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Quantification of piperazine in chicken and pig tissues by gas chromatography–electron ionization tandem mass spectrometry employing pre-column derivatization with acetic anhydride

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ABSTRACT

This paper describes a novel method that combines acetic anhydride derivatization with gas chromatography–electron ionization tandem mass spectrometry (GC–EI/MS/MS) for the sensitive and selective determination of piperazine in chicken and pig tissues. Samples were extracted using an accelerated solvent extraction (ASE) apparatus, purified by solid-phase extraction (SPE) and derivatized with acetic anhydride. This optimized method was validated according to the requirements defined by the European Union and the Food and Drug Administration. At the limit of quantification (LOQ) spiked levels of 50.0, 100.0, 500.0, 1000.0 and 2000.0 μg/kg, the average recoveries of piperazine in chicken and pig tissues were 77.46–96.26%, with relative standard deviations (RSDs) of 1.55–6.64%. The intra-day RSDs were 1.39–5.92%, and the inter-day RSDs were 2.24–8.39%. The limits of detection (LODs) and the LOQs were 1.4–1.6 μg/kg and 4.8–5.2 μg/kg, respectively. The decision limits (CC_α) were 102.02–105.17 μg/kg, and the detection capabilities (CC_β) were 104.03–109.09 μg/kg. Finally, the new approach was verified for the quantitative determination of piperazine in 30 commercial chicken and pig tissues from local supermarkets.

1. Introduction

Piperazine is currently used as an effective vermifuge and has been widely applied in veterinary clinics [1]. Piperazine exhibits various structural and pharmacological characteristics caused by differences in substituent groups, sites, and spatial structures [2,3]. However, the improper use of piperazine causes a serious issue—drug residues in animal tissues—which threatens human health after long-term consumption of animal-derived products containing piperazine. To ensure the food safety of consumers, the European Union (EU) and the Food and Drug Administration (FDA) have set a maximum residue limit (MRL) for piperazine of 100 μg/kg in animal tissues [4,5]. As a result, various methods have been used to measure piperazine or its salts in pharmaceutical preparations, including gravimetry [6,7], colorimetry [8,9], spectrophotometry [10–13], gas chromatography [14,15], high-performance liquid chromatography (HPLC) [16–19], and HPLC tandem mass spectrometry [20,21]. Gravimetry involves complex operations and cannot be used to determine trace analytes. Colorimetry is relatively simple but has a low selectivity and a high limit of detection (LOD). Rogers [22] found that more than one type of substance could cause the same color-producing reaction with piperazine; therefore, the colorimetric method may not be accurate because of its low selectivity and specificity. Lin et al. [21] reported a quantitative analysis method for piperazine phosphate in human plasma using HPLC–ESI/MS/MS after derivatization with dansyl chloride (DNS-CL). The validated HPLC–ESI/MS/MS method was successfully applied in a bioequivalence study of piperazine phosphate trochiscus in healthy Chinese male volunteers. A method using HPLC with fluorescence detection (FLD) for the quantification of piperazine in animal products was recently reported using

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pre-column derivatization [23]. This analytical method was used to separate and detect piperazine residues and was applied to monitor the piperazine concentrations in 5 beef, pork, chicken, milk, and egg samples purchased from large markets in Seoul, Republic of Korea.

In an earlier study, we developed an ultra-performance liquid chromatography-electrospray ionization tandem triple quadrupole mass spectrometry (UPLC–ESI/MS/MS) method for the detection of piperazine in chicken muscle [24]. However, to date, the quantitative determination of piperazine residues in chicken and pig tissues using gas chromatography–tandem mass spectrometry (GC–MS/MS) has not been previously reported. Therefore, the purpose of the present study was to establish a simple, rapid and specific GC–MS/MS method for the quantitative analysis of piperazine in the muscle, the liver, the kidney, and a mixture of skin and fat tissues from chicken and pig, providing a new method for the determination of piperazine residues. This experimental study holds great significance for promoting animal food safety testing, human health and international trade exports and provides a scientific basis for the determination of piperazine residues in chicken and pig tissues.

2. Materials and methods

2.1. Chemicals and reagents

A piperazine (99.0% purity) standard was purchased from Sigma–Aldrich Company (St. Louis, Missouri, USA). 1,4-Diacetyl-piperazine (95.0% purity) standard was obtained from International Laboratory USA (San Francisco, California, USA). Analytical grade methanol, acetonitrile, *n*-hexane, formic acid, methylene chloride, trichloroacetic acid, ethyl acetate, Na_2SO_4, infusorial earth, sodium sulfate and acetic anhydride (the derivatization agent) were supplied by Sinopharm Chemical Reagent Co. (Shanghai, China). HPLC-grade triethylamine was purchased from Tedia Company Inc. (Fairfield, OH, USA). Water was purified using a PURELAB Option-Q synthesis system (ELGA Lab Waters, High Wycombe, Bucks, UK).

2.2. Preparation of the standard stock and working solutions

The standard stock solution of piperazine was prepared by dissolving 10 mg of piperazine in 10 mL of methylene chloride (1.0 mg/mL). 10 μL of the standard stock solution of piperazine (1.0 mg/mL) was then diluted to 10.0 μg/mL with 990 μL of methylene chloride for calibration. The working solutions were prepared daily and were stored at 4 °C, while the stock solutions were stored at −75 °C in an ultra-low temperature refrigerator, providing stable storage for 3 months.

2.3. GC–MS/MS instrumentation and conditions

GC–MS/MS analysis was performed using a Trace 1300 and TSQ 8000 selective MS/MS detector coupled with a Triplus RSH automatic sample injector (Thermo Fisher Corp., Massachusetts, USA). The GC was operated in splitless mode (the splitless time was 1 min) with the carrier gas (helium grade 5) at a flow rate of 1.0 mL/min. The temperature program was as follows: (a) 80 °C for 1 min; (b) increase at a rate of 20 °C/min from 80 °C to 280 °C and hold for 1 min. The injector temperature was held at 280 °C. The injection volume was 1.0 μL. Fig. 1 shows that the electron impact (EI) mass spectra (MS) of the derived product has distribution characteristics and ion ratios of fragment ions identical to the MS of 1,4-diacetyl-piperazine; the characteristic fragments are *m/z* 56, 69, 85, 111, 127, 156, and 170. The formula weight of the derived product is the same as that of 1,4-diacetyl-piperazine (*m/z* 170.1), a compound that is produced by the addition of two acetyl groups to the piperazine molecule (nucleophilic substitution).

Table 1
Retention time and relevant MS parameters.

Molecular Weight	Retention Time (min)	Mass Transitions (*m/z*)	Collision Energy (eV)
170.1	8.47	170.1 > 56.1	20
		170.1 > 68.1[a]	10
		170.1 > 85.1	10

[a] Quantitative ion.

GC–MS/MS was performed using a Thermo Fisher TG-5MS Amine (30 m × 0.25 mm i.d., 0.25 μm) column with helium as the carrier gas at a flow rate of 1.0 mL/min. The tandem MS system was equipped with an EI source and TraceFinder 3.0 software (Thermo Fisher Technologies, Massachusetts, USA). The mass spectrometer was operated in selected reaction monitoring (SRM) mode at an ionization voltage of 70 eV with both source and transfer line temperatures operated at 280 °C. Detection was performed on a triple-quadrupole mass spectrometer using EI and SRM modes with the precursor to product ion transition *m/z* 170.1 → 68.1 for 1,4-diacetyl-piperazine, as shown in Table 1.

2.4. Quality parameters

The linearity of the method was determined by analyzing the standard plots associated with a 7-point standard calibration curve, which was prepared by spiking the blank tissue sample with the appropriate volume of the 10.0 mg/mL working solution mentioned above to produce curve points equivalent to 4.5, 50.0, 100.0, 500.0, 1000.0, 2000.0, and 2500.0 μg/kg piperazine. The calibration curve was generated by tracing the peak area of the analyte versus the piperazine concentration and was fitted to the equation $Y = aX + b$ using least-squares linear regression. Meanwhile, the correlation coefficient (R^2) was determined, and the value was $R^2 \geq 0.99$.

The recovery and precision were tested by analyzing six independently spiked blank tissue samples at the LOQ, 50.0, 100.0, 500.0, 1000.0, and 2000.0 μg/kg. The percent recoveries were calculated by comparing the peak area ratios between the analytes of interest and the internal standard to the spiked concentration. Precision was expressed as the relative standard deviation (RSD; %), which is the ratio between the standard deviation (SD) and the mean analyte concentration. A method is considered accurate and precise when the recovery is between 80 and 110% with RSDs not exceeding RSD_{max}. CC_α is defined as the limit at which a sample is non-compliant with an error of probability α. CC_β is defined as the lowest analyte concentration that can be detected, identified, and/or quantified in a sample with an error of probability of β. The maximum residue limit (MRL) of piperazine established by the FDA is 100 μg/kg; therefore, in this study, CC_α was calculated as MRL + 1.64 × SD (α = 5%) by analyzing 8 blank tissue samples spiked with 100 μg/kg piperazine. CC_β was calculated as CC_α + 1.64 × SD (β = 5%). The LOD is defined as the lowest piperazine concentration at which the analyte can be detected with reasonable statistical certainty using a detector with S/N ≥ 3. The LOQ is defined as the lowest concentration for which the method gives recovery and precision values that fall within the ranges recommended by the EU. The LOQ was estimated by the peak height-to-average background noise ratio, which was based on the peak-to-peak baseline near the analyte peak from the SRM chromatograms. The LOQ was calculated based on a signal-to-noise (S/N) ratio of 10 according to the FDA guidelines. The developed method was validated by measuring the linearity, recovery, precision, CC_α, CC_β, LOD, and limit of quantification (LOQ), as established by the EU and the FDA [4,5].