* **15**, 363–371.
17. Wang B, Pang M, Xie X, Xie K, Zhang Y, Cui L, Zhao X, Wang Y (2017) Quantification of piperazine in chicken and pig tissues by gas chromatography-electron ionization tandem mass spectrometry employing pre-column derivatization with acetic anhydride. *J Chromatogr A* **1519**, 9–18.
18. Xie K, Liu Y, Sun L, Pang M, Wang J (2016) Quantification of piperazine in chicken muscle by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Food Anal Method* **10**, 1736–1744.
19. Lin H, Tian Y, Zhang Z, Wu L, Yun C (2010) Quantification of piperazine phosphate in human plasma by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry employing precolumn derivatization with dansyl chloride. *Anal Chim Acta* **664**, 40–48.
20. Standardization Administration (2008) *Water for Analytical Laboratory Use-specification and Test Methods*. Standardization Administration of the People's Republic of China, China.
21. EMA (2002) *Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results*. The European Medicines Agency (EMA), EU.
22. Kanaya K, Kanda A, Suzuki Y (2016) Stabilization technology development for the liquid-liquid extraction process in a bioplant. *Chem Eng Res Des* **108**, 49–54.
23. Rungruang R, Panichakul T, Rattanathavorn W, Kaisangsri N, Kerdchoechuen O, Laohakunjit N, Chao LK (2021) Effects of extraction methods on the flavonoid and phenolic contents and anti-aging properties of *Rhyncholaeliocattleya* Haw Yuan Beauty extracts. *ScienceAsia* **47**, 698–706.
24. Lu Y, Zhu Y (2014) Combination of accelerated solvent extraction and vortex-assisted liquid-liquid microextraction for the determination of dimethyl fumarate in textiles and leathers by gas chromatography-mass spectrometry. *Talanta* **119**, 430–434.
25. Song Y, Chai T, Lou S, Zhao Y, Zhang X, Yang X, Qian Y, Jing Q (2018) Determination of synephrine in feeds by a novel quick, easy, cheap, effective, rugged, and safe solid-phase extraction method combined with UHPLC-MS/MS. *J Sep Sci* **41**, 1743–1751.
26. Richter BE, Jones BA, Ezzell JL, Porter N, Avdalovic N, Pohl C (1996) Accelerated solvent extraction: A technique for sample preparation. *Anal Chem* **68**, 1033–1039.
27. Barton SJ, Whittaker JC (2010) Review of factors that influence the abundance of ions produced in a tandem mass spectrometer and statistical methods for discovering these factors. *Mass Spectrom Rev* **28**, 177–187.
28. Taylor PJ (2005) Matrix effects: The achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clin Biochem* **38**, 328–334.
29. Bonfiglio R, King RC, Olah TV, Merkle K (1999) The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun Mass Sp* **13**, 1175–1185.
30. Skarping G, Bellander T, Mathiasson L (1986) Determination of piperazine in working atmosphere and in human urine using derivatization and capillary gas chromatography with nitrogen- and mass-selective detection. *J Chromatogr A* **370**, 245–258.
31. Gadzała-Kopciuch R (2005) Accurate HPLC determination of piperazine residues in the presence of other secondary and primary amines. *J Liq Chromatogr R T* **28**, 2211–2223.
32. Pietsch J, Hampel S, Schmidt W, Brauch HJ, Worch J (1996) Determination of aliphatic and alicyclic amines in water by gas and liquid chromatography after derivatization by chloroformates. *Fresen J Anal Chem* **355**, 164–173.