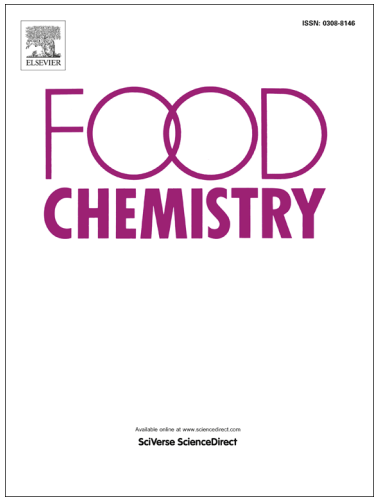

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Simultaneous enantioselective determination of seven psychoactive drugs enantiomers in multi-specie animal tissues with chiral liquid chromatography coupled with tandem mass spectrometry

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Abstract

In stock farming, illegal use of antipsychotics has caused the food safety problem. This paper presents for the first time, a multi-residues method for the simultaneous enantioselective determination of seven antipsychotics in pork, beef and lamb muscles. The behaviors of Chiralpak AGP toward changes in pH and organic modifier in mobile phase were studied, and all analytes were rapidly separated within 30 min. The calibration curves of all enantiomers were linear in the range of 1-250 ng g^{-1}, with correlation coefficient above 0.9931. The recoveries of the targeted compounds were higher than 82.1%, with repeatability and intermediate precision lower than 18.2% and 17.4%, respectively. In three matrices, the limit of detection and limit of quantification ranged from 0.20 to 0.65 ng g^{-1} and from 0.40 to 1.00 ng g^{-1}, respectively. The proposed method can be used to provide additional information for the reliable risk assessment of chiral antipsychotics.

Keywords: Antipsychotics; Chiral separation; Enantiomeric determination; Meat productions.

1. Introduction

Psychotropic drugs are illegally applied on a large scale for different purposes in modern farming. For example, antipsychotics are often used in animal husbandry to enhance the feed conversion ratio by reducing animal activity. In addition, antipsychotics are administered to farm animals (such as pig, bovine and lamb) to reduce stress during their transport to slaughterhouse. Such stress will cause the loss of meat quality and even premature death. Clinical diagnosis and forensic toxicological cases have shown that antipsychotics in animal can lead to overdose dependence, excessive sedation, anesthesia, coma, and even death (Wang, Zhao, Qiu, & Zhou, 2006). Illegal administration of drugs before slaughter gives rise to residues in edible animal tissues because these antipsychotics are often used just a few hours before slaughter. The accumulation of antipsychotics in animal meat products may enter the human body via food chain. As a result, these antipsychotics residues will cause a range of side effects, including hypotension, lethargy, nightmares or hyperprolactinaemia, and thus bring a potential risk to consumers. Previous study proposed that the psychotropic drugs had potential genotoxic activity (Gocke, 1996). The use of phenothiazine derivatives (chlorpromazine and propionylpromazine) is totally prohibited. The joint FAO/WHO Expert Committee on Food Additives (JECFA) showed concern about the safety of drug residues including antipsychotics in food in 1987, but these antipsychotics are still illegally used. Thus, from the food safety point of view, quantification and confirmation of antipsychotics residues in animal productions are necessary for human health and fair competition.

In the current market, various classes of antipsychotics have come into use, such as selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors, noradrenergic and specific serotonergic antidepressants, etc. Herein, seven chiral antipsychotics belonging to different families are chosen as the targeted analytes, including cyamemazine, promethazine, fluoxetine, oxazepam, mirtazapine, bupropion and phencynonate. The chemical structures of seven antipsychotics are shown in Fig. 1. As seen, all the targeted

seven antipsychotics contain heteroatoms in their structures. The synthesis and biological importance of different heteroatom-containing compounds have become the hot topics in recent years (Ahankar, Ramazani, lepokura, Lis, & Joo, 2016; Dayyani, Khoee, & Ramazani, 2015; Garmroodi et al., 2016; Jalili-Baleh et al., 2018; Lauro et al., 2019; Malekzadeh, Ramazani, Tabatabaei-Rezaei, & Niknejad, 2017; Shalbafan, Behbehani & Ghasemzadeh, 2019; Yavari, & Ramazani, 1997). For example, doxorubicin has shown great treatment potential, as it possesses the ability to combat rapidly dividing cells and slow disease progression (Shalbafan et al., 2019). Lauro et al. (2019) synthesize a diaza-bicyclo-naphthalen-oxiranyl-methanone derivative using some chemical tools, which could change the biological activity of cytochrome P450-17A_1 enzyme and thus be translated as good candidates for prostate cancer. The natural and synthesized chroman-4-one-based structures would be used asmulti-target-directed ligands (MTDLs) for Alzheimer's disease (Jalili-Baleh et al., 2018). For the seven heteroatom-containing antipsychotics, the quantitative determination of their contents in meat products is of great significant, to obtain more information about the toxicity and biological behaviors.

Stereo-chemistry in drugs has gained more and more attention because two enantiomers always show different pharmacokinetic profiles and biological activities. The use of racemic drugs may reduce curative effect and cause adverse effect owing to the presence of either inactive or toxic enantiomer. Lot of the used antipsychotics contain one or more chiral centers and until now they are administrated as racemates (Nageswara-Rao, & Guru-Prasad, 2015). Metabolism of fluoxetine was found to be enantioselective in human, with the (R)-fluoxetine being metabolized faster than the (S)-enantiomer. Toxicity tests of fluoxetine also proved strong enantiomer-dependent (Andrés-Costa et al., 2017). Recent finding shows that promethazine displays stereoselectivity in its pharmacokinetics, and thus two enantiomers exhibit different biological profiles toward attractive molecular targets including cytokines (Borowiecki, 2015). There are evidences that (+/-)-mirtazapine, (R)-(-)-mirtazapine and (S)-(+)-mirtazapine possess different pharmacokinetic and pharmacodynamic properties.

(S)-(+)-mirtazapine is a more potent receptor antagonist than its antipode at nearly all binding sites, except for 5-HT_3 and the muscarinic receptors (M_1–M_5) (Freynhagen, Vogt, Lipfert, & Muth-Selbacha, 2006). Trough plasma level of (R)-(-)-mirtazapine is approximately 2-3 times higher than that of the (S)-(+)-isomer, which would be attributed to the strong preference of CYP2D6 for the (S)-(+)-isomer (Dodd, Burrows, & Norman, 2000). In the case of bupropion, the pharmacological activities of two enantiomers and its metabolites are reported to be significantly different. Hydroxylation of (S)-(+)-bupropion by recombinant CYP2B6 is found to be 3-fold greater than (R)-(-)-bupropion and the same by human liver microsomes is found to be 1.5-fold greater than the (R)-(-)-isomer (Coles, & Kharasch, 2008). As is well known that pork, beef and lamb are the main meat wares in our daily live, residues of antipsychotics enantiomers, especially the highly active, toxic or even dangerous stereoisomer, in these products will be harmful to consumers. The potency and toxicity relative to the enantiomers of antipsychotics have not been fully characterized. With respect to these factors, enantioselective determination of chiral antipsychotics in animal food product is a very important branch of analytical chemistry, to provide information about chemical composition and quality control, to ensure compliance with food and trade laws, also to assure product authenticity and safety. Up to now, there is a lack of literature working on the enantioselective determination of antipsychotics in meat samples.

HPLC has become the most commonly used technique for analytical chemistry, especially in the pharmaceutical and biomedical fields (Ganni, Kumar, Jain, Kumar, Shrivastava, & Kumar, 2019; Rezaei, & Ramazani, 2019; Rezaei, Ramazani, & Hokmabadi, 2018; Rezaei, Ramazani, & Rouhani, 2017; Vyas, Nathwani, Patel, Patel, & Patel, 2019;). Recently, more sensitive and specific mass spectrometry techniques hyphenated to gas and liquid chromatography are widely applied for screening and confirmatory analysis. In cases of the seven tested analytes, a high number of GC-MS and LC-MS/MS methods are developed in order to quantify racemates or single enantiomers of antipsychotics in human matrices (including plasma, serum, urine and oral fluid). For

instance, Kirchherr, and Kü hnVelten (2006) have established a method for the quantitative determination of forty-eight antidepressants and antipsychotics in human serum by HPLC-MS/MS. da Fonseca, Moreno, Barroso, Costa, Queiroz and Gallardo (2013) developed a GC-MS/MS method for the determination of seven selected antipsychotic drugs in human plasma. LC-MS method for the enantioselective analysis of mirtazapine, dimethyl mirtazapine and 8-hydroxy mirtazapine in human plasma and urine has previously reported (De Santana, Jabor, Cesarino, Lanchote, & Bonato, 2010). The GC-MS/MS method for the determination of seven antipsychotics in plasma and oral fluid samples has been developed by Rosado et al. (2018). Based on the previous literatures, the antipsychotic residues are mostly detected in human plasma, urine, or serum samples, but relatively less attention has been paid to the antipsychotic residues in food, such as pork, beef and lamb. Only few reports on the determination of racemic antipsychotics in animal tissues have been published. For example, assay based on GC-MS has been used to detect four antipsychotics residues in swine muscle and liver tissues (Cheng, Zhang, Shen, Wu, & Zhang, 2010). He, Wang, Zhang, Liu and Fang (2012) reported a LC-MS/MS method for the simultaneous determination of the tranquilizers (chlorpromazine, promethazine, diazepam, azaperone, and its metabolite, azaperol) and a β-blocker (carazolol) in edible swine tissues. Cheng, Shen, Zhang, Zhang and Zhang (2015) presented a rapid method for the determination of three tranquillizers (diazepam, chlorpromazine, and promethazine) in lamb liver by UPLC-MS/MS. For the seven targeted antipsychotics, there have been few reports on the determination of racemic promethazine and fluoxetine in animal tissues. The quantification of other five antipsychotics in animal tissues has not been reported, which leads to lack of data for risk assessment. Furthermore, no study has reported on the enantioselective determination of chiral antipsychotics in animal tissue. Therefore, it is vital to develop the enantioseparation and enantioselective determination method for antipsychotics in animal products to provide more results for evaluating the biotoxicity and monitoring food safety.

Based on this, in the present study, we first separated the enantiomers of seven chiral antipsychotics using the α_1-acid glycoprotein (AGP) chiral column, and then a reverse phase UPLC-MS/MS method was optimized, validated and applied for the enantiomeric determination of seven antipsychotics in pork, beef and lamb meats. The different parameters affecting chiral separation and extraction recovery were studied. The method was demonstrated to be sensitive, selective and easily applicable to analyze animal tissue samples, and potentially beneficial for research in this area. To date, this is the first report for the simultaneous enantioselective analysis of seven antipsychotics in multi-specie animal tissues (pork, beef and lamb meats).

2. Materials and Methods

2.1 Chemicals and Reagents

Seven antipsychotics standards: R/S(±)-cyamemazine, R/S(±)-promethazine, R/S(±)-fluoxetine, R/S(±)-oxazepam, R/S(±)-mirtazapine, R/S(±)-bupropion and R/S(±)-phencynonate were purchased from China Standard Material Centers (Beijing, China). All standards were of >98.5% purity. The chemical structures of the seven psychoactive drugs are shown in Fig. 1. MS-grade ammonium acetate, acetic acid, methanol and acetonitrile were provided by Sigma-Aldrich (Beijing, China). All other reagents were of analytical grade and purchased from Yuwang Technology (Shandong, China). Ultrapure water was obtained from Jilin Wahaha Foods Co., Ltd. (Jilin, China) and was used in all the experiments. The Cleanert C18 solid-phase extraction columns (1000 mg, 6cc) were obtained from Agela Technologies (Tianjin, China). Stock standard solutions for the seven antipsychotics (1 mg mL^{-1}) were prepared in methanol and stored at -20 ◦C. Working mixture solution (10 μg mL^{-1}) was prepared by combining aliquots of stock solutions followed by subsequent dilution with methanol. The spiked solutions were then made immediately before the analysis by serially diluting the working mixture solution with methanol. Accordingly, matrix-fortified standard calibration curves at seven concentration levels (1, 5, 10, 50, 100, 200 and 250 ng g^{-1} of each enantiomer) were made by spiking certain amounts of

standards to the blank matrices.

2.2 Sample preparation

The pork, beef and lamb muscle tissue samples were bought from the local market in Benxi (China). These samples were homogenized and stored at -20 ◦C until use. These samples were prescreened to confirm that they did not contain the targeted antipsychotics and thus used for the method validation. Each aliquot (5.00 ± 0.01 g) of homogenized sample (pork, beef and lamb muscle tissues) was weighed and transferred into a 50 mL polypropylene centrifuge tube. About 5 g of anhydrous sodium sulfate was added until free-flowing powder could be obtained after vortex-mixing. Acetonitrile (15 mL) was also added to the mixture, which was vortex-mixed for 2 min. The sample was then placed in an ultrasonic bath for 20 min at 40 ◦C. After cooling to room temperature, the mixture was centrifuged at 4000 rpm for 10 min to form distinct liquid layer from the liquid and solid mixture. The supernatant was transferred into a 100 mL evaporation flask and then concentrated to dryness by a rotary evaporator at 60 ◦C. Residue was re-dissolved with 20 mL methanol-water (5:95, v/v) for further purification.

2.3 Sample purification

The Cleanert C18 solid-phase extraction (1000 mg, 6 cc) column was preconditioned with 6 mL of methanol and then equilibrated with 6 mL of water. Then, the 20 mL sample solution was loaded onto the SPE column. When the sample had eluted under gravity, the column was washed with deionised water (6 mL). Vacuum was applied to the cartridge for 2 min to completely dry the resin. Finally, elution was carried out with 6 mL of methanol, and the elute was evaporated to dryness under a gentle stream of nitrogen at 50 ◦C. The obtained residue was reconstituted with 0.2 mL of methanol-water (50:50, v/v) and filtered through a 0.22 μm syringe filter before LC-MS/MS analysis.

2.4 LC-MS/MS

All samples were analyzed using chiral liquid chromatography performed on Waters Acquity™ UPLC system (Waters Corp., Milford, MA, USA) composed of Acquity™ UPLC binary pump solvent management system, an Acquity™ UPLC autosampler and a thermostatic column compartment. The simultaneous enantioseparation of the seven psychoactive drugs was achieved on a Chiralpak AGP column (150 mm×4.6 mm, I.D. 5 μm), coupled to a Chiralpak AGP guard column (10 mm×4.6 mm, I.D. 5 μm), which were supplied by Daicel Chiral Technologies Co., Ltd. (Tokyo, Japan). Chiralpak AGP was protein-based chromatographic support with α_1-acid glycoprotein as the chiral selector. A gradient mode was used to separate and elute targeted analytes from the column. The optimized enantiomeric separation was obtained with the mobile phase consisting of methanol (solution A) and 10 mM ammonium acetate buffer adjusted at pH 4.5 (solution B), with a flow rate of 0.6 mL min^{-1}. The initial mobile phase was 100% solution B. From 0 to 10 min, the percentage of solution A was increased to 15% linearly and held from 10 to 30 min. The mobile phase returned to the initial composition and equilibrated for 10 min before the next injection. Column temperature and autosampler temperature were set at 30 °C and 4 °C, respectively. The injection volume was 10 μL.

Mass spectrometer was carried out on a Waters Micromass Quattromicro™ API mass spectrometer (Waters, Milford, USA) equipped with an electrospray ionization source. All data collected was processed using MassLynx 4.1 software (Waters Corp., Milford, MA, USA). Analysis was performed in positive ion mode. The ionization source conditions were as follows: capillary voltage of 3.0 kV and source temperature of 150 °C. Nitrogen gas was used as the desolvation gas and set to a flow rate of 16666.67 mL min^{-1} with a temperature of 500 °C. The collision gas by ultra-high purity argon was held at 0.12 mL min^{-1}. The mass spectrometer was operated in product ion scan mode to select the transition, and in multiple reaction monitoring (MRM) mode to quantify the analytes in samples. The MRM transition of each antipsychotic was optimized by the direct infusion of individual standard solution at a concentration of 100 ng mL^{-1}. Two MRM transitions were monitored for each analyte. The most sensitive product ion was selected for quantification, and the other transition was used for confirmation. Optimized MRM transition, collision energy and capillary voltage are depicted in Table 1.

3. Results and Discussion

3.1 Optimization of LC-MS/MS

3.1.1 Column selection

The selection of column is important in separation of chiral antipsychotics. In the preliminary experiments, we initially screened two polysaccharide-based chiral stationary phases (CSPs), namely tris-(4-methylbenzoate) of cellulose (Chiralcel OJ-RH) , tris-(3-chlorophenylcarbamate) of amylose (Chiralpak ID), to resolve the seven antipsychotics. Both columns were performed under reversed phase mode. With acetonitrile/water as the mobile phase, Chiralpak ID showed enantioselectivity for oxazepam (Rs=0.9), while no separation was observed for other analytes. On Chiralcel OJ-RH, two analytes of promethazine (Rs=2.42) and phencynonate (Rs=2.44) obtained complete separation, with acetonitrile/water/DEA (60/40/0.1, v/v/v) as the mobile phase (data not shown). The enantiomers of other five analytes were not separated on Chiralcel OJ-RH. Based on the above results, polysaccharide-typed CSPs were not capable of the simultaneous enantioseparation of the targeted antipsychotics. Due to the broad application range and LC-MS compatibility of protein-based CSPs, in particular α_1-acid glycoprotein, Chiralpak AGP column was also attempted for the separation experiments. As a result, Chiralpak AGP proved to be the most efficient column, on which all analytes were separated into individual enantiomer. Chiralpak AGP was thereby selected as the optimal CSP to further optimize the separation.

3.1.2 Mobile phase optimization

The enantioseparation performance of Chiralpak AGP may be influenced by several parameters, such as the buffer type and its concentration, the pH value of the buffer, the organic modifier type and its content (Michishita, Franco, & Zhang, 2010). The mobile phase optimization on Chiralpak AGP requires adjustment of these parameters. On the basis of all published studies on Chiralpak AGP, ammonium acetate buffer is generally applied on Chiralpak AGP because of its good buffering ability and LC-MS compatibility. The pH of 5.8 is the mean value of the recommended pH value (4.5-7.0) for Chiralpak AGP, and IPA is reported to be the most

versatile modifier. Thus, 10 mM ammonium acetate buffer adjusted at pH 5.8 admixed with 5% IPA was chosen as the initial mobile phase to start the enantioseparation (Table 2). As a consequence, five out of the seven analytes (except for mirtazapine and bupropion) were baseline-resolved along with too longer retention (over 40 min). In most cases, antipsychotics were found to be highly retained on Chiralpak AGP column. It has been reported that buffer pH is the most vital parameter to regulate the retention (Michishita et al., 2010). In acidic mobile phase, the chiral selector on Chiralpak AGP is negatively charged, which can provide ionic interaction for these tested antipsychotics (basic compounds) (Michishita et al., 2010). Thus, lower pH value is expected to decrease the retention time, and pH 4.5 was chosen in our present study. Considering that two analytes of mirtazapine and bupropion were not separated with IPA/10 mM ammonium acetate buffer as the mobile phase, other organic modifiers were subsequently tested to achieve more successful enantioresolution.

The use of acetonitrile as organic modifier was subsequently studied on Chiralpak AGP. Mobile phase containing different percentage of acetonitrile and 10 mM ammonium acetate buffer (pH=4.5) was tried, and six analytes (except for bupropion) obtained the satisfactory separation (Table 2). In the case of bupropion, the poor resolution was probably due to the fast elution ($k'<0.7$), and the complete removal of acetonitrile from the mobile phase allowed an increase in the retention and, more importantly, an improvement of the resolution up to 1.59. Besides acetonitrile, methanol is the other alternative organic modifier on Chiralpak AGP. Using methanol instead of acetonitrile as the modifier, significant decrease in analyte retention (k_1) and improvement in enantioselectivity (α) were observed for all analytes (Table 2). Based on the results obtained with three modifiers, it could be concluded that methanol was more suitable for enantioseparation of targeted antipsychotics because of the comprehensive separation ability, higher enantioselectivity as well as shorter retention.

It should be noted that the chromatographic behaviors of enantiomers were dependent on the properties of compounds and the composition of mobile phase. The enantiomers of mirtazapine and bupropion were weakly

retained on Chiralpak AGP compared with other analytes. The adjustment of methanol content to a lower value (~5%) would regulate their retention and guarantee the baseline separation. But for other five analytes, the analysis time increased significantly and their peak shapes broadened when lower methanol content was added to mobile phase. For the purpose of simultaneous enantioseparation, gradient elution was necessary, which began with 100% ammonium acetate buffer (10 mM, pH 4.5), increased linearly in 10 min to 15% of methanol, and then maintained this proportion for 20 min. Under the optimized mobile phase, baseline resolutions (Rs > 1.5) were achieved for all analytes within half an hour. MRM chromatograms of the spiked pork samples (10 ng g^{-1} for each enantiomer) are presented in Fig. 2 (b).

3.2 Sample preparation

3.2.1 Extraction

Sample preparation is the crucial step in the analysis of complicated biological matrix, which is highly influenced by the physical and chemical properties of targeted analytes. The main purposes of sample preparation are to concentrate analytes in matrix, to remove interferences from complicated matrix and also to prepare analytes in suitable form for the following chromatographic analysis. According to the previous papers, the extraction of antipsychotics from meat matrices was always accomplished with organic solvent extraction and SPE. The selection of appropriate extraction solvent and sorbent is highly related to the properties of the studied analytes. However, no paper reports the simultaneous extraction of the seven targeted antipsychotics from meat matrices. Based on this, the pretreatment procedure in our study was established by the modification of published literatures. Prior to determination by LC-MS/MS, the main parameters that affected the extraction efficiency such as the type of the extraction solvent, extraction times, the type of SPE cartridge, and the content of loading buffer were carefully studied.

Previously, experiments to extract antipsychotics from animal tissues were frequently performed with

acetonitrile and ethyl acetate (Cheng et al., 2010; He et al., 2012; Cheng et al., 2015; Zhang, Shao, Yin, Wu, & Duan, 2009; Zhang et al., 2014; Zhang et al., 2016). Cheng et al. (2015) extracted three antipsychotics (diazepam, chlorpromazine and promethazine) from lamb live with ammonia in ethyl acetate. In the study of Zhang, Shao, Yin, Wu, and Duan (2009), thirty targeted drugs (including 11 antipsychotics) were extracted with acetonitrile in animal tissues (pork kidney, liver, muscle; bovine muscle). Zhang et al. (2016) extracted eight tranquilizers residues including chlorpromazine, diazepam, nitrazepam, oxazepam etc in pork with acetonitrile as extraction solvent. It has been reported that alkaline compounds tend to dissolve in no-polar solvent (ethyl acetate) as molecular form in alkaline conditions, but dissolved in polar solvent (acetonitrile) as ionic form under acidic condition (Cheng et al., 2015). On the basis of the published literatures, our present study evaluated the extraction efficiencies of four kinds of extractants, including acetonitrile, formic acid-acetonitrile (1:99, v/v), ethyl acetate, 25% of ammonium hydroxide-ethyl acetate (10:90, v/v). As depicted in Fig. 3 (a), the use of ethyl acetate as the extractant led to the relatively low recoveries of targeted analytes. Moreover, it was found that the extracts obtained with ethyl acetate showed muddy colors, thus increasing the matrix effect for most analytes. The addition of 25% ammonium hydroxide to ethyl acetate did not improve the recoveries. In comparison, the use of acetonitrile as the extractant provided superior extraction efficiencies for seven analytes with recoveries higher than 78% (Fig. 3 (a)). This phenomenon is inconsistent with the report of Cheng et al. (2015). It could be explained by the different properties of different antipsychotics. The seven analytes possess a wide range of polarity, with logarithms of oil-water partition coefficient (log P) for the seven analytes from 2.71 (bupropion) to 4.27 (fluoxetine), and thus a polar organic solvent of acetonitrile is more workable. Considering that the seven analytes were alkaline, formic acid-acetonitrile (1:99, v/v) was then attempted, leading to poor extraction of cyamemazine, promethazine and phencynonate (recoveries<5%). Therefore, acetonitrile was selected as the optimum extractant for the seven analytes. We tried to repeat the solvent extraction twice to improve the

recoveries, but no improvement in recovery was observed. Thus, the one-step extraction with acetonitrile as the extractant was chosen for present study. In addition, anhydrous sodium sulfate was adopted to dry the samples and also improved the extraction efficiency of hydrophobic analyte.

3.2.2 Purification

Liquid-liquid extraction (LLE) and SPE are often used for the extraction and purification of antipsychotics from biological matrices. However, LLE always leads to mutual solubility and emulsion formation of analytes in two phases. For this reason, we subsequently tried to select appropriate SPE cartridges, after rotary evaporator drying and redissolving with methanol-water. The cartridges recovery experiments were performed on three SPE cartridges, including Cleanert C18 (1000 mg, 6 cc), PEP-2 (60 mg, 3cc) and NH_2 (500 mg, 6cc). Results are compared in Fig. 3(b). Through the further purification on PEP-2 cartridge, it was found that the PEP-2 was unable to absorb bupropion in the extraction solvent. For the NH_2 cartridge, poor recoveries (<20%) were observed for fluoxetine, mirtazapine, bupropion and phencynonate. However, C18 cartridge would provide higher recoveries for all the analytes. C18 is cheaper than other cartridges, so it was selected for the purification.

For the reverse-phase C18 cartridge, water-methanol was used as the loading solvent. Then, we also optimized the ratio of methanol and water, and selected the ratio of water and methanol of 85: 15, 90: 10, 95:5, 98:2 (v/v). Fig. 3(c) shows the extraction recoveries for the seven analytes. As depicted, a decrease in extraction recovery was observed with increasing the methanol content from 5 to 15%. Finally, the ratio of water-methanol was determined as 95: 5 due to the highest recoveries for seven analytes.

Under above optimization of pretreatment procedure, a simple acetonitrile extraction step, followed by a SPE cleanup using C18 cartridge was developed for the simultaneous extraction of seven antipsychotics from multiple meat matrices. We acknowledge that the pretreatment method is established based on previous method. Nevertheless, our research first report the extraction of cyamemazine, oxazepam, mirtazapine, bupropion and

phencynonate from multiple meat matrices. This procedure shows advantages compared with the published literatures for antipsychotics analysis. In our study, a total amount of 15 mL acetonitrile is used for the extraction step, which is solvent-saving than the methods developed by Cheng et al. (2015) and Zhang et al. (2009). In the course of SPE, the C18 cartridge is more efficient, low-cost and easily-operated than other absorbents, such as Oasis HLB (Zhang et al., 2016), NH_2 (Zhang et al., 2009), MCX (Zhang et al., 2014), and Sep-pak VAC alumina N (Cheng et al., 2015), leading to good cleanup and higher recoveries (>80%) for all the targeted analytes. Nevertheless, the sample preparation with SPE procedure is time-consuming, which also encounters in other literatures. Due to the complexity composition of meat matrices (including protein and lipids), the use of SPE for the cleanup is necessary.

3.3 Method and validation

The performance of the developed method was validated in terms of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision, matrix effects (MEs), as well as the stability. Blank muscle tissue samples were analyzed to verify the specificity of the method. Chromatograms of blank sample and spiked sample are compared in Fig. 2. It was evident that no interfering specie was observed at the retention of targeted analytes. Therefore, the method was proved to be selective for the determination of the targeted antipsychotics in meat samples.

To prevent matrix suppression or sometimes enhancement effects, which may cause difference in absolute response from the matrix compared to that of the standard, matrix-spiked samples were used as calibration standards for evaluating response linearity. The matrix-fortified calibration curves were prepared using blank meat samples spiked in the range of 1-250 ng g^{-1} at seven concentration levels. Each level was prepared in triplicate. The spiked samples were extracted and cleaned up following the above-mentioned procedure. Calibration lines were conducted from the peak areas of analytes (y-axis) against the nominal drug

concentrations (x-axis). Linear regression analysis was used to calculate the slope, intercept, and the correlation coefficient (R^2) of each calibration. The data calculated are listed in Table 3. It was clear that accepted linearities ($R^2 > 0.9931$) were achieved for all enantiomers in the range of 1-250 ng g^{-1}, indicating a good correlation for each targeted analyte.

The LOD was defined as the lowest concentration of analyte spiked in the meat sample which could be detected with a signal-to-nosie (S/N) ratio of 3, while the LOQ was determined at the S/N of 10. The LODs and LOQs of the present method for the antipsychotics enantiomers are shown in Table 3. LOQs of 0.40-1.00 ng g^{-1} and LODs of 0.20-0.65 ng g^{-1} were obtained.

The recovery experiments were carried out to evaluate the accuracy (expressed as recovery) and precision (expressed as RSD). Five replicates of spiked samples at three different concentration levels (5, 10, 50 ng g^{-1} for each enantiomer) were prepared on three different days. For recovery studies, 5.00 ± 0.01 g portion of meat was placed in the container and then spiked with the standards, taken care to uniformly spread them on the sample. Individual spiked sample was left for 30 min at room temperature to ensure the sufficient distribution in the meat matrix, and then prepared according to the above preparation method. Method recovery was calculated from the ratio of the mean values of the detected concentration (Cdet) and the nominal concentration (Cnom) as follows: (Cdet/Cnom)×100%. Repeatability (or intra-day precision) was based on the RSD of the determined responses of five replicated spiked samples at three concentrations on the same day. Intermediate precision (or inter-day precision) was calculated using fifteen spiked samples (five replicates per concentration), which were analysed one three consecutive days and also expressed as RSD. The within-laboratory reproducibility was obtained using the fifteen spiked samples (five replicates at the lowest spiked concentration of 5 ng g^{-1} and analysed at three occasions with three different operators). The results of average recovery and RSDs are presented in Table 4. The method provided satisfactory average recoveries of 82.1-113.9% for all targeted enantiomer independently

of the matrix under study. The analytical method showed good repeatability from 2.0 to 18.2%, and also good intermediate precision from 2.6 to 17.4%. The results of the recovery studies indicated that our method would provide satisfactory accuracy and precision for the enantiomeric determination of antipsychotics in multi-specie animal tissue samples.

In LC-MS/MS analysis, the response of analyte in biological matrix can be affected by interfering species in the original biological sample, which is the matrix effects (MEs). This effect could result in signal suppression or sometimes in enhancement of the analyte response. The peak areas for standards prepared in sample extracts (post-extraction standard) divided by the corresponding peak areas of standards in methanol are defined as the MEs. The post-extraction standards were prepared by extracting blank meat samples in according with the procedure in Section 2.2 and 2.3. The final extracts were evaporated and re-dissolved in standards prepared in methanol at appropriate concentrations. The matrix-suppression effect occurs when the value is lower than 100%, and a ME value above 100% represents the matrix-enhancement. The MEs values of all analytes in three animal tissues are listed in Table 3. The general suppression of signal in different meat matrices was observed. Expecially, the MEs was more pronounced for bupropion, which was more polar than other analytes. It has been reported that ionization efficiency of polar analyte is more affected by the coeluates of the matrix than that of apolar analyte, because most of the coeluates of the matrix are polar (Zhao, Wang, Gao, Guo, & Zhao, 2019). To compensate the signal suppression, matrix-matched standard curves were therefore adopted for the validation of the analytical method and for the quantitative analysis of real samples.

The stability was checked by storing standard solutions, spiked samples and the extracts at 4 ◦C and -20 ◦C at different periods. The mixed standard solutions (100 ng mL^{-1} of each enantiomer, prepared in methanol) of the seven antipsychotics were stores at -20 ◦C for three months. The solutions were analyzed every month, and the peak areas were compared with that obtained after preparation. The peak areas after storage were found to be

between 90% and 110% of the initial area. When stored at 4 ◦C, the mixed solution standards remained stable for one month. Meanwhile, the spiked samples at the lowest fortification level of 1 ng g^{-1} and the extracts of the spiked sample at 1 ng g^{-1} were stored at 4 ◦C and -20 ◦C after preparation, and measured immediately, 24h, 48h, one week and two weeks later. The recoveries of the targeted analytes were within the accepted limit of ±15% of the nominal concentration when stored at 4 ◦C for two weeks or at -20 ◦C for one month.

3.4 Application to real samples

Meat samples (a portion from the leg of slaughtered animals) were purchased from different markets of Benxi (China). A total of thirty meat samples of pork, beef and lamb (ten of each) were determined. These samples of about 100 g were preserved at 4 ◦C until arrival at laboratory. Then, each of the samples (5.00 ± 0.01 g portion) were homogenized and transferred to polypropylene centrifuge tube, labeled and stored in the -20 ◦C freezer. After storage for about 3 hours, these samples were extracted and cleaned-up according to the above-mentioned sample preparation and purification procedure, followed by the enantioselective determination of enantiomers of seven antipsychotics by UPLC-MS/MS. Results showed that no antipsychotic was detected in any of the animal tissues samples.

4 Conclusion

The present method, based on one-step acetonitrile extraction, SPE clean-up with Cleanert C18 cartridges and chiral LC-MS/MS determination, is suitable for the enantioselective determination of seven antipsychotics residues in pork, beef and lamb muscle tissue samples. With Chiralpak AGP as the chiral selector, chiral separation of the seven antipsychotics was accomplished within half an hour. The sample preparation procedure, including one-step acetonitrile extraction and C18 SPE, provided high extraction efficiencies for all the analytes. Quantification at 1ng g^{-1} for the different kinds of meat samples by LC-MS/MS was readily achieved. In conclusion, the developed method is fast, simple and sensitive, which can be advantageously adopted as the

routine procedure to monitor the concentration of antipsychotics enantiomers in the laboratories for food quality and safety control because of its robustness and feasibility.

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Conflicts of interest

The authors have declared that there are no conflicts of interest.

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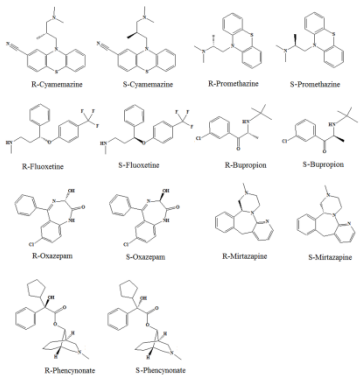
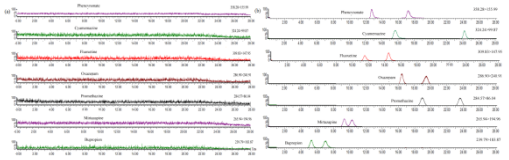
Figure captions

Fig. 1. Chemical structures of the seven chiral antipsychotics.

Fig. 2. MRM chromatograms: (a) control pork sample; (b) 10 ng g^{-1} of standard spiked pork sample.

Fig. 3 Effect of the extraction solvent (a), sorbent (b) and loading solvent (c) on the recoveries of the seven antipsychotics (n=3). Experimental conditions: (a) sorbent, C18; loading solvent, methanol-water (5:95, v/v); (b) extraction solvent, acetonitrile; loading solvent, methanol-water (5:95, v/v); (c) extraction solvent, acetonitrile; sorbent, C18. Other experimental conditions were indicated as “Materials and Methods” part.





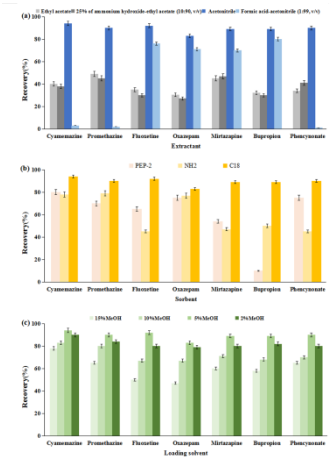


Table 1 Retention time, ion pairs, cone voltage and collision energy of MRM for the optimized LC-MS/MS.

Compound	Precursor ion	Retention time (t_1 / t_2, min)[a]	MRM transitions[b]	CV (V)[c]	CE (eV)[d]
Cyamemazine	$[M+H]^+$	15.6 / 24.2	324.24 > 99.87	44	18
			324.24 > 57.96		22
Promethazine	$[M+H]^+$	19.0 / 23.6	284.57 > 86.04	9	14
			284.57 > 197.87		22
Fluoxetine	$[M+H]^+$	11.5 / 14.4	309.83 > 147.95	9	6
			309.83 > 66.93		18
Oxazepam	$[M+H]^+$	16.5 / 19.6	286.90 > 240.91	5	22
			286.90 > 268.89		14
Mirtazapine	$[M+H]^+$	9.3 / 10.3	265.94 > 194.96	35	26
			265.94 > 71.99		16
Bupropion	$[M+H]^+$	5.2 / 7.0	239.79 > 183.87	12	12
			239.79 > 130.94		24
Phencynonate	$[M+H]^+$	12.8 / 17.2	358.28 > 155.99	26	30
			358.28 > 341.08		10

[a] t_1 and t_2 means the retention time of the first and second eluted enantiomers.

[b] The quantification ion transitions are underlined.

[c] CV means cone voltage;

[d] CE means collision energy

Table 2 Enantioseparation of seven antipsychotics on Chiralpak AGP column with different organic modifier.

Analytes	IPA				Acetonitrile				Methanol			
	%	k_1	α	Rs	%	k_1	α	Rs	%	k_1	α	Rs
Cyamemazine	5	15.07	1.67	4.52	10	1.62	1.67	3.14	15	2.39	1.96	3.70
Promethazine	5	15.60	1.10	1.56	5	7.24	1.37	2.51	10	5.55	1.39	2.78
Fluoxetine	5	12.66	1.11	1.76	5	3.05	1.50	2.62	10	2.96	1.50	2.47
Oxazepam	5	14.32	1.50	3.90	5	4.0	1.38	2.63	15	2.49	1.55	2.75
Mirtazapine	5	5.24	1.00	-	2	1.72	1.29	1.53	5	1.19	1.32	1.61
Bupropion	5	1.59	1.00	-	0	0.60	1.87	1.59	5	0.21	2.57	1.66
Phencynonate	5	11.31	1.41	3.77	2	6.66	1.64	3.79	15	1.64	1.51	2.23

Mobile phase: organic modifier/10 mM ammonium acetate buffer (pH 5.8 for IPA, pH 4.5 for acetonitrile and methanol)

"-": no separation could be observed under the tested conditions;

Capacity factor of the first eluted enantiomer (k_1), separation factor (α) and resolution (Rs).

Table 3 Correlation coefficient (R^2), linearity range, matrix effect, LOQ and LOD of seven antipsychotics in animal tissues.

Analyte	Matrix	Linearity range (ng g^{-1})	Matrix Effect (%)	R^2	LOQ (ng g^{-1})	LOD (ng g^{-1})
Cyamemazine E1	Pork	1-250	88.0	0.9982	0.44	0.20
	Beef	1-250	79.6	0.9960	0.57	0.31
	Lamb	1-250	95.7	0.9984	0.80	0.45
Cyamemazine E2	Pork	1-250	81.9	0.9961	0.44	0.20
	Beef	1-250	88.2	0.9975	0.57	0.31
	Lamb	1-250	96.4	0.9951	0.80	0.45
Promethazine E1	Pork	1-250	79.4	0.9978	0.40	0.28
	Beef	1-250	85.9	0.9989	0.45	0.30
	Lamb	1-250	83.6	0.9963	0.66	0.40
Promethazine E2	Pork	1-250	78.8	0.9975	0.40	0.28
	Beef	1-250	80.9	0.9978	0.45	0.30
	Lamb	1-250	80.0	0.9986	0.66	0.40
Fluoxetine E1	Pork	1-250	88.5	0.9950	0.80	0.38
	Beef	1-250	78.3	0.9931	0.90	0.50
	Lamb	1-250	91.4	0.9982	0.68	0.45
Fluoxetine E2	Pork	1-250	83.0	0.9967	0.80	0.38
	Beef	1-250	89.7	0.9943	0.90	0.50
	Lamb	1-250	76.6	0.9950	0.68	0.45
Oxazepam E1	Pork	1-250	78.0	0.9969	0.85	0.50
	Beef	1-250	96.4	0.9971	0.88	0.40
	Lamb	1-250	91.5	0.9970	1.00	0.65
Oxazepam E2	Pork	1-250	87.4	0.9981	0.85	0.50
	Beef	1-250	80.5	0.9985	0.88	0.40
	Lamb	1-250	73.9	0.9948	1.00	0.65
Mirtazapine E1	Pork	1-250	77.1	0.9967	0.88	0.53
	Beef	1-250	95.8	0.9940	0.75	0.40
	Lamb	1-250	86.0	0.9957	0.95	0.55
Mirtazapine E2	Pork	1-250	82.7	0.9981	0.88	0.53
	Beef	1-250	89.9	0.9970	0.75	0.40
	Lamb	1-250	76.1	0.9979	0.95	0.55
Bupropion E1	Pork	1-250	43.1	0.9988	0.78	0.44
	Beef	1-250	54.9	0.9964	0.90	0.56
	Lamb	1-250	41.1	0.9946	0.96	0.60
Bupropion E2	Pork	1-250	30.5	0.9969	0.78	0.44
	Beef	1-250	45.0	0.9980	0.90	0.56
	Lamb	1-250	50.8	0.9968	0.96	0.60
Phencynonate E1	Pork	1-250	87.4	0.9945	0.50	0.35
	Beef	1-250	90.6	0.9947	0.67	0.40
	Lamb	1-250	79.5	0.9987	0.70	0.51

Phencynonate E2	Pork	1-250	83.4	0.9979	0.50	0.35
	Beef	1-250	86.1	0.9983	0.67	0.40
	Lamb	1-250	93.4	0.9957	0.70	0.51

Table 4 Recovery and RSDs of the seven analytes in multi-species animal tissues (n=5)

Analyte	Spiked level ($ng\ g^{-1}$)	Pork				Beef				Lamb			
		Recovery (%)	Precision (%)			Recovery(%)	Precision (%)			Recovery(%)	Precision (%)		
			Intra-day	Inter-day	Within-laboratory		Intra-day	Inter-day	Within-laboratory		Intra-day	Inter-day	Within-laboratory
Cyamemazine E1	5	91.3	4.1	8.3	6.9	98.1	12.9	3.1	7.1	84.9	3.0	4.1	4.7
	10	94.6	10.9	7.1	-	98.4	5.0	2.6	-	103.2	6.9	9.8	-
	50	102.7	3.7	10.3	-	101.6	9.1	6.5	-	85.8	3.7	6.9	-
Cyamemazine E2	5	96.8	6.6	17.4	5.5	103.0	9.6	5.1	8.8	94.5	5.3	8.8	7.5
	10	99.1	12.1	9.0	-	90.0	8.8	7.0	-	86.9	9.8	14.8	-
	50	93.8	5.8	4.3	-	95.8	8.3	11.8	-	85.8	5.4	7.7	-
Promethazine E1	5	105.9	6.3	8.9	7.0	93.7	11.7	5.0	9.4	91.0	10.9	8.0	8.9
	10	90.5	13.5	4.9	-	100.9	10.9	5.1	-	99.1	11.6	8.9	-
	50	93.0	7.9	11.0	-	88.4	3.8	3.7	-	105.9	6.9	4.6	-
Promethazine E2	5	94.3	8.9	9.3	5.9	89.0	4.0	8.0	5.9	100.9	9.0	8.0	10.4
	10	98.2	11.0	12.2	-	102.8	9.6	7.2	-	89.2	12.9	10.9	-
	50	96.8	4.5	14.7	-	100.9	5.9	11.8	-	104.4	10.9	15.0	-

Fluoxetine E1	5	103.8	9.6	3.6	4.9	89.2	8.0	15.0	10.5	109.1	11.6	8.9	9.4
	10	92.6	6.0	8.1	-	92.3	13.3	9.5	-	93.2	9.1	10.0	-
	50	95.7	18.2	7.9	-	90.5	17.4	10.9	-	86.0	11.0	9.6	-
Fluoxetine E2	5	111.7	3.9	16.2	8.7	94.9	7.6	8.0	8.8	88.9	9.0	6.4	7.1
	10	99.1	9.4	10.2	-	88.0	7.9	4.9	-	93.9	8.3	5.0	-
	50	93.4	10.9	3.5	-	86.2	14.6	3.8	-	97.1	6.0	6.6	-
Oxazepam E1	5	89.2	11.3	5.9	7.9	104.3	11.4	5.5	6.3	88.0	9.5	3.9	7.4
	10	92.7	9.8	8.3	-	98.9	10.9	5.9	-	106.9	13.5	10.6	-
	50	112.9	7.8	9.6	-	98.0	12.8	7.2	-	101.5	9.9	8.3	-
Oxazepam E2	5	96.9	7.6	8.0	6.4	109.2	5.6	8.7	6.0	95.8	4.8	8.0	7.5
	10	92.4	9.2	11.7	-	101.1	8.0	10.6	-	90.4	7.2	11.6	-
	50	109.7	10.2	7.3	-	93.5	3.9	12.3	-	92.1	5.9	10.9	-
Mirtazapine E1	5	91.0	13.4	10.8	9.4	85.3	9.9	13.0	11.2	84.0	2.0	4.7	5.8
	10	113.9	4.0	10.3	-	90.9	11.0	9.2	-	89.4	13.7	10.1	-
	50	95.5	8.5	6.7	-	89.8	10.9	6.0	-	90.0	11.9	15.1	-
Mirtazapine E2	5	96.3	15.5	16.2	12.2	95.0	8.2	6.8	5.9	108.9	9.1	6.9	6.0
	10	101.1	7.9	13.2	-	91.3	8.6	8.0	-	110.0	14.2	10.4	-
	50	89.2	11.9	9.9	-	108.5	6.9	10.9	-	92.5	3.9	5.3	-
Bupropion E1	5	95.0	9.2	10.5	10.3	85.7	9.4	4.8	7.6	98.3	4.9	8.6	9.3
	10	104.9	8.8	6.7	-	87.0	4.0	3.0	-	82.1	4.3	7.7	-
	50	103.3	10.9	8.0	-	104.7	17.3	8.2	-	89.3	7.8	10.2	-
Bupropion E2	5	111.8	4.2	8.9	6.6	84.2	11.4	4.7	8.3	107.6	9.5	8.0	10.5
	10	89.9	7.3	3.4	-	91.8	10.7	13.1	-	97.0	10.0	16.4	-
	50	90.5	9.0	10.0	-	89.7	5.0	9.2	-	87.9	12.3	10.7	-
Phencynonate E1	5	99.0	9.6	8.5	9.4	109.2	8.0	5.1	4.8	94.0	14.6	9.2	11.2

	10	105.6	14.5	14.9	-	95.0	6.7	7.0	-	109.5	11.9	10.0	-
	50	92.1	10.9	12.2	-	110.5	7.8	7.8	-	100.9	5.7	6.4	-
Phencynonate	5	82.7	8.4	4.4	7.4	107.4	7.0	10.0	6.6	87.2	6.0	9.2	9.8
E2													
	10	89.4	10.4	5.8	-	99.0	10.3	12.7	-	88.8	6.0	10.9	-
	50	90.9	3.9	9.2	-	96.0	13.8	9.9	-	98.0	8.8	14.8	-

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Enantiomeric determination of antipsychotics residues in multiple meat productions.