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Application News

No. AD-0062

LCMS-8030Plus UFMS

Analysis of Nitrofuran Metabolites in Animal Tissues by LC/MS/MS with QuEChERS Pre-treatment Method

❑ Abstract

An LC/MS/MS method for quantitative analysis of four nitrofuran metabolites, AH, SC, AOZ and AMOZ in shrimp, chicken and chicken liver was established using Shimadzu LC/MS/MS. Automated MRM optimization was applied to each compound using electrospray ionization (ESI) in positive mode to determine the multiple reaction monitoring (MRM) transitions for subsequent analysis. Sample clean-up and analyte enrichment were performed with QuEChERS prior to LC/MS/MS analysis. Linear calibration curve was established with concentrations ranging from 0.25 to 25 ng/g and linearity was between 0.995 and 0.999. Limit of detection (LOD) of the compounds was calculated by LabSolutions software. LOD of the compounds in shrimp, chicken and chicken liver matrices for AH is between 0.05 - 0.07 ng/g, SC is between 0.07 - 0.08 ng/g, AOZ is between 0.05 - 0.07 ng/g and AMOZ is between 0.002 - 0.02 ng/g. Limit of quantitation (LOQ) for the compounds are less than 0.25 ng/g which is below the minimum required performance limit (MRPL) set at 1 µg/kg by the European Union. Process recovery of the derivatisation reaction and QuEChERS sample clean-up for all three matrices at 0.5 ng/g is between 70.2–131.9%.

❑ Introduction

Nitrofuran is a class of antibiotics which is commonly used as veterinary drugs or feed additives for food-producing animals such as poultry, swine, cattle and shrimps. The common nitofurans used are nitrofurantoin, nitrofurazone, furazolidone and furaltadone. In 1997, these four drugs were banned by the European Union (EU) as veterinary drugs for food-producing animals due to their carcinogenic and mutagenic activity towards human health. As nitrofurans are rapidly metabolized in animal tissues, analysis of these compounds is based on the determination of their main metabolites, 1-aminohydantoin (AH), semicarbazide (SC), 3-Amino-2-oxazolidinone (AOZ) and 3-Amino-5-morpholinomethyl-2-oxazolidinone (AMOZ).

Considering the extremely low MRPL for nitrofurans (1 µg/kg), the main challenge for the analysis is to achieve very low LOD. Here, a UHPLC-MS/MS method is developed for the quantitative determination of the four metabolites, AH, SC, AOZ and AMOZ in shrimp, chicken and chicken liver. Sample preparation includes combined hydrolysis of the protein-bound drug metabolites and derivatisation of the metabolites with 2-nitrobenzaldehyde. QuEChERS is introduced here as an alternative sample clean-up process instead of traditional liquid-liquid extraction.

❑ Experimental

Preparation of standards and samples

All standards used were obtained from Sigma Aldrich. Derivatized nitrofuran metabolites, 2-NP-AH, 2-NP-SC, 2-NP-AOZ and 2-NP-AMOZ were used for LC/MS/MS method development and post-spiked calibration. AH, SC, AOZ and AMOZ were used for recovery studies. Stock solutions of the standards were prepared in methanol. The standards were diluted in series to the required calibration concentration with methanol as diluent. The animal tissue samples were pre-treated using a modified QuEChERS method as illustrated in Figure 1. The final clear sample solution was injected into LC/MS/MS for analysis.

A LCMS-8030 triple quadrupole LC/MS/MS (Shimadzu Corporation, Japan) was used in this work. The system consisted of a high pressure binary gradient UHPLC coupled with a LCMS-8030 Plus system. A Shim-pack HR-ODS column was used for separation of nitrofuran metabolites with a gradient program developed in house. The details of the LC and MS conditions are shown in Table 1.

Table 1: Analytical conditions of nitrofuran metabolites in animal tissue on LCMS-8030 Plus

LC conditions

Column	Shim-pack HR-ODS 3µ, 150 x 2.1mm
Flow Rate	0.3 mL/min
Mobile Phase	A :0.1 % formic acid in water B :Methanol
Elution Mode	Gradient elution: 10%B (0.01 to 0.5 min) ➔ 70%B (0.5 to 10 min) ➔ 70%B (10 to 12min) ➔ 10%B (12.01 to 17min)
Oven Temperature	45°C
Injection Volume	40 uL

MS conditions

Interface	ESI
MS mode	Positive
Block Temperature	450°C
DL Temperature	300°C
CID Gas	Ar (230kPa)
Nebulizing Gas Flow	N_2, 2.0L/min
Drying Gas Flow	N_2, 20.0L/min

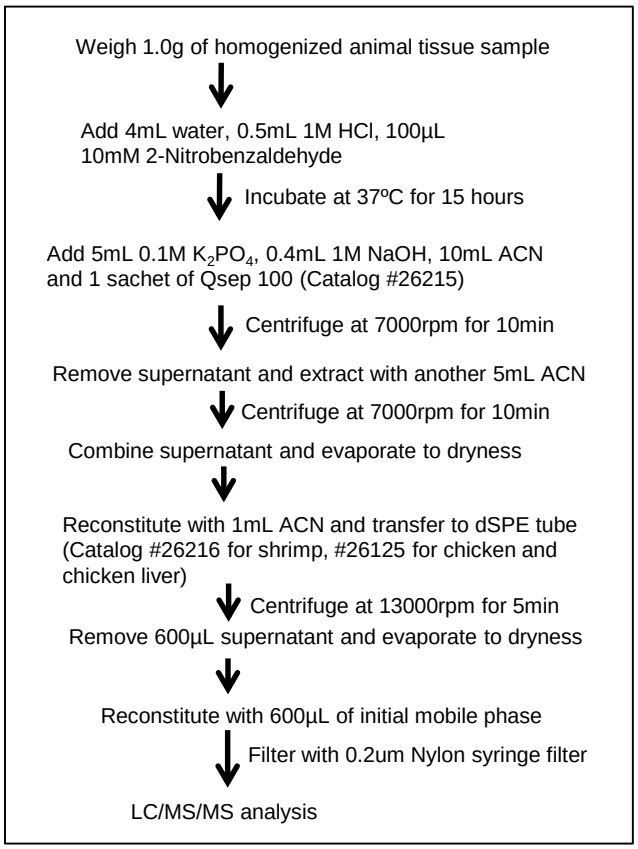


Figure 1: Flow chart of sample pre-treatment method

❑ Results and Discussion

MRM optimization

MRM optimization of the nitrofuran metabolites were performed using an automated MRM optimization program of the LabSolutions. The precursors were the protonated ions. Two optimized MRM transitions of each compound were selected and used for quantitation and confirmation. The MRM transitions and parameters are shown in Table 2.

Table 2: MRM transitions and optimized parameters

Name	RT (min)	Transition (m/z)	Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
AH	8.19	249.1 > 134.2	-11	-13	-17
		249.1 > 104.2	-11	-24	-19
SC	8.47	209.1 > 166.1	-18	-11	-12
		209.1 > 192.0	-18	-13	-14
AOZ	8.38	236.1 > 134.0	-10	-14	-16
		236.1 > 104.0	-10	-23	-20
AMOZ	6.83	335.1 > 291.1	-14	-13	-21
		335.1 > 262.1	-14	-18	-19

Method & Performance Evaluation

A LC/MS/MS method was developed for quantitation of nitrofuran metabolites based on the MRM transitions in Table 2. Figure 2 shows the chromatogram of the standards at 1 ng/mL in neat solution under the separation conditions in Table 1.

Figures 3 and 4 show the calibration curves of nitrofuran metabolites standards in neat solutions and in animal tissue matrices (spiked). The linearity with correlation coefficient (R^2) more than 0.995 across the calibration range of 0.25~25.0 ng/g was obtained for the standards in neat solution and matrices (spiked).

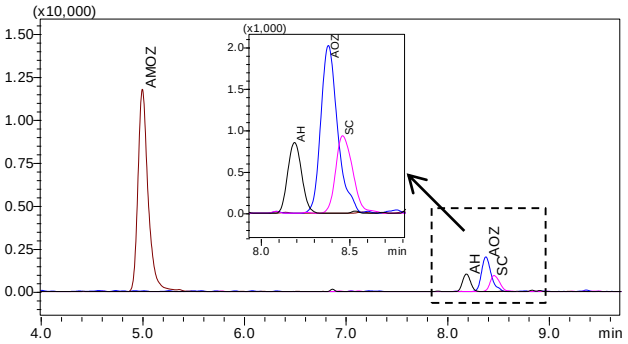


Figure 2: MRM chromatogram of standards at 1 ng/mL in neat solution

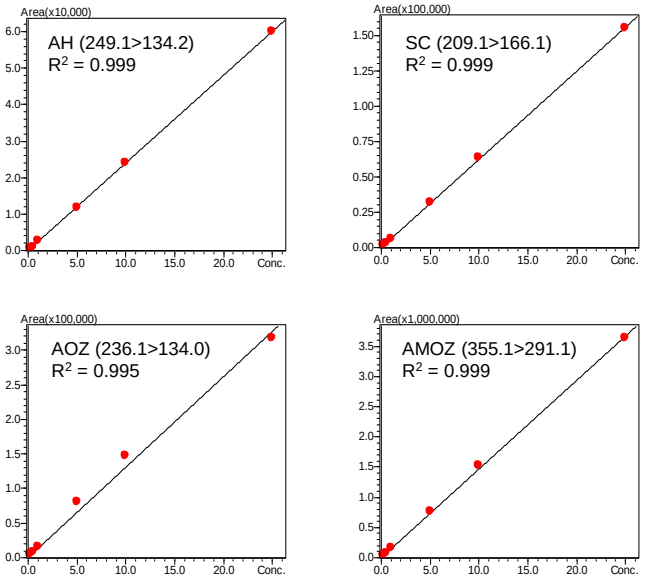
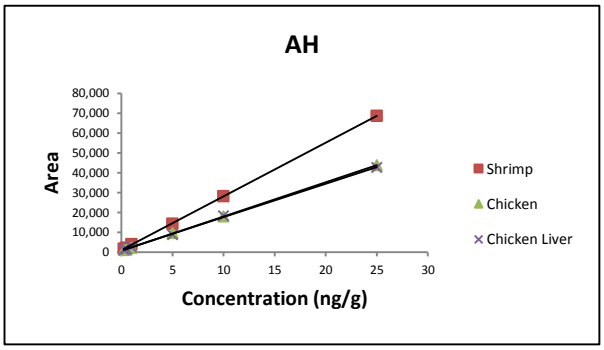
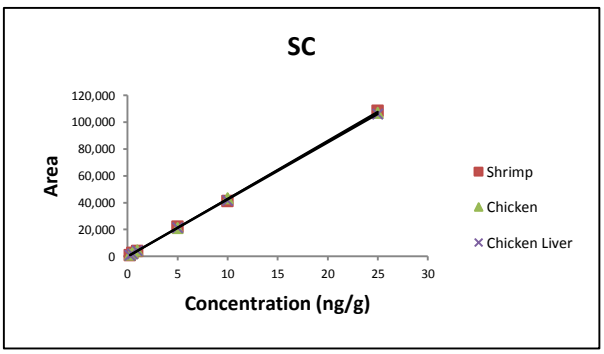
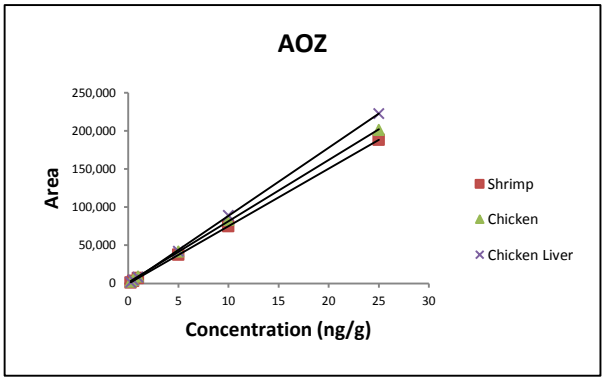


Figure 3: Calibration curves of nitrofuran metabolites in neat solution with concentration range from 0.25 to 25.0 ng/mL







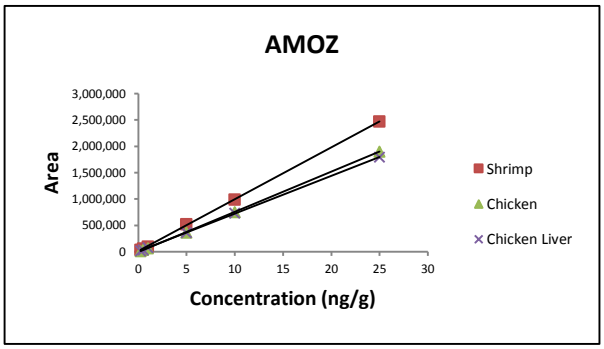


Figure 4 : Calibration curves of nitrofuran metabolites in matrices with concentration range from 0.25 to 25.0 ng/g

Repeatability of the method was evaluated at 5 ng/g spiked in neat and animal tissue matrices. The LOD and LOQ were estimated from the results of 0.25 ng/g in both neat and matrix solution. The LOD and LOQ results were summarized in Table 3

Table 3: Results of repeatability and sensitivity evaluation of nitrofuran metabolites in neat solution and matrices (n=5)

Compound	Sample	Linearity (R2)	%RSD	LOQ (ng/g)	LOD (ng/g)
AH	Neat	0.999	5.4	0.15	0.05
	Shrimp	0.999	4.1	0.18	0.06
	Chicken	0.999	10.2	0.18	0.05
	Chicken Liver	0.999	6.3	0.23	0.07
SC	Neat	0.999	3.8	0.05	0.02
	Shrimp	0.999	2.9	0.21	0.07
	Chicken	0.999	4.8	0.24	0.08
	Chicken Liver	0.999	5.7	0.21	0.07
AOZ	Neat	0.995	3.8	0.25	0.08
	Shrimp	0.999	5.7	0.16	0.05
	Chicken	0.999	4.7	0.15	0.05
	Chicken Liver	0.999	5.1	0.22	0.07
AMOZ	Neat	0.999	1.0	0.01	0.003
	Shrimp	0.999	8.4	0.01	0.004
	Chicken	0.999	2.7	0.05	0.02
	Chicken Liver	0.999	2.7	0.006	0.002

Process recovery of the method was done by spiking 0.5 ng/g of non-derivatized nitrofuran metabolites into different matrices. The samples were subjected to derivatization procedures and sample clean-up was done using QuEChERS. Recovery study was done in triplicates for each matrix. Table 4 shows the results of the process recovery. The recovery was determined using the formula as shown below. Recovery of the nitrofuran metabolites in the different matrices were determined to be between 70.2–131.9%.

Formula for Process recovery calculation :

Process recovery of nitrofuran metabolite in matrix =

$$\frac{\text{(Calculated concentration of nitofuran metabolite)}}{\text{(Actual concentration spiked)}} \times 100\%$$

Table 4: Recovery of nitrofuran metabolites determined with spiked sample of 0.5 ng/g (n=3)

Compound	Sample	Recovery (Average) (%)	% RSD
AH	Shrimp	97.5	3.0
	Chicken	117.6	2.3
	Chicken Liver	105.3	1.3
SC	Shrimp	99.7	3.4
	Chicken	119.7	7.1
	Chicken Liver	101.6	6.3
AOZ	Shrimp	125.7	3.9
	Chicken	82.1	10.9
	Chicken Liver	78.4	7.6
AMOZ	Shrimp	75.1	3.2
	Chicken	92.7	5.7
	Chicken Liver	81.8	2.6

❑ Conclusions

An LC/MS/MS method for simultaneous quantitative analysis of four nitrofuran metabolites in shrimp, chicken and chicken liver was developed and optimized. Limit of quantitation (LOQ) for the compounds are less than 0.25 µg/kg which is below the minimum required limit (MRL) set at 1 µg/kg by the European Union. Process recovery of the derivatisation reaction and QuEChERS sample clean-up for all three matrices at 0.5 ng/g is between 70.2–131.9%.

❑ References

1. Valera-Tarifa et al. Determination of nitrofuran metabolites in seafood by ultra high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry, Journal of Food Composition and Analysis (2013)

2. Leitner et al. Determination of the metabolites of nitrofuran antibiotics in animal tissue by high-performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 939 (2001) 49–58

3. P. Mottier et al. Quantitative determination of four nitrofuran metabolites in meat by isotope dilution liquid chromatography–electrospray ionisation–tandem mass spectrometry, J. Chromatogr. A 1067 (2005) 85–91

4. C. Bock et al. Matrix-comprehensive in-house validation and robustness check of a confirmatory method for the determination of four nitrofuran metabolites in poultry muscle and shrimp by LC–MS/MS / J. Chromatogr. B 856 (2007) 178–189

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SHIMADZU (Asia Pacific) Pte. Ltd
79 Science Park Drive, #02-01/08 Cintech IV, Singapore 118264
www.shimadzu.com.sg
Tel: +65-6778 6280 Fax: +65-6778 2050