Application Note

Veterinary Drugs

# Analysis of Nitroimidazoles in Egg Using Agilent Captiva EMR—Lipid and LC/MS/MS


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## Abstract

This Application Note describes the determination of four nitroimidazoles (metronidazole, dimetridazole, ronidazole, and ipronidazole) and their hydroxy-metabolites in chicken egg with an easy, robust pass-through cleanup and LC/MS/MS analysis. The sample preparation procedure with QuEChERS extraction and Captiva EMR—Lipid cartridge cleanup achieved premium results to remove lipids and pigment. The method delivers excellent accuracy (85.6–118.3 %) and precision (RSD <6 %) at all three QC levels.

## Introduction

Nitroimidazoles are a class of active antibiotics used in poultry that are also used as growth promoters in food-producing animals. However, they have been prohibited in many countries because of potential risk[1]. There are some reports on the analysis of nitroimidazoles using solvent extraction[2,3] and solid phase extraction (SPE)[4]. However, solvent extraction often uses strong solvents such as ethyl acetate or dichloromethane, and can extract additional matrix, which can cause interference during LC/MS analysis[4]. An SPE method including an acetonitrile extraction followed with cation exchange cleanup can deliver clean eluent, but requires multiple steps and pH adjusting during the sample preparation.

Captiva EMR—Lipid, which is a lipid removal product, combines size exclusion and hydrophobic interaction to selectively capture lipid hydrocarbon chains without the loss of target analytes. Compared with the SPE multistep operation, EMR's unique pass-through functionality simplifies the sample preparation workflow significantly. This Application Note used Captiva EMR—Lipid for sample cleanup in the analysis of nitroimidazoles and their hydroxy-metabolites in chicken eggs, resulting in excellent recovery and precision. Figure 1 shows the chemical structures of the four nitroimidazoles and their metabolites.

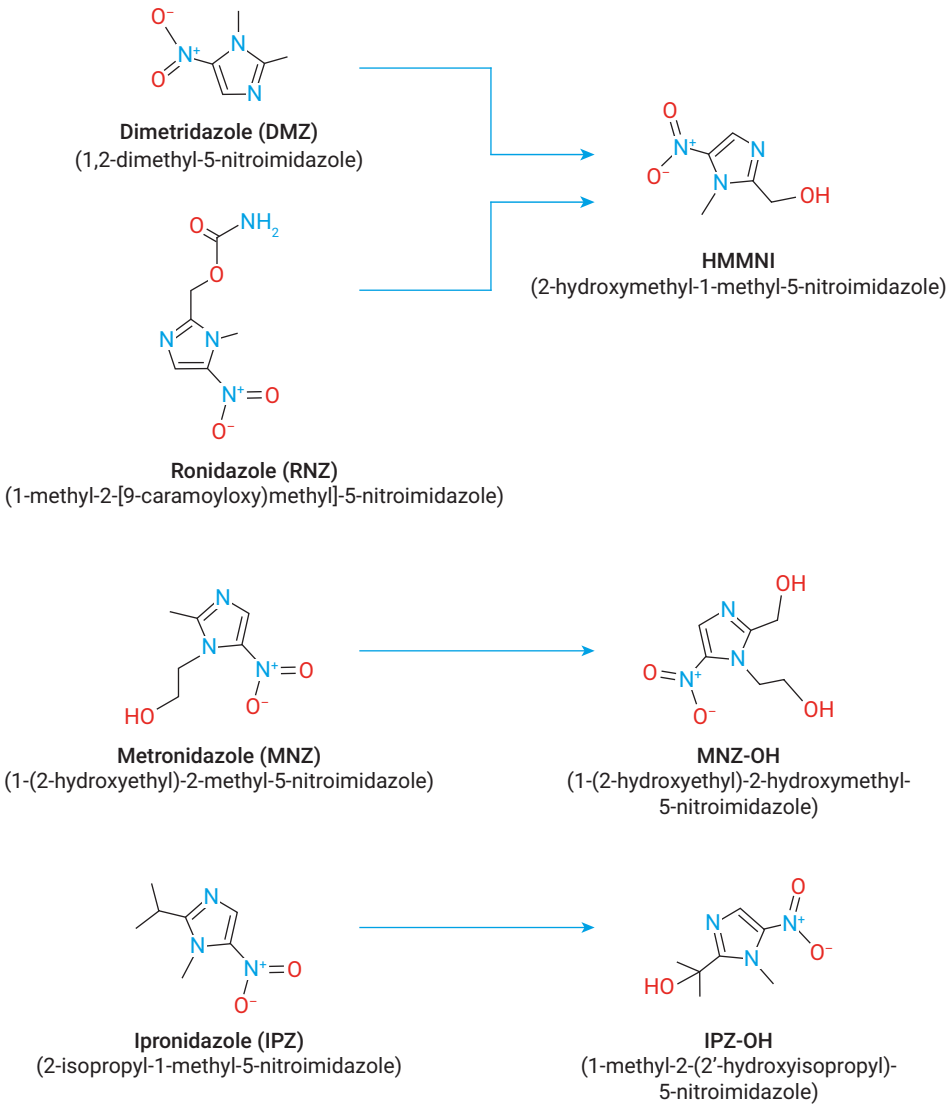


**Figure 1.** Chemical structures of the four nitroimidazoles and their metabolites.

# Experimental

## Reagents and chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN) and methanol (MeOH) were from Honeywell (Muskegon, MI, USA). Formic acid (FA) was from J&K Scientific Ltd. (Beijing, China). The nitroimidazoles and internal standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

## Solution and standards

Individual standard stock solutions were made in MeOH at 10 mg/mL in amber glass vials, and stored at –20 °C. The combined standard spiking solution (1 mg/mL) and the internal standards mixture (1 mg/mL) were prepared in MeOH before use.

## Equipment and materials

Separation was carried out using an Agilent 1290 Infinity LC coupled with an Agilent 6495B triple quadrupole LC/MS system with an Agilent Jet Stream electrospray ionization (EI) source. Removal of matrix was evaluated using GC/MS full scan by an Agilent 9000 GC system combined with an Agilent 7000 GC/MS triple quadrupole. Agilent MassHunter workstation software was used for data acquisition and analysis.

Other equipment and materials used for sample preparation included:

- SPEX sample preparation 2010 Geno/Grinder (Metuchen, NJ, USA)
- Eppendorf Centrifuge 5810R (Hamburg, Germany)
- Agilent Captiva EMR—Lipid cartridge, 3 mL, 300 mg (p/n 5190-1003)
- Agilent Vac Elut 20 Manifold (p/n 12234101)
- Agilent QuEChERS extraction kit for veterinary drugs (p/n 5982-0032)

## Instrument conditions

Figure 2 shows typical MRM chromatograms of the seven nitroimidazoles and metabolites and the three internal standards with 1 ng/g spiking concentration in egg. The internal standards were spiked at 10 ng/g concentration.

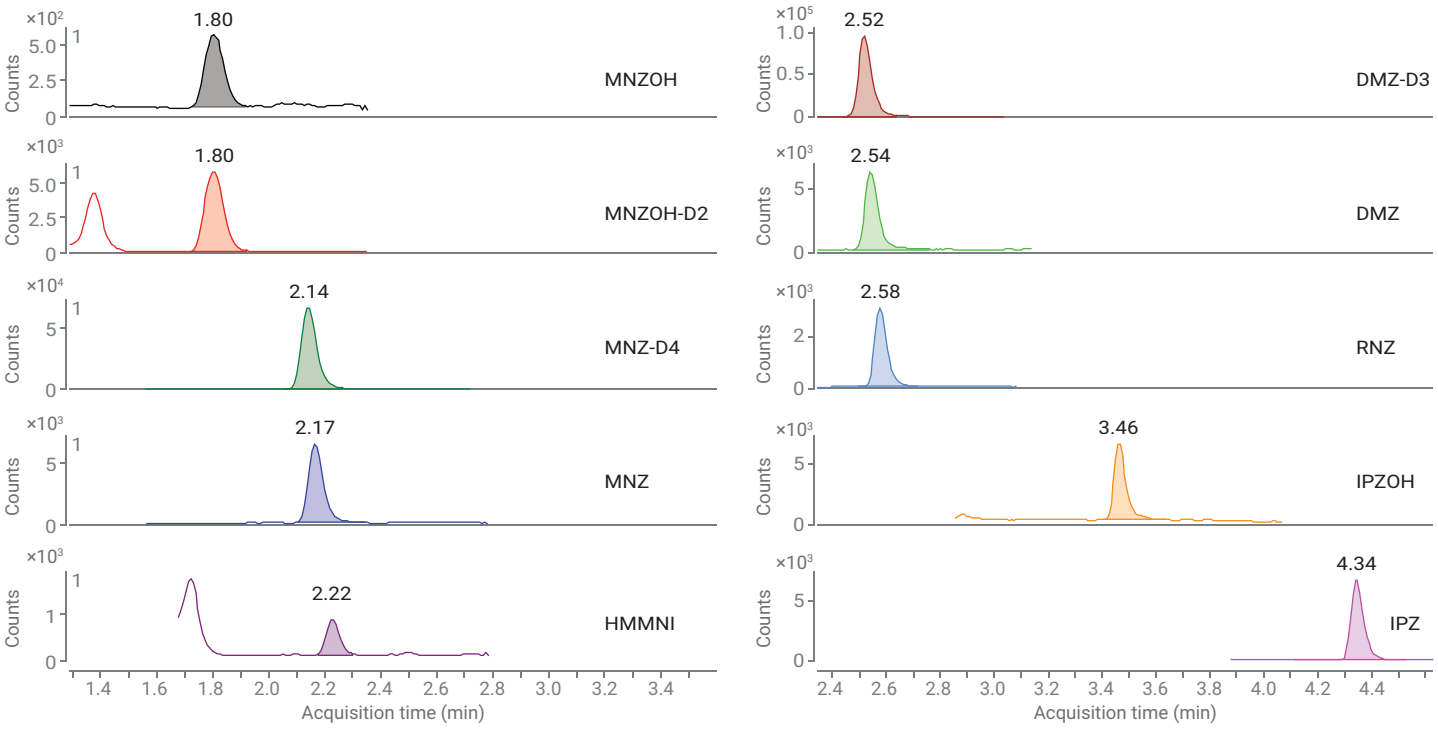


**Figure 2.** Typical chromatograms of egg spiked with 1 ng/g of each nitroimidazole and 10 ng/g internal standard.

## HPLC conditions

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302) |
| Column temperature | 35 °C |
| Autosampler temperature | 15 °C |
| Injection volume | 1 µL |
| Mobile phase | A) 0.1 % FA in water<br>B) 0.1 % FA in ACN |
| Gradient | Time (min) %B Flow rate (mL/min)<br>0 10 0.5<br>0.5 10 0.5<br>4.5 50 0.5<br>5.0 95 0.5<br>6.0 95 0.5 |
| Stop time | 8.5 minutes |

## MS conditions

| Parameter | Value |
|---|---|
| Positive/negative mode | Positive |
| Fragmentor | 380 V |
| Cell accelerator voltage | 3 |
| Gas temperature | 210 |
| Gas flow | 13 L/min |
| Nebulizer | 35 psi |
| Sheath gas heater | 400 |
| Sheath gas flow | 12 |
| Capillary | 3,500 V |
| Delta EMV (+) | 400 |

## MRM parameters

| Analyte | Retention time (min) | Ion transition (*m/z*) | Collision energy (eV) |
|---|---|---|---|
| DMZ | 2.54 | 142 → 96<br>142 → 81 | 21<br>33 |
| DMZ-D3 | 2.52 | 145 → 99 | 17 |
| MNZ | 2.17 | 172 → 128<br>172 → 82 | 13<br>25 |
| MNZ-D4 | 2.15 | 176 → 128 | 15 |
| MNZOH | 1.83 | 188 → 123<br>188 → 126 | 13<br>21 |
| MNZOH-D2 | 1.82 | 190 → 128 | 17 |
| HMMNI | 2.18 | 158 → 55<br>158 → 140 | 18<br>13 |
| IPZ | 4.35 | 170 → 109<br>170 → 124 | 25<br>33 |
| IPZOH | 3.46 | 186 → 168<br>186 → 121 | 13<br>29 |
| RNZ | 2.58 | 201 → 140<br>201 → 55 | 9<br>25 |

### Sample preparation

Figure 3 shows the sample preparation procedure. Egg samples were mixed well and weighed accurately (5 g). Then, 5 % formic acid was added to ACN for extraction. A high percentage of formic acid was necessary to achieve better protein precipitation, which can be visualized by the larger amount of precipitant formed. QuEChERS extraction with 4 g of $Na_2SO_4$ and 1 g of NaCl was chosen to clean up polar interference from the egg sample. After centrifugation, the upper ACN layer (2.4 mL) was transferred to a clean tube, diluted with 0.6 mL of water (20 % water by volume), and vortexed well. Then, the mixed solution was transferred to a 3-mL Captiva EMR—Lipid tube and allowed to flow under gravity. The cartridge was then dried with vacuum. Finally, the eluent was collected and vortexed, before it was ready for injection.

### Calibration standards and quality control (QC) samples

The solvent calibration standards for the standard curve were prepared at 0.1, 0.5, 1, 5, 10, 50, and 100 ng/g. The internal standard mixture, which included DMZ-D3, MNZ-D4, and MNZOH-D2 was spiked at 10 ng/g.

Prespiked QC samples were fortified by spiking the appropriate standard working solution into the homogenized egg sample with six replicates at low (1 ng/g), mid (5 ng/g), and high (10 ng/g) levels.

## Results and discussion

### Linearity

The data were processed with Agilent MassHunter quantification software. A calibration curve gave $R^2$ values between 0.993 and 0.995 for the seven nitroimidazoles and metabolites using linear regression fit and 1/x weighting.

### Recovery and precision results

Table 1 shows that the QuEChERS extraction followed with the Captiva EMR—Lipid cleanup method was verified by running spiked samples at three QC levels. The recovery was 86.5~118.3 %, and RSD was <6 %. Due to the limited availability of internal standards, MNZOH was calibrated with MNOH-D3, MNZ was calibrated with MNZ-D4, and DMZ, RNZ, IPZ, and IPZOH were calibrated with DMZ-D3. HMMNI was not calibrated with any of the internal standards due to its different chemistry characterization.

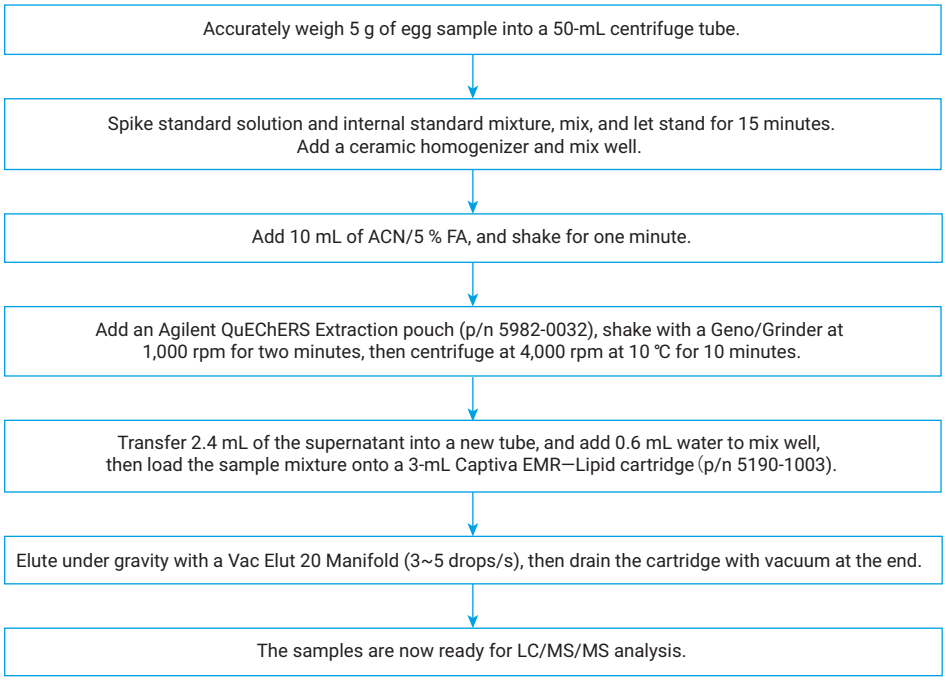


**Figure 3.** Egg sample extraction and subsequent cleanup using an Captiva EMR—Lipid 3-mL cartridge.

**Table 1.** Method quantitation results for three QC levels in egg.

| Analyte | $R^2$ | Linear range (ng/g) | 1 ng/g QCs (n = 6) | | 5 ng/g QCs (n = 6) | | 10 ng/g QCs (n = 6) | |
|---|---|---|---|---|---|---|---|---|
| | | | Rec% | RSD% | Rec% | RSD% | Rec% | RSD% |
| DMZ | 0.994 | 0.1~50 | 89.6 | 3.2 | 89.6 | 2.4 | 88.8 | 2.6 |
| HMMNI | 0.993 | 0.5~100 | 118.3 | 4.7 | 103.9 | 4.3 | 110.9 | 2.5 |
| IPZ | 0.994 | 0.1~50 | 93.3 | 3.5 | 93.1 | 2.9 | 93.0 | 3.0 |
| IPZOH | 0.995 | 0.1~50 | 94.2 | 5.0 | 91.3 | 3.5 | 90.0 | 3.3 |
| MNZ | 0.994 | 0.1~50 | 91.5 | 3.5 | 87.6 | 3.7 | 86.5 | 3.6 |
| MNZOH | 0.995 | 0.1~50 | 94.3 | 5.4 | 88.4 | 4.8 | 87.3 | 3.4 |
| RNZ | 0.994 | 0.1~50 | 99.3 | 5.9 | 99.7 | 3.5 | 98.9 | 3.4 |

## Monitoring matrix removal by GC/MS

Egg contains protein and various classes of lipids. We compared the GC/MS full scan of samples with and without Captiva EMR—Lipid cleanup in Figure 4. The red chromatogram represents acidic acetonitrile extraction only, and the black chromatogram represents the result of acidic acetonitrile extraction followed by Captiva EMR—Lipid cleanup.

The chromatogram shows that the EMR—Lipid removed 76 % of the matrix. This was calculated by comparing the total peak area under two conditions. Figure 5 shows the dried samples following preparation using the two different conditions. it can clearly be seen that the Captiva EMR—Lipid removed most of the matrix and pigment.

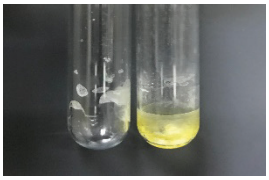

**Figure 5.** Left: Dried egg sample extracted with acidic ACN, followed by Captiva EMR—Lipid cleanup; Right: Dried egg sample extracted with acidic ACN only.

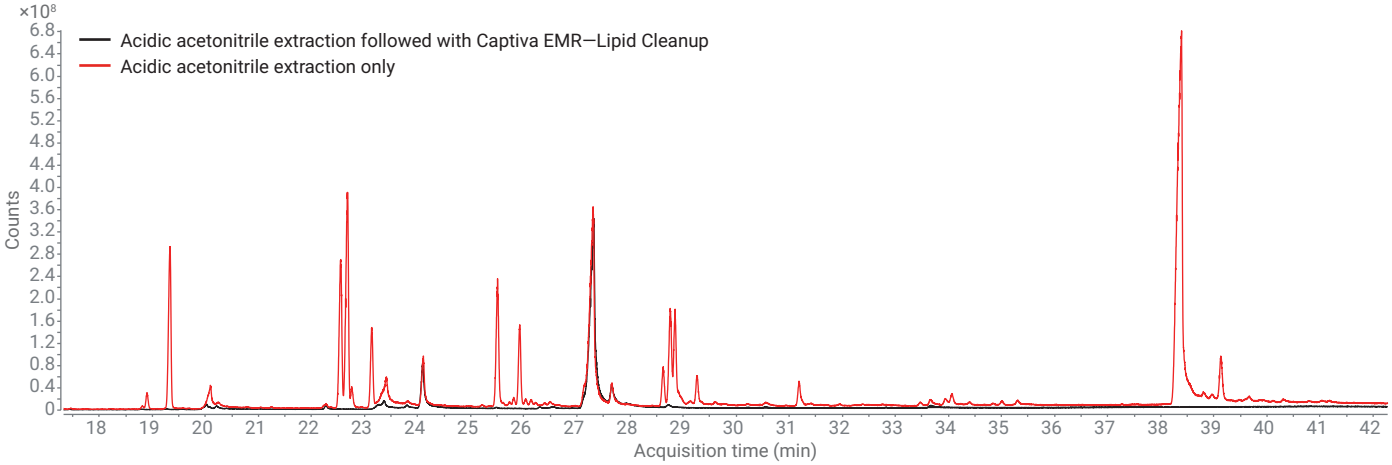


**Figure 4.** Matrix removal evaluation using a GC/MS full scan chromatogram comparison of egg sample after solvent extraction only (red) and after Captiva EMR—Lipid cleanup (black).

## Conclusion

This work demonstrates that Captiva EMR—Lipid is an easy and efficient cleanup method for the analysis of veterinary drugs in egg. Validation of seven nitroimidazoles and metabolites gave excellent recovery (86.5~118.3 %) and precision (RSD <6 %). A GC/MS full scan showed highly efficient cleanup of lipids and pigments using Captiva EMR—Lipid. Overall, Captiva EMR—Lipid provides simple, selective, and efficient lipid/matrix removal for veterinary drug analysis in egg without negatively impacting analyte recovery.

## References


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