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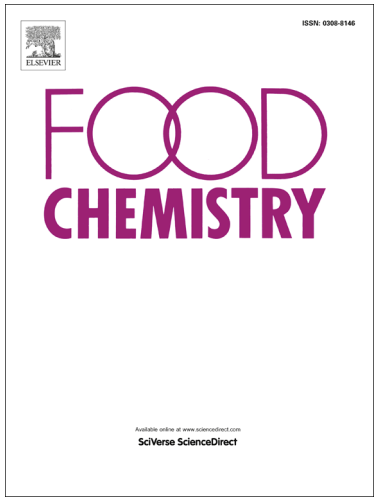

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PII: S0308-8146(19)31948-X
DOI: https://doi.org/10.1016/j.foodchem.2019.125814
Reference: FOCH 125814

To appear in: *Food Chemistry*

Received Date: 3 August 2018
Revised Date: 5 October 2019
Accepted Date: 27 October 2019

Please cite this article as: Wu, S., Yang, B., Yu, H., Li, Y., A rapid derivatization method for analyzing nitrofuran metabolites in fish using ultra-performance liquid chromatography–tandem mass spectrometry, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.125814

A rapid derivatization method for analyzing nitrofuran metabolites in fish using ultra-performance liquid chromatography–tandem mass spectrometry

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Introduction

Nitrofurans are a class of broad-spectrum veterinary drugs used for the treatment of microbial infections. They contain a 5-nitrofuran ring and a variety of substituents at the 2 position (Radovnikovic, Moloney, Byrne, & Danaher, 2011). Because of their efficiency, availability, and relatively low cost, nitrofurans quickly gained worldwide popularity, with furaltadone (FTD), furazolidone (FZD), nitrofurantoin (NFT), and nitrofurazone (NFZ) once being the most widely used veterinary drugs and feed additives (Sarmah, Meyer, & Boxall, 2006). However, nitrofurans and their metabolites are suspected carcinogens and mutagens of human (McCalla, 1983; Jung, Le, Wengenmayer, Wolf, & Kramer, 1985; Shahin, 1987). Hence, the use of nitrofurans in animals for human consumption has been banned in many countries and regions, including the European Union (EU), United States, and China (Commission Regulation (EC) 1995/1442/EC; Khong, Gremaud, Richoz, Delatour, Guy, Stadler, et al., 2004).

Even though their use in food animal production is prohibited in many countries, 854 notifications of nitrofuran contamination were reported in the years 2002–2017, according to the database of the Rapid Alert System for Food and Feed (RASFF) (Sniegocki, Giergiel, Sell, & Posyniak, 2018). Unlike most other veterinary drugs, nitrofurans are extensively metabolized to tissue-bound metabolites, including 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), and semicarbazide (SEC). Consequently, the concentration of the parental drug quickly drops below the detection capability of

modern analytical methods. Therefore, newer analytical methods have focused on the determination of hydrolyzed free residues of tissue-bound metabolites (Sniegocki, Giergiel, Sell, & Posyniak, 2018; Wang, Liu, Wang, Chen, & Jiang, 2018) that accumulate in proteins and form stable adducts that can be detected in tissues for up to 56 days (Chu, Lopez, Abraham, El Said, & Plakas, 2008). Following nitrofuran ban, nitrofuran metabolites act as marker residues for the detection of illegal use of nitrofurans, at the minimum required performance limit (MRPL) of 1 $\mu g \cdot kg^{-1}$, set by the EU (Commission Decision (EC) 2003/181/EC).

Because of its sensitivity and selectivity, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is now widely used for analyzing nitrofuran metabolites in animal tissues and other matrices (Palaniyappan, Nagalingam, Ranganathan, Kandhikuppam, Kothandam, & Vasu, 2013; Veach, Baker, Kibbey, Fong, Broadaway, & Drake, 2015; Wang, Lin, Huang, & Chen, 2017; Wang, Chan, & Chan, 2017; Sniegocki, Giergiel, Sell, & Posyniak, 2018). However, hydrolyzed nitrofuran metabolites are very polar (with poor retention on reverse-phase column), with poor ionization properties in the electrospray interface of mass spectrometer (Leitner, Zöllner, & Lindner, 2001). Therefore, derivatization has been recognized as essential for LC-MS detection of nitrofuran metabolites (Du, Chen, Sheng, Chen, Xu, Liu, et al., 2014). Most of the reported derivatization approaches use 2-nitrobenzaldehyde (2-NBA) as the derivatization reagent, with 16 h or overnight incubation required for the completion of reaction (Radovnikovic, Moloney, Byrne, & Danaher, 2011; An, Parrales, Wang, Cain, Hollins, Forrest, et al., 2015; Zhang, Qiao,

Chen, Wang, & Xia, 2016; Park, Kim, & Kang, 2017; Sniegocki, Giergiel, Sell, & Posyniak, 2018; Øye, Couillard, & Valdersnes, 2019). Further, a liquid/liquid clean up step or additional reversed-phase solid phase extraction step are used to minimize matrix interference (Lopez, Feldlaufer, Williams, & Chu, 2007; Du, Chen, Sheng, Chen, Xu, Liu, et al., 2014).

The aim of the current study was to develop a rapid derivatization method for analyzing nitrofuran metabolites. Accordingly, the derivatization of nitrofuran metabolites was optimized, focusing on such parameters as the concentrations of 2-NBA and HCl, methanol volume, derivatization temperature, and derivatization time. The rapid derivatization method was used for the analysis of four types of nitrofuran metabolites in fish samples by UPLC–MS/MS. The method was validated and successfully applied to actual fish samples. The optimized method offers several advantages over reported analytical methods: (1) the derivatization time was significantly shortened, from overnight or 16 h to 5 min; (2) after extraction by methanol spiked with HCl and 2-NBA, the samples are directly placed in a bath for derivatization; and (3) a satisfactory lower limit of quantification (LLOQ) of 0.1 $\mu g \cdot L^{-1}$ for nitrofuran metabolites in a fish homogenate sample, equal to 0.4 $\mu g \cdot kg^{-1}$ for fish, was achieved, which meets the regulatory requirements of the EU (Commission Decision (EC) 2003/181/EC).

1. Experimental

2.1 Chemicals and reagents

Analytical standards of AHD, AOZ, AMOZ, and SEC (Fig. 1) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2-NBA and wogonin (IS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the National Institutes for Food and Drug Control (Beijing, China), respectively. Reagent-grade HCl was supplied by Beijing Chemical Works (Beijing, China). HPLC-grade methanol and acetonitrile were both purchased from Honeywell (Morristown, NJ, USA). HPLC-grade formic acid was purchased from ROE Scientific Inc. (Newark, USA). Ultra-pure water (18.2 MΩ) was prepared using a Millipore Milli-Q purification system (Bedford, USA). Individual stock solutions were prepared by dissolving each standard in methanol at 1000 $mg \cdot L^{-1}$; the stock solutions were stored in a freezer at −80 °C. Other chemicals used were of analytical or HPLC grade.

Insert Figure 1 here.

2.2 Sample preparation and derivatization

Nine fish species, with six animals per species, were purchased from a local farmers' market and supermarket (Beijing, China). These were: bluntnose black bream (*Megalobrama amblycephala*), carp (*Cyprinus carpio*), catfish (*Silurus asotus*), grass carp (*Ctenopharyngodon idellus*), tilapia (*Oreochromis mossambicus*), basa fish (*Paugusiushamiltoa*), turbot (*Scophthalmus maximus*), weever (*Lateolabrax japonicus*), and large yellow croaker (*Larimichthys crocea*). The fish were authenticated by Dr. Binghu Yang (Dongcheng Monitoring Center for the Safety of Food and Drug, Beijing, China), and weighed at least 0.5 kg each.

Approximately 1 g of the fish epaxial muscle was cut off, weighed, and blended using a mechanical blender with three volumes of saline (*v:w*). The homogenized samples were stored at –20 °C until use.

For the analysis, 400-µL aliquot of fish homogenate was transferred into a 2-mL Eppendorf vial. Thereafter, 10 µL of IS (100 µg·L^{-1}) was added and the sample was vortex-mixed for 1 min. Next, 1200 µL of methanol, 80 µL of HCl (1 M), and 80 µL of 2-NBA (0.1 M) were sequentially added. After vortex-mixing at 2000 rpm for 2 min, the mixture was centrifuged at 14,000 × *g* and 4 °C for 5 min. Subsequently, 1200 µL of the supernatant was transferred into a 1.5-mL EP tube and incubated at 37 °C for 30 min for derivatization. The reaction solution was then evaporated to dryness under a gentle stream of nitrogen at 40 °C. The obtained residue was dissolved in 100 µL of 50% acetonitrile and vortex-mixed at 2000 rpm for 5 min. After centrifugation at 14,000 × *g* and 4 °C for another 5 min, 5 µL of the supernatant was injected into the UPLC–MS/MS for analysis.

2.3 Optimization of the derivatization conditions

The optimal concentrations of 2-NBA and HCl, methanol volume, derivatization temperature, and derivatization time, as the main factors influencing the derivatization process, were individually determined. To identify the appropriate derivatization conditions, the following were tested: 2-NBA concentration: 0.001, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 M; methanol volume: 400, 600, 800, 1000, and 1200 µL; HCl concentration: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 M; derivatization

121 temperature: 0, 20, 40, 50, 60, and 70 °C; and derivatization time: 0, 2, 5, 10, 15, and
122 30 min. The different derivatization conditions were tested in triplicate.

123 2.4 UPLC–MS/MS analysis

124 Sciex API 5500 Q Trap mass spectrometer (Toronto, ON, Canada) interfaced
125 with a Waters Acquity UPLC separation module (Milford, MA, USA) was used for
126 the detection and quantification of the 2-NBA derivatives of nitrofuran metabolites,
127 specifically, AHD-NB, AOZ-NB, AMOZ-NB and SEC-NB (Fig 1). Empower 3.0
128 (Milford, MA, USA) and Analyst 1.62 (Concord, ON, Canada) software were used to
129 control the UPLC and mass spectrometer, respectively.

130 Chromatographic separation was achieved using an Agilent Eclipse Plus C_{18}
131 column (2.1 mm × 50 mm, 5 μm; maintained at 23 °C), using a mobile phase
132 containing 0.04 ‰ formic acid that consisted of solvent A (acetonitrile/water, 1:99,
133 *v/v*) and solvent B (acetonitrile/water, 99:1, *v/v*). The following gradient program was
134 used: 0–1 min, linear gradient from 10% to 15% solvent B (curve 6); 1–2 min,
135 gradient from 15% to 50% (curve 5); 2–3.5 min, linear gradient from 50% to 80%
136 solvent B (curve 6); 3.5–4.3 min, 80% solvent B; and 4.3–5.0 min, 10% solvent B
137 (curve 1). The mobile phase was delivered at 0.3 $mL \cdot min^{-1}$.

138 MS detection was performed in the positive ion mode, with the ion spray voltage
139 set to 5.5 kV and the turbo spray temperature maintained at 500 °C. Nebulizer gas
140 (gas 1), heater gas (gas 2), and curtain gas were set at 344,738, 379,212, and 241,316
141 Pa, respectively. The interface heater was on. Data acquisition for the four derivatives

of nitrofuran metabolites (AHD-NB, AOZ-NB, AMOZ-NB, and SEC-NB) and IS was performed in the multiple reaction monitoring (MRM) mode (Table 1).

Insert Table 1 here.

2.5 Validation experiments

2.5.1 Specificity and selectivity

The specificity of the method was determined by analyzing six different fish samples to demonstrate the lack of chromatographic interference from endogenous fish components.

2.5.2 Calibration curves, linearity, and LLOQ

Calibration curves were acquired by plotting the peak area ratio of derivatives to that of IS against the nominal concentrations of calibration standards under the optimum conditions. The concentrations of AHD, AOZ, AMOZ, and SEC used for the construction of the calibration curve were 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 $\mu g \cdot L^{-1}$, accordingly. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value, except for LLOQ, which was set at ±20%. In addition, LLOQ was defined as the signal ≥10 times the noise ratio, respectively.

2.5.3. Accuracy and precision

The inter- and intra-day precision and accuracy of the method were evaluated through recovery experiments by spiking three concentrations of nitrofuran

metabolites (0.25, 5, and 80 μg·L^{-1}) into blank cowpea fish homogenate, with six replicates per concentration, before adding the derivatization solvent. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value.

2.5.4. Recovery and matrix effect

The recovery was assessed by supplementing six blank samples with three concentrations of the analytes (0.25, 5, and 80 μg·L^{-1}). The matrix effects of nitrofuran metabolite derivatives were evaluated using a post-derivatization spiked method (Rebane, Oldekop, & Herodes, 2014). Briefly, the IS-normalized peak areas of derivatized analytes spiked with the blank sample extract were compared with those of analytes derivatized in a standard solution. The coefficient of variation (CV) of IS-normalized matrix effect of each derivative was within ±15%.

2.6 Data analysis

The LC–MS/MS peak areas were used for the assessment of the effects of different derivatization conditions on the responses of derivatives. Statistical significance ($p < 0.05$) was determined by using the Student's *t*-test, unless otherwise stated.

2. Results and discussion

3.1 Optimization of derivatization conditions

3.1.1 Effect of 2-NBA concentration

182 The effect of 2-NBA concentrations in the range of 0.001–0.1 M on the
183 derivatization was evaluated (Fig. 2A, Table S1). The maximum responses (peak
184 areas) of the nitrofuran metabolite derivatives were obtained at the 2-NBA
185 concentration of 0.01 M. The responses of nitrofuran metabolite derivatives increased
186 significantly ($p < 0.05$) with the 2-NBA concentration increasing from 0.001 M to
187 0.01 M. Increasing the 2-NBA concentration beyond 0.01 M resulted in decreased
188 responses of the four derivatives. Therefore, 0.01 M was selected as the optimal
189 derivatization concentration of 2-NBA for further experiments.

190 3.1.2 Effect of methanol volume

191 Methanol was used as both the 2-NBA solvent and protein precipitator. Although
192 protein precipitation is the simplest approach for removing the majority of protein
193 matrix in biological samples, the volume ratio of methanol to precipitated sample may
194 greatly affect the mass response because of the co-elution interference. The
195 optimization was performed by testing the reaction performance with 400–1200 μL of
196 methanol (Fig. 2B, Table S1). Compared with 400 μL of methanol, the responses of
197 all nitrofuran metabolite derivatives increased 1.20-fold to 1.41-fold, and were
198 significantly ($p < 0.05$) higher than when 1200 μL of methanol was used. No
199 significant change in peak areas for AOZ, AMOZ, and AHD derivatives was noted
200 when 800–1200 μL of methanol was used. By contrast, for SEC derivatives, there was
201 no significant change in peak areas for methanol volumes of 400–1000 μL. In
202 agreement with the published work, the precipitant-to-plasma volume ratio of 3:1 (*v:v*)
203 could significantly reduce the co-elution isobaric interference and improves the lower

limit of detection (Xu, Zhou, & Korfmacher, 2005). Finally, 1200 μL of methanol was selected (methanol:fish homogenate sample = 3:1, *v:v*) for further experiments.

3.1.3 Effect of HCl concentration

Typically, HCl is used to hydrolyze protein-bound nitrofuran metabolites to form free small molecular compounds for 2-NBA derivatization, at a final concentration of 0.081–0.174 M (Radovnikovic, Moloney, Byrne, & Danaher, 2011; An, Parrales, Wang, Cain, Hollins, Forrest, et al., 2015; Zhang, Qiao, Chen, Wang, & Xia, 2016; Park, Kim, & Kang, 2017; Sniegocki, Giergiel, Sell, & Posyniak, 2018). However, different concentrations of HCl result in different pH values. Therefore, controlling the pH in a sample solution is very important since it greatly influences the extent of the derivatization reaction. Generally, the sample pH should be above the pKa of the analyte to allow its deprotonation. In the current study, HCl was mixed with methanol for protein precipitation. HCl concentrations from 0.1 to 1 M were investigated (Fig. 2C, Table S1). Responses of the derivatives increased 1.25-fold to 1.98-fold ($p < 0.05$) as the HCl concentration increased from 0.1 M to 0.4 M. No significant changes in peak areas of the four derivatives were observed as the HCl concentration was further increased from 0.4 M to 0.7 M. Although no significant changes in the peak areas were noted, a downward trend for the four derivatives was apparent as the HCl concentration increased from 0.7 M to 0.9 M. Considering the above, 0.5 M was selected as the suitable HCl concentration for the following analyses.

3.1.4 Effect of derivatization temperature

225 Reaction temperature provides the necessary activation energy to accelerate the
226 derivatization reaction to completion, increasing the derivative yield. Derivatization
227 using 2-NBA is usually performed at 37 °C (Radovnikovic, Moloney, Byrne, &
228 Danaher, 2011; An, Parrales, Wang, Cain, Hollins, Forrest, et al., 2015; Zhang, Qiao,
229 Chen, Wang, & Xia, 2016; Park, Kim, & Kang, 2017). In the current study, the
230 optimal derivatization temperature was investigated between 0 and 70 °C (Fig. 2D,
231 Table S1). The derivatization occurred even at 0 °C, but decreased peak areas were
232 observed as the reaction temperature increased from 0 and 70 °C. In addition,
233 significant differences ($p < 0.05$) in peak areas were observed for AHD-NB, AOZ-NB,
234 SEC-NB, and AMOZ-NB, when the reaction temperature exceeded 50 °C, 60 °C, 40
235 °C, and 50 °C, respectively. Considering the above, 0 °C was chosen at the optimal
236 reaction temperature.

237 3.1.5. Effect of derivatization time

238 The optimization of derivatization time was performed for the time window of
239 0–30 min (Fig. 2E, Table S1). No significant changes in peak areas of the four
240 derivatives were apparent as the reaction time increased from 0 to 30 min. The largest
241 peak areas for AHD-NB, AOZ-NB, and AMOZ-NB were observed when the
242 derivatization proceeded for 5 min. However, the largest peak area for SEC-NB was
243 achieved after a 10-min reaction, and was 1.005-fold that obtained after a 5-min
244 reaction. The earliest reported 2-NBA derivatization time for nitrofuran metabolites is
245 16 h, originally reported by Horne et al. (Horne, Cadogan, O'Keeffe, & Hoogenboom,
246 1996) and also used in subsequent studies (Radovnikovic, Moloney, Byrne, &

247 Danaher, 2011; An, Parrales, Wang, Cain, Hollins, Forrest, et al., 2015; Zhang, Qiao,
248 Chen, Wang, & Xia, 2016; Park, Kim, & Kang, 2017; Sniegocki, Giergiel, Sell, &
249 Posyniak, 2018; Øye, Couillard, & Valdersnes, 2019). However, in the current study,
250 5 min allowed sufficient derivatization of nitrofuran metabolites. Consequently, 5 min
251 was employed as the optimal derivatization time.

252 Insert Figure 2 here.

253 3.2. Method validation

254 3.2.1. Selectivity

255 In the current study, the presence of nitrofuran metabolites in fish was
256 determined by using an analytical LC–MS method. Typical MRM chromatograms of
257 a blank fish homogenate, blank fish homogenate spiked with the analytes and IS, and
258 the fish sample are presented in Fig. S1. No significant interference were apparent at
259 the retention time of 2.18, 2.31, 2.12, 1.51, and 2.96 min for the derivatives of AHD,
260 AOZ, SEC, and AMOZ, and for IS, respectively.

261 3.2.2. Linearity and LLOQ

262 The calibration curves were linear for all analytes over the concentration range of
263 0.1–100 $\mu g \cdot L^{-1}$, with the correlation coefficient $r > 0.99$ and LLOQ = 0.1 $\mu g \cdot L^{-1}$.
264 The calibration curves are shown in Fig S2.

265 3.2.3. Precision and accuracy

266 The intra-day and inter-day precision and accuracy of AHD, AOZ, AMOZ, and

267 SEC were investigated by analyzing three concentration levels of quality control (QC)
268 samples. The data are shown in Table S2. All data for the tested samples were within
269 the acceptable criterion of ±15%.

270 3.2.4. Recovery and matrix effect

271 The mean recovery of the analyzed drugs was calculated at all QC levels. It
272 varied from 64.6 to 68.9% for AHD; from 55.0 to 73.2% for AOZ; from 71.8 to
273 75.6% for AMOZ; and from 69.8 to 75.8% for SEC (Table S3).

274 The effect of fish constituents on analyte ionization was determined by
275 comparing the IS-normalized responses of derivatized analytes spiked into a blank
276 sample extract with those of analytes derivatized in a standard solution. The relative
277 standard deviations of the IS-normalized matrix effects were 4.72 to 8.73% for AHD;
278 3.34 to 10.8% for AOZ; 2.63 to 9.30% for AMOZ; and 4.72 to 9.27% for SEC (Table
279 S3).

280 3.3 Analysis of fish samples

281 The validated method relying on the optimized experimental conditions was then
282 used to detect four types of nitrofuran metabolites in 54 fish samples. For catfish
283 samples, no nitrofuran metabolites were detected above the LLOQ (0.4 $\mu g \cdot kg^{-1}$).
284 Further, AHD residues were detected above the LLOQ in 6/54 fish samples.
285 Specifically, AHD was detected in two bluntnose black breams (batch 1 and batch 5,
286 at concentrations of 1.34 and 0.58 $\mu g \cdot kg^{-1}$, respectively), one carp (batch 1, 0.44
287 $\mu g \cdot kg^{-1}$), one tilapia (batch 1, 0.52 $\mu g \cdot kg^{-1}$), one turbot (batch 1, 0.52 $\mu g \cdot kg^{-1}$), and

288 one large yellow croaker (batch 1, 0.40 μg·kg^{-1}). AHD below the LLOQ was detected
289 in grass carp (batch 3). Only one sample (1/54) of the weever (batch 4) contained
290 detectable AOZ residue (0.65 μg·kg^{-1}). Although AOZ was detected in one carp
291 (batch 1) and one weever (batch 1), the concentrations in both samples were below
292 the LLOQ. Further, SEC residue was detected above the LLOQ in 6/54 fish samples.
293 Specifically, SEC was detected in two turbot samples (batch 1 and batch 4, at
294 concentrations of 0.55 and 1.08 μg·kg^{-1}, respectively), one grass carp (batch 6, 0.72
295 μg·kg^{-1}), one tilapia (batch 3, 0.67 μg·kg^{-1}), one basa fish (batch 1, 0.41 μg·kg^{-1}), and
296 one weever (batch 5, 0.75 μg·kg^{-1}). Furthermore, SEC below the LLOQ was detected
297 in four fish samples, namely, two bluntnose black breams (batch 1 and batch 5), one
298 tilapia (batch 1), and one weever (batch 6). More than 0.40 μg·kg^{-1} AMOZ was
299 detected in 3/54 fish samples, i.e., in one carp (batch 1, 0.48 μg·kg^{-1}), one catfish
300 (batch 5, 0.80 μg·kg^{-1}), and one turbot (batch 6, 0.49 μg·kg^{-1}) (Table 2). Detection of
301 the four nitrofuran metabolites in fish samples obtained from local markets indicates
302 illegal use of nitrofurans in fish breeding or preservation.

303 Insert Table 2 here.

304 3. Conclusions

305 In the current study, the derivatization of nitrofuran metabolites using 2-NBA as
306 the reaction reagent was optimized to reduce the reaction time from over 16 h (or
307 overnight) to 5 min, with the analyte detection achieving acceptable sensitivity, with
308 LLOQs of 0.1 μg·L^{-1} in fish homogenate samples (equivalent to 0.4 μg·kg^{-1} in fish).

Accordingly, 400 μL of fish homogenate was transferred to a 2-mL EP vial, 10 μL of IS (100 μg·L^{-1}) was added, and the sample was vortex-mixed for 1 min. Thereafter, a mixture of 1200 μL of methanol, 80 μL of HCl (0.5 M), and 80 μL of 2-NBA (0.01 M) was added. After vortex-mixing at 1200 rpm for 1 min, the mixture was centrifuged at 14,000 × *g* and 4 °C for 5 min. Subsequently, 1200 μL of the supernatant was placed in a 1.5-mL EP tube and incubated for 5 min in an ice water bath to derivatize the nitrofuran metabolites. The reaction solution was then evaporated to dryness under a gentle stream of nitrogen at 40 °C. After centrifugation at 14,000 × *g* and 4 °C for 5 min, 5 μL of the supernatant was injected into the UPLC–MS/MS system for analysis. This approach efficiently removed protein and lipid matrix compounds, and guaranteed precision of detection. Consequently, the four analytes were satisfactorily recovered (64.6% to 75.8% recovery), demonstrating the reliability and practicability of the developed method. The developed method shows great promise for simultaneous monitoring of nitrofuran metabolites to ascertain food safety.

Acknowledgements

The authors acknowledge the financial support of the National Science and Technology Major Project of China "Key New Drug Creation and Manufacturing Program" (Grant Number: 2015ZX09501004–003–005) and the National Natural Science Foundation of China (Grant Number: 81773990).

Notes

The authors declare no competing financial interest.

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Figure captions

Figure 1 Derivatization reaction of nitrofuran metabolites with 2-NBA.

Figure 2 Effects of different derivatization reaction conditions on the metabolite derivatization of nitrofuran metabolites, as assessed by LC/MS/MS analysis. A: Effect of different concentrations (0.001–0.1 M) of 2-NBA on the analyte responses; B: effect of different volumes (400–1200 μL) of methanol on the analyte responses; C: effect of different concentrations (0.1–1.0 M) of HCl on the analyte responses; D: effect of different derivatization temperatures (0–70 °C) on the analyte responses; and E: effect of different derivatization time (0–30 min) on the analyte responses.

Highlights

1. The derivatization conditions of nitrofuran metabolites, including the concentrations of 2-nitrobenzaldehyde and HCl, methanol volume, and derivatization temperature and time, were optimized.
2. The derivatization time was considerably shortened, from overnight or 16 h to 5 min.
3. The lower limit of quantification of nitrofuran metabolites is 0.1 $\mu g \cdot L^{-1}$, which meets the regulatory requirements of the EU.

Table 1. Precursor ions, product ions and MS parameters of MRM transitions used for 2-NBA-derivatized NF metabolites.

Compound	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	CE (V)
AHD-NB	249.1	134.0	140	17
AOZ-NB	236.0	134.0	120	17
AMOZ-NB	335.1	290.1	120	16

SEC-NB	209.1	78.1	56	25
Wogonin (IS)	285.0	270.0	20	30

428 Table 2. Detection of NF metabolites in the fish samples collected from markets
429 (μg·kg^{-1})

Fish species	Batch											
	1	2	3	4	5	6	1	2	3	4	5	6
	AHD						SEC					
Bluntnose black bream (*Megalobrama amblycephala*)	1.34	N.D.	N.D.	N.D.	0.58	N.D.	BLQ	N.D.	N.D.	N.D.	BLQ	N.D.
Carp (*Cyprinus carpio*)	N.D.	N.D.	N.D.	0.44	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Catfish (*Silurus asotus*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Grass carp (*Ctenopharyngodon idellus*)	N.D.	N.D.	BLQ	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.72
Tilapia (*Oreochromis mossambicus*)	0.52	N.D.	N.D.	N.D.	N.D.	N.D.	BLQ	N.D.	0.67	N.D.	N.D.	N.D.
Basa fish (*Paugusiushamiltoa*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.41	N.D.	N.D.	N.D.	N.D.	N.D.
Turbot (*Scophthalmus maximus*)	0.52	N.D.	N.D.	N.D.	N.D.	N.D.	0.55	N.D.	N.D.	1.08	N.D.	N.D.
Weever (*Lateolabrax japonicus*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.75	BLQ
Large yellow croaker (*Larimichthys crocea*)	0.40	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	AOZ						AMOZ					

Bluntnose black bream (*Megalobrama amblycephala*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Carp (*Cyprinus carpio*)	BLQ	N.D.	N.D.	N.D.	N.D.	N.D.	0.48	N.D.	N.D.	N.D.	N.D.	N.D.
Catfish (*Silurus asotus*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.80	N.D.
Grass carp (*Ctenopharyngodon idellus*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Tilapia (*Oreochromis mossambicus*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Basa fish (*Paugusiushamiltoa*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Turbot (*Scophthalmus maximus*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	BLQ	N.D.	N.D.	0.49
Weever (*Lateolabrax japonicus*)	N.D.	BLQ	N.D.	0.65	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Large yellow croaker (*Larimichthys crocea*)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D.: not detected; BLQ: below the LLOQ (equal to 0.4 μg·kg^{-1} in fish).