

# Highly Selective Detection and Identification of Nitrofurans Metabolites in Honey Using LC-MS/MS

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## Introduction

Nitrofurans are broad-spectrum antibiotics used to treat bees and other animals with bacterial infections. As a result of dosing bees with these antibiotics their metabolites are sometimes found in honey. Female rats given nitrofurans in both low and high doses have exhibited increased incidences of ovarian granulose cell tumors. In the same study, newborn mice showed an increased incidence of pulmonary papillary adenomas.<sup>2</sup> As a result, nitrofurans have been banned from use in food-producing animals in Australia (1993), the European Union (EU) (1995), The Philippines (2001), the United States (2002), Brazil (2002), Thailand (2002), and other countries.

Several studies have shown that animals rapidly metabolize nitrofurans within a few hours so detection has focused on the metabolites rather than the native drug.<sup>3</sup> The metabolites accumulate in tissue where they are stable and can be analyzed long after the nitrofurans have been administered. The EU has established a harmonized minimum required performance limit (MRPL) for the detection of residues of nitrofurans at one part per billion (ppb). Some European laboratories have been working to a detection limit of 0.3 ppb for several of the nitrofurans metabolites.<sup>4</sup> The EU recently tightened its inspection policy for food imports after nitrofurans residues were found in shrimp, fish, and poultry imports. This significantly reduced the volume of those imports.

As a result, food exporting countries are required to detect nitrofurans metabolites at very low levels. There are several challenges that must be overcome. The first is that honey, as well as other food products, provides a complex matrix which increases the difficulty of sample preparation. Second, efficient chromatography is critical in order to provide good separation of the various metabolites from each other and any contaminants that might be present. The third and most important requirement is a very high level of sensitivity and linearity in the mass spectrometer in order to achieve the required high levels of accuracy in quantifying the metabolites. This note describes LC-MS methods developed on the Thermo Scientific TSQ Quantum Discovery by the Food Science Laboratories and Eidomet in Argentina in cooperation with Thermo Fisher Scientific. The method exceeds all current detection limits as set by the EU.

## Goal

To demonstrate the ability to accurately quantitate nitrofurans metabolites at levels as low as 0.3 ppb in a matrix consisting of honey using the TSQ Quantum Discovery.

## Experimental Conditions

In this study, 2 grams of honey samples were treated with four nitrofurans metabolites, AOZ, AMOZ, SEM, and AHD.<sup>5</sup> An aliquot of honey was dissolved in 125 mM HCl and derivatized with 2-nitrobenzaldehyde and the mixture was shaken for 3 minutes. The slurry was then incubated at 37°C in a water bath for 17 hours. The mixture was then cooled to room temperature and neutralized by adding potassium phosphate to adjust the pH to approximately 7.0. Ethyl acetate was added to the slurry and it was hand shaken for 2 minutes and centrifuged for 15 minutes. The organic phase was collected into a tube, water added, and the mixture centrifuged. The supernatant was evaporated to dryness under a stream of nitrogen. The dry residue was reconstituted with water and injected into a filter cartridge. The residue was then washed with water and eluted with hexane, then analyzed by LC-MS/MS.

HPLC was performed on a Thermo Scientific Surveyor™ MS Pump with a Surveyor Autosampler from Thermo Fisher Scientific. A 100 × 2.1 mm, 3 μm HPLC column was used. The mobile phase consisted of A (water containing 0.05% acetic acid) and B (methanol containing 0.05% acetic acid). The gradient program was as follows: 0-3.0 min. 90% A 10% B; 3.0-5.0 min. 85% A 15% B; 5.0 to 10.0 min. 75% A 25% B; 10.0-15.0 min. 70% A 30% B; 15.0-17 min. 65% A 35% B; 15-17 min. 65% A 35% B; 17.0-21.0 min. 60% A 40% B; and 21.0-25.0 min. 90% A 10% B.

Sample analysis was performed on a TSQ Quantum Discovery mass spectrometer. The 0-13.4 min segment eluted AMOZ and d5-AMOZ while the 13.4 to 25 min segment eluted AOZ, d4-AOZ, SEM, and AHD. Samples were analyzed using positive electrospray ionization (ESI) in SRM mode. The scan width was 0.002 *m/z* and the scan time was 0.1 second. A peak width of 0.7 FWHM was used in both Q1 and Q3. Argon was used as the collision gas at a pressure of 1.5 mTorr.

## Key Words

- TSQ Quantum Discovery™
- LC-MS/MS
- Quantitative Analysis

Analyte	Precursor Ion	Product Ion	Collision Energy
AMOZ	335	262	15
	335	291	10
d5-AMOZ	340	296	10
	236	78	15
AOZ	236	134	8
	240	134	8
SEM	209	166	6
	209	192	5
AHD	249	104	16
	249	134	7

Table 1: Transition reactions for MS/MS

The nitrofurantolone metabolites were quantified with five calibration standards at nominal concentrations of 0.63 ppb, 1.04 ppb, 2.09 ppb, 4.17 ppb, and 8.34 ppb. The area ratio of the analyte versus the quality control (QC) samples was plotted against the standard concentration ratio. The linearity of the MS response was determined by calculating the relative standard deviation (RSD) of

the average of results from a series of injections at a single concentration. The method is generally considered to be validated if the RSD is less than 15%. The recovery ratio was also calculated by injecting a known amount of sample and comparing it with the calculated amount delivered by the detector.

## Results and Discussion

Tables 2 through 5 report the results obtained with the standard and QC samples in honey for each metabolite. Note that for clarity purposes, all areas reported in the tables are divided by 1000. The concentrations of the QC samples were calculated by comparing the area to the standards. Then the relative standard deviation for each set of QC samples was calculated. The RSD for AOZ ranged from 6.7% at 0.3 ppb to 2.7% at 4 ppb. The RSD for AMOZ ranged from 3.60% at 0.3 ppb to 2.50% at 4 ppb. The RSD for AHD ranged from 9.0% at 0.3 ppb to 2.9% at 4 ppb. The RSD for SEM ranged from 8.3% at 0.3 ppb to 4.2% at 4 ppb.

Equation		IDENT. LEVEL					NOMINAL CONC.			AREA AOZ			AREA ISTD (d4-AOZ)			AREA RATIO		
Y = 0.5424 X + 0.0973		R <sup>2</sup> = 0.9998					(ppb)			(ppb)			(ppb)			(ppb)		
Std 0.6 ppb	0.63	217.3	559	0.39														
Std 1 ppb	1.04	344.1	524.3	0.66														
Std 2 ppb	2.09	671.2	532.3	1.26														
Std 4 ppb	4.17	1286.3	567.2	2.27														
Std 8 ppb	8.34	2601.2	580.6	4.48														

IDENT. LEVEL	AREA AOZ	AREA ISTD (d4-AOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	1609.6	356.5	4.52	4.172	4.204	0.77%		100.8
QC-4ppb	1728.1	381.1	4.53	4.172	4.222	1.20%		101.2
QC-4ppb	1849.5	392.6	4.71	4.172	4.253	1.94%	2.7%	101.9
QC-4ppb	1743.8	378.7	4.60	4.172	4.155	-0.41%		99.6
QC-4ppb	1919.4	389.7	4.93	4.172	4.451	6.69%		106.7
QC-2ppb	864.7	372.9	2.32	2.086	2.111	1.20%		101.2
QC-2ppb	912.9	370.1	2.47	2.086	2.252	7.96%		108.0
QC-2ppb	789.1	358.8	2.20	2.086	1.938	-7.09%	5.5%	92.9
QC-2ppb	924.2	377.7	2.45	2.086	2.166	3.84%		103.8
QC-2ppb	912.2	375.5	2.43	2.086	2.150	3.07%		103.1
QC-1ppb	436.6	356.7	1.22	1.043	1.068	2.40%		102.4
QC-1ppb	466.2	390.9	1.19	1.043	1.038	-0.48%		99.5
QC-1ppb	431.3	359.3	1.20	1.043	1.017	-2.49%	4.7%	97.5
QC-1ppb	477.7	358.6	1.33	1.043	1.138	9.11%		109.1
QC-1ppb	451.5	346.4	1.30	1.043	1.112	6.62%		106.6
QC-0.5ppb	258.3	376.1	0.69	0.521	0.556	6.72%		106.7
QC-0.5ppb	271.1	388.1	0.70	0.521	0.567	8.83%		108.8
QC-0.5ppb	260.2	366.4	0.71	0.521	0.565	8.45%	7.4%	108.4
QC-0.5ppb	228.8	372.2	0.61	0.521	0.477	-8.45%		91.6
QC-0.5ppb	249.0	380.6	0.65	0.521	0.513	-1.54%		98.5
QC-0.3ppb	162.6	357.3	0.46	0.313	0.335	7.03%		107.0
QC-0.3ppb	146.9	369.5	0.40	0.313	0.280	-10.54%		89.5
QC-0.3ppb	145.5	341.0	0.43	0.313	0.304	-2.88%	6.7%	97.1
QC-0.3ppb	171.5	412.6	0.42	0.313	0.293	-6.39%		93.6
QC-0.3ppb	156.0	369.4	0.42	0.313	0.300	-4.15%		95.8

Table 2: AOZ data

Equation	
$Y = 0.6123 X + 0.0413$	$R^2 = 1.0000$

LEVEL	CONC. (ppb)	AMOZ	ISTD (d5-AMOZ)	AREA RATIO
Std 0,6 ppb	0.6	760.3	1799.0	0.40
Std 1 ppb	1.0	1320.8	1936.8	0.69
Std 2 ppb	2.0	2811.8	2170.0	1.37
Std 4 ppb	4.0	5582.3	2166.6	2.68
Std 8 ppb	8.0	11265.8	2231.2	5.32

IDENT. LEVEL	AREA AMOZ	AREA ISTD (d5-AMOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	8814.0	1700.1	5.18	4.000	4.200	5.00%		105.0
QC-4ppb	6866.9	1376.6	4.99	4.000	4.040	1.00%	2.50%	101.0
QC-4ppb	9431.2	1894.0	4.98	4.000	3.938	-1.55%		98.5
QC-4ppb	7438.6	1464.6	5.08	4.000	4.017	0.43%		100.4
QC-4ppb	7235.0	1439.7	5.03	4.000	3.974	-0.65%		99.4
QC-2ppb	3117.9	1246.4	2.50	2.000	2.000	0.00%	3.45%	100.0
QC-2ppb	3951.3	1511.5	2.61	2.000	2.100	5.00%		105.0
QC-2ppb	3617.4	1477.9	2.45	2.000	1.914	-4.30%		95.7
QC-2ppb	3214.0	1263.0	2.54	2.000	1.991	-0.45%		99.6
QC-2ppb	4164.8	1662.7	2.50	2.000	1.959	-2.05%	98.0	
QC-1ppb	1742.6	1357.0	1.28	1.000	1.010	1.00%	2.90%	101.0
QC-1ppb	2178.9	1687.2	1.29	1.000	1.020	2.00%		102.0
QC-1ppb	1864.6	1476.0	1.26	1.000	0.967	-3.30%		96.7
QC-1ppb	2307.9	1853.7	1.25	1.000	0.952	-4.80%		95.2
QC-1ppb	2281.8	1781.8	1.28	1.000	0.981	-1.90%	98.1	
QC-0.5ppb	1153.4	1710.4	0.67	0.500	0.517	3.40%	4.63%	103.4
QC-0.5ppb	850.2	1345.0	0.63	0.500	0.482	-3.60%		96.4
QC-0.5ppb	1067.5	1602.7	0.67	0.500	0.489	-2.20%		97.8
QC-0.5ppb	830.8	1331.6	0.62	0.500	0.455	-9.00%		91.0
QC-0.5ppb	1067.2	1640.8	0.65	0.500	0.477	-4.60%	95.4	
QC-0.3ppb	548.5	1289.1	0.43	0.300	0.314	4.67%	3.60%	104.7
QC-0.3ppb	485.0	1139.9	0.43	0.300	0.314	4.67%		104.7
QC-0.3ppb	559.5	1309.3	0.43	0.300	0.298	-0.67%		99.3
QC-0.3ppb	727.5	1719.1	0.42	0.300	0.295	-1.67%		98.3
QC-0.3ppb	529.3	1264.3	0.42	0.300	0.291	-3.00%	97.0	

Table 3: AMOZ data

Equation	
$Y = 0.1396 X + 0.0174$	$R^2 = 0.9955$

IDENT. LEVEL	NOMINAL CONC. (ppb)	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO
Std 0,6 ppb	0.61	70.9	559.0	0.127
Std 1 ppb	1.02	112.2	524.3	0.210
Std 2 ppb	2.03	200.3	532.3	0.380
Std 4 ppb	4.06	416.4	567.2	0.730
Std 8 ppb	8.13	797.2	580.6	1.370

IDENT. LEVEL	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	436.6	356.5	1.22	4.063	3.585	-11.76%	2.9%	88.2
QC-4ppb	461.5	381.1	1.21	4.063	3.543	-12.80%		87.2
QC-4ppb	397.0	392.6	1.01	4.063	3.559	-12.40%		87.6
QC-4ppb	408.3	378.7	1.08	4.063	3.799	-6.50%		93.5
QC-4ppb	402.6	389.7	1.03	4.063	3.638	-10.46%	89.5	
QC-2ppb	222.6	372.9	0.60	2.034	1.683	-17.26%	2.6%	82.7
QC-2ppb	224.4	370.1	0.61	2.034	1.711	-15.88%		84.1
QC-2ppb	179.1	358.8	0.50	2.034	1.726	-15.14%		84.9
QC-2ppb	194.8	377.7	0.52	2.034	1.785	-12.24%		87.8
QC-2ppb	181.8	375.5	0.48	2.034	1.672	-17.80%	82.2	
QC-1ppb	123.0	356.7	0.34	1.017	0.919	-9.64%	5.5%	90.4
QC-1ppb	126.8	390.9	0.32	1.017	0.857	-15.73%		84.3
QC-1ppb	100.6	359.3	0.28	1.017	0.941	-7.47%		92.5
QC-1ppb	90.6	358.6	0.25	1.017	0.843	-17.11%		82.9
QC-1ppb	98.3	346.4	0.28	1.017	0.954	-6.19%	93.8	
QC-0.5ppb	78.7	376.1	0.21	0.508	0.509	0.20%	9.2%	100.2
QC-0.5ppb	70.6	388.1	0.18	0.508	0.425	-16.34%		83.7
QC-0.5ppb	57.7	366.4	0.16	0.508	0.502	-1.18%		98.8
QC-0.5ppb	50.8	372.2	0.14	0.508	0.427	-15.94%		84.1
QC-0.5ppb	52.6	380.6	0.14	0.508	0.433	-14.76%	85.2	
QC-0.3ppb	44.5	357.3	0.12	0.305	0.251	-17.70%	9.0%	82.3
QC-0.3ppb	45.8	369.5	0.12	0.305	0.249	-18.36%		81.6
QC-0.3ppb	35.4	341.0	0.10	0.305	0.309	1.31%		101.3
QC-0.3ppb	39.6	412.6	0.10	0.305	0.281	-7.87%		92.1
QC-0.3ppb	35.1	369.4	0.10	0.305	0.278	-8.85%	91.1	

Table 4: AHD data

Equation
$Y = 0.1396 X + 0.0174$ $R^2 = 0.9955$

IDENT. LEVEL	NOMINAL CONC. (ppb)	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO
Std 0,6 ppb	0.61	70.9	559.0	0.127
Std 1 ppb	1.02	112.2	524.3	0.210
Std 2 ppb	2.03	200.3	532.3	0.380
Std 4 ppb	4.06	416.4	567.2	0.730
Std 8 ppb	8.13	797.2	580.6	1.370

IDENT. LEVEL	AREA AHD	AREA ISTD (d4-AOZ)	AREA RATIO	SPECIFIED CONC. (ppb)	CALCULATED CONC. (ppb)	Diff %	RSD %	RECOVERY %
QC-4ppb	436.6	356.5	1.22	4.063	3.585	-11.76%	2.9%	88.2
QC-4ppb	461.5	381.1	1.21	4.063	3.543	-12.80%		87.2
QC-4ppb	397.0	392.6	1.01	4.063	3.559	-12.40%		87.6
QC-4ppb	408.3	378.7	1.08	4.063	3.799	-6.50%		93.5
QC-4ppb	402.6	389.7	1.03	4.063	3.638	-10.46%		89.5
QC-2ppb	222.6	372.9	0.60	2.034	1.683	-17.26%	2.6%	82.7
QC-2ppb	224.4	370.1	0.61	2.034	1.711	-15.88%		84.1
QC-2ppb	179.1	358.8	0.50	2.034	1.726	-15.14%		84.9
QC-2ppb	194.8	377.7	0.52	2.034	1.785	-12.24%		87.8
QC-2ppb	181.8	375.5	0.48	2.034	1.672	-17.80%		82.2
QC-1ppb	123.0	356.7	0.34	1.017	0.919	-9.64%	5.5%	90.4
QC-1ppb	126.8	390.9	0.32	1.017	0.857	-15.73%		84.3
QC-1ppb	100.6	359.3	0.28	1.017	0.941	-7.47%		92.5
QC-1ppb	90.6	358.6	0.25	1.017	0.843	-17.11%		82.9
QC-1ppb	98.3	346.4	0.28	1.017	0.954	-6.19%		93.8
QC-0.5ppb	78.7	376.1	0.21	0.508	0.509	0.20%	9.2%	100.2
QC-0.5ppb	70.6	388.1	0.18	0.508	0.425	-16.34%		83.7
QC-0.5ppb	57.7	366.4	0.16	0.508	0.502	-1.18%		98.8
QC-0.5ppb	50.8	372.2	0.14	0.508	0.427	-15.94%		84.1
QC-0.5ppb	52.6	380.6	0.14	0.508	0.433	-14.76%		85.2
QC-0.3ppb	44.5	357.3	0.12	0.305	0.251	-17.70%	9.0%	82.3
QC-0.3ppb	45.8	369.5	0.12	0.305	0.249	-18.36%		81.6
QC-0.3ppb	35.4	341.0	0.10	0.305	0.309	1.31%		101.3
QC-0.3ppb	39.6	412.6	0.10	0.305	0.281	-7.87%		92.1
QC-0.3ppb	35.1	369.4	0.10	0.305	0.278	-8.85%		91.1

Table 5: SEM data

Figure 1 shows the chromatograms of a negative and a positive unknown sample set in which the AOZ metabolite is clearly identified at the 0.6 ppb level. Table 6 summarizes the average method results for the four metabolites over multiple sample sets. The limits of detection (LODs) and

limits of quantification (LOQs) are reported and the data shows good accuracy at the LOQ levels for all the metabolites. The LODs and LOQs achieved on the four nitrofurantol metabolites are all at sub ppb levels.

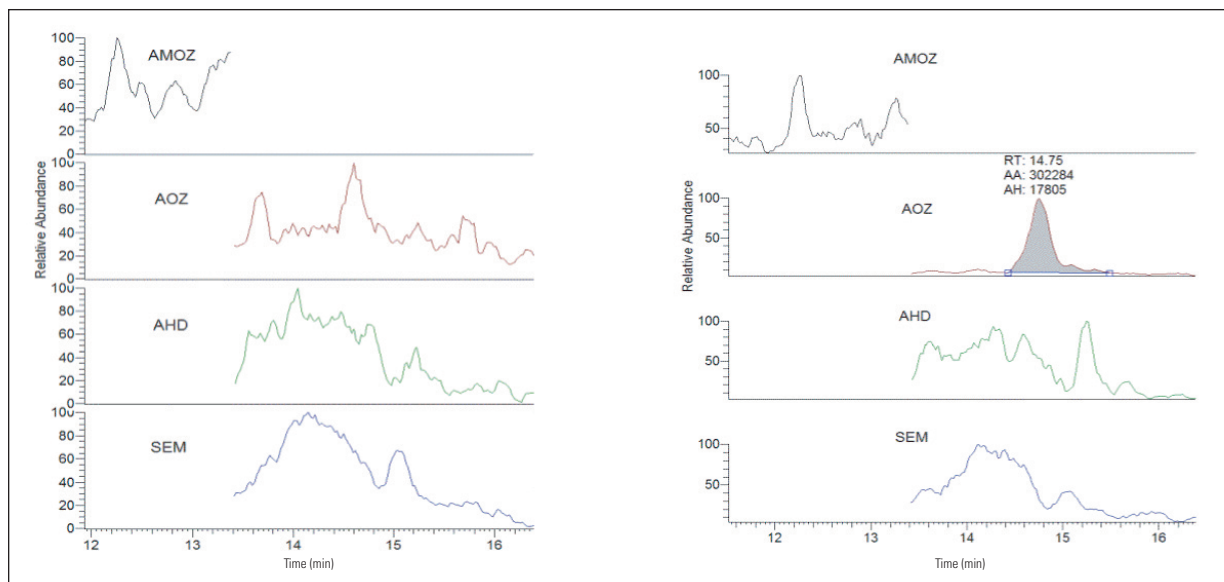


Figure 1: Identification of AOZ metabolite in honey at 0.6 ppb level

ANALYTE	MATRIX	LOD (ppb)	LOQ (ppb)	ANALYTICAL RANGE (ppb)	% RECOVERY	RANGE % REC	CV %	INCERTITUDE %
AMOZ	Honey	0.04	0.09	0,04-4,0	99.3	88,8-109.8	3.5	7.1
AHD	Honey	0.06	0.16	0,06-4,063	88.5	71.2-105.8	6.5	13.0
AOZ	Honey	0.06	0.14	0,06-4,172	101.2	84.2-118.5	5.6	11.3
SEM	Honey	0.08	0.18	0,08-4,064	94.3	70.6-117.8	8.3	16.6

Table 6: Summary of Method Results

## Conclusion

An LC-MS/MS assay to detect and identify nitrofuran metabolites was developed using the TSQ Discovery. The extraction method appears to be extremely robust and reliable with good recovery efficiency (better than 80%), allowing unambiguous routine identification and quantification of all nitrofuran metabolites in honey. The LC-MS/MS-based method described here provides high speed, excellent sensitivity, and specificity of detection. The assay demonstrated the ability to easily meet the 0.3 ppb limit of quantitation that is required by the most stringent current requirements of food monitoring applications operating under FDA and EC regulations.

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