Determination of Nitrofuran Metabolites in Fish by Ultraperformance Liquid Chromatography-Photodiode Array Detection with Thermostatic Ultrasound-Assisted Derivatization

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ABSTRACT: Nitrofuran (NF) is a class of broad-spectrum antibiotics that are used illegally in animal feeding. NF and its metabolites have proven to pose potential risk to human health. To address the current analytical needs to quantify low levels of NF metabolites in animal foods, a sensitive method was developed for simultaneous detection of four NF metabolites in fish products by an ultraperformance liquid chromatography-diode array detector (UPLC-DAD). With 2-nitrobenzaldehyde (2-NBA) as the derivatizing reagent, the metabolites were hydrolyzed and derivatized under the assistance of thermostatic ultrasound. Compared with the current detection methods, the time of the derivatization reaction has been shortened from 16 to 2 h. The relative coefficient of four NF metabolite derivatives reached more than 0.998, with excellent linear relationship. The limits of detection (LODs) and limits of quantification (LOQs) of six repeated determinations reached 0.25–0.33 and 0.80–1.10 μg/kg, respectively. For all four NF metabolites, the limit of detection of the method was below the minimum required performance limit (MRPL) of 1.0 μg/kg, which makes it compatible with the EU requirements. The recoveries ranging from 89.8 to 101.9% with relative standard deviation below 6.5% were obtained for all of the NF metabolites. What’s more, this method was successfully applied for the determination of four NF metabolites in fish products. As a promising approach, this method could also be extended for the quantitation of NF metabolites in aquaculture and poultry products.

1. INTRODUCTION

Nitrofuran (NF), mainly including furazolidone (FZD), nitrofurazone (NFZ), nitrofurantoin (NFT), and furaltadone (FTD),1 are a class of synthetic broad-spectrum antibacterial drugs with a 5-nitro structure that were commonly used in veterinary drugs for the treatment of protozoan and gastrointestinal infections.2,3 These drugs metabolize rapidly within a few hours after ingestion, and the residual metabolites could remain in the body for weeks, possibly even months, as protein-bound compounds.4–6 These NF metabolite compounds have been proven to pose potential risk to human health because of their carcinogenic, teratogenic, and mutagenic potency.5,7 Based on food safety needs, the use of NF drugs was banned by the European Union during the cultivation of animal products.8,9 Since then, many countries have followed the suit, such as the United States, China, and Japan.10–12 However, because of the low price and significant efficacy, NF drugs are still allowed or illegally used as veterinary drugs in some developing countries.13,14 At present, the minimum performance limit (MRPL) of these residual compounds in meat products stipulated by the EU is 1.0 μg/kg.2,15 In this context, it is essential to develop a fast and sensitive detection method for NF metabolite compounds for solving food safety problems.

At present, the analytical strategy for the quantitation of NF is based on the determination of four stable and persistent metabolites that can be released from proteins-bound compounds under mildly acidic conditions and then derivatized in situ with a derivatization reagent.16,17 These persistent metabolites include 3-amino-2-oxazolidinone (AOZ, a metabolite of FZD), 3-aminomorpholinomethyl-2-oxazolidinone (AMOZ, a metabolite of FTD), 1-aminohydantoin (AHD, a metabolite of NFT), and semicarbazide (SEM, a metabolite of NFZ).18 In general, acid hydrolysis and derivatization reactions are carried out in a 37 °C water bath.
for 16 h, but it is impossible to get results quickly.\textsuperscript{15,19,20} Compared with water bath derivatization, ultrasonic-assisted derivatization has a better relative response ratio, which can significantly shorten the reaction time and reduce the complexity of the target compound analysis.\textsuperscript{21,22} Ultrasonic energy can accelerate the derivatization reaction because ultrasonic energy can increase the number of effective collisions between the reactants and make the derivatization reaction more efficient.\textsuperscript{23−25} Therefore, the reaction time can be reduced from several hours to a few minutes with the assistance of ultrasound with heating. To our knowledge, ultrasound-assisted derivatization has many advantages, but it cannot maintain a thermostatic system during the reaction. The process of derivatization of NF metabolites requires a constant temperature to obtain stable results, which has led to rare reports on the use of assistance of ultrasound for derivatization of the NF metabolites. To minimize the effects of temperature changes during ultrasound, we planned to use constant temperature ultrasound-assisted derivatization of NF metabolites to shorten the reaction time.

Many analytical methods have been developed for the detection of NF metabolites in many matrices, such as shrimp,\textsuperscript{26} animal feed,\textsuperscript{27} meat,\textsuperscript{14} seafood,\textsuperscript{28} milk,\textsuperscript{29} honey,\textsuperscript{30,31} and so on.\textsuperscript{13,32} These analytical methods include liquid chromatography-tandem mass spectrometry (LC-MS),\textsuperscript{33} high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS),\textsuperscript{30,34} ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS),\textsuperscript{35,36} enzyme-linked immunosorbent assay (ELISA),\textsuperscript{37} etc.\textsuperscript{38,39} Although many methods have been developed, they still cannot meet the detection requirements because of the extraordinarily low detection limit requirements of NF metabolites (MRPL = 1.0 μg/kg).\textsuperscript{40} HPLC-MS/MS has been widely used because it has high sensitivity and accuracy so it can be used for the detection of NF metabolites.\textsuperscript{41} However, the analysis method is limited due to the expensive equipment, professional operation, and relatively high cost.\textsuperscript{18,32}

Because of this, it still cannot be promoted and used in some economically affected developing countries.\textsuperscript{42} In this case, it is an important and urgent need to develop a cheap and simple analytical method for the detection of NF metabolites in animal foods. UPLC with a diode array detector (UPLC-DAD) is relatively inexpensive and convenient compared with other well-established coupled detection techniques conventionally used.\textsuperscript{35,43} The detection of NF metabolites by UPLC-DAD has rarely been reported, to the best of our knowledge. The reason may be that it is difficult to obtain an ideal detection limit by DAD detection compared to conventional methods.\textsuperscript{40} To make the experimental process simple, fast, and the detection can meet the MRPL requirements for NF metabolites in fish products, a new method combining thermostat ultrasound-assisted derivatization technology with UPLC-DAD is established.

In this work, a sensitive and fast UPLC-DAD analysis method was developed for the simultaneous determination of NF metabolites (AMOZ, SEM, AHD, and AOZ) in fish samples. In addition, 2-nitrobenzaldehyde (2-NBA) and NF metabolites in the samples were rapidly derived by thermostat ultrasound-assisted derivatization technology. More importantly, it is more practical to optimize the experimental conditions using the prepared positive fish samples. This method could be successfully applied to the simultaneous quantification of four NF metabolites in fish products. The establishment of this method guarantees the safety and export of food items in developing countries and regions.

2. RESULTS AND DISCUSSION

Positive fish samples for experiments can better reflect the actual process of acid hydrolysis of NF metabolites in proteins and derivatization. So, the positive fish samples were used to discuss derivatization conditions and the effect of organic reagent cleaning during sample pretreatment.
2.1. Optimization of the Derivatization Conditions. To ensure optimal NF derivatization efficiency in ultrasound-assisted derivatization and experimental repeatability, the temperature and time of ultrasound-assisted derivatization were chosen at an ultrasonic frequency of 45 kHz and a water level of 9 cm. The requirement of temperature for derivatization in different literature reports was referenced for better comparison. Excessive temperature may cause decomposition of the product, so 37, 50, and 60 °C were selected as the screening temperatures for ultrasound-assisted derivatization. To avoid erroneous conclusions about the interaction between the derivatization temperature and time, we tested different temperatures and simultaneously screened the derivatization time. As shown in Figure 1, based on the ultrasound-assisted derivatization under the same conditions, the derivatization temperature was determined to be 60 °C by comparing the peak intensities of the NF metabolites derivatives at the three derivatization temperatures. Furthermore, we examined the derivatization time in the range of 0–360 min at 60 °C. It can be seen that the peak intensity of the derivatives of NF metabolites increased with increasing derivatization time and reached a maximum at 180 min. When the reaction time exceeded 120 min, the peak intensity of the NF metabolite derivative almost remained invariable and approached a constant value. Thus, 120 min had been used as the reaction time in our experiments. From a time saving perspective, the derivatization time is set to 120 min.

2.2. Effects of Organic Reagent Cleaning. It was found that the use of organic reagents to clean the sample can significantly reduce impurity peaks in the chromatogram during sample preparation. The influence of organic reagent cleaning on the peak intensity of the target was further explored. Both no washing and reagent washing treatments were performed on existing positive samples. As shown in Figure 2, washing with 20 mL of different organic reagents would significantly reduce the appearance of impurity peaks but would decrease NF metabolites to varying degrees. Even in the absence of acidic conditions, washing positive samples with organic reagents can lead to the loss of NF metabolites. It can be seen from the experimental results that after performing the same experimental steps on the unwashed sample during the pretreatment process, the impurity peaks do not interfere with the peaks of the NF derivatives. Therefore, organic reagents will not be used to wash the tissue during sample pretreatment.

Although the same amount of four NF veterinary drugs was fed when preparing a positive fish sample, the peak intensities of the four NF metabolite derivatives in the chromatogram obtained when using them for experiments were different. The reason may be the different abilities of the four drugs to be absorbed by the fish. It can be seen from the experiment that the thermostatic ultrasound-assisted method can not only accelerate the acid hydrolysis of NF metabolites in the sample but also enable the derivatization reaction to proceed quickly. Compared with 16 h of the routine derivatization method, the derivatization process took 2 h by this method. The pretreatment step becomes faster and simpler because acid hydrolysis and derivatization are performed simultaneously in the thermostatic ultrasound-assisted derivatization system and organic reagents are not used to wash the samples during the experiment. It is very clear that our method significantly increases the throughput of large inspection projects.

3. METHOD VALIDATION

The developed method was verified through a series of experiments, including linearity, limits of detection (LODs), limits of quantitation (LOQs), recovery, accuracy, and precision under the optimized conditions.

Blank samples were fortified with all four NF metabolites (AMOZ, SEM, AHD, and AOZ) to obtain final tissue concentrations of 1, 2, 5, 10, and 20 μg/kg to verify linearity. The calibration curve was obtained by plotting the peak area against the theoretical concentration of NF metabolite derivatives. As can be seen from Table 1, the correlation coefficients of the four NF metabolite derivatives range from 0.9982 to 0.9993. The LODs and LOQs of the four NF metabolite derivatives were obtained by 3 and 10 times the signal-to-noise ratio (S/N), ranging from 0.25 to 0.33 and 0.80 to 1.10 μg/kg, respectively. It can be seen that the limit of detection of this method is lower than the MRPL of 1.0 μg/kg established by the EU.

The accuracy and relative standard deviation (RSD, %) of the method were obtained by evaluating six replicate analyses (the standard solution was prepared in six replicates, each injected once) of blank fish samples and blank fish samples fortified at 1, 5, and 10 μg/kg. After analysis and determination of the concentration of each sample, the recovery rates were calculated as follows:

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\text{recovery (\%)} = \frac{\text{observed concentration}}{\text{fortification level}} \times 100
\]

Table 2 summarizes the recovery and RSD values obtained at each concentration level. The average recovery of all NF metabolites ranges from 89.8 to 101.9%, and the relative standard deviation (RSD, %) does not exceed 6.5%.

4. REAL SAMPLE ANALYSIS

We selected four types of fish samples from the local market to verify the availability of the method, including Grass carp, Crucian carp, Tilapia, and Carp. After testing the content of NF metabolites in real fish samples, fortified tests (3 μg/kg) were performed on the fish samples to further verify the
accuracy of the method. The test results are shown in Table 3. As can be seen from Table 3, AOZ and SEM were found in the Grass carp and Crucian carp samples, with the contents of 2.53 and 5.26 μg/kg, respectively. The presence of AOZ and SEM may result from either illegal use of the banned veterinary drugs or the use of polluted raw materials as mentioned above. All carps and tilapias are negative samples, which can be explained by the fact that they come from the same legal pond. It is not difficult to see that excellent fortified recovery was obtained in the 3 μg/kg fortified experiments on real fish samples. Moreover, it can be seen from the experimental results that this analysis method can be well applied to the detection of real samples.

5. CONCLUSIONS

A quick and sensitive analytical method was developed, optimized, and validated for the simultaneous determination of four NF metabolites in fish samples by UPLC-DAD. When
the sample was acidified and derivatized using ultrasound-assisted technology, under acidic conditions at 60 °C, acid hydrolysis and derivatization reactions proceeded quickly and completed within 2 h. During the pretreatment process, a one-pot method was used for acid hydrolysis and derivatization, which simplifies the pretreatment steps, and constant-temperature ultrasound assisted derivatization is used, which shortens the derivatization time. For the determination of NF metabolites in fish, the UPLC-DAD detection method is cheaper and simpler than the LC-MS method, so this analysis method can be better popularized and used in developing countries and regions. This method can easily and accurately detect the abuse of NF veterinary drugs, thereby ensuring the food safety of local residents and normal export trade.

6. MATERIALS AND METHODS

6.1. Reagents and Standards. The analytical standards AOZ, AMOZ, SEM, and AHD (purities >99.0%) and their 2-NBA derivatives (NP-AOZ, NP-AMOZ, NP-SEM, and NP-AHD), as well as some HPLC-grade reagents (methanol, hexane, ethyl acetate, acetonitrile, and dimethyl sulfoxide (DMSO)), were purchased from Sigma-Aldrich (Steinheim, Germany). High-purity water purified with a Milli-Q water purification system (Millipore, Bedford, MA) was used throughout the experiment. 2-NBA was purchased from Aladdin (Shanghai, China). The fish samples were purchased from the local supermarket in Urumqi (Xinjiang, China). NF parent drugs FTD, NFZ, NFT, and FZD were purchased from Aladdin (Shanghai, China). Molecular structures of the NF parent drugs, their metabolites, and 2-NBA derivatives are shown in Figure 3.

The derivatization reagent solution (25 mmol/mL) was freshly prepared as follows: 37.8 mg of 2-NBA was dissolved in 10 mL of DMSO.

6.2. Preparation of Positive Fish Tissue. For adapting to the actual detection needs, a batch of positive fish samples was selected to explore the temperature and time required for simultaneous acid hydrolysis and derivatization reactions in the ultrasound-assisted derivatization process.

The experimental samples were ten blank Crucian carps that were 15–20 cm long and were not fed for 2 days. The prepared feed contains FTD, NFZ, NFT, and FZD, all of which have a mass fraction of 200 mg/kg. The feed was fed twice in total and once every 6 h, and the quantity of the feed was fixed at 1% body weight of the fish samples. The fish samples were collected 2 days after feeding was stopped. After homogenization, all collected fish tissue samples were stored at −20 °C until analysis.

6.3. Extraction Procedure for Fish Tissue Samples. Sample preparation methods used in the previously published literature were referred and had been improved. Each test sample consisted of homogenized fish muscle tissue without the head and scales. Five grams (5 g ± 0.05) of the sample was weighed and added into a 50 mL polypropylene tube. Then, 10 mL of 0.2 mol/L hydrochloric acid solution and 200 μL of 25 mmol/L fresh 2-NBA were added to the sample. After thoroughly vortexing for 2 min, it was derivatized in a 60 °C ultrasonic cleaner (45 kHz, the height of water surface was 9 cm) for 2 h. Subsequently, after cooling the sample to room temperature, 1 mL of 0.4 mol/L K2HPO4 and 2 mol/L sodium hydroxide were added to ensure neutrality. Subsequently, 10 mL of ethyl acetate was added to the sample twice for extraction, and after thorough vortex extraction, the organic phase was transferred to a 15 mL centrifuge tube. This extract was evaporated to dryness at 45 °C in a heat block chamber under a mild flow of nitrogen gas. Thereafter, the dissolved residue was centrifuged at 13 000 rpm and 4 °C for 10 min and the intermediate clear liquid was collected. The clear liquid was extracted three times with 3 mL of acetonitrile saturated n-hexane (C6H14) to remove excess 2-NBA. The final solution was filtered through a 0.22 μm nylon filter (NY) directly into a UPLC-DAD system for analysis. To avoid light-sensitive decomposition of the sample, light was avoided as much as possible during the entire pretreatment process.

6.4. Instruments and Apparatus. Chromatographic analyses were performed in an UltiMate 3000 ultraperformance liquid chromatography system with an autosampler (model WPS-3000) and a photodiode array detector (model DAD-3000(RS)) from Dionex (CA). Chromatography separations were carried out using a Syncronis C18 column (100 × 2.1 mm2, 1.7 μm particle size) from Dionex (CA). The ChemStation system with Chameleon 7 software was used to control devices and analyze the output data. The column oven (model TCC-3000RS) and the autosampler (model WPS-3000) were maintained at 35 and 15 °C during operation, respectively.

The ultrasonic bath was used was a KQ-300GVGDV from Kunshan Ultrasound Instrument Co., Ltd ( Jiangsu, China) with an ultrasonic frequency of 45 kHz and an effective power of 300 W.

6.5. UPLC-DAD Analysis. Mobile phase A (acetonitrile/water/glacial acetic acid, 95:5:0.5, v/v/v) and phase B (water/glacial acetic acid, 100:0.1, v/v) in a gradient that started with 10% of A and 90% of B were used. Gradients of 0–10.5 min from 90 to 65% B, 10.5–11 min from 65 to 10% B, and 11–13 min at 10% B were used. Subsequently, equilibration was carried out with the initial mobile phase for 3 min before next injection. The flow rate was 0.3 mL/min, and the column was operated at 35 °C. The injection volume required for the experimental analysis was 20 μL. A wavelength of 275 nm was used in the analysis.

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Notes

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