

Analysis of Nitrofurán Metabolites in Tilapia Using Agilent 6410 Triple Quadrupole Application

Food Safety

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Abstract

The metabolites of nitrofurán antibiotics banned in meat and meat products are analyzed by LC/MS/MS with the new Agilent 6410 triple quadrupole. The method is shown to be highly sensitive, to 0.01 ppb (10 ppt), for each of the four analytes. Calibration from 0.1 ppb to 10 ppb is presented with all criteria for confirmation as set by the European Union decisions for analytical method performance. Extracts of tilapia are used to show the performance of the LC/MS/MS method for aquaculture samples.

Introduction

Nitrofurans are inexpensive antibiotics used for Gram positive and Gram negative bacteria. They have been used to treat gastrointestinal and dermatological infections in farm animals and fish. In addition, they have been used to treat bacteria in bees. Because both parent compounds and their metabolites are suspect carcinogens, they have been banned around the world. The Rapid Alert System for Food and Feed Annual report for 2005 [1] shows that these compounds continue to be detected in food samples and remains a major concern for food safety. The four compounds—furazolidone, furaltadone, nitrofurantoin, and nitrofurazone—have been found to metabolize rapidly, and the metabolites bind to muscle tissue. Thus the

analytical detection of the metabolites and not the parent compounds are required in samples of animal origin.

The criteria for detection and confirmation of veterinary drugs in animal and animal products established by the European Union (EU) [2] has been accepted in much of the world. This criteria mandates a separation technique combined with a spectrometric technique. For banned substances such as the nitrofurans, no maximum residue limit (MRL) could be set. Therefore a minimum required performance level (MRPL) was set at 1 µg/kg for each metabolite [3]. Only LC/MS could meet these criteria, and very good methods have been reported [4–6]. However, the most widely accepted methodology employs triple quadrupole tandem mass spectrometers. This is the first report showing analysis of these metabolites using the new Agilent triple quadrupole LC/MS system.

Experimental

Chemicals

Derivatized standards of nitrofurán metabolites and all chemicals for sample preparation were received from a food manufacturing company. Acetonitrile was HPLC grade from Merck (Darmstadt, Germany). Formic acid was reagent grade from Merck (Darmstadt, Germany).

Sample Preparation

The accepted procedure for sample preparation was followed. To 2 g of tilapia was added 15 mL 0.125 M HCl and the mixture homogenized.



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To this solution, a 50- μ L solution of 2-nitrobenzaldehyde (50 mM in DMSO) was added and shaken. The solution was then incubated at 37 °C for 16 hours. This was followed by neutralization to pH -7 with NaOH and K₂HPO₄. The neutral derivatized sample was then extracted with ethyl acetate, concentrated to dryness, and reconstituted in 100 L of initial LC mobile phase. Standards of the four metabolites were spiked into 0.125 M HCl, derivatized, and extracted for calibration using the same procedure as was used for the samples of tilapia.

LC/MS/MS Method

LC Conditions

Instrument:	Agilent 1100 LC
Column:	C18, 2.1 mm \times 150 mm, 3 μ m
Column temp.:	40 °C
Mobile phase:	A = 0.1% formic acid in water B = acetonitrile
Gradient:	22% B at 0 min 99% B at 6 min 99% B at 9 min
Flow rate:	0.3 mL/min
Injection volume:	50 nL

MS Conditions

Instrument:	Agilent 6410 LC/MS Triple Quadrupole
Ionization mode:	Positive ESI
Drying gas flow:	10 L/min
Nebulizer:	35 psig
Drying gas temp.:	350 °C
V _{cap} :	5000 V

Quantitation

Quantitative analysis was done with the first transition listed in the MRM parameter table. The second transition was used as a qualifier ion for confirmation as per the confirmation criteria. Quantitative results were performed with the new MassHunter quantitative analysis software.

Results and Discussion

The instrument sensitivity is an important performance parameter for this analysis when considering the derivatization and extraction needed to meet the required detection limit of 1 ppb for each metabolite, aminohydantoin (AH), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidinone (AOZ), and semicarbazide (SC). To demonstrate this performance, a standard of each 2-nitrobenzaldehyde (2-NBA) derivatized metabolite is shown at 0.01 ppb (10 ppt) in Figure 1. The structure of each derivatized metabolite is given and each is shown with a signal-to-noise ratio of greater than 3:1. Another indicator of performance is linearity. Calibration curves from this concentration (10 ppt) to 10 ppb are displayed in Figure 2 showing the linearity for each compound.

Treatment of fish with nitrofurans is a continual problem for food safety and import into EU member countries. To demonstrate the capability of the Agilent triple quadrupole LC/MS, tilapia samples were spiked with the four metabolites, hydrolyzed, derivatized, and extracted. An analysis of a tilapia extract at 500 ppt is shown in Figure 3 and demonstrates the signal obtained at half the MRPL. In addition to meeting the sensitiv-

MRM Mode Parameters

Compound	Transition	Dwell time (ms)	Fragmentor voltage (V)	Collision energy (V)	MS2 resolution
AMOZ	335.1 \rightarrow 291.4	60	100	5	Unit
	335.1 \rightarrow 262.4	60	100	5	Unit
SC	209.1 \rightarrow 192.3	60	100	5	Unit
	209.1 \rightarrow 166.3	60	100	5	Unit
AH	249.1 \rightarrow 134.2	60	100	5	Unit
	249.1 \rightarrow 104.2	60	100	5	Unit
AOZ	236.0 \rightarrow 134.1	60	100	5	Unit
	236.0 \rightarrow 104.1	60	100	5	Unit

ity requirement, the analysis must also meet the confirmation criteria, including both chromatographic retention time match with the standards and measuring a qualifying ion with a relative intensity ratio within a specified tolerance of the quantitation ion. This tolerance is set by the ratio obtained when analyzing standards and increasing as that ratio decreases. This tolerance ranges from 20% for ions with relative ratio intensities above 0.5 and to 50% for ratios below 0.1.

Table 1 shows tilapia samples spiked with the metabolites, derivatized and extracted. The spikes

were used as the calibrants, so the final concentration is obtained from the curve. The table is produced as the batch using the MassHunter software results with outliers highlighted in blue (low) and red (high). The table shows that in the blank a peak is found within the tolerance set for the retention time of AMOZ but the qualifier ratio is low. For AOZ and SC, retention times for suspect peaks are below the specified retention in the same blank. For AH, the 0.5 ppb spike, the qualifier ion ratio is outside the 35% tolerance limit set for this ion (again low).

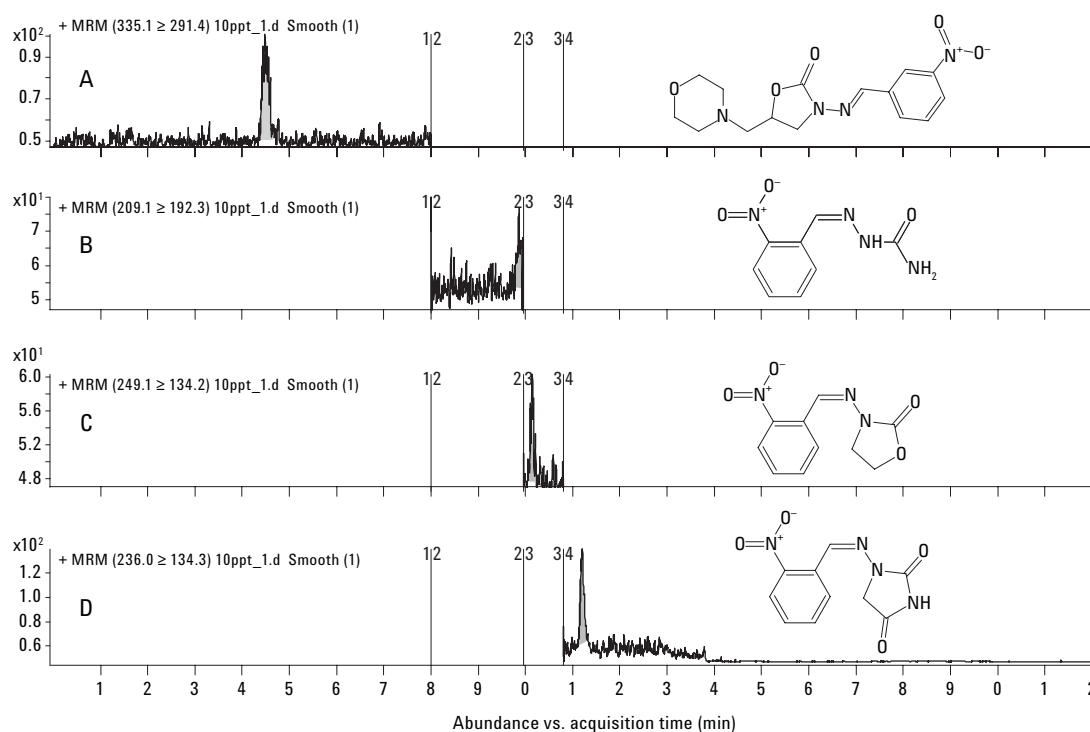


Figure 1. The MRM quant ion chromatogram for each derivatized metabolite at 10 ppt of A) 2-NBA AMOZ, B) 2-NBA SC, C) 2-NBA AH, and D) 2-NBA-AOZ

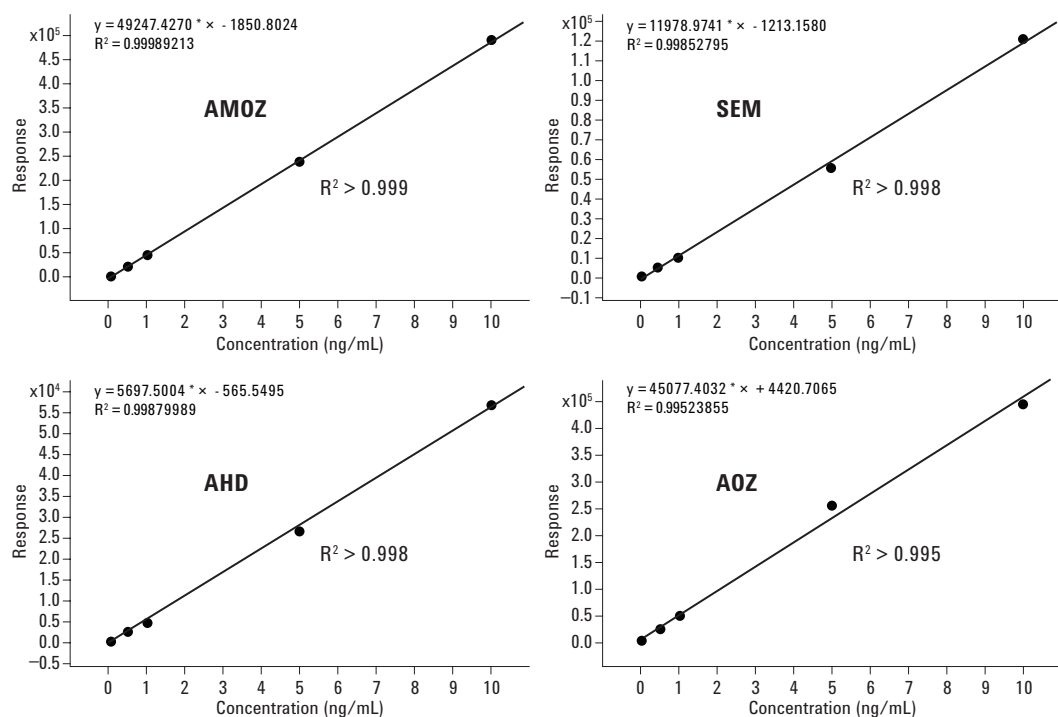


Figure 2. Calibration curves of nitrofurans metabolites linear range from 10 ppt to 10 ppb.

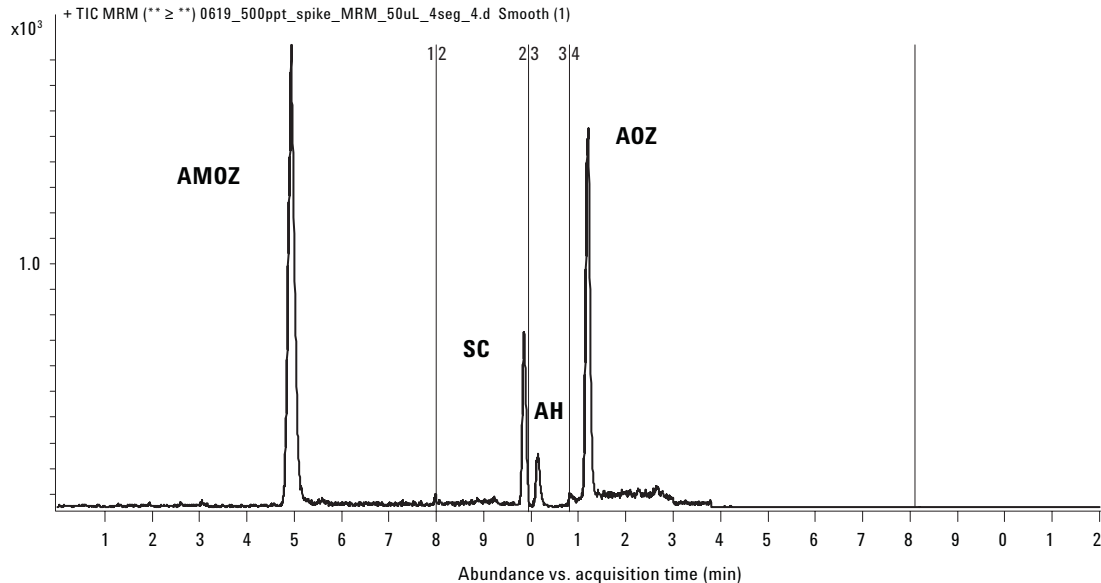


Figure 3. Spiked tilapia sample extract at 500 ppt each metabolite.

Table 1. Analysis of Talapia Spikes Self-Calibrated. Note Qualifier Ratios and Retention Times Reported.

		AH		Qualifier		AMTZ		Qualifier		AOZ		Qualifier		SC		Qualifier	
Name	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.	Ratio	RT	Final Conc.
Nitrofurantoin Blank		0.00		2.72	0.02	8.70	8.71	0.00		7.94	0.00						
Nitrofurantoin 0.5 ppb	8.66	0.54	6.07	2.62	0.51	17.44	9.19	0.37	7.99	8.44	0.50	65.32					
Nitrofurantoin 1 ppb	8.66	1.07	15.83	2.64	0.92	20.42	9.19	1.06	10.19	8.44	1.05	70.85					
Nitrofurantoin 3 ppb	8.67	3.42	13.78	2.65	3.41	18.67	9.20	3.18	9.51	8.44	2.89	67.47					
Nitrofurantoin 5 ppb	8.66	5.71	14.24	2.66	4.66	19.28	9.19	4.89	8.61	8.44	5.05	69.06					

Conclusions

This work shows the high performance of the new Agilent 6410 LC/MS triple quadrupole system for the sensitive analysis of the nitrofurantoin metabolites in fish samples. The system readily meets the performance requirements and provides advanced quantitation software for calculating and reporting all confirmation parameters specified by the European Commission decision.

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