APPLICATION NOTE

Mass Spectrometry

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Determination of the Nitrofuran Metabolites in Seafood using Ultra High-Performance Liquid Chromatography - Tandem Mass Spectrometry

Introduction

Nitrofurans are a class of broad-spectrum antibiotics that are widely used to kill or slow down the growth of bacteria in the aquaculture industry. The use of nitrofurans and their metabolites has been restricted by a number of countries and organizations within the European Union, United States and China, due to their harmful side effects to human health. Nitrofurans have been defined as Class A prohibited drugs in many countries, and a Minimum Required Performance Limit (MRPL) 0f 1.0 µg/kg has been set for food, animal and aquaculture products.

According to previous studies, nitrofurans transform rapidly to metabolites which readily bind to protein tissues. The bound metabolites are very stable, and are used as an indicator of nitrofuran residues in various food, animal and aquatic products. The most widely used nitrofurans and their metabolites are furazolidone as 3-amino-2-oxazolidinone (AOZ), nitrofurazone as semicarbazide (SCA), furaltadone as 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) and nitrofurantoin as 1-aminohydantoin (AHD).

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LC/MS/MS methods have been used for the detection of nitrofuran metabolites in many studies owing to the high sensitivity, selectivity and specificity of the methods. However, nitrofuran metabolites demonstrate low ionization efficiency with the electrospray ion source used in LC/MS/MS systems. Moreover, the lower molecular weight of nitrofuran metabolites results in higher background noise in many food matrices. 2-nitrobenzaldehyde (2-NBA) has been used for derivatization of metabolites to enhance ionization efficiency, reduce the background noise resulting from the lower molecular weight of underivatized nitrofuran metabolites, and improve their detection limits with LC/MS/MS[1]. Therefore, the use of a derivatization step for nitrofuran metabolites before extraction and LC/MS/MS analysis is crucial to meet the low regulatory limits of 1.0 µg/kg in food samples.

The main purpose of this work is to determine the detection limits for nitrofurans using QSight® 210 LC/MS/MS system.

Experimental

Hardware and Software

Chromatographic separation and subsequent detection were conducted utilizing the PerkinElmer QSight LX 50 ultra-high-performance liquid chromatography instrument, coupled to the QSight 220 series tandem mass spectrometer. All instrument control, data acquisition and processing were performed using the Simplicity 3Q™ software platform.

Preparation of Standard Solutions

HPLC-grade solvents and reagents were used in the preparation of solutions. SEM, AOZ, AHD, AMOZ and stable isotopically labelled internal standards SEM-^{13}C-$^{15}N\neg_2$, AHD-$^{13}C_3$, AOZ-D_4, AMOZ-D_5 were purchased from Sigma-Aldrich Inc. The stock solutions were prepared by dissolving 10 mg of AOZ, 10 mg of AMOZ, 14.9 mg of SEM and 13.2 mg of AHD in 10 mL of methanol. The four stock standards were diluted to make an intermediate mixed stock solution, which was subsequently used for the preparation of calibration curve standards. Internal standard solutions of each compound with concentrations of 100 µg/mL were diluted to prepare 100 ng/mL mixed internal standard working solutions. All stock solutions were kept in the refrigerator to prevent decomposition.

Sample Preparation and Extraction

To make a homogenized sample, edible parts of a fish sample were cut into small pieces and subsequently blended. Two grams of homogenized fish sample, and 0.05 mL of mixed working internal standard solution were added into the 50 mL centrifuge tube and vortexed for 50 seconds. Next, 5 mL of 0.2 mol/L hydrochloric acid solution, and 0.15 mL of 0.05 mol/L 2-NBA solution were also added into the same tube and vortexed for 50 seconds. The tube was then placed into the water-bath for 16 hours with a constant temperature and vibration for complete derivatization of the nitrofuran metabolites.

The tube was cooled to room temperature, and approximately 3 to 5 mL of 1.0 mol/L dipotassium hydrogen phosphate solution was added to adjust the pH between 7 to 7.5. Once the pH was adjusted, 4 mL of ethyl acetate was added, and the sample was vortexed for 50 seconds and then centrifuged for 5 minutes at 4000 rpm. The supernatant was transferred into a 10 mL clean centrifuge tube. Another 4 mL of ethyl acetate was then added to the sample tube, and the supernatant was transferred to a 10 mL centrifuge tube after vortexing for 50 seconds and centrifuging for 5 minutes at 4000 rpm. The combined extract was shaken and centrifuged for 5 minutes at 4000 rpm. The ethyl acetate from the extract was dried by blowing nitrogen over it at 40 °C. The dried extract was reconstituted by dissolving it in 1 mL of 95/5 (V/V) methanol/water solution, and was filtered before LC/MS/MS analysis.

LC Condition and MS parameter settings

The LC and MS/MS method parameters are listed in Table 1, 2 and 3.

Table 1. LC Method Parameters.

Column	**PerkinElmer Quasar™ AQ, 100×2.1 mm, 1.7 µm** **Product Number:** N9308857 **Note:** PerkinElmer Quasar AQ colum=n was selected due to the best performance in terms of analyte retention, signal intensity and selectivity.				
Mobile Phase	Solvent A: 0.5 mM ammonium acetate in water Solvent B: Methanol				
	Step	Time (min)	Flow Rate (mL/min)	%A	%B
	1	Initial	0.4	60	40
	2	0.25	0.4	60	40
	3	6.25	0.4	40	60
	4	7.00	0.4	1	99
	5	7.10	0.4	60	40
	6	10.00	0.4	60	40
Analysis Time	7.0 min; Re-equilibration time: 3.0 min				
Oven Temp.	40 °C				
Injection Volume	20 µL				

Table 2. MS/MS Method Parameters.

Compound	Precursor Ion(m/z)	Product Ion(m/z)	EV(V)	CCL2(V)	CE (eV)
NBA-SEM	209.1	166.00*	26	-44	-11
	209.1	192.00	18	-40	-13
NBA- SEM-^{13}C-$^{15}N\neg_2$	212.1	168.00*	26	-44	-11
NBA-AHD	249.1	104.00	28	-48	-30
	249.1	134.00*	30	-44	-14
NBA- AHD-$^{13}C_3$	252.1	134.00*	30	-44	-14
NBA-AOZ	236.1	104.00	28	-48	-32
	236.1	134.00*	30	-48	-14
NBA- AOZ-D_4	240.1	134.00*	30	-48	-14
NBA-AMOZ	335.2	128.00	12	-72	-32
	335.2	291.00*	6	-72	-13
NBA- AMOZ-D_5	340.2	296.00*	6	-72	-13

*The asterisk labeled is quantitative ion.

Table 3. MS/MS Source Parameters

Parameter	Setting Value
Ionization Mode	ESI positive
Drying Gas Setting	75
HSID Temperature (℃)	320
Nebulizer Gas Setting	220
Electrospray Voltage(V)	5500
Source Temperature (℃)	500
Multipole 1 RF	300

Results and Discussion

The objective of this work was to assess the performance of the QSight 210 with a validated extraction method for the determination of nitrofuran residues in seafood.

A typical MRM chromatogram of 1 μg/L NBA-SEM, NBA-AHD, NBA-AOZ and NBA-AMOZ in neat solution is displayed in Figure 1.

Generally, a good and wide range of linearity was obtained for all analytes. Calibration curves over the concentration range from 0.05 to 10 μg/L, with regression coefficient (r^2) ≥ 0.995 in neat solution are shown in Figure 2 for all 4 of nitrofuran metabolites.

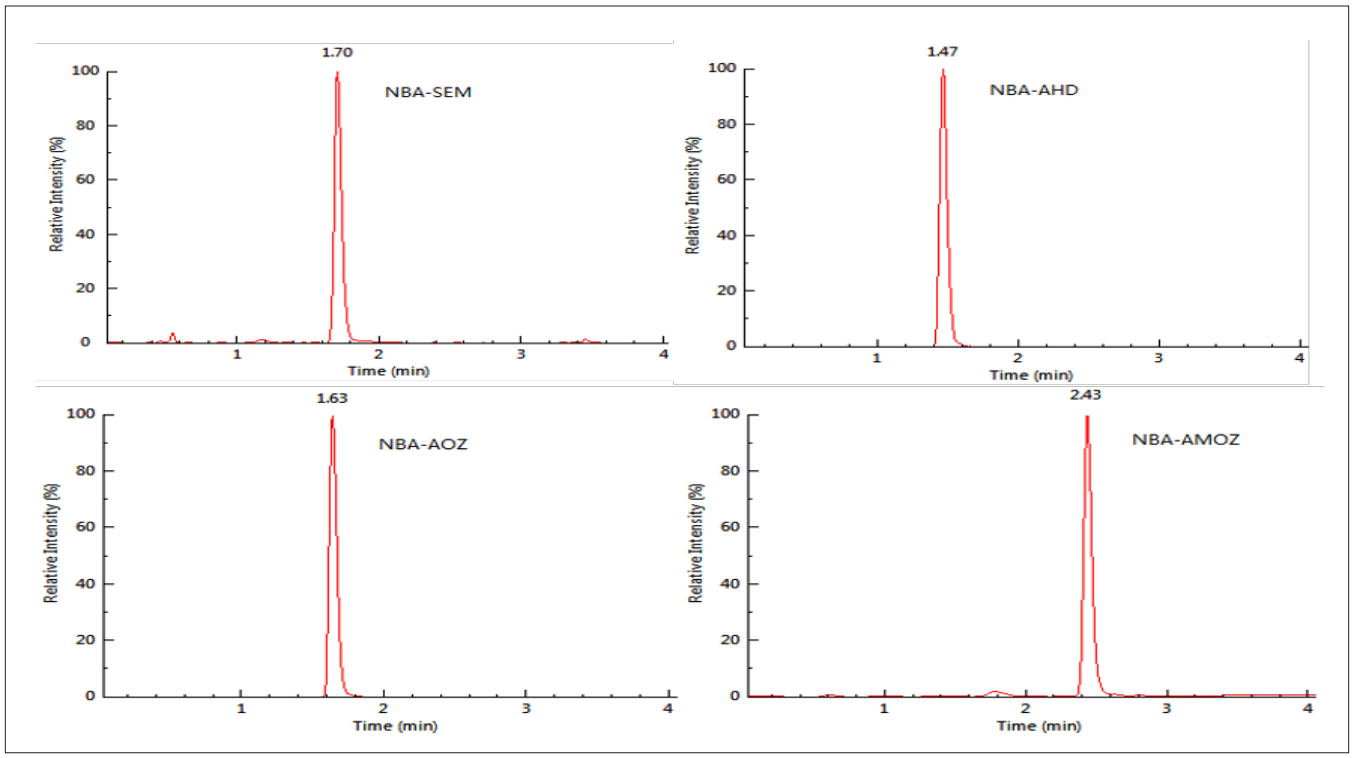

Figure 1. EIC of NBA-SEM, NBA-AHD, NBA-AOZ and AMOZ for quantitative transitions of spiked 0.5 g/kg in fish sample.

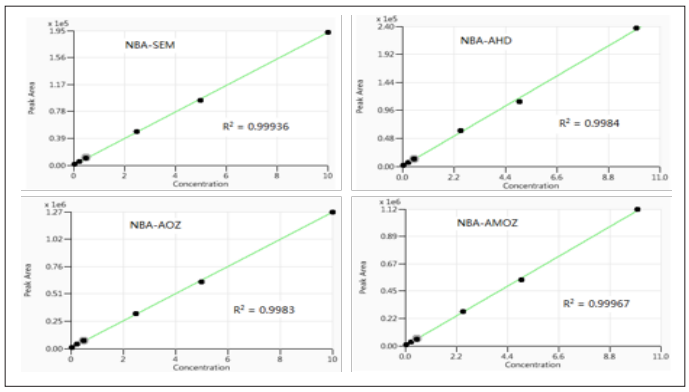

Figure 2. Calibration curves for NBA-SEM, NBA-AHD, NBA-AOZ and AMOZ.

	Sample	Sample Type	Ion Ratio (128/291) Area	Expected Ion Ratio (128/291) Area Range	Extrapolat by Area %
1	20190429NJF...	Standard	0.63	0.50, 0.76	N/A
2	20190429NJF...	Standard	0.60	0.50, 0.76	N/A
3	20190429NJF...	Standard	0.65	0.50, 0.76	N/A
4	20190429NJF...	Standard	0.60	0.50, 0.76	N/A
5	20190429NJF...	Standard	0.62	0.50, 0.76	N/A
	20190429NJF...	Standard	0.65	0.50, 0.76	N/A
7	20190429NJF...	Standard	0.65	0.50, 0.76	N/A
8	20190429NJF...	Standard	0.63	0.50, 0.76	N/A
9	20190429NJF...	Fish sample	0.60	0.50, 0.76	In Range
10	20190429NJF...		0.58	0.50, 0.76	In Range

Figure 4. NBA-AMOZ identified in fish sample by measuring ion ratio using Simplicity software.

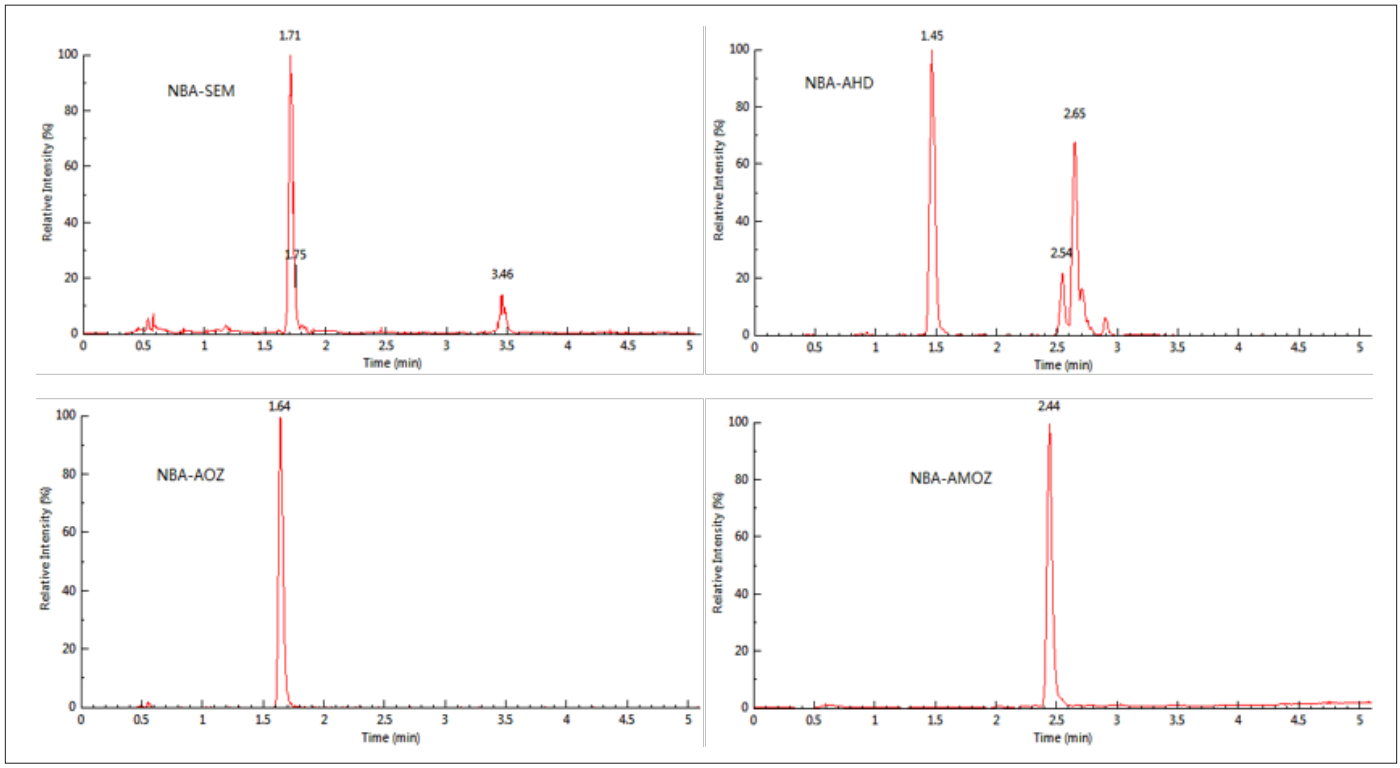

Figure 3. EIC of NBA-SEM, NBA-AHD, NBA-AOZ and AMOZ for quantitative transitions of spiked 0.5 g/kg in fish sample.

The limit of quantitation (LOQ) was calculated based on a signal-to-noise ratio ≥ 10 for the analyte's quantifier transition. Overall, the calculated LOQs for the each of the nitrofurans were lower than 0.05 µg/L, which is 20 times lower than the regulated MRPL amounts (1.0 µg/L).

The developed LC/MS/MS method was applied for the analysis of nitrofurans in fish samples. Example chromatograms of nitrofuran metabolites spiked at 0.5 µg/kg of nitrofurans in the fish sample are shown in Figure 3.

Simplicity software provided automatic calculation of ion ratios, and the capability of setting specific acceptance criteria of ion ratio for confirmation of analytes[2]. The presence of each of the nitrofuran metabolites in the fish sample was determined by confirming that the product ion ratios (qualifier vs. quantifier) were within the 20% tolerance windows of the expected ion ratio.

Figure 4 shows an example of the Simplicity software used to confirm the identification of NBA-AMOZ in the fish sample by comparing ion ratio for analyte within acceptance criteria of expected ion ratio.

Conclusion

The PerkinElmer LX 50 coupled to the QSight 210 system provides good sensitivity for the identification and quantification of nitrofuran metabolites in seafood. This efficient method meets the requirements of the low regulated limits of quantification for routine screening and quantitation analysis, making it an ideal application for regulatory compliance.

References

1. Announcement No. 783 of the Ministry of Agriculture, Jan 2016, China.
2. Guidance document on the analytical quality control and method validation procedures for pesticides residues analysis in food and feed. European Commission SANTE document 11945/2015.

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