Rapid LC-MS/MS Method for the Analysis of Fipronil and Amitraz Insecticides and Associated Metabolites in Egg and Other Poultry Products

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Background

During August 2017, Fipronil was detected in eggs produced from poultry farms in Belgium and The Netherlands. 15 other European countries were also affected prompting the recall of millions of eggs from human consumption. By the end of August 2017, contaminated eggs were also found across the globe including China and Hong Kong. Fipronil, a broad spectrum insecticide which belongs to the phenylpyrazole chemical family, is used in the application of red mite, flea, cockroach and ant control. It is the main active ingredient in many flea prevention pet care products. The World Health Organisation (WHO) has classed Fipronil as class II moderately hazardous pesticide. For eggs, European legislation sets the maximum residue limit (MRL) in Regulation (EC) No. 396/2005 at 0.005mg/kg. The European Food Safety Authority (EFSA) defines an Acute Reference Dose (AFrD) of 0.009mg/kg body weight. AFrD refers to the maximum amount of a substance that can be ingested with no health hazard. At 0.72mg/kg, the EU Commission proposes this level of contamination of fipronil could present an acute health risk.

Application Overview

Within this application note, we describe a fast and sensitive multi-component single method using LC-MS/MS for the detection and quantitation of Fipronil and its associated metabolites, along with Amitraz and its associated metabolites in eggs and other poultry products. Both compounds are used as insecticides on control of fleas and ticks. The developed assay uses a modified QuEChERS sample preparation method for the extraction of the egg and poultry matrices. Chromatography was performed using a reversed phase water/methanol gradient at a complete runtime of 7mins injection to injection using a Phenomenex Kinetex Polar C18 column. Mass Spectrometry is performed on a SCIEX Triple Quad™ 6500+ LC-MS/MS instrument using electrospray ionization, scheduled MRM detection with simultaneous positive/negative ionization switching throughout the run. Results from the assay easily meet the EU regulation MRL of 5µg/kg in terms of LODs, LOQs, signal to noise, MRM ion ratio accuracy and CV and we will show data

Partnering with Phenomenex and TLR to develop a rapid method in the analysis of Fipronil.

to highlight such on both spiked samples and 'real' samples where positive results were found.

Analytical Method

Sample Preparation

A modified and optimized QuEChERS protocol was employed to extract the target compounds from egg. The procedure is as follows :

Step 1: Take 5 g of homogenized egg (no shell)

Step 2: Add 5 mL of water and shake for 10 min

Step 3: Add 10 mL of MeCN and shake for 10 min

Step 4: Add roQ salts (4.0 g $MgSO_4$, 1.0 g NaCl, 1.0 SCTD and 0.5 g SCDS) and shake for 10 min (Phenomenex P/N KS08909)

Step 5: Centrifuge at 2500 g for 5 min

Step 6: Take 4 mL of the MeCN layer, add 0.6 g of calcium chloride and shake for 10 min

Step 7: Centrifuge at 2500 g for 5 min

Step 8: Take 1 mL of the MeCN layer, transfer to an HPLC vial and add 1 mL of water

LC-MS/MS Conditions

Column: Kinetex Polar 2.6 µm C18

Dimensions: 100 x 2.1 mm

Part No.: 00D-4759-AN

Mobile Phases:-

A: 5mM Ammonium Formate in Water

B: 5mM Ammonium Formate in Methanol + 0.05 %

Formic Acid

Injection Volume: 5 µl

Flow Rate: 0.9 mL/min

Temperature: 40 °C

Detection: MS/MS (MRM)

Detector: SCIEX Triple Quad™ 6500+, Pos/Neg switching

LC System: SCIEX Exion LC

LC Gradient

Time (min)	%B
0.5	5
1.5	40
3.0	*80*
5.0	*87*
5.5	*95*
5.6	*5*
7.0	*5*

Table 1. LC Gradient conditions

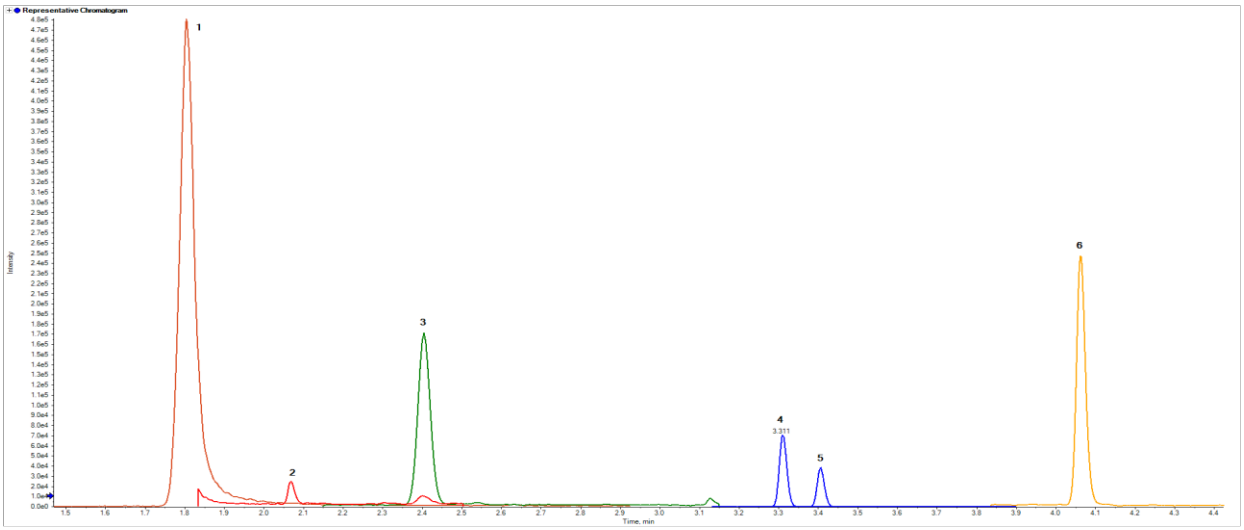

Figure 1. Chromatographic separation of analytes, 1. DPMF, 2. DMA, 3. DMF, 4. Fipronil, 5. Fipronil Sulfone, 6. Amitraz

Also included in this experiment, to monitor sample performance and enable accuracy, are 4 isotopically labeled internal standards (IS). Samples were spiked with an IS solution and followed the extraction procedure in the sample preparation part of this workflow.

MS/MS Conditions

MRM Conditions for Positive Ion Mode

Q1	Q3	Rt	Name
294.097	163.1	3.99	Amitraz-1
294.097	122.2	3.99	Amitraz-2
294.097	107.1	3.99	Amitraz-3
163.058	107.1	1.74	DMPF-1
163.058	106	1.74	DMPF-2
163.058	117	1.74	DMPF-3
150.027	107.1	2.35	DMF-1
150.027	106	2.35	DMF-2
150.027	132.1	2.35	DMF-3
122.03	107	1.99	DMA-1
122.03	77	1.99	DMA-2

Figure 2. Details of the MRM transitions used for each analyte in this experiment

MRM Conditions for Negative Ion Mode

Q1	Q3	Rt	Name
434.83	329.9	3.26	Fipronil-1
434.83	249.9	3.26	Fipronil-2
436.816	331.9	3.26	Fipronil-3
436.816	251.9	3.26	Fipronil-4
450.815	414.9	3.36	Fipronil Sulfone-1
450.815	282	3.36	Fipronil Sulfone-2
452.786	416.9	3.36	Fipronil Sulfone-3
452.786	281.9	3.36	Fipronil Sulfone-4

Figure 3. Details of the MRM transitions used for Fipronil and Fipronil Sulfone in negative ion mode in this experiment

Ion Source Conditions

CAD	Medium
Cur	35
GS1	*55*
GS2	*55*
IS	*4500/-4500*
Temp °C	*550*

Table 2. Source Conditions

Results and Discussion

The chemical structures of fipronil and its metabolite and amitraz and its metabolites are shown in Figure 4 and Figure 5 respectively.

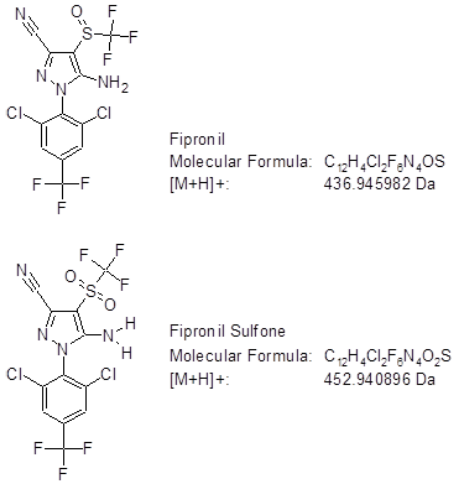

Figure 4. Chemical Structures of Fipronil and Fipronil Sulfone

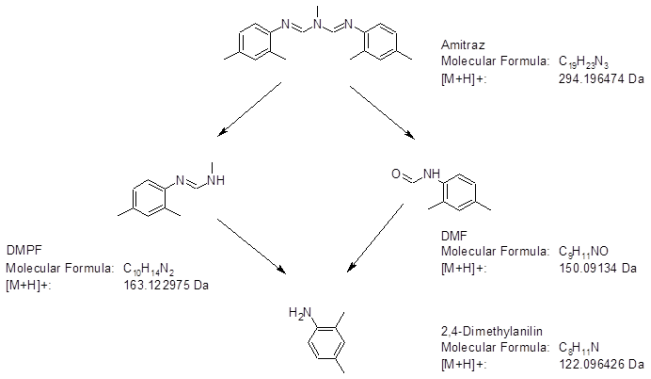

Figure 5. Chemical Structure of Amitraz and its metabolites

During the development of the analytical method, it was determined that the addition of acid in mobile phase A is not

possible as this has a detrimental impact on the peak shape of DMPF. Also the elution order of DMA and DMPF swaps around and makes the separation more challenging. The importance of column temperature was found to be high also as this affects the chromatographic separation of DMA and DMPF

Analytical Performance – Sensitivity

The assay developed shows that it is possible to reach the MRL level of 5µg/kg for all analytes (note DMA was less sensitive than all other analytes in the method but still able to achieve the 5µg/kg MRL but with with lower signal to noise values). Figure 5 below shows all of the analytes.

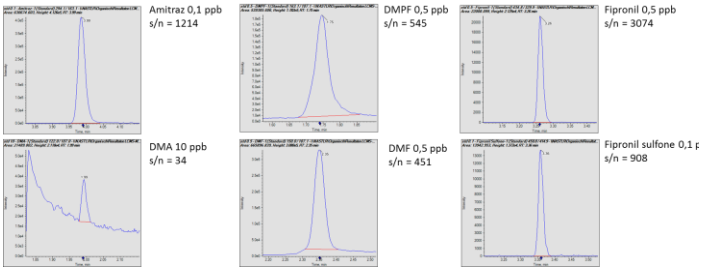

Figure 6. Example of the Assay Sensitivity for All Analytes

Analytical Performance – Reproducibility (%CV)

As part of the analytical method validation, the reproducibility of measurement was evaluated. Extracted standards at 5µg/kg were injected 12 times and Figure 7 below shows the %CV results for each analyte.

	Amitraz	DMPF	DMF	Fipronil	Fipronil sulfone	DMA
Std dev (ppb)	0,08345	0,05241	0,08303	0,15732	0,14076	0,74572
RSDR(%)	1,7%	1,0%	1,7%	3,1%	2,8%	3,0%

Figure 7. %CV for each analyte

Analytical Performance – Linearity

Using the SCIEX Triple Quad™ 6500+ instrument in MRM acquisition mode, all compounds within the assay showed good linearity of measurement between 0.5ppb to 50ppb which was a suitable level of measurement. Figure 8 below shows some reproducibility data for each analyte at the different concentrations measured in matrix.

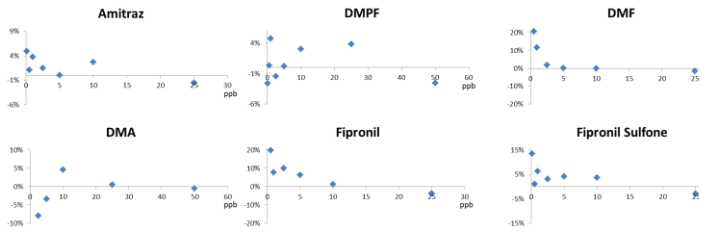

Figure 8. Linearity for each analyte with reproducibility measurement

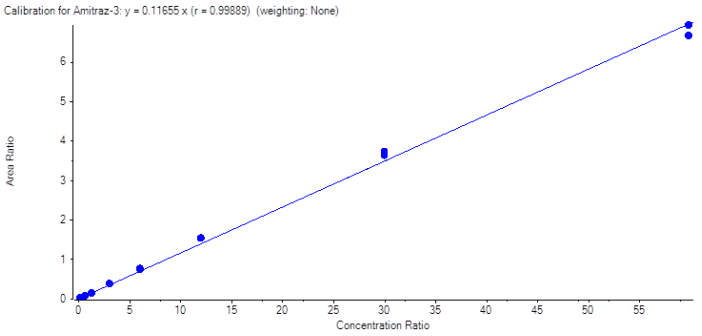

Figure 9. Calibration curve for Amitraz

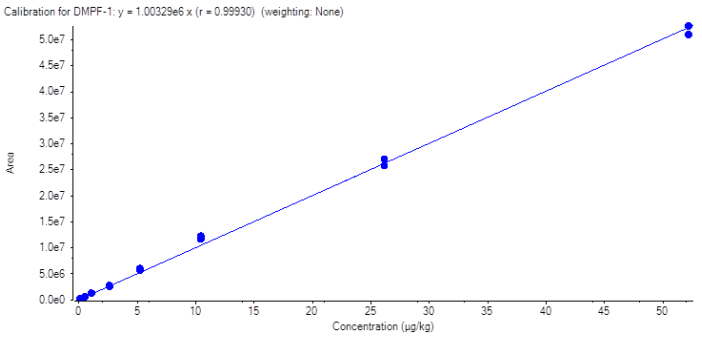

Figure 10. Calibration curve for DMPF

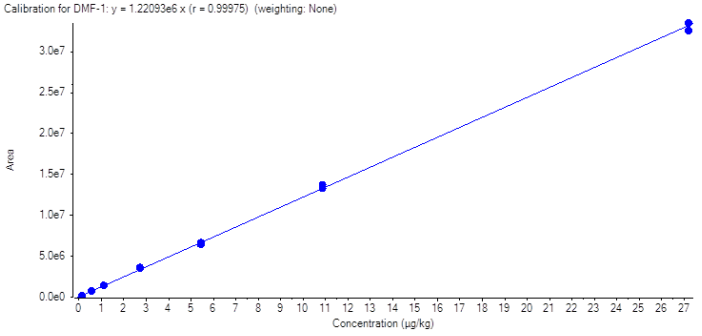

Figure 11. Calibration curve for DMF

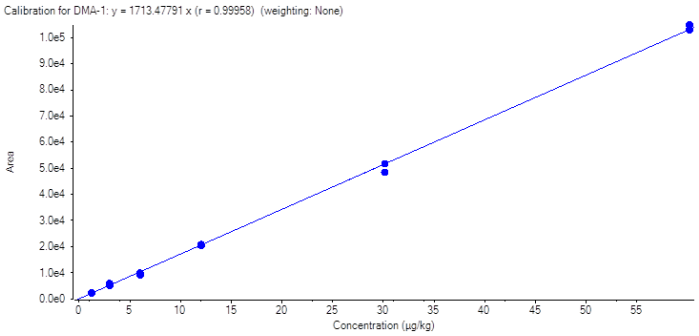

Figure 12. Calibration curve for DMA

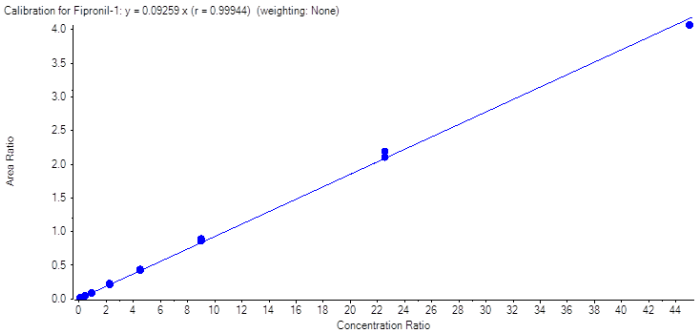

Figure 13. Calibration curve for Fipronil

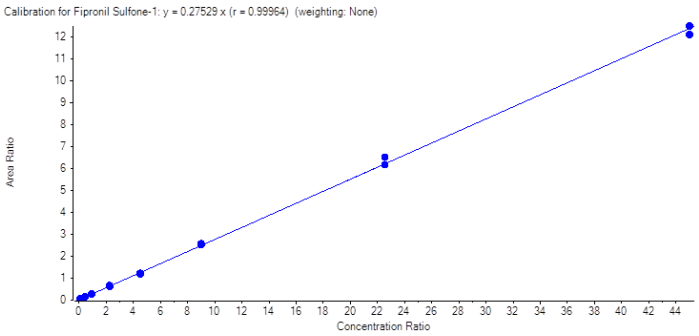

Figure 14. Calibration curve for Fipronil Sulfone

Finally, the effect of the matrix on the analyte response, post-extraction, was evaluated. The matrices tested were egg, egg powder, chicken meat and chicken fat. Figure 15 below shows the overall effect of each matrix on each analyte in terms of MS response.

Component Name	Egg	Egg Powder	Chicken	Chicken Fat
Amitraz	2%	6%	3%	7%
DMPF	4%	8%	6%	8%
DMF	3%	0%	6%	3%
DMA	1%	0%	3%	1%
Fipronil	-3%	-7%	-3%	3%
Fipronil Sulfone	2%	8%	-3%	3%

Figure 15. Effect of matrix on the analyte response.

Conclusions

The analytical method described here is found to be suitable to detect and confirm the presence of the insecticides fipronil, amitraz and their associated metabolites in egg and poultry products. Using a modified and optimised QuEChERS protocol,

samples of egg, egg powder, chicken meat and fat were extracted prior to LC-MS/MS. Using a Phenomenex Kinetex Polar 2.6 µm C18 column with a reversed phase gradient provide by a SCIEX Exion LC systems, the chosen analytes were detected using MRM acquisition on a SCIEX 6500+ triple quadrupole LC-MSMS instrument. Detection was performed using simultaneous pos/neg ion switching.

The results of the analytical assay showed excellent linearity and reproducibility of detection for all compounds. Detection limits were in line with the EU set MRL levels of 5µ/kg, with compounds being able to be detected below this level with high reproducibility in the presence of matrix.

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