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Determination of Nitrofurans Metabolites Residues in Aquatic Products by Ultra-performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract:

A method was described for monitoring four nitrofuran metabolites including 5-methylmorpholino-3-amino-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidone (AOZ), 1-amino-hydantoin (AHD) and semicarbazide (SEM) in aquatic products. The analytes were quantified by ultra-performance liquid chromatography-tandem mass spectrometry operating in positive ion multiple monitoring mode (MRM) after 2-nitrobenzaldehyde derivatization. The matrix calibration curve was established with correlation coefficient ($R^2 > 0.99$) in the range of 1~100 ng·ml$^{-1}$. Limit of detection and limit of quantification for all analytes were 0.5 and 1.5 µg·kg$^{-1}$, respectively. Recovery rates and relative standard deviations ranged from 88 ~ 112% and 2 ~ 4%, respectively. The validated method was successfully applied to detect nitrofuran metabolites in 120 fish samples. The analytes were detected in 6/16 species of fish samples, with a total detection rate of 6.5%. AOZ was most frequently detected (8.3%), followed by AMOZ (7.5%), AHD (5.0%) and SEM (5.0%). The method is proposed for monitoring nitrofuran metabolites in aquatic products.

Keywords:
Aquatic products;
Nitrofuran metabolites;
Residue determination;
Ultra-performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS);
1. Introduction

Nitrofurans (NFs) belong to a series of synthetic broad spectrum antibiotics which all contain 5-nitrofururan ring and various substituents in the 2-position. The history of NFs application as pharmacologically active substances began in 1944, when the antibiotic properties of nitrofurazone (NFZ) were first confirmed (Dodd M C, et al, 1944). In addition to their use as veterinary drugs to prevent and control diseases, they are often applied to animal feed to stimulate growth of animals like swine, poultry and bovine.

Four pharmacologically active compounds commercially successful are furaltadone (FTD), furazolidone (FZD), nitrofurantoin (NFT) and nitrofurazone (NFZ). NFs are resorbed, metabolised and distributed rapidly, even shortly after the uptake of NFs only their metabolites are detectable as tissue-bound residues (Cooper K M, et al. 2005). The marker residues identified are 3-amino-5-methyl-morpholino-2-oxazolidinone (AMOZ) for furaltadone, 3-amino-2-oxazolidone (AOZ) for furazolidone, 1-amino-hydantoin (AHD) for nitrofurantoin and semicarbazide (SEM) for nitrofurazone. These metabolites act as marker residues for the detection of an illegal use of NFs.

NFs have displayed significant toxicity in human health manifested through carcinogenic, mutagenic and teratogenic effects (Bogiali S, et al. 2009). For this reason, application of NFs compounds in food and animal production was banned in the EU in 1995 and in the USA in 2002 (Commission Regulation (EC) 1995/1442/EC). However, because of their efficiency, availability and low cost, illegal or imprudent use of NFs still exists (Greenwood D. 2008). A minimum required performance level (MRPL) of 1 µg·kg⁻¹ for each metabolite has been set by the EU (Commission Decision (EC) 2003/181/EC) and all laboratories entrusted with
the determination of NFs residues must meet this requirement.

Previous studies have demonstrated that parent NFs deplete rapidly in animals and that NFs are extensively metabolized to tissue-bound metabolites (McCracken R J, et al. 2000). As such, newer analytical methods have been directed to the determination of the intact side-chains of tissue-bound residues, instead of the parent NFs. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methods are now used throughout the world for analyzing NFs in animal tissues and other matrices. These methods are based on those described by Leitner et al. (2001) and Conneely et al. (2003) and were developed under the auspices of the multinational EU research project FoodBRAND. Xia et al. (2008) described a simultaneous identification and quantification of NFs in pork using ultra-performance liquid chromatography (UPLC) coupled with ESI-MS/MS, which can detect the NFs metabolites as their nitrophenyl (NP) derivatization using the isotopically labeled analogues (d₅-AMOZ, d₄-AOZ, ¹³C₃-AHD, ¹³C-¹⁵N-SEM), 2-NP-AMOZ, 2-NP-AOZ, 2-NP-AHD, 2-NP-SEM are marker residues of FTD, FZD, NFT, NFZ, respectively, which need to be derivatised to finally attach a mass portion for enhancing their detection.

In this study, a method of UPLC-MS/MS for simultaneous detection and quantitation of residues of four kinds of NFs metabolites in aquatic products was described. The method was validated according to the EU guidelines pertaining to the performance of analytical methods and the interpretation of results (Commission Regulation (EC) 2002/657/EC). In addition, this method was applied successfully to actual fish samples.

2. Materials and methods

2.1. Chemicals and reagents
NFs metabolites (AMOZ, AOZ, AHD, SEM), nitrophenyl derivatives (2-NP-AMOZ, 2-NP-AOZ, 2-NP-AHD, 2-NP-SEM), internal standard (d₅-AMOZ, d₄-AOZ, ¹³C₃-AHD, ¹³C-¹⁵N-SEM), derivatives of isotopically labeled internal standards (2-NP-d₅-AMOZ, 2-NP-d₄-AOZ, 2-NP-¹³C₃-AHD, 2-NP-¹³C-¹⁵N-SEM, chemical purity > 99 %, isotopic purity > 99 %), 2-nitrobenzaldehyde (2-NBA), were obtained from Sigma-Aldrich (USA). Methanol, acetonitrile, ammonium acetate were of HPLC grade and were obtained from Merck (Darmstadt, Germany).

All other solvents and reagents as well as Oasis HLB solid phase extraction (SPE) cartridges (60 mg, 3 mL) were obtained from Waters Corp. (Milford, MA, USA).

2.2. Standard solutions

Individual standard stock solutions (1 mg·mL⁻¹) of AMOZ, AOZ, AHD, SEM and the labelled internal standards, d₅-AMOZ, d₄-AOZ, ¹³C₃-AHD, ¹³C-¹⁵N-SEM, were prepared in methanol. Mixed-standard working solutions (1µg·mL⁻¹) were prepared by diluting a stock solution with methanol. Mixed-internal standard working solutions (100 ng·mL⁻¹) were prepared to be used for sample spiking during method validation. A series of calibration standards were prepared by diluting 1µg·mL⁻¹ of the mixed standard solution to produce final concentrations of 1, 5, 10, 20, 50, 100 ng·mL⁻¹ with methanol-water (50:50, v/v). Stock and working solutions were protected from light, and stored at 4 °C until use.

2.3. Samples preparation

2.3.1. Samples collection

A total of 120 fish samples, including 16 types of fish such as grass carp, carp, crucian carp, catfish, chub, bluntnose block bream etc., were selected randomly from the local market of Shaanxi Province, China. The sample wet weight was at least 1 kg for small and medium-size fresh product.
The representative portion of the sample was blended using a food processor and mixed thoroughly. The homogenised samples were stored at -20 °C. Before using, the samples were thawed at 4 °C overnight.

2.3.2 Extraction

A well homogenised sample (1 g) was weighed and placed into a 50 mL centrifuge tube. Internal standard working solution (100 ng·mL⁻¹, 200 µL) was added and contents were briefly vortexed. Hydrochloric acid (0.2 mol·L⁻¹, 10 mL) and 2-NBA (0.05 mol·L⁻¹ solution in DMSO, 0.3 mL) were added and the sample vortexed. The sample was derivatised overnight at 37 °C. Following derivatization, the sample was cooled to room temperature and neutralised by the addition of trisodium phosphate (0.3 mol·L⁻¹, 500 µL). The pH value was adjusted to pH 7.2~7.5 using sodium hydroxide (1 mol·L⁻¹, 1.8 mL) and the sample then centrifuged for 10 min at 10000 rpm.

2.3.3 Clean-up

The supernatant (10 mL) was applied to Oasis HLB SPE cartridges (60 mg, 3 mL) conditioned previously with methanol (3 mL) and water (3 mL). The column was washed successively with distilled water (3 mL), and dried by sucking through air (Visiprep vacuum manifold, Supelco, Switzerland) after each solvent addition. The derivatised NFs metabolites were finally eluted with ethyl acetate (6 mL) and collected in a 10 mL centrifuge tube. The elutate was evaporated to dryness under a stream of nitrogen at 40 °C and reconstituted with 1 mL of acetonitrile: formic acid (10:90, v/v). The final solution was filtered through a 0.22 µm nylon filter (Nalgene, Rochester, NY, USA) directly into an UPLC vial for analysis.

2.4 UPLC-MS/MS analysis

2.4.1 UPLC conditions
UltiMate™ 3000 ultra performance liquid chromatography (Dionex, USA) was used to analyze the samples. Analyses were performed at 40 °C on an Acclaim® RSLC C18 column (100 mm×2.1 mm, 2.2 μm particle size; Dionex, USA). The injection volume was 10 μL. The mobile phase A consisted of acetonitrile and the mobile phase B consisted of ammonium acetate (5 mmol·L⁻¹) and acetic acid (0.1 %), (A:B =25:75, v/v). The isocratic elution was applied at a flow rate of 0.5 mL·min⁻¹. The retention times for 2-NP-AMOZ, 2-NP-AOZ, 2-NP-AHD and 2-NP-SEM under these conditions were listed in Table 1.

2.4.2. Tandem mass spectrometry

The triple quadrupole mass spectrometer (API 3200, Applied Biosystems, Foster City, CA, USA), equipped with a direct online inlet system and an ESI interface was operated in the positive mode with a TurboIon Spray voltage of 5000 V. The source temperature was adjusted to 650°C. Nitrogen was used as nebulizer gas (70.0 psi), curtain gas (10.0 psi) and collision gas (30.0 psi). The detection was carried out in the multiple reaction monitoring (MRM) by analysing two transitions with a dwell time of 100 ms with the resolution of Q1 and Q3 set to “unit”. Transition reactions were given in Table 1, as well as the adjustments of the collision energy (CE), declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP).

2.5. Method validation

Since NFs are banned substances and listed in Annex IV of Council Reg 2377/90, there is no safe limit for presence of these compounds in food. A reference point for action (RPA) for NF metabolites has been established as 1 μg·kg⁻¹.

Validation for the methodology presented in this work was performed according

Recovery was assessed by fortification of 10 blank samples with the addition of analytes at concentrations of 50, 100 and 200 ng·mL\(^{-1}\). The recovery assays were replicated ten times. After analyzing and determining the concentrations for each sample, recovery rates were calculated as follows:

\[
\%\text{Recovery} = \frac{\text{observed concentration}}{\text{fortification level}} \times 100
\]

The standard curve was established by using the ratio of the peak area of the analyte and the internal standard (\(Y\)) and the ratio of the concentration of the analyte and the internal standard (\(X\)).

LOD and LOQ were defined, respectively, as the signal corresponding to 3 and 10 times the noise ratio, determined experimentally from fortified samples (European Commission (EC) SANCO/2013/12571), obtained from peak areas of 10 independent blank aquatic product samples fortified with metabolites standard mixture at a level of 1 ng·mL\(^{-1}\), respectively.

3. Results and discussion

3.1. Optimization of UPLC-MS/MS conditions

Hydrolysis and derivatization are key steps of extraction. Endogenous NFs metabolites exist in animals in protein binding forms, and are released in acidic conditions generating free state small molecular compounds. The relative molecular mass of AOZ, AMOZ, AHD and SEM are 102.1, 201.2, 115.1 and 75.1, respectively, which do not generate characteristic ion debris in the mass spectrometer condition, and bring difficulties to the qualitative and quantitative analysis. Derivatization increased the relative molecular mass of NFs metabolites to 248.2, 334.3, 235.2 and 208.2, respectively. In appropriate collision voltage conditions, these derivatives may
generate at least two characteristic ion peaks, which improves the mass spectrometer response capacity.

We used 2-nitrobenzaldehyde (2-NBA) as derivatization agent with reference to GB/T 21311-2007. Metabolites were treated with acid hydrolysis and derivatization agent simultaneously to increase the relative molecular mass of target compound. Through the optimization of experimental conditions, we have obtained the satisfactory experimental results.

Selection of chromatographic column is essential in UPLC-MS/MS, and the test efficiency is related to specifications of C\textsubscript{18} column. C\textsubscript{18} column with particle size of 1.7 \(\mu\)m has been commonly used because this column has large specific surface and small particle size, which are beneficial to improve the test efficiency. However, due to the complex matrix composition of real samples, column blockage and high column pressure occur frequently, affecting the working-life span of column. Therefore, we have chosen C\textsubscript{18} columns with particle size of 2.2 \(\mu\)m to avoid column blockage and high pressure.

3.2. Validation

Specificity assays are necessary to clearly and unequivocally identify the analytes among related NFs metabolites. The technique of UPLC-MS/MS itself provides a high degree of specificity. To determine the specificity of the method, fish samples were fortified with the four metabolites and the internal standards and non-fortified samples were also analysed. Interfering peaks were observed at the retention time for some transitions but the peaks were so low as to be of little significance (Fig. 1).

Mean recovery values were calculated for each level and are listed in Table 2. Recovery rates and relative standard deviation (RSD) ranged from 88 ~ 112 % and
from 2 ~ 4 %, respectively, indicating high efficiency of the derivatization, neutralization, extraction and cleanup steps.

The linearity was investigated over a concentration range of 1 ~ 100 ng·mL⁻¹. A good linearity and high correlation coefficients of regression (R² > 0.9959) were obtained for all the compounds.

The LOD and LOQ were 0.5 µg·kg⁻¹ and 1.5 µg·kg⁻¹ for all analytes (Table 3). The LOQ achieved allows for the identification and quantification of target analytes below the maximum residue limits (MRLs) established by the EU. The minimum required performance limit (MRPL) for nitrofurans in poultry muscle and shrimps is set at 1.0 µg·kg⁻¹ by Commission Decision (EC) 2003/181/EC amending Commission Regulation (EC) 2002/657/EC.

3.3. Application in fish samples

Using optimized experimental conditions, we applied the method to detect four kinds of NFs metabolites in 120 fish samples. It was shown that the total detection rate was 6.5%. The AOZ was most frequently detected (8.3%), followed by AMOZ (7.5%), AHD (5.0%) and SEM (5.0%). AOZ was detected in 10 fish samples with a median value of 56.3 µg/kg; AMOZ was detected in 9 fish samples with a median value of 31.5 µg/kg; and AHD and SEM were detected in 6 fish samples with a median value of 55.7 and 58.7 µg/kg, respectively.

NFs metabolites were detectable in 6/16 species of fish samples, including bluntnose block bream, tilapia, grass carp, catfish, carp and crucian carp samples, with detection rates of 100.0%, 100.0%, 20.6%, 15.4%, 15.2% and 6.3%, respectively. Detection rates of AMOZ, AOZ, AHD, SEM were showed in Fig. 2.

SEM residues was detected in tilapia sample with a rate of 100%. Both SEM and AHD residues were detected in bluntnose block bream samples with a rate of 100%,
which were due to the small number of samples. However, detection of four NFs metabolites in fish samples collected from markets indicates the possible usage of NFs in aquatic breeding or preservation and the need for regular monitoring.

4. Conclusion

A UPLC–MS/MS has been developed and validated to determine residues of four NFs metabolites in aquatic products. The optimized procedure includes a simple and efficient extraction and derivatization. Compare to previous methods, the method developed in this study has improved sensitivity, shorten the detection time, and made analytes achieve the best response value. At the same time, UPLC-MS/MS method has integrated the advantage of liquid chromatography (high analysis speed, high resolution) and tandem mass spectrometry (high selectivity, high sensitivity) simultaneously. The usage of MRM mode provides characteristics of parent ions and daughter ions and makes analytes reaching satisfactory outcome quality. The target compounds have been qualitatively and quantitatively determined accurately, meeting the testing requirements of import and export of aquatic products.

The method is proposed as tool for the simultaneous monitoring the presence of four nitrofuran metabolites in a number of aquatic products.

References:


the performance of analytical methods and the interpretation of results, *Official Journal of the European Union, L221, 8-36.*


Table 1: Mass spectrometry conditions of UPLC-MS/MS method

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Retention times (min)</th>
<th>Q1 mass(m/z)</th>
<th>Q3 mass(m/z)</th>
<th>CE(V)</th>
<th>DP(V)</th>
<th>EP(V)</th>
<th>CEP(V)</th>
<th>CXP(V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-NP-AMOZ</td>
<td>2.56</td>
<td>335.2</td>
<td>(a)291.4</td>
<td>18</td>
<td>50</td>
<td>5</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2.56</td>
<td>335.2</td>
<td>(b)262.4</td>
<td>20</td>
<td>50</td>
<td>5</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>2-NP-AOZ</td>
<td>2.73</td>
<td>236.0</td>
<td>(a)134.1</td>
<td>17</td>
<td>48</td>
<td>5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2.73</td>
<td>236.0</td>
<td>(b)104.1</td>
<td>30</td>
<td>48</td>
<td>5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>2-NP-AHD</td>
<td>2.18</td>
<td>249.1</td>
<td>(a)104.2</td>
<td>30</td>
<td>48</td>
<td>5</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2.18</td>
<td>249.1</td>
<td>(b)134.3</td>
<td>16</td>
<td>48</td>
<td>5</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2.21</td>
<td>209.1</td>
<td>(a)192.1</td>
<td>16</td>
<td>40</td>
<td>4</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>2-NP-SEM</td>
<td>2.21</td>
<td>209.1</td>
<td>(b)166.3</td>
<td>16</td>
<td>40</td>
<td>4</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>2-NP-d5-AMOZ</td>
<td>2.43</td>
<td>340.3</td>
<td>(a)296.4</td>
<td>18</td>
<td>50</td>
<td>4.5</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>2-NP-d4-AOZ</td>
<td>2.08</td>
<td>240.0</td>
<td>(a)134.1</td>
<td>18</td>
<td>48</td>
<td>4</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>2-NP-13C3-AHD</td>
<td>2.18</td>
<td>252.2</td>
<td>(a)104.2</td>
<td>32</td>
<td>50</td>
<td>3</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>2-NP-13C3-15N-SEM</td>
<td>2.21</td>
<td>212.2</td>
<td>(a)168.3</td>
<td>16</td>
<td>45</td>
<td>3</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

(a)=quantifier transition  (b)=qualifier transition

CE: collision energy, DP: declustering potential, EP: entrance potential, CEP: collision cell entrance potential, CXP: collision cell exit potential

Table 2: Recovery rates of nitrofuran metabolites from fortified samples (n=10)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Standard (50 ng·mL⁻¹)</th>
<th>Standard (100 ng·mL⁻¹)</th>
<th>Standard (200 ng·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery rate (%)</td>
<td>RSD⁴ (%)</td>
<td>Recovery rate (%)</td>
</tr>
<tr>
<td>AMOZ</td>
<td>94</td>
<td>3</td>
<td>88</td>
</tr>
<tr>
<td>AOZ</td>
<td>107</td>
<td>4</td>
<td>112</td>
</tr>
<tr>
<td>AHD</td>
<td>112</td>
<td>3</td>
<td>107</td>
</tr>
<tr>
<td>SEM</td>
<td>122</td>
<td>2</td>
<td>109</td>
</tr>
</tbody>
</table>

RSD⁴: relative standard deviation

Table 3: Linear regression equation and correlation coefficient, LOD, LOQ for four analytes (n=10)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Linear regression equation</th>
<th>Line range (ng·mL⁻¹)</th>
<th>Correlation coefficient</th>
<th>LOD⁵ (µg·kg⁻¹)</th>
<th>LOQ⁵ (µg·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOZ</td>
<td>Y=0.0111x + 0.013</td>
<td>1~100</td>
<td>0.9985</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>AOZ</td>
<td>Y=0.00644x + 0.0102</td>
<td>1~100</td>
<td>0.9988</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>AHD</td>
<td>Y=0.00567x + 0.00497</td>
<td>1~100</td>
<td>0.9981</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>SEM</td>
<td>Y=0.0129x + 0.017</td>
<td>1~100</td>
<td>0.9959</td>
<td>0.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

LOD⁵: limit of detection;  LOQ⁵: limit of quantification
335.2 → 291.4
2-NP-AMOZ

236.0 → 134.1
2-NP-AOZ
Fig. 1 MRM chromatograms of AMOZ, AOZ, AHD and SEM in fish samples.
Fig. 2 The detection rates of nitrofurans metabolites in fish samples (MRLs: maximum residue limits)
Highlights:

1. A method is proposed for simultaneous detection of four NFs metabolites in aquatic products.

2. The correlation coefficient ($R^2$) for matrix calibration curve, in the range of 1~100 ng·ml$^{-1}$, was above 0.99.

3. Recovery rates and RSD ranged from 88 ~ 112% and from 2 ~ 4%, respectively.

4. LOD and LOQ for all four metabolites were 0.5 µg·kg$^{-1}$ and 1.5 µg·kg$^{-1}$, respectively.