

# Technical Report

## Extraction of Nitroimidazoles from Milk and Egg Samples using SupelMIP SPE Nitroimidazoles and LC-MS-MS

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### Introduction

In this report, a new SPE phase was evaluated for extraction of the nitroimidazoles dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ) and their hydroxylated metabolites (DMZOH and MNZOH). Using this molecularly imprinted polymer (SupelMIP) and associated method, nitroimidazoles were extracted from milk and fresh egg samples with high and reproducible recoveries with low RSD.

### Background

Nitroimidazoles are antibacterial and anticoccidial drugs used for treatment of cattle, poultry and pigs. These compounds and their metabolites are suspected to be carcinogens and mutagens. Consequently, their use in veterinary practice is thus strictly regulated within the countries in the European Union (Council Regulation 2377/90, Annex IV Banned Compounds).

Dimetridazole (DMZ), metronidazole (MNZ) and ronidazole (RNZ) are prohibited and belong to the list of pharmacologically active substances for which no maximum residue limit (MRL) can be fixed, meaning that their use is forbidden in food-producing animals and that any residue of these compounds found in food producing animals or products intended for human consumption has to be considered as a violation of the regulations. MNZ and ipronidazole (IPZ) are not licensed as veterinary drugs and are considered forbidden compounds. The surveillance of nitroimidazole drug residues requires the detection of these compounds as well as their main metabolites at trace levels in animal products

### Molecularly Imprinted Polymers

Molecularly imprinted polymers (MIPs) are a class of highly cross linked polymer-based molecular recognition elements engineered to bind one specific target compound or a class of structurally related compounds with high selectivity. The MIP material is designed with cavities that are sterically and chemically complementary to the target analyte(s). As a result, multiple interactions (e.g., hydrogen bonding, ionic, Van der Waals, hydrophobic) can take place between the MIP cavity and the analyte.

### Method

#### Sample Pre-treatment

One egg was homogenized. 10 g of the mix was spiked at 1, 2 and 5 µg/kg with nitroimidazoles, dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ) and their hydroxylated metabolites (DMZOH and MNZOH), and 2 µg/kg with deuterated standards for each compound, respectively. For MNZ and MNZOH, DMZOH-d<sub>3</sub> was used as internal standard.

The spiked sample was extracted by combining the sample with 10 mL acetonitrile followed by centrifugation for 5 minutes. 2 g sodium chloride was mixed with the supernatant, centrifuged, and the extract was evaporated to dryness in a silanized glass tube. The residue was reconstituted in 2 mL water and sonicated for 3 min. prior to SPE processing.

Similar pre-treatment protocol was followed for milk samples except liquid-liquid extraction using acetonitrile and sodium chloride was done as one step.

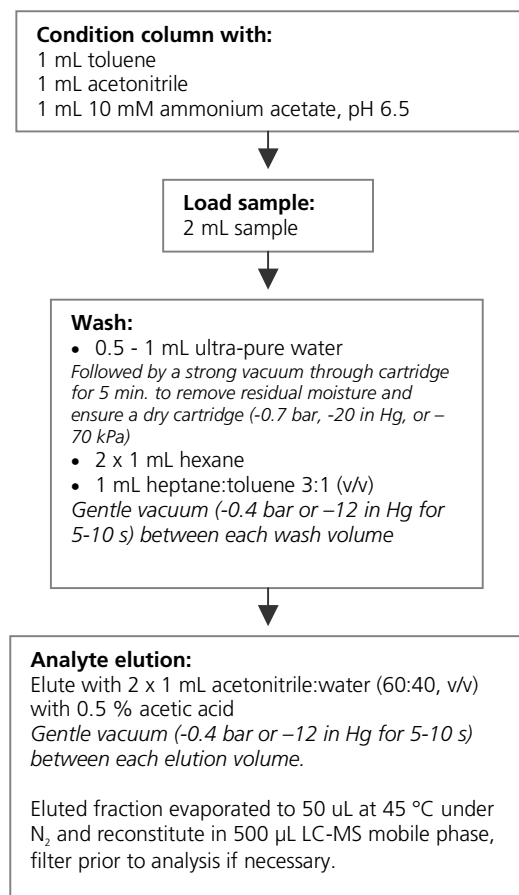
#### SupelMIP SPE Nitroimidazole Procedure

The SPE material used was SupelMIP Nitroimidazoles SPE tubes 50 mg/3 mL (Cat. No. 52734-U). The column was conditioned with 1 mL toluene followed by 1 mL acetonitrile and 1 mL buffer, pH 6.5. 2 mL sample was loaded using gravity and the columns were then washed to remove interfering contaminants. Initially, 1 mL water was applied followed by the application of strong vacuum for 5 minutes to dry the column. 2 aliquots of 1 mL hexane were added followed by 1 mL heptane:toluene (3:1 V/V). Between each wash step, a brief vacuum was applied and after the last heptane:toluene wash step, 3 minutes of strong vacuum was applied to dry the column. To elute the nitroimidazoles, 2 aliquots of 1 mL 60:40 acetonitrile:water containing 0.5% acetic acid was applied. The flow during the elution was controlled to 0.2 mL/minutes. The eluted fractions were evaporated to 50 µL in silanized glassware and reconstituted to 500 µL with 0.1% formic acid in water. See also Figure 1.

#### Performance Evaluation

Relative recovery was determined against internal standards for each of the spike levels tested (n = 5).

The data was also used to determine reproducibility and to estimate LLOD. The influence of matrix (ionization effects) was tested by spiking nitroimidazoles into blank post-SPE extracts prior to LC-MS analysis. The results were quantified against an external calibration curve in buffer. LC-MS conditions are described in Table 4.

**Figure 1. SupelMIP SPE Nitroimidazole Procedure**

## Results

### High recoveries, reproducibility and clean extracts allow low detection levels

The reported recoveries are high and reproducible, allowing high quality analysis. Relative recoveries are presented in Table 1. The recovery and relative standard deviation were 95% resp. 7% in average. Representative ion-chromatograms of egg samples (1 ng/g spike) are presented in Figure 2. These chromatograms illustrate low background.

Samples spiked into post-SPE extracts revealed good sample cleanup and low ion-suppression. The matrix ionization effects were minimal as evident from 90-120% recovery values in the post-spiked samples (Table 2).

The estimated detection levels for nitroimidazoles ranged from 0.010-0.16 µg/kg in milk and 0.016-0.12 µg/kg in egg. The LODs for the different nitroimidazoles for each sample matrix are shown in Table 3. The LODs were estimated based on the analyte and background response levels of the lowest spike concentration. Using this information the LOD concentration was estimated based on signal-to-noise calculation of 3:1.

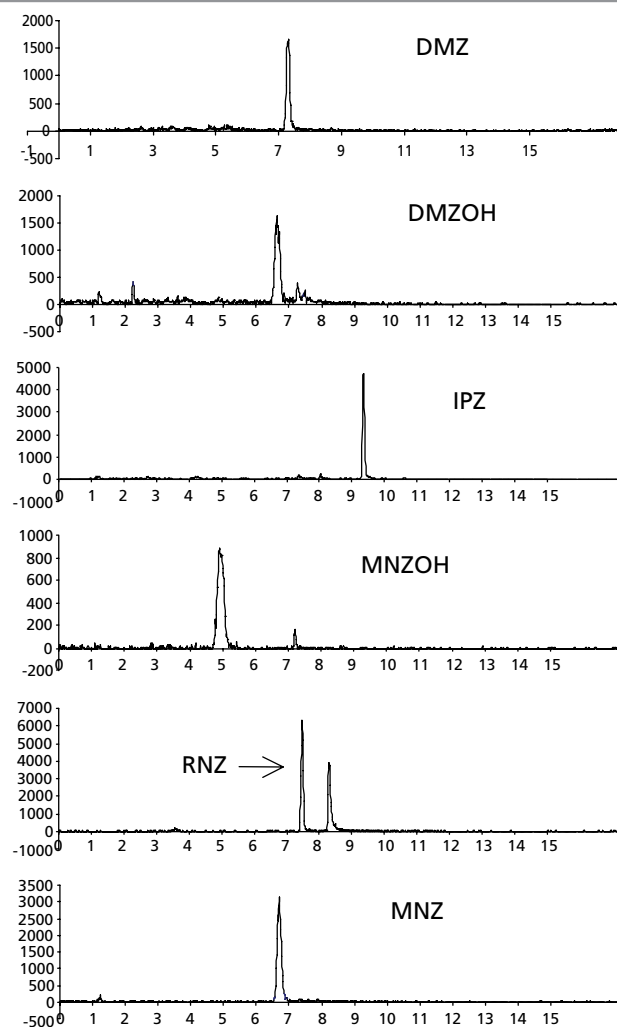
**Table 1. Recoveries of Nitroimidazoles from Spiked Egg and Milk Samples (n=5)**

Sample	DMZ <sup>1</sup>	DMZOH <sup>1</sup>	IPZ <sup>1</sup>	MNZ <sup>2</sup>	MNZOH <sup>2</sup>	RNZ <sup>1</sup>
Egg 1 µg/kg	111(7)	106(5)	105(6)	109(3)	83(6)	105(7)
Egg 2 µg/kg	84(5)	90(11)	94(5)	91(9)	58(8)	113(20)
Egg 5 µg/kg	88(5)	92(8)	90(1)	92(8)	57(13)	134(3)
Milk 1 µg/kg	100(8)	107(6)	108(6)	105(2)	76(4)	104(3)
Milk 2 µg/kg	88(7)	94(3)	97(10)	92(14)	59(7)	111(10)
Milk 5 µg/kg	96(10)	101(10)	95(5)	107(9)	62(8)	123(4)

<sup>1</sup>Deuterated standard is available, <sup>2</sup>Used DMZOH-d<sub>3</sub> as an internal standard

**Table 2. Absolute Recovery of Nitroimidazoles Spiked Post-SPE – Determination of Matrix Effects after SPE Cleanup**

Sample	DMZ	DMZOH	IPZ	MNZ	MNZOH	RNZ
Egg	91	92	103	96	99	89
Milk	118	110	123	116	98	109

**Figure 2. MS-MS Chromatograms of the Nitroimidazole Extract from Egg at 1 ng/g, Dimetridazole (DMZ), Iprnidazole (IPZ), Metronidazole (MNZ), and Ronidazole (RNZ) and their Hydroxylated Metabolites (DMZOH and MNZOH)**

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**Table 3. Estimated LOD for Nitroimidazoles in Egg and Milk Samples**

LOD (ng/g)	RNZ	MNZ	DNZ	DMZOH	MNZOH	IPZ
Egg	0.016	0.020	0.066	0.124	0.061	0.026
Milk	0.010	0.023	0.063	0.162	0.076	0.015

## Conclusion

The high recoveries, reproducibility and clean extracts using the SupelMIP SPE for Nitroimidazoles allow low detection levels and increased food safety

In this report the SupelMIP SPE Nitroimidazole was evaluated for extraction of the nitroimidazoles dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ), and ronidazole (RNZ) and their hydroxylated metabolites (DMZOH and MNZOH). Using molecularly imprinted polymer technology, nitroimidazoles were extracted from milk and fresh egg samples with high and reproducible recoveries with low RSD (avg.  $95 \pm 7\%$ ). The LOD was in the ppt to low ppb level. The SupelMIP method described in this report is highly robust and reproducible offering the necessary selectivity to achieve the detection limits required in food residue analysis.

**Table 4. Analytical Method**

Recommended Analytical Technique: LC-MS-MS or LC-MS

column	Ascentis® C18, 15 cm x 2.1mm I.D. 3 µm particle size (581302-U)		
instrument	Sciex API 3200		
mobile phase A:	0.1% formic acid in LC-MS grade water		
mobile phase B:	0.1% formic acid in acetonitrile		
gradient:	<b>Min.</b>	<b>A%</b>	<b>B%</b>
	0.0	95	5
	1.0	95	5
	8.0	0	10.0
	12.0	0	10.0
	13.0	95	5
	18.0	95	5
flow rate:	0.3 mL/min.		
temp.:	ambient		
det.:	MS/MS, MRM transitions		
	DMZ (142/96)	IPZOH (186/168)	
	DMZ-d <sub>3</sub> (145/99)	IPZOH-d <sub>3</sub> (189/171)	
	DMZOH (158/140)	MNZ (172/128)	
	DMZOH-d <sub>3</sub> (161/143)	MNZOH (188/126)	
	IPZ (170/124)	RNZ (201/140)	
	IPZ-d <sub>3</sub> (189/171)	RNZ-d <sub>3</sub> (204/143)	
polarity:	Positive		
ion source:	Turbospray		
ion spray voltage:	1200 V		
source temp:	350 °C		
collision gas:	4 psi		
curtain gas:	50 psi		
inj.:	30 µL		

## Ordering Information

Description	Oty.	Cat. No.
SupelMIP SPE - Nitroimidazoles, 50 mg/3 mL	50	52734-U
Ascentis C18 Column, 15 cm x 2.1 mm I.D., 3 µm particle size	1	581302-U

## Trademarks

Ascentis, SupelMIP — Sigma-Aldrich Biotechnology LP

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