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Enantioseparation and determination of flumequine enantiomers in multiple food matrices with chiral liquid chromatography coupled with tandem mass spectrometry

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Abstract

The present work firstly described the enantioseparation and determination of flumequine enantiomers in milk, yogurt, chicken, beef, egg, and honey samples by chiral liquid chromatography-tandem mass spectrometry. The enantioseparation was performed under reversed-phase conditions on a Chiralpak IC column at 20°C. The effects of chiral stationary phase, mobile phase components, and column temperature on the separation of flumequine enantiomers have been studied in detail. Target compounds were extracted from six different matrices with individual extraction procedure followed by cleanup using Cleanert C18 solid phase extraction cartridge. Good linearity (R^2>0.9913) was obtained over the concentration range of 0.125 to 12.5 ng g^{-1} for each enantiomer in matrix-matched standard calibration curves. The limits of detection and limits of quantification of two flumequine enantiomers were 0.015-0.024 and 0.045-0.063 ng g^{-1}, respectively. The average recoveries of the targeted compounds varied from 82.3 to 110.5%, with relative standard deviation less than 11.7%. The method was successfully applied to the determination of flumequine enantiomers in multiple food matrices, providing a reliable method for evaluating the potential risk in animal productions.

KEYWORDS

chiral separation, enantiomeric determination, flumequine, food matrices, LC-MS/MS

1 | INTRODUCTION

Fluoroquinolones (FQs) are synthetic antibiotics widely used in veterinary medicine with the purposes of inhibiting the growth of microorganisms, preventing or treating infections, and promoting growth when used at subtherapeutic doses.[1] In recent years, the public concern about the presence of antibiotic residues in food-producing animals has increased because they can lead to the growth of antibiotic-resistant bacteria, allergic reactions, or even toxic effects. Flumequine, a first-generation FQ, can selectively inhibit type II topoisomerase and bacterial DNA gyrase, which is essential for bacterial DNA replication and transcription in the process of cell growth and division.[2,3] Owing to the high antimicrobial activity against a wide range of Gram-negative and Gram-positive pathogens, flumequine has become an indispensable part of the treatment of infections in food-producing animals such as cattle, turkey, pig, and poultry. Usually, flumequine is administered orally to food-producing animals and distributed

Abbreviations: CSP, chiral stationary phase; Na_2EDTA, acidethylenediamine tetraacetic acid disodium salt; TCA, trichloroacetic

widely in the tissue. Research studies have shown that high doses of flumequine can distort the embryonic development in rats.[4] There is no report indicating that low doses of flumequine are harmful; however, the widespread use of flumequine inevitably leads to the significant and harmful residues in animal edible tissues and later sold as food products, such as meat, milk, and egg. Thus, concerning the amount of flumequine residue found within food and food safety, it is necessary to quantify and confirm residues of flumequine in animal productions to protect the consumer.

Stereochemistry of chiral drugs has gotten widespread attention because enantiomers of drugs always tend to display quite different pharmacological and toxicological activities. Many chiral drugs available for clinical use have at least one chiral centre in chemical structure, and most are used as racemates. In some cases, the bioactivity is only presented in one of the enantiomers, while the other enantiomer has no effect. Furthermore, degradation products may also be enantioselective, which may exhibit potential risk. Flumequine contains one chiral centre and exists as a mixture of two enantiomers (shown in Figure 1). Study has suggested that the enantiomers of flumequine exhibit significant differences in the antibacterial activity.[5] Therefore, the sensitive and reliable resolution and determination method of flumequine enantiomers should be developed to control the enantiomer occurrence and quantify the contaminants in animal productions and accurately evaluate the potential risk.

According to previous papers, a variety of analytical methods for the racemic determination of flumequine in animal productions (including milk, honey, egg, fish, shrimp, chicken, pork, and beef) have been published using capillary electrophoresis (CE),[6,7] CE tandem mass spectrometry (CE-MS/MS),[8,9] liquid chromatography-ultraviolet detection (LC-UV),[10-15] and liquid chromatography tandem mass spectrometry (LC-MS/MS).[16-21] LC-UV and LC-MS have been widely utilized for quantification analysis of flumequine. For example, Zhao et al[10] developed a method for 10 quinolones in swine, chicken, and shrimp muscle by LC-UV. The trace analysis of flumequine and several other quinolones in muscle and egg has been investigated using LC-MS.[16] In the present, there is a trend on enantioselective determination of chiral drugs in various matrices. Nevertheless, a few papers have reported on the enantioselective quantification of flumequine in different matrices, including in rat plasma, sediment, and different water samples.[22-25] But, to our best knowledge, no literature has been published on enantioselective determination of flumequine in multiple food matrices by LC-MS/MS.

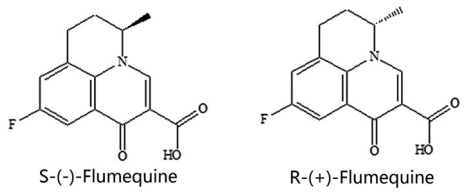

FIGURE 1 Chemical structures of flumequine enantiomers

Therefore, this study aimed to develop a new robust method for the enantioselective determination of flumequine in multiple matrices (milk, yogurt, honey, egg, chicken, and beef) using a reversed-phase HPLC-MS/MS with the Chiralpak IC column. The effects of chiral stationary phase (CSP), mobile phase composition, and the temperature on the separation and retention of flumequine enantiomers have been investigated in detail. To date, this paper is the first report on the enantioselective analysis of flumequine in multiple food matrices using chiral LC-MS/MS. The method developed was validated by its application to the analysis of flumequine authentic samples. The developed method was demonstrated to be sensitive, selective, and reliable for the determination of flumequine enantiomers in multiple food matrices, which could facilitate further research in this area.

2 | MATERIALS AND METHODS

2.1 | Chemical and reagents

The racemic flumequine (purity>98.0%) was supplied from the National Institute for Food and Drug Control (Beijing, China). MS-grade ammonium acetate, acetic acid, formic acid, methanol, and acetonitrile were purchased from Sigma-Aldrich (Beijing, China). Analytical grade trichloroacetic (TCA) acid (purity≥99.0%), ethylenediamine tetraacetic acid disodium salt (purity≥98.0%), NaOH (purity≥96.0%), n-hexane, disodium hydrogen phosphate dihydrate (purity≥99.0%), citric acid monohydrate (purity≥98.0%), and potassium dihydrogen phosphate (purity≥99.5%) were provided by Yuwang Technology (Shandong, China). Ultrapure water was used throughout our study. The Cleanert C18 cartridge (500 mg, 3 mL), Cleanert PAX cartridge (60 mg, 3 mL), and Cleanert PEP-2 (60 mg, 3 mL) cartridge were purchased from Agela Technologies (Tianjin, China). Standard stock solution of flumequine (1 mg mL^{-1}) was prepared in pure methanol. Working standard solutions were prepared daily by serial dilution in methanol from the standard stock solutions. All solutions were stored at 4°C prior to the analysis.

2.2 | Sample preparation

The multiple blank samples were purchased from the local market in Benxi (China). Preliminary analysis showed that these samples were analyte-free and thus were used for the method validation. These samples were homogenized and spiked with minimum volumes of solutions containing the necessary concentrations of the flumequine assayed. The samples were stored at 4°C for 24 hours, thus allowing the incorporation of the flumequine on the matrix.

2.3 | Milk and yogurt samples

Aliquots of 2 g of milk (or yogurt) were transferred to 50 mL of polypropylene centrifuge tubes, and 2.5 mL of TCA in acetonitrile (25%, w/v) was added. Then, 5-mL acidethylenediamine tetraacetic acid disodium salt (Na_2EDTA) solution (0.1 mM) was added to the tube and pH was adjusted to 4 with NaOH solution (5 M). The mixture was vortexed and left to settle in the dark for 15 min. The solution was then ultrasonic bath for 15 minutes and directly centrifuged at 4000 rpm for 15 minutes. The supernatant was transferred to a new glass tube, and the precipitate was rinsed and centrifuged again with 2.5 mL of ultrapure water. The entire supernatant was used for further clean-up. The clean-up was carried out using a Cleanert C18 cartridge (500 mg, 3 mL). The column was conditioned with 3 mL of methanol and 3 mL of water. After application of the extract, the cartridge was rinsed with 3 mL of water and vacuum dried for 2 minutes. The flumequine was eluted from the column with 3 mL 1% formic acid in methanol. The eluate was evaporated to dryness at 45°C under a stream of nitrogen. The dry residue was dissolved in 200-μL mobile phase and filtered through a 0.22-μm syringe filter before LC-MS/MS analysis.

2.4 | Chicken and beef samples

Aliquots of 2 g of thawed and minced muscle tissues were weighed and placed in a 50 mL of polypropylene centrifuge tube. Then, 15 mL of 0.2-M ammonium acetate buffer (pH 7.4) was added. Subsequently, 2 mL of acetonitrile was added. The sample was allowed to stand for 15 minutes at room temperature and then sonicated (15 min) before centrifugation for 15 minutes at 4000 rpm. The supernatant was transferred to another 50 mL of centrifuge tube. About 2 mL of n-hexane was added to each tube, followed by 10 seconds of vortex mixing and 5 minutes of centrifugation at 4000 rpm. The top hexane layer was discarded. The remaining solution was used for further clean-up, which resembled the purification described for milk and yogurt products.

2.5 | Egg samples

Aliquots of 2 g of whole egg were placed into a 50 mL of polypropylene centrifuge tube, and 8 mL of 1% acetic acid in ethanol solution was added. The sample was shaken for 5 minutes, and 500 μL of acetonitrile and 4 mL of 1% acetic acid in ethanol solution were added. Before centrifugation step, the suspension was vortexed and put into ultrasonic bath for 15 minutes; next, the mixture was centrifuged at 4000 rpm for 15 minutes. The upper layer was transferred into a new centrifuge tube, and the extraction was repeated with 4 mL of 1% acetic acid in ethanol solution. The entire supernatant was evaporated under nitrogen stream at 45°C; the residue was reconstituted with 1 mL of methanol. After mixing on a vortexer, 2 mL of n-hexane was added. After shaking and sonication, the upper layer was discarded. And the suspension was treated for further clean-up, which was analogous to one described for milk and yogurt products.

2.6 | Honey samples

An aliquot of 2 g homogenized honey was weighed and placed into 50 mL of polypropylene centrifuge tube. Then, 10 mL of 0.2 M ammonium acetate buffer (pH 7) was added. Subsequently, 3 mL of 5% formic acid in acetonitrile was added and the mixture was vortexed until the honey was completely diluted. After sonication (15 min) and centrifugation (15 min, 4000 rpm), the entire sample was used for further clean-up. The purification was similar to the one described for milk and yogurt products.

2.7 | LC-MS/MS

The chromatographic analysis of the flumequine enantiomer was conducted using a Waters AcquityTM UPLC system (Waters Corp., Milford, MA, USA), coupled to a Micromass QuattromicroTM API mass spectrometer (Waters, MA, USA) with an electrospray ionization (ESI) interface. Six CSPs, including four cellulose-based columns (ChiralpakIB and Chiralpak IC, 250 mm × 4.6 mm, i.d. 5 μm; Chiralcel OD-RH and Chiralcel OJ-RH, 150 mm × 4.6 mm, i.d. 5 μm) and two amylose-based chiral columns (Chiralpak IA and Chiralpak ID, 250 mm × 4.6 mm, i.d. 5 μm), have been evaluated for the chiral separation of flumequine. All the seven chiral columns were provided by Daicel Chiral Technologies (China)

Co., Ltd (Shanghai, China). The optimized chromatographic condition for the enantioseparation of the flumequine on Chiralpak IC column was achieved by running the reversed phase condition consisting of 20% aqueous phase (0.1% formic acid and 5 mM ammonium formate in water) and 80% organic phase (0.1% formic acid and 5 mM ammonium formate in acetonitrile). Separation was performed under isocratic mode with a flow rate of 0.6 mL min^{-1}. Column temperature and autosampler temperature were set at 20 and 4°C, respectively. The injection volume was 10 μL.

All data collected were processed using MassLynx 4.1 software (Waters Corp., Milford, MA, USA). Optimized collision energy and other parameters were obtained based on the direct injection of individual standard solutions at the concentration of 100 ng mL^{-1}. 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 1000 L $hour^{-1}$ with a temperature of 500°C. The collision gas by ultrahigh-purity argon was held at 0.12 mL min^{-1}. Detection was performed in multiple reaction monitoring (MRM) mode and in the positive ionization mode. Two MRM transitions were monitored for flumequine. The most abundant product ion was selected for quantification, while the other transition was used for confirmation. Typical conditions were as follows: m/z 262 > 243.88 was used for quantification and m/z 262 > 243.88 and m/z 262 > 201.88 were used for confirmation when the collision energy was set at 28 and 12 eV, respectively. The optimized cone voltage of flumequine was 36 V.

3 | RESULTS AND DISCUSSION

3.1 | Method development

3.1.1 | Effect of CSPs

According to previous studies, enantioselective separation of chiral drugs is extensively carried out using a CSP. Owing to the poor ionization and potential hazard, the normal-phase LC is generally considered to be incompatible to MS. Hence, the reversed phase mode is applied to determination. The selection of chiral column with different stationary phases is a key factor to achieve efficient enantioseparation. Among the various CSPs, the polysaccharide-based CSPs are extensively used, which show good chiral recognition ability toward many chiral compounds.[26-30]

In the preliminary experiments, the enantioseparation of flumequine on four cellulose-based columns (Chiralpak IB and Chiralpak IC, Chiralcel OD-RH and Chiralcel OJ-RH) and two amylose-based chiral columns (Chiralpak IA and Chiralpak ID) was tested using a variety of reversed-phase mobile phase combinations. The initial column screening was conducted under the same mobile condition, with a organic solvent of 80% methanol or 60% acetonitrile at a flow rate of 0.6 mL min^{-1}. If enantioseparation was unsatisfactory, optimization steps by changing the mobile phase compositions were considered. And mobile phase additives such as ammonium acetate and formic acid were also tested for good resolution. From the results obtained from the selected six columns, Chiralpak IA (amylose tris-(3, 5-dimethylphenylcarbamate)), Chiralpak IB (cellulose tris-(3, 5-dimethylphenylcarbamate)), Chiralpak ID (amylose tris-(3-chlorophenylcarbamate)), and Chiralcel OD-RH (cellulose tris-(3, 5-dimethhylphenylcarbamate)) could be capable for partial separation of the enantiomers. Only Chiralpak IC and Chiralcel OJ-RH column were effective for baseline separation of the flumequine enantiomers. Chiralpak IC is an immobilized column, which is produced by immobilization of the cellulose tris- (3,5-dichlorophenylcarbamate) on silica gel surface. While, Chiralcel OJ-RH is a coated column, of which the chiral selector is cellulose tris-(4-methylbenzoate). Though the baseline separation could be achieved on the two columns, Chiralpak IC column was preferable because of the higher enantioselectivity and the shorter retention times.

It can be seen that flumequines contain carboxyl, carbonyl, phenyl moiety, fluorine atom, and nitrogen atom. There might be hydrogen bonding, π-π interaction, and dipole-dipole interaction between flumequine and CSP during the chiral resolution process. For Chiralpak IC, a chloro-substituted CSP, the higher enantioselectivity abilities may be due to the existence of chlorine atoms on the phenyl moiety. Owing to the electron-withdrawing inductive effect, chlorine atoms might improve the acid strength of the NH group (carbamate group) and contribute to the formation of hydrogen bonding with flumequine.[31] Besides, the weak π-π interaction may exist between phenyl moiety of CSP and the phenyl ring of flumequine.

3.1.2 | Effect of mobile phase composition

Composition of mobile phase plays an important role in enantiomeric separation in terms of retention behaviours, elution, and resolution.[32] Acetonitrile and methanol as the commonly used organic modifiers were tested in the preliminary experiments. It was found that the baseline separation of flumequine could not be achieved, and the column pressure greatly increased when methanol was

used. Whereas, a satisfactory separation was obtained when using acetonitrile as an organic modifier. As increasing the percentage of acetonitrile, shorten retention times, and decreased resolution was observed, so 80% of acetonitrile was finally used. Generally, small quantities of additives could remarkably improve peak shapes and enhance MS response and resolutions. Different ammonium acetate concentrations (2, 5, and 10 mM) were investigated for this purpose. It was found that the concentration of ammonium acetate exhibited no significant effect on the enantioselective separation, whereas the MS response was the highest when the buffer concentration was 5 mM. Consequently, 5-mM concentration of ammonium acetate was selected. In addition, 0.1% formic acid was added in mobile phases that could enhance the $[M+H]^+$ responses and improve sensitivity for the target analytes. And lower flow rate resulted in better resolution but longer analysis time. For the aim of good resolution and reasonable analysis time, the flow rate was set at 0.6 mL min^{-1}. In consequence, the final mobile phase was composed of 20% aqueous phase (0.1% formic acid and 5mM ammonium formate in water) and 80% organic phase (0.1% formic acid and 5mM ammonium formate in acetonitrile) delivered with a flow rate of 0.6 mL min^{-1}.

3.1.3 | Effect of column temperature

Column temperature is regarded as a significant parameter with reference to chiral separations.[33] The thermodynamic parameters could be calculated according to the capacity factor (k), separation factor (α), and resolution (Rs) by classical Van't Hoff equations,[34-36] which assumes that analyte retention is only due to enantioselective interactions with the stationary phase and does not distinguish between chiral and nonchiral interactions.[37] The classical Van't Hoff equations are exhibited as follows:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\Phi = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^*}{R}, \quad (1)$$

$$\ln\alpha = -\frac{\Delta\Delta H^\circ}{RT} + \frac{\Delta\Delta S^\circ}{R}. \quad (2)$$

In the classical Van't Hoff equations, ΔH° and ΔS° represent the enthalpy and entropy changes of the analyte from the mobile phase to the stationary phases, Φ is the phase ratio, T is the absolute temperature (K), and R is the universal gas constant (8.314 $J{\cdot}mol^{-1}K^{-1}$). The $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ are on behalf of the differences of ΔH° and ΔS° between the two enantiomers. Equation 1 predict that if plots of ln k versus $1/T$ were linear, the slope and intercept were $-\Delta H^\circ/R$ and $\Delta S^\circ/R + \ln\Phi$. Due to the fact that the value of Φ is unknown, $\Delta S^*/R$ will take place of $\Delta S^\circ/R + \ln\Phi$. For a linear plot of ln α versus $1/T$, the slope and intercept are $-\Delta\Delta H^\circ/R$ and $\Delta\Delta S^\circ/R$.

In the present work, the effect of column temperature on separation of flumequine enantiomers was carried out on Chiralpak IC with step increasing of temperature from 20 to 40°C with 5°C increments using the optimized mobile phase. With the increase of column temperature, the capacity factor and separation factor decreased. Hence, 20°C was chosen as the optimal temperature. The calculated thermodynamic parameters are listed in Table 1. The plots of ln k and ln α versus $1/T$ were linear (linear correlation coefficient R^2>0.99), suggesting that the retention mechanism was independent of temperature in the studied range and the enantioselective interaction remained unchanged. The negative ΔH° values revealed that the distribution of the two enantiomers between the mobile phase and the stationary phase was exothermic. Moreover, both $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ were negative showing that separation of flumequine was enthalpy-driven.[34,36]

3.1.4 | Identification of enantiomeric elution orders

Commercially available enantiopure standards of flumequine were not available, and their elution order on the Chiralpak IC column, which was utilized for chiral separation in our work, has not been reported. The elution order of the flumequine enantiomers was determined according to previous literature, that demonstrated S-(-)- flumequine was eluted first from the Chiralcel OJ-RH column.[22] Firstly, we achieved the separation of flumequine under the same condition as depicted by Zhao et al.[22] The first eluting peak from Chiralcel OJ-RH column was collected, then the solvent was evaporated. The residue was dissolved in methanol and injected into the Chiralpak IC column by optimum condition. By comparing the retention time of single enantiomer with the racemic flumequine, the enantiomer

TABLE 1 The thermodynamic parameters of flumequine enantiomers.

Analyte	ln k	R^2	ΔH°, kJ/mol	ΔS*, J/K/mol	ΔΔH°, kJ/mol	ΔΔS°, J/K/mol
S-enantiomer	$\ln k_1$=954.83/T−2.2939	0.9950	−7.86	−19.07		
R-enantiomer	$\ln k_2$=1080/T−2.546	0.9937	−8.98	−21.17	−1.12	−2.10

elution order was established as S-(-)-flumequine followed by its R-(+)-enantiomer.

3.1.5 | Optimization of extraction

Food matrices (milk, yogurt, chicken, beef, honey, and egg) are notably complex consisting of various endogenous substance. For the purpose of developing a sensitive method to analyse flumequine at trace concentration level in food matrices, it is of great importance to isolate the analytes from matrices as much as possible. Since different food matrices were quite distinct and complex, and in order to improve the extraction efficiency, individual extraction methods were applied to different food matrices.

3.1.6 | Extraction of milk and yogurt samples

Dairy products contain abundant fats and proteins. Therefore, it is necessary to precipitate the protein and remove the fat to enhance the sensitivity. Based on the published literatures,[12,38,39] TCA in acetonitrile (25%, w/v), TCA in methanol (25%, w/v) and Mcllvaine buffer were evaluated as the extraction solvents. As indicated in Figure S1, the extraction efficiency of TCA in acetonitrile (25%, w/v) was better than others. In some cases, the addition of EDTA could form the complexes with the analyst, which was beneficial for extraction. Results demonstrated that the recovery was obviously improved when the EDTA was added. The pH of sample also be tested, and the pH 4 provided higher recovery.

3.1.7 | Extraction of chicken and beef samples

To date, previous research studies showed many extraction solvents, which were effective in extracting meat matrix,[2,40-42] including acid-acetonitrile, ammonium acetate buffer, phosphate buffer solution, and dichloromethane. In this work, we investigated acid-acetonitrile, 0.2 M ammonium acetate buffer, and 0.01 M phosphate buffer as extraction solvents. It was found that the recovery of 0.2 M ammonium acetate buffer was higher than others (Figure S1). Based on this, 0.2-M ammonium acetate buffer was selected as the optimum extraction procedure for chicken and beef samples.

3.1.8 | Extraction of egg samples

According to previous study, three extraction solutions were preliminarily investigated, including acetonitrile, TCA in acetonitrile (10%,w/v), and ammonium hydroxide in acetonitrile (10%,v/v). But the recovery of all these solutions was poor and the recovery provided by alkalized acetonitrile approached zero indicating that alkalized solvent was not efficient for flumequine extraction from egg samples. Therefore, the extraction produce had to be modified by adding acid in solvent to improve the recovery. Finally, the sample preparation produce was adopted 1% acetic acid in ethanol, providing a satisfactory recovery for egg samples[43] (Figure S1).

3.1.9 | Extraction of honey samples

Honey is a weakly acidic complex matrix that contains sugars, proteins, and pigments. On account of the published reports, the 0.03-mM NaH_2PO_4 buffer, 0.1-M Na_2EDTA solution, and 0.2-M ammonium acetate buffer were investigated as extraction solvent. Among the three extraction solutions, 0.2-M ammonium acetate buffer showed best extraction efficiency and was chosen as extraction solvent. (Figure S1)

3.1.10 | Optimization of purification

Owing to the complexity of the food matrices, following the extraction process, the purification step is essential before the LC-MS/MS analysis. Previously, SPE has been widely applied for preconcentration and purification of flumequine from different food matrices.[10,16,19] Based on the properties of the flumequine, three commonly used cartridges, namely, Cleanert C18 (500 mg, 3 mL), Cleanert PEP-2 (60 mg, 3 mL), and Cleanert PAX (60 mg, 3 mL) have been selected to investigate the extraction recovery. Results are revealed in Figure 2. It was found

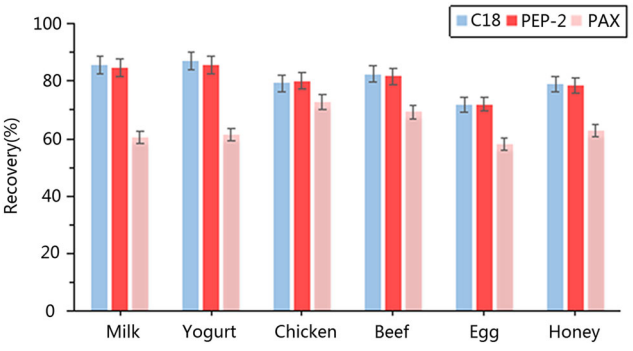

FIGURE 2 Effect of the solid-phase extraction cartridge on the recoveries of the flumequine

that poor recoveries were provided by Cleanert PAX. The recoveries of Cleanert C18 and Cleanert PEP-2 were very similar. Hence, considering satisfactory recoveries and lower cost, a C18 cartridge was chosen in current study. Because of the acidic character of flumequine, acidic elution solvent helps to improve the extraction recoveries. So 1% formic acid in methanol, 1% formic acid in acetonitrile,and 1% formic acid in ethanol were studied as elution solvent. The results illustrated in Figure 3 indicated that methanol provided the highest recovery, which may be due to the reason that methanol can form strong hydrogen bonding with analyte and then elute it from cartridge. Thus, 1% formic acid in methanol was selected as optimum elution solvent.

3.1.11 | Method validation

The performance of the developed method was validated according to a conventional validation procedure that included the following parameters: specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ), accuracy, precision, matrix effect (ME), and stability. Blank samples (free of the flumequine studied) were used for the method validation.

Ten blank samples (milk, yogurt, chicken, beef, eggs, and honey) were extracted and analysed to evaluate the specificity of the method, and it was shown that no interference was observed at the retention of target analytes. The typical chromatograms of the blank sample and spiked sample are acquired and compared in Figure 4. It can be concluded that validated method is specific for the target analytes.

The linearity of the method was performed using matrix-matched calibration curves by spiking standards before extraction at six concentration levels ranging from 0.125 to 12.5 ng g^{-1}. Each level was prepared in triplicate. The spiked samples were pretreated following the above-mentioned procedures. Calibration curves were generated using linear regression analysis, and obtained linearity was accepted when the correlation coefficients (R^2) was higher than 0.99. The

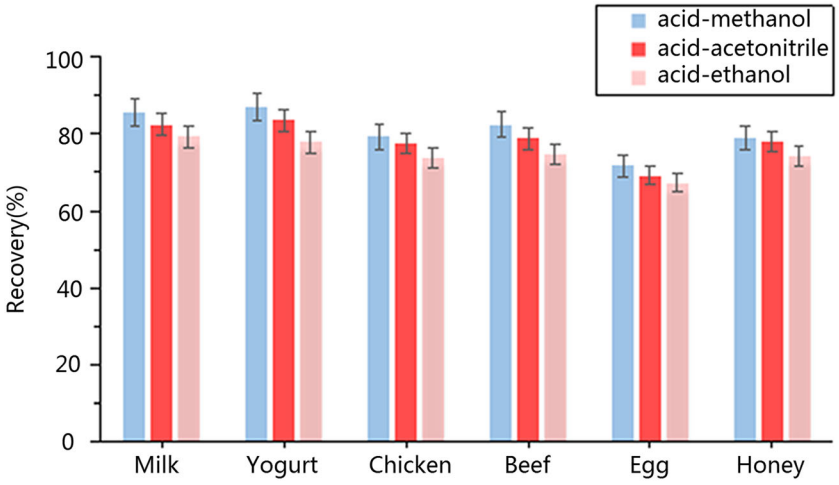

FIGURE 3 Effect of the elution solvent on the recoveries of the flumequine

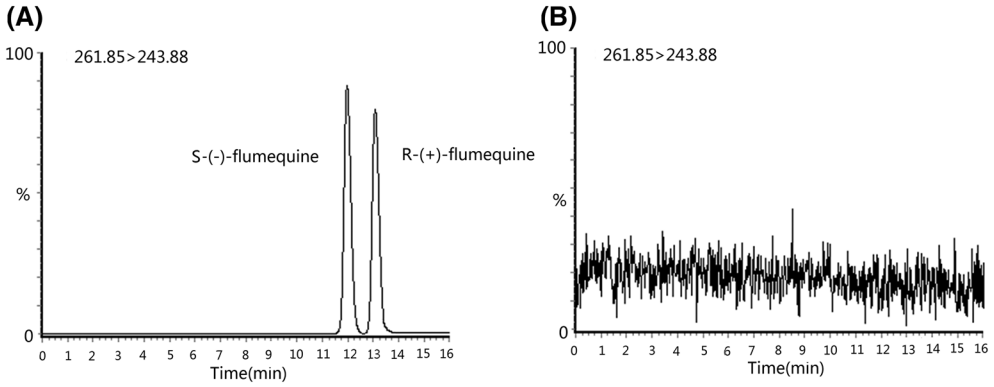

FIGURE 4 Typical enantioselective liquid chromatography tandem mass spectrometry multiple reaction monitoring chromatograms of A, blank milk sample and B, milk spiked with 0.25 ng g^{-1}

data are listed in Table 2. Satisfactory linearities were obtained (R^2 >0.9913) in the range of 0.125 to 12.5 ng g^{-1}.

The LOD of the method is defined as the lowest concentration that produced signal-to-noise (S/N) ratios of 3, and the LOQ is defined based on the S/N of 10. The results showed that the LODs and LOQs for flumequine ranged from 0.015 to 0.024 ng g^{-1} and 0.045 to 0.063 ng g^{-1}, respectively (Table 2).

The recovery assay was performed to investigate the accuracy and precision (expressed as relative standard deviation, RSD) of the method by spiking the analytes into various matrices (before extraction). Three replicates of the spiked samples at three different levels (0.25, 0.5, and 5 ng g^{-1} for each enantiomer) were prepared on three successive days. All samples were extracted and cleaned up according to the above-mentioned procedures. Method recovery was determined by comparing the back-calculated concentration with the nominal value. Intraday precision was obtained by analyzing the three levels of the spiked samples with three replicates in the same day on the LC-MS/MS, while interday precision was obtained by repeating this experiments on three consecutive days. The results are summarized in Table 3. The relative recoveries S-enantiomer of flumequine varied from 83.7 to 110.5% in all studied matrices, intraday RSD, and interday RSD were in the range of 1.0 to 11.7% and 2.2 to 11.0%. The relative recoveries of R-enantiomer of flumequine varied from 82.3 to 108.3% in all studied matrices. And intraday RSD and interday RSD were in the range of 0.6 to 10.2% and 1.4 to 10.2%.

TABLE 2 Correlation coefficient (R^2), linearity range, matrix effect, LOQ, and LOD of flumequine enantiomers in food matrices

Matrix	Analyte	Linearity Range, ng g^{-1}	R^2	LOD, ng g^{-1}	LOQ, ng g^{-1}	Matrix Effects, %
Milk	S-enantiomer	0.125–12.5	0.9924	0.017	0.045	132.4
	R-enantiomer	0.125–12.5	0.9927	0.015	0.049	128.9
Yogurt	S-enantiomer	0.125–12.5	0.9980	0.018	0.048	131.6
	R-enantiomer	0.125–12.5	0.9975	0.021	0.055	130.7
Chicken	S-enantiomer	0.125–12.5	0.9915	0.015	0.050	126.8
	R-enantiomer	0.125–12.5	0.9913	0.017	0.046	129.0
Beef	S-enantiomer	0.125–12.5	0.9984	0.020	0.056	131.4
	R-enantiomer	0.125–12.5	0.9978	0.019	0.050	127.3
Egg	S-enantiomer	0.125–12.5	0.9960	0.020	0.063	139.1
	R-enantiomer	0.125–12.5	0.9950	0.018	0.059	143.7
Honey	S-enantiomer	0.125–12.5	0.9926	0.021	0.052	95.4
	R-enantiomer	0.125–12.5	0.9937	0.024	0.055	97.8

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

TABLE 3 Recovery and RSDs of flumequine enantiomers in food matrices (n=3)

		Recovery, %			Intraday RSD, %			Interday RSD, %		
Matrix	Analyte	0.25 ng g^{-1}	0.5 ng g^{-1}	5 ng g^{-1}	0.25 ng g^{-1}	0.5 ng g^{-1}	5 ng g^{-1}	0.25 ng g^{-1}	0.5 ng g^{-1}	5 ng g^{-1}
Milk	S-enantiomer	103.2	96.3	83.7	1.9	5.6	3.5	3.4	5.9	3.0
	R-enantiomer	106.7	92.5	86.2	4.2	4.4	1.0	5.6	8.4	5.5
Yogurt	S-enantiomer	87.4	88.6	91.8	4.6	11.7	4.3	4.3	6.5	3.8
	R-enantiomer	86.2	87.3	85.8	4.8	6.9	3.4	8.7	5.2	4.9
Chicken	S-enantiomer	88.9	100.8	83.9	6.3	3.5	2.1	6.1	3.3	9.5
	R-enantiomer	87.1	101.1	82.3	5.2	5.0	2.4	9.5	7.8	3.7
Beef	S-enantiomer	110.5	97.5	100.4	6.4	6.2	1.2	2.2	11.0	6.4
	R-enantiomer	108.3	96.4	101.5	8.9	4.3	0.8	7.3	8.1	4.9
Egg	S-enantiomer	102.6	85.7	96.7	1.6	1.6	2.1	4.3	5.0	6.7
	R-enantiomer	101.8	88.4	98.3	5.0	5.7	3.1	10.2	2.8	4.5
Honey	S-enantiomer	92.1	102.4	94.7	1.0	8.1	9.2	5.3	6.6	3.3
	R-enantiomer	97.5	106.2	94.2	0.6	10.2	9.9	1.4	9.8	5.2

Abbreviation: RSDs: relative standard deviation.

It is well known that ME is a widely existing phenomenon when ESI is used, which is attributed to the ionization competition from other matrix constituents. Both signal enhancement and signal suppression can influence the accuracy of and precision of the method. Hence, it is essential to evaluate the ME. In the current study, the ME was investigated for flumequine in different matrices by comparing peak areas obtained in postextraction spiked samples with that of corresponding standard solution. If the ME equals to 100%, no ME is present. In contract, the ME >100 % indicates an ionization enhancement and the ME <100 % indicates an ionization suppression. Table 3 gave the values of ME for flumequine in six matrices. As seen, the ME observed for honey was negligible ranging from 95.4 to 97.8%. However, in other five matrices, a quite strong signal enhancement was observed with ME from 126.8 to 143.7%, due to the high complexity of the sample matrix. When all these problems were considered together, we adopted the matrix-matched standard curves, which could eliminate the effect of the matrix and could meet the requirement of quantitative analysis.

The stability of the enantiomers of flumequine was evaluated in solvent and in matrix. The stock solutions prepared in methanol were stored at 4°C for 3 months. The stability of the stock solutions was tested monthly by injection of a newly prepared working solution. Spiked blank samples were also stored at 4°C, and then analysed after 3, 7 and 14 days. The responses did not change obviously, revealing good stability of flumequine in both solvent and matrix.

3.1.12 | Application to real samples

For purpose of evaluating the potential residues of flumequine in food matrices, the proposed method was successfully applied to analysis of real samples. Six kinds of matrices, a total of 36 real samples obtained from different local market (Benxi, China), were prepared and analysed in accordance with above-mentioned procedure. Results showed that no flumequine was detected in any the purchased food.

4 | CONCLUSION

In this study, a novel and reliable enantioselective method using chiral LC-MS/MS for the determination of flumequine enantiomers in six food matrices has been successfully established and validated. A series of CSPs were evaluated, and the chromatographic conditions were also optimized. Duo to the high complexity of the sample matrix, individual extraction methods were applied to different food matrices. The sample preparation procedure, including extraction and purification, was assessed for higher extraction efficiencies. The proposed method demonstrated good linearity and accuracy, and the LOD was at low ng g^{-1} levels. In conclusion, this method is suitable and reliable for monitoring the flumequine enantiomers occurrence and quantifying the residues in animal productions and evaluating the potential risk.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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