International Food Research Journal 28(3): 467 - 478 (June 2021)
Journal homepage: *http://www.ifrj.upm.edu.my*

# Analysis of metabolites of nitrofuran antibiotics in animal-derived food by UPLC-MS/MS

[1,2,3]Lv, Z., [1,4]Luo, Z., [1]Lu, J., [1]Xu, Z., [1,2,3]*Zhang, W. and [3]Chen, A.

[1]*School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang 621010, China*
[2]*Key Lab of Meat Processing of Sichuan Province, Chengdu University, Chengdu 610106, China*
[3]*Key Laboratory of Agrifood Safety and Quality, Ministry of Agriculture of China, Beijing 100081, China*
[4]*Sichuan Huasheng Agricultural Ltd, Mianzhu 618200, China*

**Article history**

*Received: 7 February 2020*
*Received in revised form: 21 October 2020*
*Accepted: 15 November 2020*

**Keywords**

*antibiotics, nitrofuran (NF), ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), animal-derived*

**Abstract**

An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was used for the simultaneous detection of four metabolites of nitrofuran (NF) antibiotics in eight animal-derived foods, namely porcine muscle, chicken, fish, duck, pork liver, crab, shrimp, and egg. Briefly, the sample was first acid-hydrolysed, derivatised, and extracted by ethyl acetate. The extract was then analysed by UPLC-MS/MS. Later, sample pre-treatment and UPLC-MS/MS conditions were optimised. The results showed that the method had good linearity over the range of 0.5~50 μg•kg$^{-1}$. The average recoveries were 80.3~119.0%, and the relative standard deviations (RSDs) were < 8.1 and < 10.9% for intra-assay and inter-assay precision, respectively. The limits of detection (LODs) for 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 5-morpholino-3-amino-2-oxazolidone (AMOZ), and 1-amino-hydantoin (AHD) were 0.1, 0.2, 0.2, and 0.4~0.5 μg•kg$^{-1}$, respectively, and the limits of quantification (LOQs) for AOZ, SEM, AMOZ, and AHD were 0.4, 0.5, 0.5, and 0.8~1.0 μg•kg$^{-1}$, respectively. The proposed method was used to detect NF residues in 100 animal-derived food samples and quality control samples. The results were close to those detected by the China national standard method GB/T 20752-2006, and the results of quality control samples were within the detectable ranges. The results can provide a theoretical basis for the detection of NF residues in different kinds of animal-derived foods.

© All Rights Reserved

## Introduction

Antibiotics were originally used in humans or animals to cure microbial infectious diseases or as food additives to improve animal production efficiency (Yamaguchi *et al.,* 2015; Wang *et al.,* 2017). Nitrofurans (NFs) are antibacterial agents that are widely used for aquaculture, livestock, and poultry breeding. These antibiotics are highly effective in suppressing and killing Gram-positive and Gram-negative bacteria, some fungi, and protozoa, and they are often used to prevent and treat diseases caused by *Salmonella* and *Escherichia coli* in animals (Mitema *et al.,* 2001; Chu *et al.,* 2008; Veach *et al.,* 2015). However, NFs have potential teratogenic, carcinogenic, and mutagenic effects. Inappropriate or abusive use of NFs would result in residues of veterinary drugs in animals, and could eventually harm human health (Barton, 2000; Friedman, 2015). NFs are not allowed to be used in any food-producing animals by the European Regulations (EC1442/95) and United States (Veach *et al.,* 2018). Currently, NFs are regulated at a target level of 1 μg kg$^{-1}$ in the United States, European Union (EU), and Canada (Aldeek *et al.,* 2017). Likewise, NFs have been explicitly prohibited from being used in all animal-derived food in China since 2002. In China, NFs are regulated at a target level of 0.5 μg kg$^{-1}$ (AQSIQ, 2007).

Four pharmacologically active compounds that have achieved significant commercial success and attracted research interest in recent decades are furazolidone (FZD), furaltadone (FTD), nitrofurantoin (NFT), and nitrofurazone (NFZ) (Radovnikovic *et al.,* 2011). NFs are extremely unstable if ingested. The metabolites of NFs include semicarbazide (SEM), 1-aminotetrahydrolurea (AHD), 3-amino-2-azole-alkyl ketone (AOZ), and 5-methylmorpholine 3-amino-2-azole-alkyl ketone (AMOZ) (El-Demerdash *et al.,* 2015).

At present, the detection methods for NFs include high-performance liquid chromatography (HPLC) (Yu *et al.,* 2013), liquid chromatography-mass spectrometry (LC-MS/MS) (Yu *et al.,* 2013; El-Demerdash *et al.,* 2015; Veach *et al.,* 2015),

*Corresponding author.
Email: zhangwayne1988@163.com
DOI: https://doi.org/10.47836/ifrj.28.3.06

enzyme-linked immunosorbent assays (ELISAs) (Liu *et al.,* 2013; Jester *et al.,* 2014), fluoroimmune assay (FIAs) (Le and Yu, 2015), and immunochromatographic assays (ICAs) (Wang *et al.,* 2018). Chromatography and mass spectrometry are still the main evaluation techniques. LC-MS/MS has the advantages of relatively high sensitivity, good selectivity and specificity, and can provide structural information of compounds in the tested sample. LC-MS/MS has unique advantages in the analysis of antibiotics in food for both screening and quantitative methods (Shendy *et al.,* 2016; Chen *et al.,* 2016; Guidi *et al.,* 2017; Zhang *et al.,* 2017; Song *et al.,* 2018). However, such instrumental analytical methods are usually time-consuming and costly (Moragues and Igualada, 2009). The methods used for extraction, clean up, and instrumental analysis are different for various matrices (El-Demerdash *et al.,* 2015; Kim *et al.,* 2015; Fernando *et al.,* 2017; Park *et al.,* 2017). Thus, establishing an accurate and rapid detection method for NF residues in different matrices is essential.

Therefore, eight animal-derived foods namely porcine muscle, chicken, fish, duck, pork liver, crab, shrimp, and egg were chosen as different matrices in the present work. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used as the detection method. The objective of the present work was to optimise the UPLC-MS/MS method for the simultaneous determination of four metabolites of NFs in eight animal-derived foods. The pre-treatment procedure and UPLC-MS/MS parameters were optimised. Finally, the proposed method was applied in the analysis of NFs in 100 randomly purchased animal-derived samples from local market and control chicken samples. The results obtained by the proposed method were also compared with standard methods in China (AQSIQ, 2006; 2007).

## Materials and methods

*Materials and reagents*

Fresh porcine muscle, chicken, fish, duck, pork liver, crab, shrimp, and egg were purchased from a local farm. The skin, bone, and shell of samples were removed once the samples were transported to the laboratory. The remaining edible parts were crushed and mixed by a tissue disintegrator, and stored in a polyethylene bottle at -18°C prior to use. The prepared samples were thawed and weighed before testing.

NF metabolite standards including 3-amino-2-oxazolidone (AOZ), 5-morpholine methyl-3-amino-2-oxazolidone (AMOZ), 1-amino-ethylurea (AHD), and semicarbazide (SEM) were at a concentration of 100 mg $L^{-1}$ in methanol. Internal standards including AOZ-$D_4$, AMOZ-$D_5$, AHD-$^{13}C_3$, and SEM-$^{13}C^{15}N_2$ were at a concentration of 50 mg $L^{-1}$ in methanol. These standards were purchased from the Agro-Environmental Protection Institute, Ministry of Agriculture (Tianjin, China). Control chicken samples containing AOZ (calibration value of 7.60 μg $kg^{-1}$) and AMOZ (calibration value of 7.75 μg $kg^{-1}$) were purchased from the Analysis Capability Assessment System of CAIQ (Beijing, China). Methanol, acetonitrile (ACN), *n*-hexane, and ethyl acetate were of analytical reagent grade, and purchased from Merck (Darmstadt, Germany). Formic acid (85.0%) and 2-nitrobenzaldehyde (> 99%) were purchased from Puzhen (Shanghai, China). Ammonium acetate (> 98.0%) was purchased from Bailingwei (Beijing, China). Concentrated hydrochloric acid, sodium hydroxide, and tripotassium phosphate trihydrate were of analytical reagent grade, and purchased from Kelong (Chengdu, China). Reagent water was prepared by a UV-R ultrapure water system.

*Standard preparation*

NF analytical standards and internal standards were prepared at concentrations of 100 and 50 mg $L^{-1}$ in methanol, respectively. The stock solutions were prepared at a concentration of 1 mg $L^{-1}$ from standard solutions at concentrations of 100 and 50 mg $L^{-1}$. All stock solutions in the work were stored at -20°C. Mixed intermediate analytical and internal standards were prepared daily at a concentration of 0.1 mg $L^{-1}$ from the stock solutions.

The derivative reagent (0.1 M) was prepared by dissolving 1.5 g of 2-nitrobenzaldehyde in 100 mL of methanol. The derivative reagent was prepared fresh daily, and stored in the dark. The potassium phosphate solution (0.3 M) was prepared by adding 79.893 g of tripotassium phosphate trihydrate into 1 L of $H_2O$.

*Instruments and equipment*

For liquid chromatography-tandem mass spectrometry, a 1290 ultrahigh-performance liquid chromatograph (Agilent, Palo Alto, CA, USA) was utilised. A Q-TRAP 4500 triple quadrupole linear ion trap mass spectrometer equipped with an electrospray ionisation source (ESI) and MultiQuantTM software (AB SCIEX, USA) was also utilised. A UV-R ultrapure water system (Millipore, France) was used. A TGL-16A table high-speed freezing centrifuge (Changsha Pingfan Instrument, China), a VG3 S025 vortex oscillator (IKA, Germany), an MS205DU electronic balance

(Mettler-Toledo, Swiss), an S220 pH meter (Mettler-Toledo, Swiss), and pipettes with ranges of 10~100 µL, 100~1000 µL, and 1~10 mL (Eppendorf, Germany) were used. A T25 high-speed tissue homogeniser (IKA, Germany), a ZWYR-240 thermostat culture oscillator (Shanghai Zhicheng, China), an MTN-5800A nitrogen blowing concentrator (Tianjin Automatic Science Instrument, China), and a VP6122050 suction filter device (Cole-Parmer, USA) were also used.

*Sample pre-treatment*

Two grams of prepared sample was weighed into a 50 mL polypropylene centrifuge tube, and 8 mL of 0.2 M hydrochloric acid was added. After continuous vortexing for 1 min, the mixed internal standard solution was added to obtain a final volume concentration of 10 ng $mL^{-1}$. Then, 100 µL of 0.1 M 2-nitrobenzaldehyde was added. The sample was vortexed for 30 s, and incubated overnight at 37°C with gentle shaking for 16 h.

The sample was removed to cool to room temperature. Then, 1 mL of 0.3 M potassium phosphate solution was added, followed by 2 M sodium hydroxide solution to adjust the pH to 7.5 ± 0.5. Next, 8 mL of ethyl acetate was added. The sample was subsequently vortexed for 5 min and centrifuged at 10,000 rpm for 5 min. The upper ethyl acetate layer was collected, and the residue was extracted again by using 8 mL of ethyl acetate. The collected solutions were evaporated under a gentle stream of nitrogen to dry. The residue was redissolved using 1 mL of initial mobile phase (90% mobile phase A and 10% mobile phase B), and 1 mL of *n*-hexane saturated with ACN was added for liquid-liquid extraction. After the lipids were eliminated, the lower layer was filtered through a 0.22 µm polyethersulfone (PES) filter. The filtrate was collected for UPLC-MS/MS analysis. The whole procedure was conducted away from light.

*UPLC-MS/MS analysis*

Separation was carried out on an Agilent Eclipse Plus $C_{18}$ RRHD column (100 × 2.1 mm, 1.8 µ m) at 30°C. The injection volume was 10 µL, and the flow rate was 0.4 mL $min^{-1}$. Mobile phase A was a 0.1% formic acid solution containing 0.0005 M ammonium acetate, while mobile phase B was ACN. The gradient elution program was applied as follows: 0~6 min, 10~75% B; 6~6.5 min, 75~95% B; 6.5~9.5 min, 95% B; 9.5~10 min, 95~10% B; and 10~13 min, 10% B.

For MS/MS detection, the ESI source was operated in positive ionisation mode (ESI+) under the following conditions: multiple reaction monitoring (MRM) mode; ion spray voltage (IS), 5,500 V; curtain gas (CUR), 35 psi; collision gas (CAD), medium; and ion source temperature (TEM), 550°C. The MS/MS parameters for the analysis are shown in Table 1.

*Method validation*

All the samples were subjected to strict quality control procedures. The concentrations of target compounds were calculated based on a matrix-matched calibration method. First, 0.01, 0.05, 0.1, 0.5, and 1 mL of 0.1 mg $L^{-1}$ mixed intermediate standards of target analytes were spiked into 2 g of blank sample matrices in 50 mL of polypropylene centrifuge tubes. The concentration of calibration standards against the matrix weight was 0.5, 2.5, 5, 25, and 50 µg $kg^{-1}$. Then, 0.1 mL of 0.1 mg $L^{-1}$ mixed intermediate internal standards was spiked into the samples prior to UPLC-MS/MS analysis to eliminate fluctuations in the instrument. Quantitation for each analyte was performed by calculating the

Table 1. MS/MS parameters for the analysis of four nitrophenyl (NP) derivatives of nitrofuran (NF) metabolites and their corresponding internal standards.

| Compound | Retention time ($t_R$)/min | Precursor ion (*m*/*z*) | Product ions (*m*/*z*) | Declustering potential / V | Collision energy / V |
|---|---|---|---|---|---|
| NPAOZ | 4.49 | 236.0 | 236.0 > 134.0*, 236.0 > 104.0 | 65, 65 | 17, 27 |
| NPAOZ-$D_4$ | 4.47 | 240.0 | 134.0 | 65 | 17 |
| NPSEM | 3.98 | 209.0 | 209.0 > 166.0*, 209.0 > 191.9 | 50, 50 | 13, 15 |
| NPSEM-$^{13}C^{15}N_2$ | 3.98 | 212.0 | 168.1 | 55 | 14 |
| NPAMOZ | 3.02 | 335.2 | 335.2 > 291.1*, 335.2 > 262.0 | 65, 65 | 16, 23 |
| NPAMOZ-$D_5$ | 3.00 | 340.4 | 296.0 | 65 | 16 |
| NPAHD | 4.10 | 249.0 | 249.0 > 133.9*, 249.0 > 178.0, 249.0 > 103.9 | 65, 65, 65 | 16, 20, 27 |
| NPAHD-$^{13}C_3$ | 4.10 | 252.0 | 134.0 | 65 | 17 |

* = Quantitative ion.

ratio of chromatographic area of quantitation ion to that of corresponding isotopically labelled internal standards: AOZ to AOZ-$D_4$, AMOZ to AMOZ-$D_5$, AHD to AHD-$^{13}C_3$, and SEM to SEM-$^{13}C^{15}N_2$. Each representative ratio was plotted against the corresponding matrix-matched calibration standard. The matrix-matched calibration curve was obtained by linear regression.

The proposed method was validated according to AQSIQ (2006; 2007). Moreover, controlled chicken samples were also used to validate the method. Signal-to-noise ratio calculations were used to determine the limit of quantification (LOQ, S/N = 10) and limit of detection (LOD, S/N = 3) of this method (Brombacher *et al.,* 2002; Meyer *et al.,* 2012; Zhao *et al.,* 2015). Recovery and relative standard deviations (RSDs) were calculated at three spiking levels (1, 2, and 5 μg $kg^{-1}$) by performing both intra-assay and inter-assay studies. The intra-assay study was performed at the same concentration level on a single day, analysing six replicates for each level, whereas the inter-assay variation was determined by six replicates on three different days.

For positive confirmation, the detection of all product ions was required. The associated chromatographic peak was required to exhibit a retention time within 2.5% of the retention time of the calibration standard. The maximum permitted tolerance for the relative abundance of ions was ± 20~50% according to the value of the relative abundance of ions.

## Results and discussion

*UPLC-MS/MS optimisation*

The mobile phase and chromatographic column were optimised in the present work. The separation effects and peak shapes of NF metabolites were investigated in two different length columns, including an Agilent Eclipse Plus $C_{18}$ RRHD column (2.1 × 100 mm, 1.8 μm) and an Agilent Eclipse SB $C_{18}$ RRHD column (2.1 × 50 mm, 1.8 μm). Moreover, methanol and ACN were implemented as mobile phases separately, and their separation effects were compared. The results showed that sharp, symmetrical chromatographic peaks, and a better separation effect of four NF metabolites could be obtained by using a $C_{18}$ column with a length of 100 mm, and by using ACN as the mobile phase when utilising the other column and mobile phase.

Adding a certain concentration of formic acid into the mobile phase could effectively improve the ionisation efficiency and chromatographic separation of the analytes in positive ionisation mode detection (Becker *et al.,* 2004; Veach *et al.,* 2015; 2018). Therefore, a 0.1% formic acid solution containing 0.0005 M ammonium acetate and ACN were used for gradient elution of NF metabolites. The ratio of ACN was 10% initially, and then increased linearly to 75% in the first 6 min to obtain chromatographic peaks with good shapes and high sensitivity. Due to the complex matrix of animal-derived samples, the ACN ratio was increased to 95% during 6.5~9.5 min to prevent the residues of unknown substances in the chromatographic column. The ACN ratio was reduced to 10% during 9.5~13 min to equilibrate the column for the next injection. The total run time for one sample was 13 min after optimisation. The total ion chromatogram of four kinds of nitrophenyl derivatives of NF metabolites is shown in Figure 1. As compared to China national standards (AQSIQ, 2006; 2007), the method optimised in the present work could save time, prolong the column life, and could be applied for large-scale sample analysis.

The NF metabolites were detected in positive mode. The declustering potential and collision energy were optimised to maximize the strength of selected characteristic fragment ions. According to EU Commission (657/2002/EC), a compound should have four identification points. The results showed that the response of two fragment ions at *m/z* 133.9 and 103.9 of AHD was high. However, a small chromatographic peak was found around the main chromatographic peak for porcine muscle and pork liver (shown in Figure 1). To further study the phenomenon, the same pre-treatment was conducted, but no sample matrix was used. The results showed that only one chromatographic peak was found for the fragment ions at *m/z* 133.9 and 103.9 for AHD. The fragment ions with strong and stable abundance were further screened. The fragment ion at *m/z* 178.0 showed only one chromatographic peak for AHD. Therefore, fragment ions at *m/z* 133.9, 103.9, and 178.0, and the quasi-molecular ion at *m/z* 249.0 were taken as identification points to ensure accurate qualitative and quantitative results.

*Optimisation of the extraction method*

According to China standards (AQSIQ, 2006; 2007), methanol and $H_2O$ were used for sample washing to remove impurities such as protein and lipid. However, few studies have focused on the selection of sample washing solutions. In the present work, sample washing solutions with different ratios of $H_2O$/methanol (v/v) and no sample washing were compared. The tested ratios of $H_2O$/methanol (v/v)

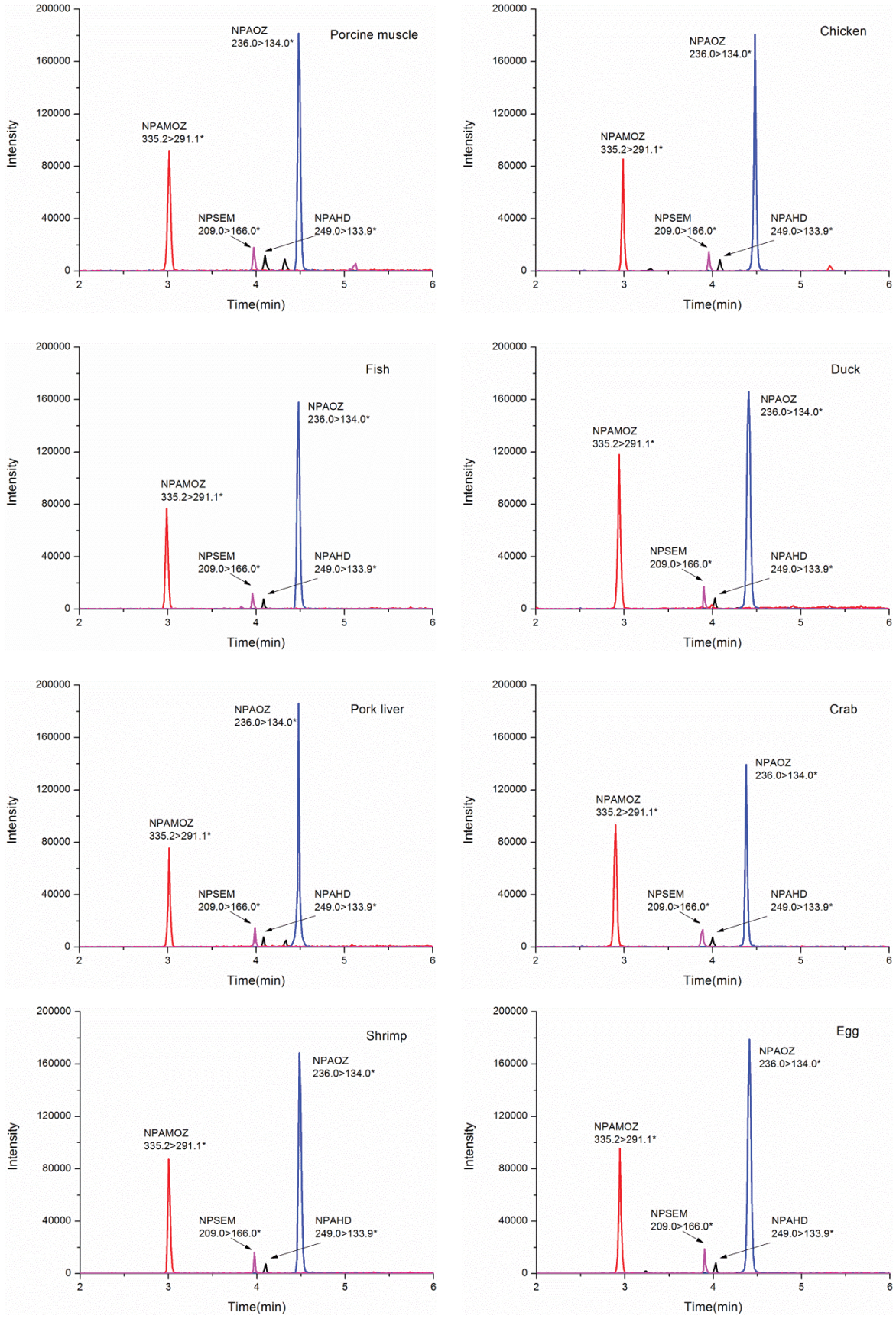


Figure 1. LC-MS/MS nitrofuran (NF) metabolite quantitation ion chromatograms of 0.5 μg $kg^{-1}$ extracted fortified control porcine muscle, chicken, fish, duck, pork liver, crab, shrimp, and egg, respectively. Four kinds of nitrophenyl (NP) derivatives of NF metabolites includes the nitrophenyl derivative of 3-amino-2-oxazolidinone (NPAOZ), nitrophenyl derivative of semicarbazide (NPSEM), nitrophenyl derivative of 5-morpholino-3-amino-2-oxazolidone (NPAMOZ), and nitrophenyl derivative of 1-amino-hydantoin (NPAHD).

were 1:1, 1:2, 1:4, 1:8, and 1:10, and no sample washing was used in the control group. Each test was conducted in six replicates. The results showed that there were no obvious differences in recovery between using and not using the sample washing solution. The recoveries were all between 80 and 120%. The aim of using a sample washing solution is to remove impurities such as proteins and lipids. The subsequent extraction steps in the sample pre-treatment could have removed the impurities as the sample washing solution did. Therefore, no sample washing solution was used in the present work, thus effectively saving time and costs.

Endogenous NF metabolites existed in the form of protein conjugates in the sample. NF metabolites were released by the hydrochloric acid hydrolysis method, and 2-nitrobenzaldehyde was added for derivatisation. The amine (-$NH_2$) of the NF metabolite reacts with the derivative keto aldehyde group (-CHO) due to the nucleophilic addition reaction under acidic conditions (Li *et al.,* 2008; Lu *et al.,* 2018). The mass-to-charge ratio (*m/z*) of the molecular ions of the four metabolite derivatives increased to more than 200 after derivatisation, which is suitable for mass spectrometric detection.

The pH of the solution for extraction has a great influence on compound recovery after hydrolysis and derivatisation (Zhang *et al.,* 2017; Øye *et al.,* 2019). Different pH values varying from 5 to 8.5 at an interval of 0.5 were investigated in the present work. The other operations were maintained as described previously. The recoveries of the four NF metabolites at different pH values are shown in Figure 2. The recovery was higher in the pH range of 7 - 8 than in the other ranges. In addition, the recoveries at a low pH (5~6.5) were lower than those at a high pH (8.5). This may be because the hydrochloride formed from the derivatives containing -N and -$NH_2$ under acidic conditions could not be extracted. Therefore, the pH value was adjusted to 7.5 ± 0.5 after hydrolysis and derivatisation to obtain optimised matrix calibration curves and recoveries.

The results also showed that the pH adjustment by directly adding sodium hydroxide solution was time-consuming. It was more efficient to add 1 mL of 0.3 M potassium phosphate solution to form a buffer system, and then add 2 M sodium hydroxide solution to adjust the pH. Moreover, the solution was fully vortexed, and the sample matrix was stirred with a glass rod so that the acid was fully released. Optimised matrix calibration curves and recoveries were obtained by adjusting the pH as described above.

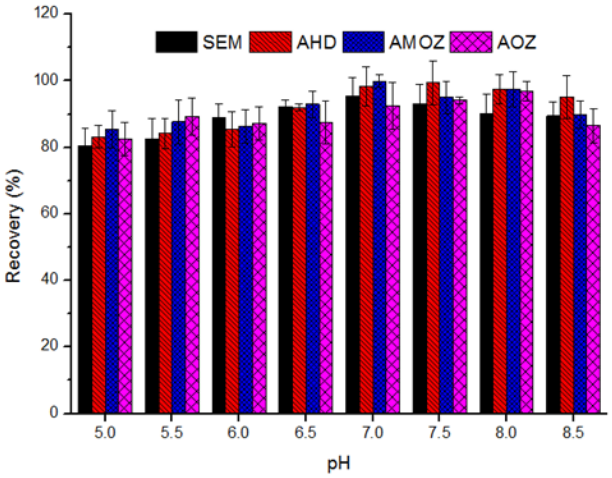


Figure 2. Recoveries of four kinds of nitrofuran (NF) metabolites (3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM), 5-morpholino-3-amino-2-oxazolidone (AMOZ), and 1-amino-hydantoin (AHD)) at different pH values.

Short extraction times to obtain high recovery rates can effectively save costs and time. The extraction was conducted using ethyl acetate once, twice and thrice. Each test was repeated six times, and 8 mL of ethyl acetate was used in each test. The results showed that the recoveries obtained by extracting the samples twice and thrice were higher than that when extracting only once. This was because not all NF metabolites were extracted in the first extraction. In addition, no obvious difference was found between the recoveries obtained by twice and thrice extractions. Therefore, the sample was extracted by 8 mL of ethyl acetate twice.

The solution for dissolving residues may produce solvation which would affect the retention effect and ionisation efficiency of some compounds in the chromatographic column, and then affect the chromatographic peak type and sensitivity. Three kinds of solutions were compared in the present work, namely, 0.1% formic acid, 0.1% formic acid containing 0.0005 M ammonium acetate, and the initial mobile phase for gradient elution (90% mobile phase A and 10% mobile phase B). The results showed that the response of SEM was low, and the peak of AHD bifurcated when 0.1% formic acid was used. When 0.1% formic acid containing 0.0005 M ammonium acetate was used instead of 0.1% formic acid, the response of SEM slightly improved. However, the AHD peak also bifurcated. The response of SEM was the highest when the initial mobile phase for gradient elution was used. Moreover, the AHD peak could be effectively separated from other impurity peaks.

Table 2. Matrix-matched calibration curve, regression coefficients ($R^2$), limit of detection (LOD, S/N = 3), and limit of quantification (LOQ, S/N = 10) of the four nitrofuran (NF) metabolites in eight sample matrices.

| Sample matrix | AOZ | | | | SEM | | | | AMOZ | | | | AHD | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Regression equation | $R^2$ | LOD (μg kg$^{-1}$) | LOQ (μg kg$^{-1}$) | Regression equation | $R^2$ | LOD (μg kg$^{-1}$) | LOQ (μg kg$^{-1}$) | Regression equation | $R^2$ | LOD (μg kg$^{-1}$) | LOQ (μg kg$^{-1}$) | Regression equation | $R^2$ | LOD (μg kg$^{-1}$) | LOQ (μg kg$^{-1}$) |
| Fish | y = 0.2930x + 0.0101 | 0.9997 | 0.1 | 0.4 | y = 0.4496x + 0.0098 | 0.9997 | 0.2 | 0.5 | y = 0.3966x + 0.0171 | 0.9994 | 0.2 | 0.5 | y = 0.2175x + 0.0073 | 0.9999 | 0.4 | 0.8 |
| Shrimp | y = 0.2179x + 0.0229 | 0.9994 | 0.1 | 0.4 | y = 0.4095x + 0.0153 | 0.9998 | 0.2 | 0.5 | y = 0.3049x + 0.0711 | 0.9957 | 0.2 | 0.5 | y = 0.1821x + 0.0207 | 0.9981 | 0.4 | 0.8 |
| Crab | y = 0.1283x + 0.0239 | 0.9955 | 0.1 | 0.4 | y = 0.3407x + 0.0177 | 0.9997 | 0.2 | 0.5 | y = 0.2721x + 0.0310 | 0.9986 | 0.2 | 0.5 | y = 0.1308x + 0.0130 | 0.9996 | 0.4 | 0.8 |
| Duck | y = 0.1317x + 0.0181 | 0.9934 | 0.1 | 0.4 | y = 0.4165x + 0.0130 | 0.9967 | 0.2 | 0.5 | y = 0.2381x + 0.0230 | 0.9927 | 0.2 | 0.5 | y = 0.1580x + 0.0104 | 0.9982 | 0.5 | 1.0 |
| Pork liver | y = 0.2656x + 0.0170 | 0.9945 | 0.1 | 0.4 | y = 0.6892x + 0.0118 | 0.9995 | 0.2 | 0.5 | y = 0.5581x + 0.0206 | 0.9978 | 0.2 | 0.5 | y = 0.2653x + 0.0101 | 0.9993 | 0.5 | 1.0 |
| Porcine muscle | y = 0.2309x + 0.0014 | 0.9964 | 0.1 | 0.4 | y = 0.4324x + 0.0028 | 0.9999 | 0.2 | 0.5 | y = 0.4177x- 0.0027 | 0.9992 | 0.2 | 0.5 | y = 0.2189x + 0.0098 | 0.9989 | 0.5 | 1.0 |
| Chicken | y = 0.2144x- 0.0079 | 0.9998 | 0.1 | 0.4 | y = 0.2964x + 0.0107 | 0.9994 | 0.2 | 0.5 | y = 0.2750x + 0.0110 | 0.9998 | 0.2 | 0.5 | y = 0.1522x + 0.0142 | 0.9971 | 0.5 | 1.0 |
| Egg | y = 0.2924x + 0.0013 | 0.9998 | 0.1 | 0.4 | y = 0.4531x + 0.0028 | 0.9995 | 0.2 | 0.5 | y = 0.4143x + 0.0057 | 0.9992 | 0.2 | 0.5 | y = 0.1842x + 0.0074 | 0.9998 | 0.4 | 0.8 |

Table 3. Recoveries and relative standard deviations (RSDs) of four nitrofuran (NF) metabolites spiked in eight blank sample matrices.

| Compound | Spiking level (μg kg$^{-1}$) | Recovery/% (RSD/%) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Intra-assay[a] | | | | | | | | Inter-assay[b] | | | | | | | |
| | | Fish | Shrimp | Crab | Duck | Pork liver | Porcine muscle | Chicken | Egg | Fish | Shrimp | Crab | Duck | Pork liver | Porcine muscle | Chicken | Egg |
| AOZ | 1 | 91.6 | 92.6 | 89.4 | 88.5 | 93.5 | 97.5 | 92.1 | 112.5 | 93.4 | 87.4 | 93.4 | 98.3 | 85.3 | 97.8 | 83.4 | 108.5 |
| | | (3.5) | (2.9) | (3.5) | (5.0) | (3.8) | (4.1) | (3.9) | (6.1) | (5.7) | (3.4) | (2.5) | (3.9) | (3.4) | (3.9) | (8.7) | (3.4) |
| | 2 | 96.5 | 95.1 | 87.2 | 93.4 | 92.9 | 98.6 | 90.9 | 108.4 | 93.0 | 95.9 | 94.3 | 83.7 | 98.5 | 90.8 | 93.4 | 102.8 |
| | | (2.6) | (6.5) | (5.4) | (2.8) | (4.0) | (5.2) | (5.1) | (5.7) | (7.6) | (4.0) | (5.0) | (4.3) | (6.5) | (5.4) | (5.6) | (2.3) |
| | 5 | 94.2 | 96.4 | 96.5 | 92.6 | 94.3 | 95.3 | 93.6 | 115.7 | 94.7 | 94.3 | 95.7 | 94.3 | 98.7 | 94.7 | 92.2 | 113.2 |
| | | (4.2) | (4.3) | (4.5) | (3.5) | (5.6) | (7.0) | (4.6) | (4.4) | (2.6) | (8.3) | (6.8) | (8.6) | (5.2) | (2.3) | (6.8) | (6.8) |
| AMOZ | 1 | 89.9 | 91.6 | 92.5 | 94.5 | 91.5 | 90.6 | 91.2 | 101.8 | 93.4 | 89.3 | 91.2 | 94.8 | 83.4 | 93.4 | 93.1 | 103.1 |
| | | (3.9) | (6.4) | (3.9) | (6.1) | (7.1) | (2.9) | (3.8) | (3.4) | (3.2) | (7.8) | (2.6) | (2.8) | (6.7) | (6.8) | (6.7) | (6.7) |
| | 2 | 92.8 | 104.4 | 93.7 | 106.2 | 94.8 | 102.7 | 92.0 | 108.1 | 95.8 | 103.7 | 93.7 | 103.2 | 102.1 | 95.3 | 100.4 | 103.8 |
| | | (5.6) | (4.8) | (5.1) | (5.4) | (5.9) | (5.2) | (7.0) | (4.0) | (5.2) | (4.6) | (6.7) | (2.3) | (6.8) | (2.6) | (8.6) | (3.5) |
| | 5 | 107.1 | 105.2 | 110.6 | 103.1 | 115.7 | 118.1 | 109.8 | 103.5 | 108.5 | 109.7 | 109.4 | 102.5 | 107.8 | 114.9 | 119.0 | 108.4 |
| | | (4.8) | (6.9) | (7.0) | (4.7) | (6.1) | (4.4) | (5.4) | (4.5) | (8.2) | (8.0) | (10.1) | (5.4) | (8.7) | (8.3) | (7.7) | (5.7) |
| AHD | 1 | 88.5 | 87.5 | 85.9 | 86.4 | 87.2 | 89.3 | 89.7 | 109.7 | 83.7 | 87.8 | 88.3 | 91.2 | 93.4 | 88.7 | 99.4 | 103.4 |
| | | (3.3) | (5.2) | (5.4) | (3.6) | (4.0) | (6.5) | (3.3) | (7.2) | (5.3) | (2.8) | (4.7) | (3.9) | (5.7) | (8.3) | (2.7) | (9.6) |
| | 2 | 89.1 | 89.1 | 84.6 | 89.1 | 89.7 | 91.7 | 90.9 | 114.6 | 96.3 | 83.4 | 85.2 | 83.2 | 90.8 | 88.3 | 94.3 | 112.4 |
| | | (5.1) | (4.7) | (3.3) | (7.0) | (5.5) | (7.4) | (6.4) | (4.7) | (5.3) | (6.2) | (2.7) | (6.0) | (2.1) | (9.4) | (8.9) | (9.8) |
| | 5 | 106.4 | 107.5 | 102.4 | 100.5 | 105.8 | 103.0 | 97.3 | 101.4 | 107.3 | 103.3 | 95.6 | 103.4 | 108.5 | 100.3 | 93.4 | 109.7 |
| | | (5.6) | (3.8) | (3.8) | (2.9) | (6.1) | (6.7) | (5.9) | (5.1) | (7.8) | (9.8) | (4.8) | (7.9) | (7.0) | (2.9) | (6.2) | (4.9) |
| SEM | 1 | 92.4 | 92.2 | 89.6 | 91.5 | 92.4 | 91.6 | 89.6 | 88.9 | 88.9 | 93.1 | 90.8 | 98.9 | 94.3 | 90.8 | 83.1 | 80.3 |
| | | (6.8) | (5.7) | (4.1) | (4.1) | (4.5) | (5.9) | (5.5) | (3.9) | (7.4) | (3.6) | (9.4) | (9.4) | (10.9) | (6.9) | (4.3) | (8.4) |
| | 2 | 91.6 | 93.5 | 91.1 | 96.4 | 96.1 | 92.7 | 93.4 | 89.7 | 93.2 | 98.9 | 89.3 | 88.9 | 93.9 | 93.9 | 93.8 | 93.9 |
| | | (4.5) | (8.1) | (6.2) | (6.4) | (6.2) | (2.8) | (4.6) | (5.8) | (7.3) | (9.7) | (3.4) | (2.5) | (7.8) | (4.3) | (6.7) | (7.4) |
| | 5 | 95.3 | 94.1 | 93.3 | 93.9 | 92.6 | 94.6 | 92.9 | 86.5 | 93.2 | 89.3 | 93.4 | 93.4 | 93.8 | 98.3 | 83.4 | 83.9 |
| | | (6.2) | (5.5) | (4.4) | (5.2) | (3.9) | (4.5) | (6.7) | (2.6) | (4.3) | (4.3) | (4.9) | (7.3) | (9.8) | (7.4) | (9.8) | (7.8) |

[a] = Intra-assay variation was determined by six replicates on a single day.

[b] = Inter-assay variation was determined by six replicates on three different days.

*Method validation*

Four isotopically labelled internal standards of NF metabolites were used to reduce the influence of operations on recovery. Matrix-matched calibration curves of four NF metabolites in eight sample matrices are summarised in Table 2. Linearity was assessed by statistical analysis of calibration curves (five points) obtained from spiking blank matrices with 0.5~50 μg $kg^{-1}$ target compounds. Overall, in the range of 0.5~50 μg $kg^{-1}$, all the target compounds had good linearity with regression coefficients ($R^2$) higher than 0.99 for matrix-matched calibration curves.

The LODs (S/N = 3) for AOZ, SEM, AMOZ, and AHD in the eight sample matrices were 0.1, 0.2, 0.2, and 0.4~0.5 μg $kg^{-1}$, respectively, and the LOQs (S/N = 10) for AOZ, SEM, AMOZ, and AHD were 0.4, 0.5, 0.5, and 0.8~1.0 μg $kg^{-1}$, respectively. The LODs (S/N = 3) and LOQs (S/N = 10) met the requirements of China and most international standards.

Three spiking levels of NF metabolites (1, 2, and 5 μg $kg^{-1}$) were added into eight blank sample matrices separately, and a mixed intermediate internal standard was added to achieve a final concentration of 10 ng $mL^{-1}$. The average recoveries and RSDs of each target compound at three concentrations are shown in Table 3. The intra-assay and inter-assay recoveries of NF metabolites at three spiking levels were 83.7~108.5%, 83.4~109.7%, 84.6~110.6%, 83.2~106.2%, 83.4~115.7%, 88.3~118.1%, 83.1~119.0%, and 80.3~115.7% for fish, shrimp, crab, duck, pork liver, porcine muscle, chicken, and egg, respectively. The precision of the method was evaluated by RSDs which were < 8.1 and < 10.9% for intra-assay and inter-assay precision, respectively. The results proved the good precision and stability of the proposed method.

To further verify the reliability of the method, the proposed method and the national standard method (AQSIQ, 2006) were used to detect NFs in 100 randomly purchased animal-derived samples and control chicken samples. As compared to the national standard method (AQSIQ, 2006), extraction without an Oasis HLB solid-phase extraction (SPE) cartridge or sample washing solution ($H_2O$/methanol) was used in our proposed method, which effectively saved a large amount of time and costs. The samples included five batches of freshwater shrimp, three batches of freshwater crab, four batches of seawater shrimp, four batches of seawater crab, two batches of crayfish, 23 batches of fish, 25 batches of pork, eight batches of chicken, four batches of duck, 20 batches of pork liver, and two batches of eggs.

All the samples were analysed by both the national standard method (AQSIQ, 2006) and our proposed method. All the samples met the required confirmation criteria for NF metabolites. The positive results obtained by the two methods were the same. Except for four samples that were positive, no NFs were detected in the other samples. Most positive samples were aquatic products, including freshwater shrimp and crabs. Positive detections were mainly of AOZ and SEM. This was because AOZ is commonly used for aquaculture environment disinfection, and can also be used to prevent intestinal infections in livestock and poultry. SEM is used for the prevention and treatment of aquatic animal diseases (Zhang *et al.,* 2016; Xing *et al.,* 2017). All the results of quality control samples were within their appropriate value ranges, thus demonstrating that the proposed method can be used for quantitative detection of NF metabolites.

## Conclusion

Simultaneous determination of four metabolites of NFs in eight animal-derived foods by UPLC-MS/MS was conducted in the present work. Sample pre-treatment and UPLC-MS/MS conditions were optimised. As compared to the methods by AQSIQ (2006; 2007), no sample washing solution was used in the present work. In addition, no Oasis HLB SPE cartridge was used in the present work, unlike the national standard method (AQSIQ, 2006). Therefore, the proposed method can effectively save costs and time.

The proposed method showed good linearity over the range of 0.5~50 μg/kg. The average recoveries were 80.3~119.0%, and RSDs were < 8.1 and < 10.9% for intra-assay and inter-assay precision, respectively. The LODs (S/N = 3) for AOZ, SEM, AMOZ, and AHD in the eight sample matrices were 0.1, 0.2, 0.2, and 0.4~0.5 μg/kg, respectively, and the LOQs (S/N = 10) for AOZ, SEM, AMOZ, and AHD were 0.4, 0.5, 0.5, and 0.8~1.0 μg/kg, respectively. Moreover, the proposed method was used to detect 100 randomly purchased animal-derived samples and control chicken samples. The results demonstrated that the proposed method can be used for the quantitative detection of NF metabolites.

## Acknowledgement

The authors gratefully acknowledge the Program of Key Laboratory of Meat Processing of Sichuan Province, Chengdu University (grant no.: 20-R-04 and 19-R-07), Program of Key Laboratory

of Agrifood Safety and Quality, MOA (grant no.: 2018-KF-04), and Longshan Academic Talent Research Supporting Program of Southwest University of Science and Technology (grant no.: 17LZX546).

## References

Aldeek, F., Hsieh, K. C., Ugochukwu, O. N., Gerard, G. and Hammack, W. 2017. Accurate quantitation and analysis of nitrofuran metabolites, chloramphenicol, and florfenicol in seafood by ultrahigh-performance liquid chromatography-tandem mass spectrometry: method validation and regulatory samples. Journal of Agricultural and Food Chemistry 66: 5018-5030.

Barton, M. D. 2000. Antibiotic use in animal feed and its impact on human health. Nutrition Research Reviews 13: 279-299.

Becker, M., Zittlau, E. and Petz, M. 2004. Residue analysis of 15 penicillins and cephalosporins in bovine muscle, kidney and milk by liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 520: 19-32.

Brombacher, S., Oehme, M. and Dye, C. 2002. Qualitative and quantitative analysis of carbonyl compounds in ambient air samples by use of an HPLC-$MS^n$ method. Analytical and Bioanalytical Chemistry 372: 622-629.

Chen, D., Yu, J., Tao, Y., Pan, Y., Xie, S., Huang, L., … and Yuan, Z. 2016. Qualitative screening of veterinary anti-microbial agents in tissues, milk, and eggs of food-producing animals using liquid chromatography coupled with tandem mass spectrometry. Journal of Chromatography B 1017-1018: 82-88.

Chu, P., Lopez, M. I., Abraham, A., El Said, K. R. and Plakas, S. M. 2008. Residue depletion of nitrofuran drugs and their tissue-bound metabolites in channel catfish (*Ictalurus punctatus*) after oral dosing. Journal of Agricultural and Food Chemistry 56: 8030-8034.

El-Demerdash, A., Song, F., Reel, R. K., Hillegas, J. and Smith, R. E. 2015. Simultaneous determination of nitrofuran metabolites and chloramphenicol in shrimp with a single extraction and LC-MS/MS analysis. Journal of AOAC International 98: 595-601.

Fernando, R., Munasinghe, D. M. S., Gunasena, A. R. C. and Abeynayake, P. 2017. Determination of nitrofuran metabolites in shrimp muscle by liquid chromatography-photo diode array detection. Food Control 72: 300-305.

Friedman, M. 2015. Antibiotic-resistant bacteria: prevalence in food and inactivation by food-compatible compounds and plant extracts. Journal of Agricultural and Food Chemistry 63: 3805-3822.

General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ). 2007. GB/T 21311-2007 - determination of residues of nitrofuran metabolites in foodstuffs of animal origin-HPLC-MS/MS method. China: AQSIQ.

General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ). 2006. GB/T 20752-2006 - method for the determination residues of the metabolites of nitrofuran in pork, beef, chicken, porcine liver and aquatic products-LC-MS-MS method. China: AQSIQ.

Guidi, L. R., Santos, F. A., Ribeiro, A. C. S. R., Fernandes, C., Silva, L. H. M. and Gloria, M. B. A. 2017. A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish. Talanta 163: 85-93.

Jester, E. L. E., Abraham, A., Wang, Y., El Said, K. R. and Plakas, S. M. 2014. Performance evaluation of commercial ELISA kits for screening of furazolidone and furaltadone residues in fish. Food Chemistry 145: 593-598.

Kim, J., Kim, B., Kim, D., Hyung, S. and Lee, C. H. 2015. An optimized method for the accurate determination of nitrofurans in chicken meat using isotope dilution-liquid chromatography/mass spectrometry. Journal of Food Composition and Analysis 40: 24-31.

Le, T. and Yu, H. 2015. Determination of 3-amino-2-oxazolidinone in animal tissue by an enzyme-linked immunosorbent assay and a time-resolved fluoroimmunoassay. Analytical Letters 48: 1275-1284.

Li, Y., Lin, Y., Jia, D., Yang, F., Yu, K., Liu, Z., … and Chunping, C. 2008. A research to establish a new quick determination of nitrofuran metabolite residues in aquatic products. Journal of Instrumental Analysis 27: 712-717.

Liu, Y., Jiang, W., Chen, Y., Xiao, Y., Shi, J., Qiao, Y., … and Wang, Q. 2013. A novel chemiluminescent ELISA for detecting furaltadone metabolite, 3-amino-5-morpholinomethyl-2-oxazolidone (AMOZ) in fish, egg, honey and shrimp samples. Journal of Immunological Methods 395: 29-36.

Lu, Y., Sun, X., Song, F., Wang, L., Song, M. and Hang, T. 2018. Impurity profiling of varenicline tartrate by LC-QTOF mass spectrometric

techniques during drug development. Journal of Pharmaceutical and Biomedical Analysis 155: 306-313.

Meyer, A., Grotefend, S., Gross, A., Wätzig, H. and Ott, I. 2012. Total reflection X-ray fluorescence spectrometry as a tool for the quantification of gold and platinum metallodrugs: determination of recovery rates and precision in the ppb concentration range. Journal of Pharmaceutical and Biomedical Analysis 70: 713-717.

Mitema, E. S., Kikuvi, G. M., Wegener, H. C. and Stohr, K. 2001. An assessment of antimicrobial consumption in food producing animals in Kenya. Journal of Veterinary Pharmacology and Therapeutics 24: 385-390.

Moragues, F. and Igualada, C. 2009. How to decrease ion suppression in a multiresidue determination of β-agonists in animal liver and urine by liquid chromatography-mass spectrometry with ion-trap detector. Analytica Chimica Acta 637: 193-195.

Øye, B. E., Couillard, F. D. and Valdersnes, S. 2019. Complete validation according to current international criteria of a confirmatory quantitative method for the determination of nitrofuran metabolites in seafood by liquid chromatography isotope dilution tandem mass spectrometry. Food Chemistry 300: article ID 125175.

Park, M. S., Kim, K. T. and Kang, J. S. 2017. Development of an analytical method for detecting nitrofurans in bee pollen by liquid chromatography-electrospray ionization tandem mass spectrometry. Journal of Chromatography B 1046: 172-176.

Radovnikovic, A., Moloney, M., Byrne, P. and Danaher, M. 2011. Detection of banned nitrofuran metabolites in animal plasma samples using UHPLC-MS/MS. Journal of Chromatography B 879: 159-166.

Shendy, A. H., Al-Ghobashy, M. A., Gad Alla, S. A. and Lotfy, H. M. 2016. Development and validation of a modified QuEChERS protocol coupled to LC-MS/MS for simultaneous determination of multi-class antibiotic residues in honey. Food Chemistry 190: 982-989.

Song, X., Zhou, T., Li, J., Su, Y., Xie, J. and He, L. 2018. Determination of macrolide antibiotics residues in pork using molecularly imprinted dispersive solid-phase extraction coupled with LC-MS/MS. Journal of Separation Science 41: 1138-1148.

Veach, B. T., Anglin, R., Mudalige, T. K. and Barnes, P. J. 2018. Quantitation and confirmation of chloramphenicol, florfenicol, and nitrofuran metabolites in honey using LC-MS/MS. Journal of AOAC International 101: 897-904.

Veach, B. T., Baker, C. A., Kibbey, J. H., Fong, A., Broadaway, B. J. and Drake, C. P. 2015. Quantitation of chloramphenicol and nitrofuran metabolites in aquaculture products using microwave-assisted derivatization, automated SPE, and LC-MS/MS. Journal of AOAC International 98: 588-594.

Wang, K., Lin, K., Huang, X. and Chen, M. 2017. A simple and fast extraction method for the determination of multiclass antibiotics in eggs using LC-MS/MS. Journal of Agricultural and Food Chemistry 65: 5064-5073.

Wang, J., Chang, X., Zuo, X., Liu, H., Ma, L., Li, H. and Tao, X. 2018. A multiplex immunochromatographic assay employing colored latex beads for simultaneously quantitative detection of four nitrofuran metabolites in animal-derived food. Food Analytical Methods 12: 503-516.

Xing, L. H., Li, Z. X. and Sun, W. H. 2017. Simultaneous determination of four kinds of nitrofurans residues in aquatic products by liquid chromatography-tandem mass spectrometry. Food Quality and Safety 8: 1233-1239.

Yamaguchi, T., Okihashi, M., Harada, K., Konishi, Y., Uchida, K., Do, M. H. N., … and Yamamoto, Y. 2015. Antibiotic residue monitoring results for pork, chicken, and beef samples in Vietnam in 2012-2013. Journal of Agricultural and Food Chemistry 63: 5141-5145.

Yu, W., Chin, T. and Lai, H. 2013. Detection of nitrofurans and their metabolites in pond water and sediments by liquid chromatography (LC)-photodiode array detection and LC-ion spray tandem mass spectrometry. International Biodeterioration and Biodegradation 85: 517-526.

Zhang, Y., Qiao, H., Chen, C., Wang, Z. and Xia, X. 2016. Determination of nitrofurans metabolites residues in aquatic products by ultra-performance liquid chromatography-tandem mass spectrometry. Food Chemistry 192: 612-617.

Zhang, Z., Wu, Y., Li, X., Li, H., Wang, Y., Fu, Q., … and Xia, X. 2017. Multi-class method for the determination of nitroimidazoles, nitrofurans, and chloramphenicol in chicken muscle and egg by dispersive-solid phase extraction and ultra-high performance liquid chromatography-tandem mass spectrometry. Food Chemistry 217: 182-190.

Zhao, M., Zhang, Y., Yuan, F., Deng, Y., Liu, J.,

Zhou, Y. and Zhang, X. 2015. Hydrazino-s-triazine based labelling reagents for highly sensitive glycan analysis via liquid chromatography-electrospray mass spectrometry. Talanta 144: 992-997.