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Optimization of QuEChERS Method for Antibiotic Residue Analysis in Animal Foods via Response Surface Methodology

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Abstract: The present study employed a modified QuEChERS method to systematically analyze the presence of fifteen quinolone and seven tetracycline antibiotic residues in local animal food. Additionally, a multi-level four-factor Box–Behnken design (BBD) within the framework of response surface methodology (RSM) was utilized to evaluate the factors impacting the detection efficiency of the sample pretreatment procedure. Optimization was performed via Design Expert® 10.0.3, and the factors, including the volume of the acetonitrile, the addition of formic acid, the duration of the extraction, and the addition of EDTA, were combined with experimental design until an optimal solution was reached. Finally, the sample was tested via ultra-high performance liquid chromatography-quadrupole-linear ion trap mass spectrometry (UPLC/MS/MS) in both multiple reaction monitoring (MRM) and enhanced product ion (EPI) scan modes on a QTRAP® 5500 instrument (AB SCIEX instruments, Framingham, MA, USA). The overall average recoveries from actual samples fortified with 22 antibiotics at three levels ranged from 73.8% to 98.5% based on the use of matrix-fortified calibration, with variations ranging from 5.80 to 12.4% (n = 6). The limits of detection and quantification were 0.3 μg kg^{-1} and 1.0 μg kg^{-1}, respectively. Lastly, the modified method was applied to practical sample analysis in the daily risk monitoring and assessment of food safety with satisfactory stability and robustness.

Keywords: antibiotics residue; response surface methodology (RSM); quinolones; tetracyclines; multiple reaction monitoring (MRM)

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1. Introduction

Antibiotic resistance, the ability of bacteria to withstand antibiotics, is now recognized as one of the most serious global threats to human health [1–3]. Naturally occurring resistance that can ultimately lead to incurable bacterial infections could be accelerated by the improper use of antibiotics in human beings and animals [4]. Except for the misuse of antibiotics in human medicine, antibiotics' misuse in livestock is also a major contributor to the emergence of antibiotic resistance [5,6]. To satisfy the growing global demand for animal protein, antibiotics have been massively and increasingly used in farmed animal industries for different purposes, including overdoses for disease prevention and subtherapeutic doses for growth stimulation [7–13]. In September 2017, a report from the World Health Organization (WHO) corroborated that the world is running out of antibiotics [14–17]. Antibiotic misuse, if left unchecked, can drag human beings into a post-antibiotic era whereby minor injuries or common infections become fatal diseases again [18].

To protect the public from health risks, nations and related organizations had to establish broader maximum-residue limits (MRLs) for further surveillance of antibiotic residues in animal food [19,20]. Therefore, more efficient and robust detection methods were promptly developed in the past few years to satisfy increasingly rigorous regulatory requirements [21–27]. Triple quadrupole mass spectrometer with multiple reaction monitoring (MRM) scan modes that follow the requirement of ECD 2002/657/EC should be the preferred method of detection of antibiotic residues in animal food [28–30]. The

Qtrap system (AB Sciex) with the scan mode of multiple reaction monitoring—information-dependent acquisition—enhanced product ion scan mode (MRM—IDA—EPI) was found to efficiently gather comprehensive information from samples in a single run. Consequently, the identification of antibiotic residues in locally sourced animal-derived food was reaffirmed through the successful comparison of antibiotic spectra from the samples with reference spectra. Quinolones and tetracyclines, being the most extensively employed veterinary antibiotics, have contributed to the development of antibiotic resistance, thereby adversely impacting the treatment of severe bacterial infections [31–33]. In this study, a diverse range of samples, fortified with fifteen quinolone and seven tetracycline antibiotics at the recommended concentration (RC) of 1 μg L^{-1}, were subjected to analysis using liquid chromatography-tandem mass spectrometry with a QTRAP 5500 instrument.

Sample pretreatment for the separation and concentration of antibiotic residues is also a critical step in the whole analysis process of quinolone and tetracycline detection. There are, alternatively, two purification methods, including QuEChERS and solid-phase extraction. The latter, using commercial cartridges, has been widely used in daily work based on former research (Figure A1) [34]. It can be performed in an automated SPE system (e.g., Reeko, Fotector plus, USA) during non-working hours and minimize human involvement (Table A1), but high-fat or high-protein samples should be excluded, given that they frequently cause blockage in SPE cartridges and heavily prolong the pretreatment process. Since its development in 2003, QuEChERS has gained widespread acceptance for various sample preparation techniques [35–40]. It was initially introduced as a cost-effective and time-efficient method for analyzing multi-residue samples containing relatively polar compounds. During the extraction process, the efficiency of the QuEChERS sample preparation method is known to be influenced by several factors. Thus, it is imperative to comprehensively optimize this method for the detection of quinolone and tetracycline residues in this study. As a collection of statistical and mathematical techniques, response surface methodology (RSM) has vital applications in the design, development, and improvement of novel or existing product designs [41,42], especially in multi-variable analysis [43,44]. In the present study, response surface methodology with a multi-level four-factor Box–Behnken design (BBD) was applied to simultaneously evaluate the recovery rate of quinolone and tetracycline residues in sample pretreatment.

2. Materials and Methods

2.1. Standards and Stock Solutions

Standards of the following twenty-two antibiotics were all purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany): Oxytetracycline (Oxytetracycline hydrochloride, 96.5%), Tetracycline (Tetracycline hydrochloride, 98.0%), Doxycycline (Doxycycline hyclate, 98.7%), Demeclocycline (98.0%), Methacycline (99.0%), Minocycline (99.0%), Chlortetracycline (Chlortetracycline hydrochloride, 94.6%), Enrofloxacin (99.0%), Norfloxacin (99.1%), Pefloxacin (Pefloxacin methanesulfonate dehydrate, 99.0%), Ciprofloxacin (Ciprofloxacin hydrochloride, 94.0%), Ofloxacin (99.0%), Sarafloxacin (Sarafloxacin hydrochloride, 97.0%), Enoxacin (95.8%), Lomefloxacin (Lomefloxacin hydrochloride, 99.5%), Pipemidic acid (99.1%), Nalidixic acid (99.0%), Oxolinic acid (98.0%), Flumequine (98.5%), Cinoxacin (99.0%), Danofloxacin (Danofloxacin mesylate, 94.0%), and Difloxacin (Difloxacin hydrochloride, (99.2%)). Individual standards were weighed using an electronic balance (Metter Toledo, MS 205DU), dissolved in methanol at a concentration of 1.0 mg mL^{-1} or ethanol solution supplemented with potassium hydroxide (for antibiotics that were practically insoluble in methanol), and provisionally stored at −28 °C.

2.2. Reagents and Chemicals

Methanol and acetonitrile of HPLC grade used in this study were procured from Merck (Darmstadt, Germany). Ethanol of HPLC grade and formic acid (≥98%) were acquired from Aladdin (Shanghai, China). Potassium hydroxide (G.R.) was purchased from Macklin (Shanghai, China). Cleanert® C18 for QuEChERS was purchased from

Agela Technologies (Beijing, China). Ultra-pure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA). Oasis® HLB SPE cartridges (6 cc, 200 mg) were purchased from Waters (Milford, MA, USA). Millipore filters (0.22 μm, polytetrafluoroethylene) were obtained from ANPEL Lab (Shanghai, China). Sodium chloride (A.R.) and sodium sulfate (A.R.) were calcined in a muffle furnace prior to use. Citric acid (A.R.), disodium hydrogen phosphate (A.R.), and disodium ethylenediamine tetraacetic acid (EDTA, A.R.) for sample preparation were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.3. Instrumentation and Software

HPLC analysis was conducted on a Shimadzu LC-30AD system with a Waters BEH C18 column (1.7 μm 2.1 mm × 100 mm, Waters, Milford, MA, USA). Regarding mass spectrometric detection, all experiments were carried out in the MRM-IDA-EPI scan mode using AB SCIEX QTRAP® 5500 (AB SCIEX instruments, Framingham, MA, USA). The compounds were ionized in a Turbo V™ Ion Source (ESI) interface in the positive ionization mode. An Analyst® software v. 1.6.2 (AB SCIEX instruments, Foster City, Canada) was utilized to remotely control the chromatograph and mass spectrometer. A capillary voltage of 5.50 kV and desolvation temperature of 500 °C were applied to the ESI source. Nitrogen produced by the generator (Claind Nitro35, Tremezzina, Italy) was used as the cone gas (50 psi), desolvation gas (50 psi), and collision gas. Quantitative analysis of the experiments was conducted using MultiQuant® 3.0.1. The optimization of QuEChERS using the response surface method was performed on Design Expert® 10.0.3.

2.4. Sample Collection and Processing

The samples were collected from local markets or supermarkets distributed randomly across neighborhoods of the whole city, including swine, poultry, eggs, milk, and eight cultured aquatic products (*Parabramis pekinensis*, *Carassius auratus*, *Ctenopharyngodon idella*, *Ophiocephalus argus Cantor*, *Macrobranchium nipponense*, *Macrobrachium rosenbergii*, *Penaeus chinensis*, *Procambarus clarkia*, *Eriocheir sinensis*, and *Larimichthys crocea*). The edible portion of the aforementioned samples was ground and homogenized using a Mixer (BÜCHI, B400, Eastern Switzerland) and stored in polypropylene bottles at −28 °C for the ensuing analyses.

3. Results

In the sample pretreatment process using the SPE extraction method, 0.1 mol L^{-1} EDTA-Mcllvaine buffer solution (pH = 4.0), a non-volatile, non-poisonous, low-cost, and eco-friendly solution, was used as the extraction solution for quinolone and tetracycline residues [34,45–47]. Furthermore, when handling a large amount of sample, the SPE method could enhance stability by increasing the level of automation without increasing human operational time (Table A1). However, during the flowing sample purification procedure, the aqueous solution extracted from high-fat samples, particularly egg-containing samples, may potentially increase the risk of blockage in SPE cartridges and significantly prolong the detection time.

3.1. Experimental Design

The QuEChERS sample preparation method could be applied as an alternative for a quick analysis of quinolone and tetracycline residues in high-fat samples. As documented in former research, the efficiency of the QuEChERS sample preparation method for quinolone and tetracycline residues could be affected by multifarious factors, including the volume of acetonitrile, pH value of the extracted solvent, and duration of the extraction process, among others [35–40].

Attributed to the unique chemical structure of quinolones and tetracyclines, agent–agent interactions [48] between antibiotics and metal ions from the experimental environment could influence the recovery rate. As in the EDTA-Mcllvaine buffer solution of the

SPE method, EDTA acts as a screening agent for metal ions that could also be introduced in the extraction process of the QuEChERS method. As illustrated in Figure 1, when the amount of added EDTA ranged from 0 to 0.2 g in 2 g of the sample using the QuEChERS preparation, the medium recovery rate of 22 antibiotics progressively plateaued at approximately 100 mg. According to the result of this experiment, 0.1 g of EDTA was selected as the auxiliary reagent in the extraction process.

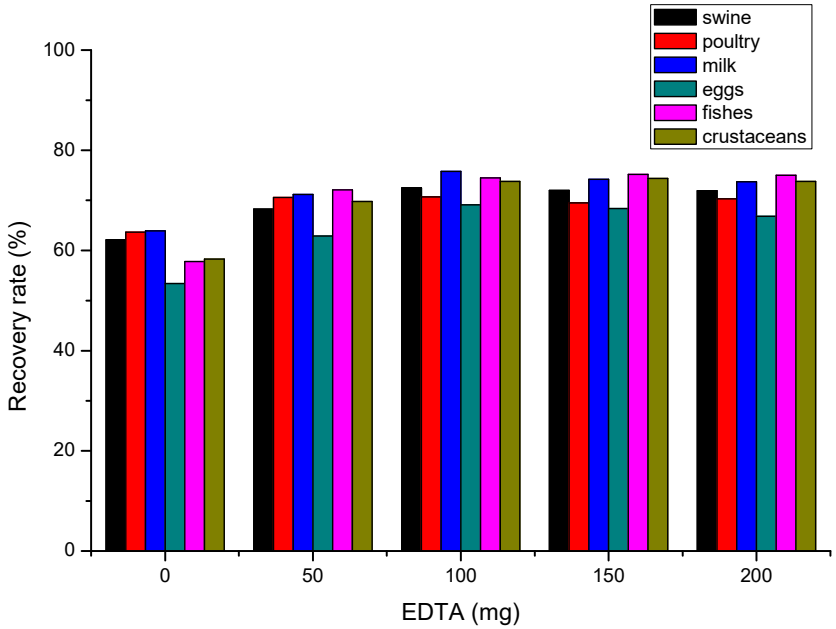


Figure 1. Variation in recovery rate with EDTA (Enrofloxacin).

3.2. Optimization of QuEChERS Method Using the Response Surface Method (RSM)

The QuEChERS sample preparation method is multivariable for the optimization of the extraction process. Its efficiency could be affected by multifarious factors, including the volume of the acetonitrile (A), the additive amount of formic acid (B), the additive amount of EDTA (C), and the extraction time (D). Nevertheless, the univariate experiment has limited ability to evaluate the interactions of the extraction conditions in sample preparation. To avoid interactions among extraction conditions while examining the optimal extraction process, possible factors were comprehensively optimized via the response surface method (RSM), including the volume of the acetonitrile (A), the additive amount of formic acid (B), the additive amount of EDTA (C) and the extraction time (D). The median recovery rate of the 22 antibiotics from standard samples (2.0 μg kg^{-1}, $n = 3$) was chosen as the response. RSM analysis was able to model the relationship between the response (recovery rate) and the four factors. Based on a previous study [48], the respective low and high levels for factors were coded.

The model's *F*-value of 15.79 implied that the model was significant. This model can be used to navigate the design space. The final equation in terms of actual factors is as follows:

$$\begin{aligned}\text{Recovery} = 84 + 21.48 \times A + 7.44 \times B + 7.58 \times C + 8.39 \times D + 5.07 \times AB + 1.95 \times AC \\ + 4.48 \times AD + 1.32 \times BC + 4.23 \times BD + 4.67 \times CD - 22.11 \times A^2 - 9.80 \times B^2 - 7.41 \times C^2 \\ - 14.45 \times D^2\end{aligned} \quad (1)$$

The *p*-value is generally employed to assess the significance of variables and can also reflect interactions among independent variables [49]. A smaller *p*-value indicates that the corresponding variable is more significant [50]. The ANOVA for the response surface quadratic model is summarized in Table 1; in this case, A, B, C, D, A^2, B^2, C^2, and D^2 were

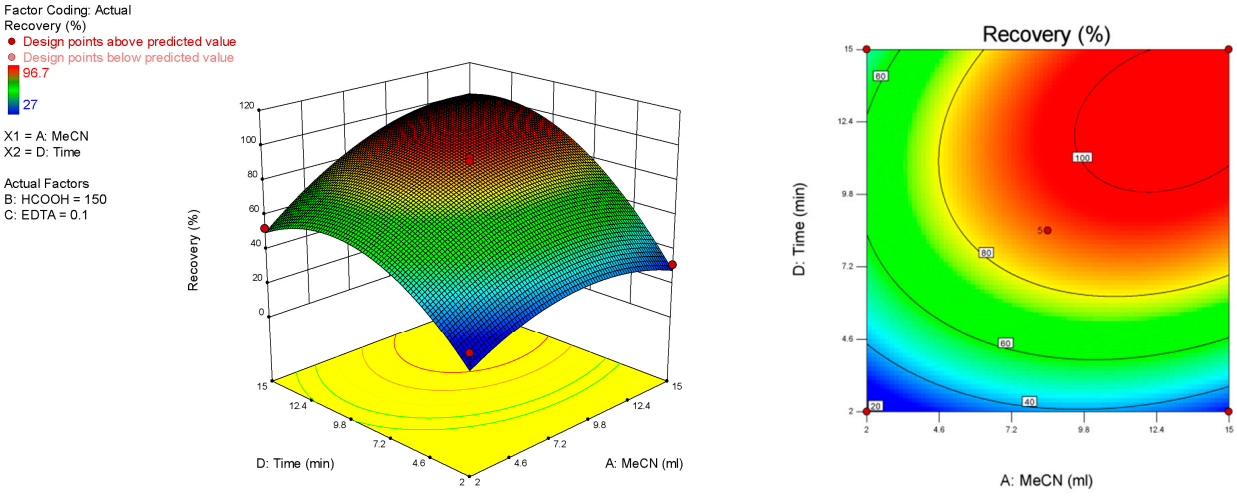
all significant model terms, and the variable volume of the acetonitrile (A) and extraction time (D) were more significant for the recovery rate.

Table 1. ANOVA for the recovery rate according to the response surface quadratic model.

Source	Sum of Squares	*df*	Mean Square	*F*-Value	*p*-Value
Model	12,099.48	14	864.25	15.79	<0.0001
A-MeCN	5538.40	1	5538.40	101.21	<0.0001
B-HCOOH	664.54	1	664.54	12.14	0.0036
C-EDTA	690.08	1	690.08	12.61	0.0032
D-Time	845.04	1	845.04	15.44	0.0015
A^2	3171.17	1	3171.17	57.95	<0.0001
B^2	622.75	1	622.75	11.38	0.0045
C^2	356.24	1	356.24	6.51	0.0231
D^2	1354.08	1	1354.08	24.74	0.0002
Cor total	12,865.58	28			

R^2 (Pred) = 0.7 R^2 (Adj) = 0.8809. Significant at a 95% confidence degree ($p < 0.05$).

As displayed in Figure 2, the recovery rate of the 22 antibiotics was superior among the solutions with the following settings: acetonitrile (A) = 8 mL, formic acid (B) = 150 µL, EDTA (C) = 0.1 g, and time for extraction (D) = 8 min. Verification tests were carried out six times under the above-mentioned optimized conditions. The median recovery rates of the 22 antibiotics from six parallel tests were 75.4%, 81.6%, 85.9%, 73.6%, 77.9%, and 82.5%, with errors ranging from 7.8% to 15.1%. The sample preparation of the QuEChERS method was finally optimized as the best solution from RSM, and the method is outlined in Figure 3.



(**a**) (**b**)

Figure 2. (**a**) Response surface plot; (**b**) response surface contour plots of optimization results.

Concerning purification, the addition of a cleaning agent, namely C18 powder, was evaluated in fish to identify the highest detection rate of antibiotic residue. The crude extraction of the spiked sample was adequately purified when 150 mg of C18 powder was added (see Figure 4).

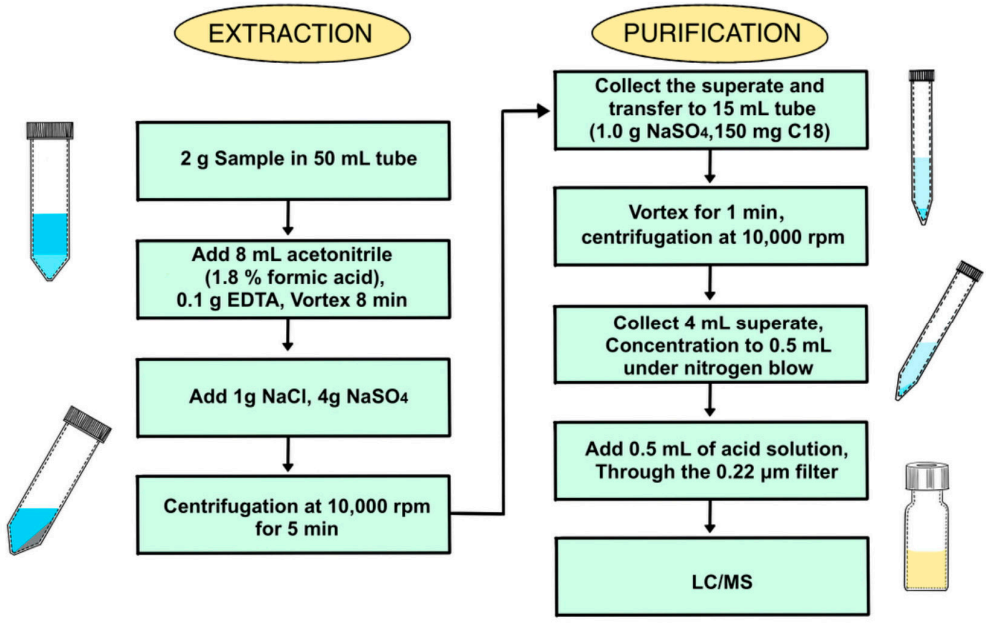


Figure 3. Scheme of QuEChERS sample preparation method.

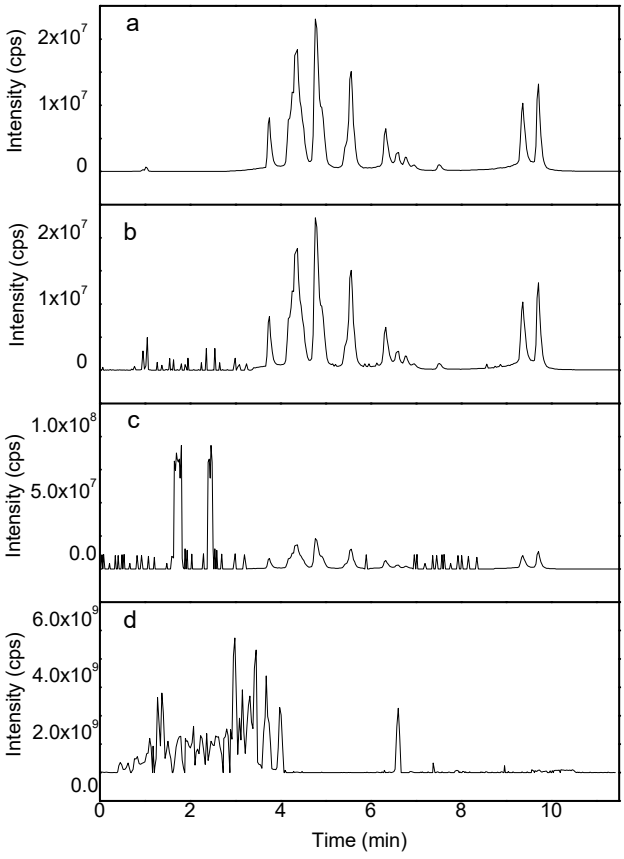


Figure 4. Variations in total ion chromatogram with C18 addition (spiked in fishes): (**a**) 150 mg; (**b**) 100 mg; (**c**) 50 mg; (**d**) no addition.

3.3. Optimization of Chromatographic Conditions and Mass Spectrometry

Quinolines and tetracyclines both contain several O and N atoms; consequently, it is easy to obtain protons and a high response in the positive ion mode. To obtain the two ion pairs for quantification, a mixed standard solution of 22 antibiotics at a concentration of

100 μg L^{-1} was infused into the QTRAP mass spectrometer at a flow rate of 7.0 μL min^{-1} to achieve automatic analyte optimization via the ESI in the positive mode. Under the optimal mass spectrometry conditions, including declustering potential and collision energy, every antibiotic was assigned two pairs of abundant ions for qualitative and quantitative analysis with high sensitivity. To achieve minimum retention time and symmetric shape of ionic peaks, the elution type, flow rate, and gradient were optimized in this study using the C18 chromatographic column. Therefore, several classical compositions of the mobile phase were performed, including acetonitrile, methanol, water, as well as water with ammonium acetates or formic acid. Finally, water (A) and acetonitrile (B), which were both supplemented with 0.1% formic acid, were chosen as the optimal mobile phase. The final gradient elution at a total flow rate of 0.3 mL min^{-1} was as follows: 0–0.5 min, 5–20% B; 0.5–2.0 min, 20–25% B; 2.0–7.0 min, 25–45% B; 7.0–10.0 min, 45–90% B; 10.0–12.0 min, 90% B; and 12.1–13.0 min, 95–5%. The column oven was maintained at a temperature of 40 °C, and the injection volume was 10.0 μL. The representative total ion chromatogram (TIC) was merged in Figure A2. The retention time (RT) and MS information for each antibiotic, including precursor and product ions, DP, and CE, are presented in Table 2.

Table 2. Retention time and MS parameters of the 22 antibiotics.

No.	Compound	Retention Time (min)	CAS No.	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Declustering Potential (V)	Collision Energy (eV)
1	Pipemidic acid	2.88	51940-44-4	304.3	217.1 * 189.0	70 70	18 27
2	Enoxacin	3.32	74011-58-8	321.4	303.3 * 233.9	80 80	22 33
3	Minocycline	3.59	10118-90-8	458.5	441.4 * 352.4	80 80	20 30
4	Norfloxacin	3.64	70458-96-7	320.3	302.3 * 276.3	80 80	26 35
5	Ofloxacin	3.66	82419-36-1	362.2	318.3 * 261.2	80 80	26 38
6	Pefloxacin	3.70	70458-92-3	334.3	290.3 * 233.2	80 80	27 25
7	Tetracycline	3.74	60-54-8	445.4	410.4 * 427.7	80 80	24 19
8	Ciprofloxacin	3.75	85721-33-1	332.2	314.3 * 288.3	80 80	25 33
9	Methacycline	3.77	914-00-1	443.3	426.4 * 201.2	60 60	18 10
10	Oxytetracycline	3.78	79-57-2	461.4	426.4 * 443.6	80 80	25 17
11	Danofloxacin	3.82	112398-08-0	358.3	340.3 * 82.0	80 80	12 35
12	Lomefloxacin	3.87	98079-51-7	352.3	265.2 * 308.3	80 80	33 28
13	Enrofloxacin	3.96	93106-60-6	360.3	316.4 * 342.3	80 80	25 35
14	Doxycycline	4.00	564-25-0	445.5	428.5 * 154.0	80 80	24 35

Table 2. *Cont.*

No.	Compound	Retention Time (min)	CAS No.	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Declustering Potential (V)	Collision Energy (eV)
15	Demeclocycline	4.07	64-73-3	465.3	430.4 * 448.4	75 75	23 28
16	Sarafloxacin	4.38	98105-99-8	386.3	342.3 * 299.3	80 80	25 38
17	Difloxacin	4.51	98106-17-3	400.1	356.1 * 299.1	80 80	28 41
18	Chlortetracycline	5.13	57-62-5	479.3	444.4 * 462.3	80 80	24 28
19	Cinoxacin	5.29	28657-80-9	263.1	244.1 * 188.8	80 80	25 35
20	Oxolinic acid	5.53	14698-29-4	262.1	244.1 * 155.9	70 70	26 40
21	Nalidixic acid	7.09	389-08-2	233.1	187.0 * 244.1	68 68	18 34
22	Flumequine	7.43	42835-25-6	262.2	244.1 * 202.1	70 70	19 32

CAS: chemical abstracts service; *: quantitative ion.

The EPI scan mode could be activated in the IDA experiment when the ionic intensity exceeded the threshold of 1000 cps. The scan time (including pauses) was 1.57 s for all MRM transitions. EPI mass spectra were acquired over a mass range of *m*/*z* 50–500 at a scan rate of 10,000 Da s^{-1}.

3.4. Fragmentation Approach for Quinolones and Tetracyclines

In the positive mode of electrospray ionization (ESI) mass spectrometry, the proton first binds to the protonation site, usually at the N atom or O atom, and then triggers cleavage by migrating to the reactive center. Although the most basic site in tetracyclines is the dimethylamino group, protonated tetracyclines initially dissociate via the loss of H_2O or NH_3 from the acylamino group. Regarding tetracycline, demeclocycline, and chlortetracycline, there is no OH at C–6 sites, and, consequently, all presented with successive losses of H_2O and NH_3. As depicted in Figure 5, tetracyclines without the tertiary OH at C–6 initially lose only NH_3 [51].

According to the spectra acquired from the EPI mode (Figure 6), the reactive center of quinolones was located in the carboxylic acid group. The abundant fragment ion $[M+H–H_2O]^+$ was formed due to the dehydration of –COOH, while another abundant fragment ion was characterized by the decarboxylation of this group. The neutral loss of *m*/*z* 20 Da and *m*/*z* 30 Da was most probably formed due to the dissociation of –HF or $–CH_2CH_3$. Moreover, another characteristic neutral loss of $–CH=CH–NH_2$ (*m*/*z* 20 Da) was produced from the cracking of the azine ring [52].

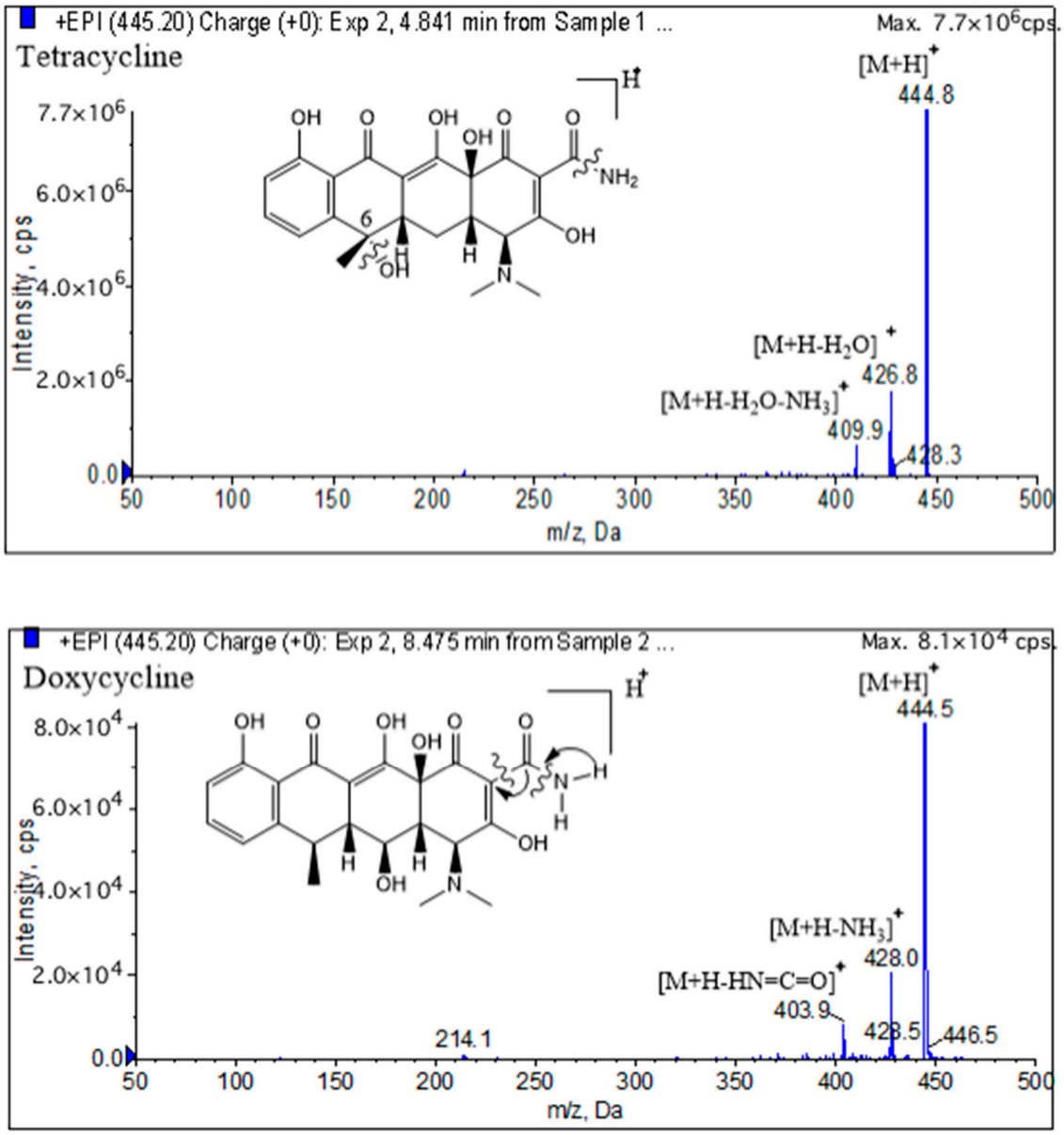


Figure 5. Production spectra and proposed fragmentation pathway of tetracycline and doxycycline.

3.5. Method Validation

3.5.1. Matrix Effect

To evaluate the matrix effects in LC-MS detection, six distinct types of antibiotic-free samples were used as matrix-matched blanks on the ionization of 22 antibiotic residues. The equation is as follows [53]:

$$ME = \frac{A_{Matrix}}{A_S} \times 100\% \quad (2)$$

where A_{Matrix} represents the peak area of the standard solution with the matrix-matched blank, and A_S stands for the peak area of the standard solution in the initial mobile phase. The percentages of the matrix effects of the 22 antibiotics at three different concentrations (2, 20, 200 ng mL^{-1}) ranged from 84.7% to 119.3%. When ion suppression and ion enhancement at the chosen levels were considered, the blank matrix-matching standard curve was adopted to eliminate the effect of the matrix.

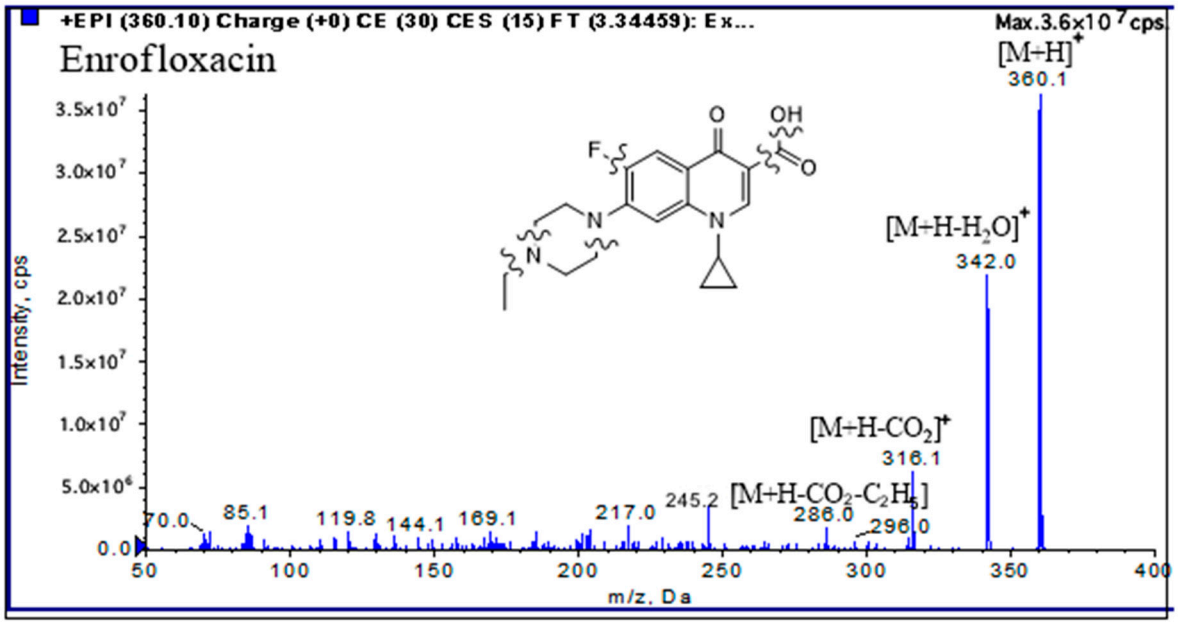


Figure 6. Production spectra and proposed fragmentation pathway of enrofloxacin.

3.5.2. Linearity and Sensitivity

Satisfactory linearities (R > 0.99) were obtained for 22 antibiotics in blank matrix-matched curves over concentrations ranging from 0.5 ng mL^{-1} to 200.0 ng mL^{-1}. The sensitivity of the proposed method was measured according to the limit of detection (LOD) and the limit of quantification (LOQ) values. LOD and the LOQ were calculated using the following equations [53]:

$$\mathrm{LOD} = \mathrm{C_S}\frac{3}{\mathrm{S/N}} \quad (3)$$

$$\mathrm{LOQ} = \mathrm{C_S}\frac{10}{\mathrm{S/N}} \quad (4)$$

where S/N denotes the average signal-to-noise ratio, and C_S represents the concentration of the specific antibiotic. The estimated values were tested using suitable spiked samples containing the 22 antibiotics at the corresponding concentrations. When the concentration ranged between 0.5 and 200.0 ng mL^{-1} (0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, with R.S.D. under 10%, n = 6), the LOD and LOQ values were 0.3 μg kg^{-1} and 1.0 μg kg^{-1}, respectively, demonstrating the sensitivity of the method for antibiotic residues.

3.5.3. Accuracy and Precision

The accuracy and precision of the method were measured using the intra- and inter-day recoveries and relative standard deviation (R.S.D.). Therefore, the standard mixed solutions of 22 antibiotics were spiked into distinct types of samples, including swine, poultry, eggs, milk, fish, and crustacea, and 18 spiked samples (six types at three concentrations of 2, 20, 200 μg kg^{-1}, shown in Table A2) were obtained. Notably, these spiked samples were detected three times intra-day and three times inter-day. As anticipated, the recovery of 22 antibiotic residues (73.8–98.5%) fell within the recommended guidelines of 60–120% (GB/T 27404-2008, China) [54]. The precision of the analysis measured as the relative standard deviation (R.S.D.) of the recovery, which ranged from 5.80% to 12.4%, was well under the criteria of 30% (GB/T 27404-2008, China) [54].

3.6. Sample Analyses

After validation of the analytical methodology through the above experimentation, it was applied for detection using various real food samples, including swine, poultry,

eggs, milk, and nine cultured aquatic products (*Parabramis pekinensis*, *Carassius auratus*, *Ctenopharyngodon idella*, *Ophiocephalus argus Cantor*, *Macrobranchium nipponense*, *Macrobrachium rosenbergii*, *Penaeus chinensis*, *Procambarus clarkia*, *Eriocheir sinensis*, and *Larimichthys crocea*). In the last six years of detection (2017–2022, total of 781 samples), quinoline or tetracycline residues from swine, eggs, milk, and *Eriocheir sinensis* were essentially not detected. Compared with the research led by Prof. Treiber [55], tetracycline residues, including tetracycline, oxytetracycline, and chlortetracycline, were also occasionally detected but never exceeded the MRL (200 ug kg^{-1}, GB31650-2019, China [56]) in poultry, *Macrobranchium nipponense*, *Macrobrachium rosenbergii*, *Penaeus chinensis* and *Procambarus clarkia*. Quinoline residues were generally detected in cultured aquatic products, with the exception of *Eriocheir sinensis*. The detection rate of quinoline residues was highest in fishes (*Parabramis pekinensis*, *Carassius auratus*, *Ctenopharyngodon idella*, *and Ophiocephalus argus Cantor*), ranging from 11.36% to 37.51%, with the over-limit rate (MRL, 100 μg kg^{-1}, GB 31650-2019, China [56]) ranging from 1.85% to 9.07%. Furthermore, enrofloxacin and ciprofloxacin were the dominant detected residues among the 22 antibiotics. Meanwhile, among the 12 types of samples, *Parabramis pekinensis*, with a medium detected concentration of enrofloxacin and ciprofloxacin of 179.9 μg kg^{-1} and 21.4 μg kg^{-1}, respectively, contributed the maximum detected frequency and value.

A simplified risk assessment of enrofloxacin and ciprofloxacin from fish could be calculated using the following equations:

$$\mathrm{EXP} = \frac{\text{Average consumption (g)} \times \text{Average detection value (μg/kg)} \times \mathrm{p}}{\mathrm{BW}} \quad (5)$$

$$\mathrm{MOS} = \frac{\mathrm{ADI}}{\text{Daily dietary exposure}} \quad (6)$$

where EXP represents the daily dietary exposure of enrofloxacin and ciprofloxacin, and MOS denotes the margin of safety. The average consumption of fish is 24.3 g/d, according to the Scientific Research Report on Dietary Guidelines for Chinese Residents of 2021. p represents the effect of food processing and was excluded from this simplified assessment; BW stands for the average body weight (60 kg and 30 kg for adults and children, respectively). According to the National food safety standard GB 31650-2019 [56], the sum of enrofloxacin and ciprofloxacin residues in fish should not exceed 100 μg kg^{-1}, and the related acceptable daily intake (ADI) is 2.0 μg/(kg·d). The results revealed that consuming fish with high levels of quinolone residues may increase the risk of adverse events in children.

4. Conclusions

The analytical approach for antibiotic residue using LC-QTRAP-MS developed in this study is reliable and effective in daily risk monitoring and assessment for food safety. The mass spectrum of each antibiotic obtained from the EPI mode could be used as a corroboration of positive samples. Furthermore, the optimization of the sample pretreatment using the response surface method (RSM) enhanced work efficiency. The analysis of real food origin samples validated the robustness and applicability of the modified QuEChERS method. Over the past six years, massive antibiotic residues have been detected in food from animal origin. Detection rates exceeding the maximum residue limits (MRL, GB 31650-2019, China) have been decreasing year by year from 2020 owing to the strict legal requirements imposed by the National Food Safety Standard—Maximum residue limits for veterinary drugs in foods (GB 31650-2019, China).

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Appendix A

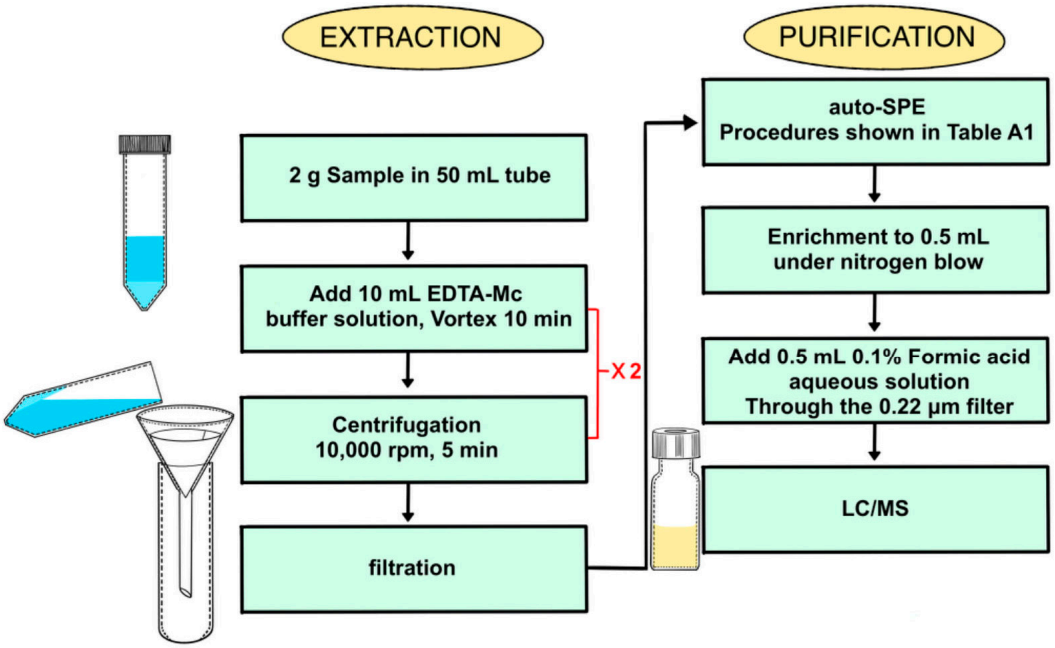

Figure A1. Scheme of solid-phase extraction.

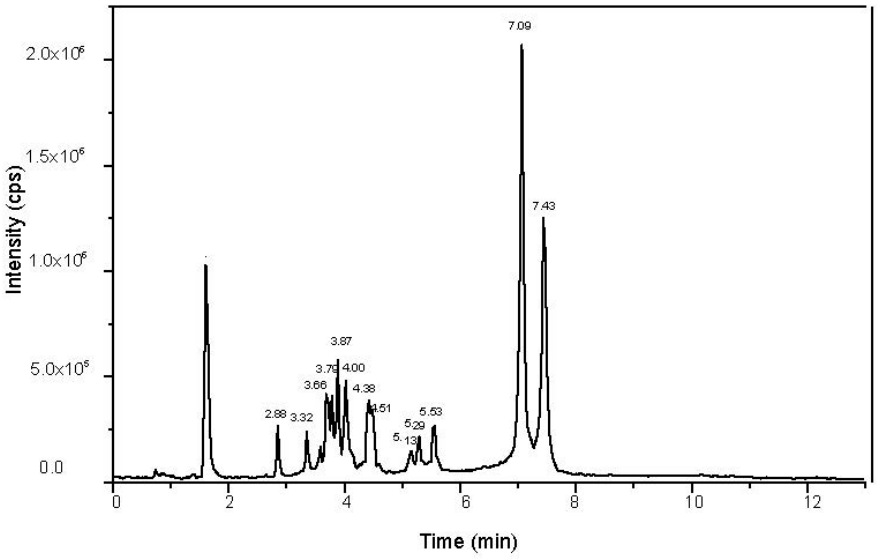

Figure A2. LC-MS/MS chromatograms of 22 antibiotics (50 ng mL^{-1}).

Table A1. Procedures for automated solid-phase extraction.

NO.	Step	Source	Output	Flow Rate (mL/min)	Volume (mL)	Time (min)
1	Rinse sample path	CH_3OH				2.8
2	Rinse sample path	H_2O				2.8
3	Rinse plunger	CH_3OH	Solvent	10	6	1.1
4	Rinse plunger	H_2O	Solvent	10	6	1.1
5	Load sample		Waste	2	20	22.3
6	Rinse	5% CH_3OH	Solvent	3	3	4.3
7	Rinse syringe	CH_3OH		10	10	1.6
8	Elute	CH_3OH	Collect	10	5	0.9
9	Air push		Collect	10	5	1.1
10	End					

Total time: 41.8 min.

Table A2. Recoveries and R.S.D. of 22 antibiotics spiked into fish at three levels.

Compound	Background µg/kg	Fortification µg/kg	Average Recovery Rate %	RSD $n = 3$ %	Compound	Background µg/kg	Fortification µg/kg	Average Recovery Rate %	RSD $n = 3$ %
Pipemidic acid	ND	2.0 20.0 200.0	74.2 78.7 77.8	8.85 6.95 5.86	Lomefloxacin	ND	2.0 20.0 200.0	82.9 89.2 86.3	9.85 9.07 8.41
Enoxacin	ND	2.0 20.0 200.0	73.9 79.1 80.4	9.12 7.63 7.45	Enrofloxacin	ND	2.0 20.0 200.0	90.2 98.5 95.4	5.87 6.15 5.98
Minocycline	ND	2.0 20.0 200.0	73.8 82.6 75.9	9.92 8.43 8.31	Doxycycline	ND	2.0 20.0 200.0	86.2 88.4 84.9	12.4 10.7 11.3
Norfloxacin	ND	2.0 20.0 200.0	80.5 78.3 85.4	9.01 7.65 6.14	Demeclocycline	ND	2.0 20.0 200.0	79.3 88.4 89.0	11.9 9.90 10.2
Ofloxacin	ND	2.0 20.0 200.0	75.2 82.7 86.4	10.2 8.72 6.96	Sarafloxacin	ND	2.0 20.0 200.0	78.9 88.9 78.5	8.68 6.12 5.99
Pefloxacin	ND	2.0 20.0 200.0	89.1 92.4 88.5	9.56 9.17 7.88	Difloxacin	ND	2.0 20.0 200.0	83.7 84.0 85.9	8.69 6.81 7.04
Tetracycline	ND	2.0 20.0 200.0	78.6 83.0 82.7	12.1 10.7 9.57	Chlortetracycline	ND	2.0 20.0 200.0	78.4 88.1 82.7	11.7 8.94 9.10
Ciprofloxacin	ND	2.0 20.0 200.0	82.2 78.6 89.5	9.07 8.19 7.54	Cinoxacin	ND	2.0 20.0 200.0	75.5 80.2 83.4	9.94 8.71 8.07
Methacycline	ND	2.0 20.0 200.0	77.3 85.1 86.4	10.8 9.76 8.33	Oxolinic acid	ND	2.0 20.0 200.0	77.3 83.9 79.8	10.4 9.29 9.38
Oxytetracycline	ND	2.0 20.0 200.0	74.8 76.1 75.5	11.8 10.1 9.78	Nalidixic acid	ND	2.0 20.0 200.0	79.2 84.6 81.7	8.93 7.10 5.81
Danofloxacin	ND	2.0 20.0 200.0	75.7 82.3 77.1	9.64 8.78 9.15	Flumequine	ND	2.0 20.0 200.0	87.4 92.1 88.3	8.94 5.89 6.37

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