

# DEVELOPMENT OF A RAPID HONEY SCREENING ASSAY FOR RESIDUES OF METRONIDAZOLE & DIMETRIDAZOLE



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## BACKGROUND

Nitroimidazoles, such as Metronidazole and Dimetridazole, are a family of antimicrobial agents used in the honeybee industry to prevent and control Nosemosis / Nosema. Although their use in the European Union (EU) is prohibited in all food-producing species (Group A6 of Annex I of Directive 96/23), Nitroimidazole - particularly Metronidazole - contamination of honey imported into the EU has become a significant issue.

To control the integrity of honey in the EU, reliable detection methods capable of detecting a minimum concentration of 3µg/kg (3ppb) - as set out in the Guidance Paper released by the Community Reference Laboratory (CRL) - are required. This study reports the use of one such method which can be used for the detection and quantification of Nitroimidazoles in honey.

## METHODS

Specific Metronidazole immunogen and antigen was prepared by using a cross linker disuccinimidyl carbonate with Jeffamine spacer between the drug and the protein molecule.

These immunogens were then injected into mice using different immunisation schemes. The best matched pair giving the best sensitivity were then chosen to be carried through into the development of this kit.

Honey samples were prepared for use on the Nitroimidazole microtitre plate (MTP) by following a simple solvent extraction protocol.

### Simple Solvent Extraction Protocol



Solvent-based extraction using sodium chloride and ethyl acetate which is evaporated at 50°C

Reconstitution of sample in buffer supplied in the assay kit

Application of samples to ELISA test kit

Samples were run on the MTP supplied in the kit using standards and antibodies also supplied in ready-to-use form.

## RESULTS

### Calibration Range: 0.625 - 20.0 ppb



Figure 1: Typical curves obtained with Metronidazole and its cross-reactants Dimetridazole and Ronidazole. The results demonstrate high cross-reactivity of Metronidazole and Dimetridazole, facilitating quantification of both Nitroimidazoles using the same Nitroimidazole kit. Cross reactivity with Ronidazole is low at only 0.79%.

## RESULTS

REPLICATE	CONCENTRATION (ppb)	
1	5.1	
2	5.3	
3	5.4	
4	5.4	
5	5.7	
6	5.5	
7	4.8	
8	5.2	
9	4.3	
10	5.4	
11	4.8	
12	5.6	
13	5.2	
14	4.8	
15	5.2	
16	4.5	
17	5.6	
18	5.5	
19	4.7	
20	5.1	
MEAN		5.160
SD		0.39
%CV		7.6

Figure 3: Inter-assay precision for Metronidazole at 5.0ppb. Inter-assay precision was examined by running one control level across twenty independent test runs. Inter-assay precision is good, with %CV ≤ 10%.

REPLICATE	SAMPLE CONCENTRATION	
1	1.6469	
2	1.1968	
3	1.3449	
4	1.2258	
5	1.0947	
6	1.2370	
7	1.1212	
8	1.7289	
9	1.5170	
10	1.3912	
11	1.7231	
12	1.2082	
13	2.0664	
14	1.7148	
15	1.5905	
MEAN		1.4538
SD		0.2844
Mean + 3 SD		2.3070

Figure 3: Limit of Detection for Metronidazole. Fifteen known negative samples were assayed using the Nitroimidazoles ELISA kit. The limit of detection (LOD) was determined by multiplying the standard deviation by three and adding to the mean concentration. The limit of detection for this ELISA is 2.3ppb.

### Intra-Assay Precision at Expected Concentration of 1.25ppb

REPLICATE	ABSORBANCE (nm)	CONCENTRATION (ppb)
1	1.595	1.524
2	1.638	1.304
3	1.630	1.342
4	1.578	1.611
5	1.609	1.449
6	1.620	1.394
7	1.662	1.187
8	1.611	1.439
9	1.552	1.753
10	1.583	1.585
11	1.606	1.465
12	1.556	1.731
13	1.539	1.827
14	1.592	1.54
15	1.619	1.397
16	1.548	1.781
17	1.534	1.855
18	1.541	1.819
19	1.562	1.698
20	1.521	1.93
MEAN		1.582
SD		0.21
%CV		13.3

### Intra-Assay Precision at Expected Concentration of 5ppb

REPLICATE	ABSORBANCE (nm)	CONCENTRATION (ppb)
1	1.061	6.128
2	1.089	5.731
3	1.108	5.49
4	1.074	5.942
5	1.060	6.13
6	1.053	6.231
7	1.050	6.289
8	1.022	6.7
9	1.059	6.145
10	1.078	5.88
11	1.111	5.449
12	1.071	5.975
13	1.085	5.787
14	1.051	6.267
15	1.038	6.453
16	1.006	6.958
17	1.036	6.491
18	1.053	6.238
19	1.081	5.84
20	1.069	6.1
MEAN		6.107
SD		0.40
%CV		6.1

Figure 2: Intra-assay precision for Metronidazole at expected concentrations of 1.25ppb and 5ppb. The results demonstrate good intra-assay precision with CV for absorbance much less than 10%.

## CONCLUSIONS

The Nitroimidazole ELISA kit exhibits high cross-reactivity between Metronidazole and Dimetridazole, and can therefore facilitate the quantification of both Nitroimidazoles, with an assay time of only 55 minutes. Combined with a simple, short sample preparation method, this kit represents a fast, low-cost option for reliable detection of Nitroimidazole contamination in honey which has been both produced in, and imported to, the EU and other countries.