Analysis of Nitrofuran Metabolites in Honey Using the SCIEX Triple Quad™ 3500 System

Chandrasekar M[1]; Santosh Kapil G[1]; Aman Sharma[1]; Anoop Kumar[1]; Manoj G Pillai[1] & Jianru Stahl-Zeng[2]
[1]SCIEX, India, [2]SCIEX, Germany

Nitrofurans are broad spectrum antibacterial agents which were used in the treatment of bacterial infections in bee colony health. Nitrofurans have been prohibited in food-producing animals in the European Union and most other Countries for public health and safety concerns. The nitrofurans are unstable and easily metabolized within a few hours but Nitrofuran metabolites are highly stable in nature. Several methods have been described in the analysis of nitrofuran metabolite in honey samples by incubation period for derivatization with nitrobenzaldehyde in overnight or 16 hours at 37 ºC.

The LC-MS/MS method developed on SCIEX Triple Quad 3500 System described here for the quantitation of nitrofuran metabolites in honey was found to meet the regulatory requirements of 1 µg/kg.

Key Feature of Nitrofuran Method

- Simple sample preparation method with no derivatization required
- Fast LC-MS/MS method with good specificity for detection of four nitrofurans from honey matrix
- Sufficient sensitivity on the SCIEX Triple Quad 3500 system for analyzing nitrofuran levels well below the MRPL levels required by the EU

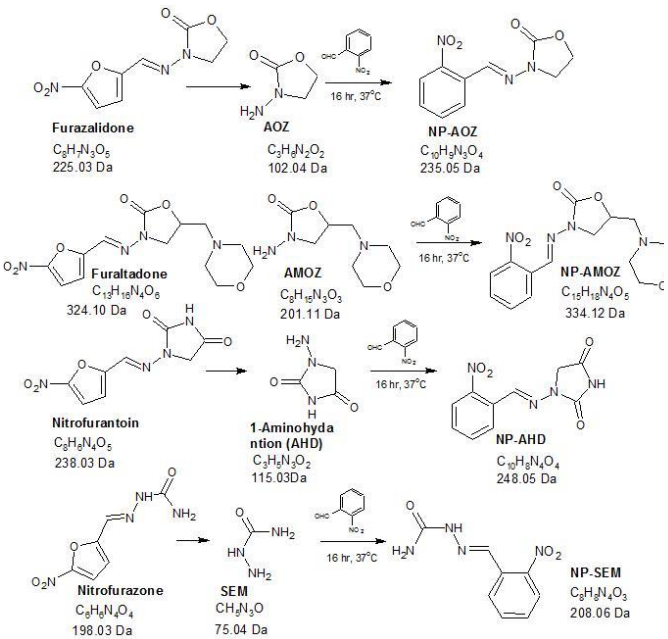

Figure 1. Structures of Nitrofuran, Nitrofuran Metabolites and Nitrophenyl Derivatives.

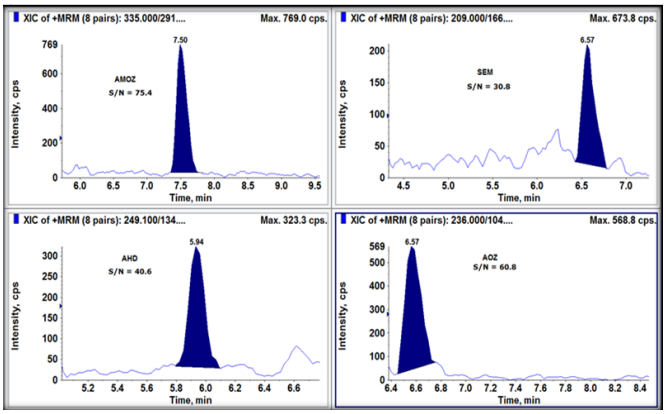

Figure 2: MRM Data. Signal to noise of AMOZ, AHD, SEM and AOZ at MRPL level (1.0 ng/ml) in honey matrix sample.

Methods

Sample Preparation: Nitrofuran metabolite standards were purchased from Clearsynth and 2-Nitrobenzaldehyde was purchased from Sigma Aldrich(≥99% Purity). All other chemicals used were of LC-MS grade, commercially available. Honey samples were procured from local market of Delhi and Gurgaon, India and were stored at room temperature until end of analysis.

Honey sample (1 gram) was mixed with 3 mL of HCl (0.1 M) and 50 mM of 2-Nitrobenzaldehyde (0.3 mL), vortexed and incubated on ultrasonic bath for 16hr. Next, 0.6 mL of 1M K_2HPO_4 solution and 10 mL of ethyl acetate was added, the sample was vortexed then centrifuged at 4000 rpm. The supernatant was evaporated to dryness, reconstituted with 1 mL of methanol:water (5:95) and 10 µL is used for LC-MS/MS analysis.

Chromatography: LC separation was achieved using the Shimadzu prominence system with an Eclipse plus C18 (4.6×150 mm) 5 µm column at flow rate of 0.4 mL/min. The injection volume was set to 10 µL. Gradient profile is give in Table 1.

Table 1. Gradient Conditions.

Time (mins)	Mobile Phase A (%)	Mobile Phase B (%)
0.01	*95*	*5*
0.5	*45*	*55*
3.5	*45*	*55*
4.0	*95*	*5*
12.0	*Controller*	*Stop*

Mobile phase A - 1 mM ammonium acetate
Mobile phase B - Methanol

Mass Spectrometry: The SCIEX Triple Quad 3500 system was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity. Two selective MRM transitions were monitored for all nitrofuran metabolites using the Analyst® software 1.6.2. MRM transition is given in Table 2.

Data Processing: Data was processed with MultiQuant™ Software.

Table 2. MRM Method.

Compound	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
AOZ	*236.0*	*104.0*	*78.0*
AMOZ	*335.0*	*291.1*	*128.2*
SEM	*209.0*	*166.0*	*192.0*
AHD	*249.1*	*134.0*	*104.0*

Results

The results of repeatability data obtained for nitrofuran metabolites in the honey matrix is given in table 3 at different levels.

For all four Nitrofuran metabolites in honey, the matrix based calibration curve shows excellent linearity (0.50 to 20.0 ppb), with a correlation coefficient r≥0.99 using linear regression and weighing factor 1/X. The SCIEX Triple Quad 3500 system was found to be capable of analyzing concentrations well below the MRPL required by EU. The Signal to noise ratio for all four nitrofuran metabolites at 1.0 ppb is ≥ 30. The signals to noise ratio and calibration curves are shown in Figure 2 and Figure 3.

Recovery experiments were performed in honey samples at ½ MRPL, MRPL and 1.5 MRPL level (n=6). The recovery of all nitrofuran metabolites was ≥ 80%. The recovery data for nitrofuran metabolites are shown in Table 3. The retention time (RT) of the AHD, AOZ, SEM, AMOZ, were 5.94, 6.57, 6.57 and 7.50 min, respectively.

Decision limit (CCα) and detection capability (CCβ) were calculated for all the four derivatives of Nitrofuran in Honey samples. The calculation was based on using linear regression model analyzing spiked honey samples at below MRPL level.[7]

Table 3. Repeatability (%CV) and Recovery Statistics in Honey Sample.

	Repeatability (%CV, n=6)			Recovery (n=6)		
Analyte	½ MRPL (0.5 ppb)	MRPL (1.0 ppb)	1.5 MRPL (1.5 ppb)	½ MRPL (0.5 ppb)	MRPL (1.0 ppb)	1.5 MRPL (1.5 ppb)
AOZ	*6.01*	*7.00*	*4.28*	*113.47*	*95.05*	*89.89*
AMOZ	*12.16*	*4.46*	*4.33*	*83.80*	*103.88*	*96.11*
SEM	*4.49*	*7.31*	*9.44*	*109.67*	*98.13*	*91.33*
AHD	*4.96*	*7.58*	*8.22*	*114.90*	*105.40*	*105.00*

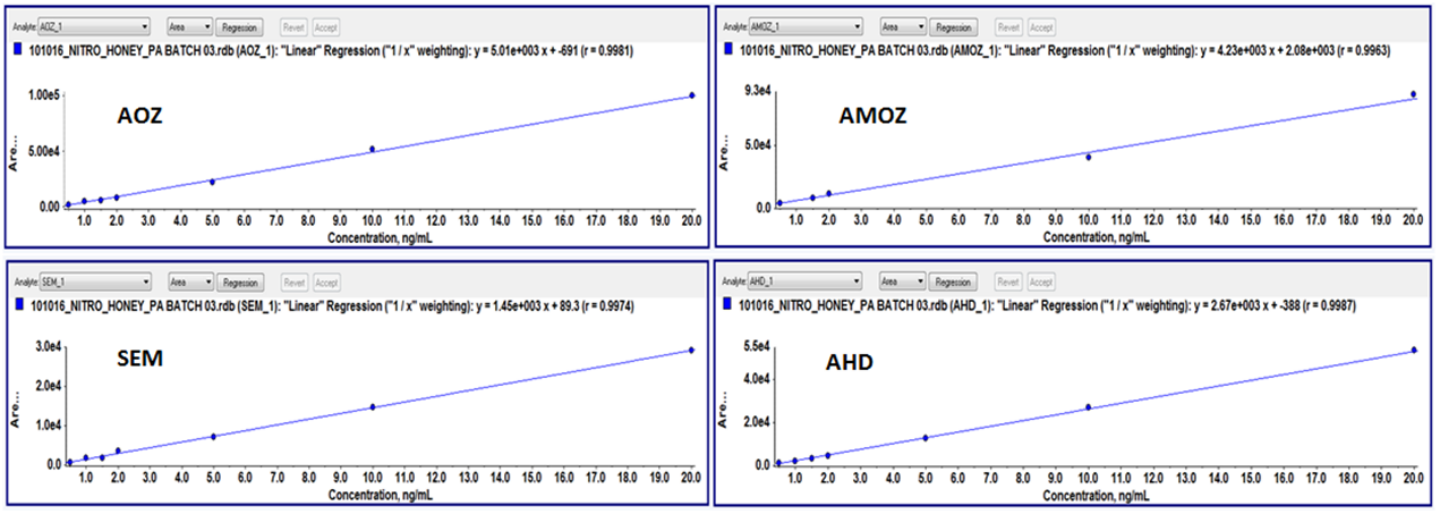

Figure 4: Calibration Curve in Matrix. Matrix based calibration curve AOZ, AMOZ, SEM and AHD in Honey sample showing r = >0.99.

The calculated value of CCα and CCβ are given in Table 4. The decision limit (CCα) and detection capability (CCβ) of all the metabolites were well below the MRPL.

Conclusions

- The developed quantitative method of Nitrofurans in honey on the SCIEX Triple Quad 3500 system was sensitive, linear, and reproducible
- Average recovery % for this method found to be ≥ 80% at various MRPL levels.
- The method and data presented showcase the fast and accurate solution for the quantitation and identification of nitrofuran metabolites in honey samples for quality control.

Table 4. Summary of CCα, CCβ and Linearity in Honey Sample.

Analyte	Calibration Range (ppb)	Linearity (r)	CCα	CCβ
AOZ	*0.5 - 20*	*0.9981*	*0.58*	*0.63*
AMOZ	*0.5 - 20*	*0.9963*	*0.62*	*0.70*
SEM	*0.5 - 20*	*0.9974*	*0.56*	*0.60*
AHD	*0.5 - 20*	*0.9987*	*0.57*	*0.61*

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Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
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