EURL-SRM - Analytical Observations Report

concerning the following…

- **Compound(s)**: 4-Amino-meta-toluic acid (main metabolite of amitraz in eggs)
- **Commodities**: Eggs
- **Extraction Method(s)**: Citrate-buffered QuEChERS
- **Instrumental analysis**: LC-MS/MS

Analysis of 4-Amino-3-methylbenzoic acid in eggs

(the main metabolite of amitraz in products of animal origin)

Version 1 (last update: 20.02.2019)

Background information / Initial Observations:

Parasitic mites, such as the blood-sucking 'poultry red mite' (*Dermanyssus gallinae*) pose one of the most worrying threats in poultry farming with affected hens suffering from anemia and lay fewer eggs. In 2005 it was estimated that the red mite leads to losses of 130 million euros per year in the European poultry industry. Red mites feed for a short time during night and the rest of the time they hide in various sheltered spots, for example in the litter. They are very difficult to get rid of as they can survive for many months even in empty farms. Once a poultry farm has been infested farmers typically hire specialized firms to exterminate the pests. Various miticides have been shown to be effective against red mites such as DDT, carbaryl, permethrin, coumaphos, malathion, fipronil and **amitraz**. These have been applied by spraying or dusting either birds directly or emptied farms. Nicotine has also been used as a fumigant and ivermectin as a systemic control agent. Due to the development of resistances there is a steady search for new products [1,2,3].

Amitraz was one of the pesticides applied in chicken farms during the so called "fipronil scandal" in 2017, which took place in several countries around the EU such as The Netherlands, Belgium, France, Italy and Germany. As a reaction to the incidence, an ad-hoc coordinated monitoring program was launched within the EU. Its main focus was on fipronil but eleven additional legal and illegal acaricides were also proposed to be included in the scope, i.e. **amitraz**, bifenthrin, cypermethrin, diazinon, etoxazole, flufenoxuron, ivermectin, pyridaben, pyriproxyfen, thiamethoxam and trichlorfon. The main commodities analyzed were eggs and egg products but other poultry products, such as fat and muscle, were also analyzed [4].

Within Reg. 396/2005/EC the residue definition for amitraz residues is established as "*Amitraz (amitraz including the metabolites containing the 2,4-dimethylaniline moiety expressed as amitraz*" and the MRLs are set at 0.01* for eggs and at 0.05* for poultry tissues. Within the veterinary drug Regulation (37/2010/EC) amitraz is regulated with focus on apicultural products with no MRLs being set for poultry products. The residue definition of the veterinary drug regulation differs in wording ("*Sum of amitraz and all metabolites containing the 2,4-DMA moiety, expressed as amitraz*"), but is essentially equivalent to that of the pesticide residues regulation. Both residue definitions are based on the compounds containing the intact 2,4-DMA moiety. Given the complexity of methods involving hydrolysis to 2,4-DMA moiety laboratories typically analyze for the parent and the two main plant metabolites DMPF[1] and DMF[2] that also entail the 2,4-DMA moiety.

According to the EFSA report [4], out of the 5508 sampes analyzed overall, of chicken products 2206 samples were analyzed for amitraz residues within the frame of the EU-ad-hoc monitoring program. No information is provided as regards the extent to which the individual analytes (amitraz, DMF and DMPF) were covered overall. Overall, only two of the samples from Italy were reported positive on amitraz, one above and one below the MRL.

Furthermore, it was reported that in certain cases amitraz formulations were encountered during farm visits and that amitraz was also detected in manure and litter sampled from some farms. However, analysis of eggs originating from such chicken farms did not show any significant residues neither of amitraz, nor for the two metabolites DMPF and DMF [personal communication].

The metabolic pathways of amitraz in animals, however, differ from those in plants. The first step with the formation of DMPF and DMF is the same for plants and animals, but in animals the methyl group in para position to the nitrogen is further oxidized into a carboxy-group. This leads to the loss of the 2,4-DMA moiety and the formation of **4-Amino-3-methylbenzoic acid** among others. As reported in the JMPR evaluation report on amitraz of 1998 [5], a metabolism study on poultry revealed that the main degradation product of amitraz in eggs was 4-amino-meta-toluic acid, which accounted for 91% of the Total Radioactive Residue (TRR) in egg white (both free acid and in form of labile conjugates) and for 34% of the TRR in egg yolk. The second important metabolite was DMPF, which accounted for 54% of the TRR in egg yolk but with very low absolute levels in the whole egg. 4-amino-meta-toluic acid was furthermore the main metabolite in chicken liver (55% of the TRR) and chicken muscle (81% of the TRR).

Based on this information it can be concluded that 4-amino-meta-toluic acid is at least as important to be monitored in eggs as amitraz, DMPF and DMF. Egg samples collected from farms for which reasonable evidence exists that amitraz was employed were of great interest for checking the presence of 4-amino-meta-toluic acid.

[1] DMPF = 2,4-dimethylphenylformamide = N-methyl-N'-(2,4-xylyl) formamidine = BTS 27271
[2] DMF = 2,4-dimethylformanilide = BTS 27919

Compound details:

Table 1: General information on amitraz

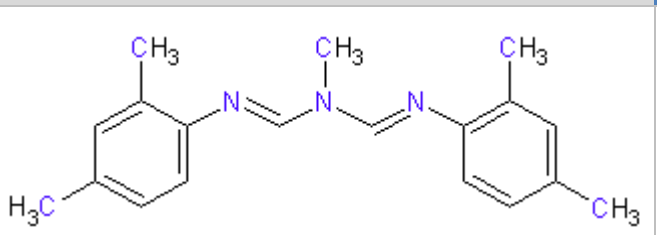
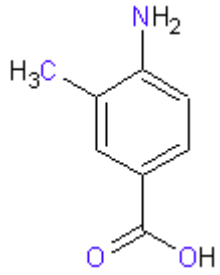
Name: Amitraz (CAS: 33089-61-1) IUPAC: N'-(2,4-dimethylphenyl)-N-{[(2,4-dimethylphenyl)imino]methyl}-N-methylmethanimidamide		
Parameter	**Value**	
Molecular Mass	293.4 g/mol	
Formula	$C_{19}H_{23}N_3$	
Exact mass	293.18919 Da	
Pka	4.2 (Basic)	
LogD	5.5 (25°C) [6]	
Residue definition EU (396/2005/EC)	Amitraz (amitraz including the metabolites containing the 2,4 -dimethylaniline moiety expressed as amitraz)	
Amitraz is approved in…	No authorisation in place in poultry farming	
ADI / ARfD	0.003 mg/kg bw per day / 0.010 mg/kg bw, SCoFCAH 4.7.03	

Table 2: General information on 4-amino meta toluic acid

Name: 4-Amino-3-methylbenzoic acid (CAS: 2486-70-6) IUPAC: 4-Amino meta toluic acid		
Parameter	**Value**	
Molecular Mass	151.2 g/mol	
Formula	$C_8H_9NO_2$	
Exact mass	151.063 Da	
Pka (computed by chemicalize.org)	Pka 1: 4.76 on carboxy group (acidic) Pka2: 2.60 on amino group (basic)	
LogD	pH3: 1.15 pH 3.6: 1.23 (maximum) pH 4: 1.21 pH 5: 0.86 pH 6: 0.04 Predominantly non ionized in the pH-range 2.6 - 4,8 Predominantly cationic at pH<2.6 Predominantly anionic at pH>4.8	
Residue definition EU (396/2005/EC)	Not regulated	

Materials

Table 3: Sources of analytical standards

Substance	Purity	CAS	Source
4-Amino-3-methylbenzoic acid	98%	286-70-6	Sigma-Aldrich
Chlorpyrifos D10	97.0%	285138-81-0	LGC (Dr. Ehrenstorfer)

Disclaimer: Names of companies are given for the convenience of the reader and do not indicate any preference by the EURL-SRM towards these companies and their products

All other materials and chemicals used as listed in EN 15662.

Experiments conducted and observations

Selection and optimization of Instrument, mobile phase and gradient

After the column was chosen, the following parameters were optimized with the aim to obtain maximum sensitivity:

- Ionization mode: ESI pos. or ESI neg.
- Mobile phase solvent: methanol/water or acetonitrile/water
- Mobile phase modifier: formic acid, acetic acid or ammonium formate
- Injection volume

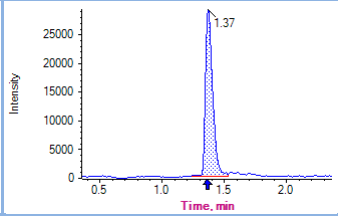
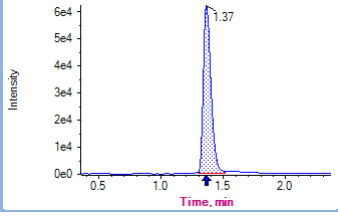
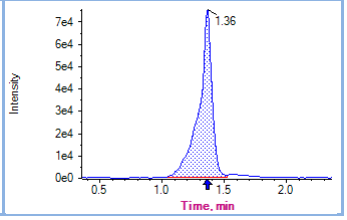
Ionization in the ESI-pos. mode proved to be more sensitive than in the negative mode during FIA analysis, thus positive mode was chosen.

The addition of 0.01% acetic acid in the mobile phase substantially increased peak height of 4-Amino-3-methylbenzoic acid compared to the use of 5 mmol NH_4formate that we typically use in the ESI pos. mode.

Methanol and acetonitrile had little influence on the sensitivity of the target analyte but methanol was favored due to the better sensitivity of chlorpyrifos D_{10}, which was used as an internal standard.

Three different injection volumes were tested as shown in Figure 1. At 10 µL injection a strong fronting appeared which kept the peak height within the same range as with 5 µL. A 2 µl injection volume resulted in a satisfying peak shape but the sensitivity of 4-Amino-3-methylbenzoic acid measured with the Sciex API 4000QTrap was not fully satisfying. Finally 5 µL injection volume was considered a good compromise.

Figure 1: *Chromatograms obtained from injections of different injection volumes (0.01 µg/mL in egg extract QuEChERS; measured by Sciex API 4000QTrap)*

Injection volume	2 µL	5 µL	10 µL
m/z 151/108 (target)			

Final Measurement conditions

Measurement was conducted by LC-MS/MS instrument (ESI-positive mode). Details are given in Table 4 and Table 5.

Table 4: *Instrumentation details*

LC	WATERS Acquity UPLC		
MS/MS	SCIEX API 4000 Q-Trap, run in ESI positive mode		
Column	Waters BEH C18 2.1 x 100 mm 1.7 µm		
Pre-column	Waters BEH C18 2.1 x 5 mm 1.7 µm		
Mobile Phase	A: 0.01% acetic acid in water + 5% methanol B: 0.01% acetic acid in methanol		
Gradient	**Time** (min)	**Mobile Phase A** (%)	**Mobile Phase B** (%)
	0	100	0
	1	10	90
	6	10	90
	6.1	100	0
	10	100	0
Flow	0.4 mL min^{-1}		
Injection volume	5 µL, partial loop with needle overfill		
Column temperature	40°C		

Table 5: *MRM details (ESI-pos. mode using Sciex API 4000 QTrap):*

Compound	Intensity ranking	Q 1	Q 3	DP	CE	CXP
4-Amino-3-methylbenzoic acid	1	152	108	56	19	6
	2	152	93	56	31	6
	3	152	134	56	21	8
Chlorpyrifos D_{10}	-	360	199	66	23	12

Analytical methods

Two QuEChERS-based analytical procedures were tested. One covering free 4-Amino-3-methylbenzoic acid, and the other one involving alkaline hydrolysis to release any bound acid.

a) **Analysis of free 4-Amino-3-methylbenzoic acid:** The QuEChERS procedure (EN-15662) was used for the extraction [6]. 3 mL of water were added to 10 g egg. No pH adjustment was considered necessary as the compound shows the highest logD values at the pH-range of the citrate buffered QuEChERS. The samples were extracted for 15 minutes using an automatic shaker. For cleanup an aliquot of the raw extract was placed in the freezer for 4 hours. The results were evaluated via matrix-matched calibration standards and using chlorpyrifos-D_{10} as internal standard.

b) **Analysis 4-Amino-3-methylbenzoic acid following hydrolysis:** This QuEChERS-based procedure differed from the previous one by conduction of a hydrolysis step at the beginning. 10 g egg sample was weighed and 10 mL acetonitrile were added followed by 1 mL 5 N NaOH and 1 mL of water. The vials were closed and placed into a hot shaking water bath at 60°C for 60 min. After cooling down 1 mL 5N H_2SO_4 were added, to neutralize the base. The internal standard chlorpyrifos-D_{10} was added after neutralization. Following addition of the QuEChERS-CEN partitioning/buffer salt mixture the vials were shaken for 1 minute and centrifuged. For cleanup, an aliquot of the raw extract was placed in the freezer for 4 hours and 1 mL of the extract was decanted for measurement. A suitable matrix-matched calibration was prepared.

Validation

Procedure a) was validated by conducting a recovery experiments at two spiking levels and in quintuplicate. Table 6 shows validation data.

Procedure b) was only checked via a duplicate recovery experiment the aim of which was to check the stability of free 4-Amino-3-methylbenzoic acid under the hydrolysis conditions. Unfortunately, the effectiveness of conjugate cleavage could not be tested lacking positive samples.

Table 6: *Recovery data for free 4-Amino-3-methylbenzoic acid in egg using the CEN-QUEChERS method (Sciex API 4000 QTrap*, 10µL inj. volume (full loop))

Matrix	SampleWeight	Spiking Level	Mass transition	QuEChERS with freezeout		
				n	Mean Rec.%	RSD %
Egg	10 g	0.01	152/108 (T)	5	**103**	**8.2**
			152/93		**111**	**9.2**
		0.1	152/108 (T)	5	**101**	**9.6**
			152/93		**104**	**10.9**

Analysis of samples from farms

In total 18 samples of homogenized eggs, that were collected from farms where amitraz use was detected or suspected, were received for analysis.

Unfortunately, it was not possible to obtain backup material of the two samples from Italy that were reported to contain amitraz residues in the EFSA report [4}.

4-Amino-3-methylbenzoic acid residues were not detected in any of the 18 Dutch samples, neither via QuEChERS nor via QuEChERS following alkaline hydrolysis.

Figure 2 shows chromatograms obtained following procedure a) (CEN-QuEChERS) as follows: a) blank extract; b) matrix-matched calibration standard at 0.01 µg/mL; c) solvent-based calibration standard at 0.01 µg/mL; d) sample extract; e) extract of recovery experiment at 0.01 mg/kg.

Figure 2 shows chromatograms obtained with procedure b) entailing alkaline hydrolysis as follows: f) blank extract; g) matrix-matched calibration standard at 0.01 µg/mL; h) sample extract; i) extract of recovery experiment at 0.01 mg/kg. Figure 3j shows a matrix-matched calibration curve in the range between 0.005 and 0.05 mg/kg.

Figure 2: *Chromatograms of 4-Amino-3 methylbenzoic acid obtained from injections of **CEN-QuEChERS** extracts as follows: a) blank extract; b) matrix-matched calibration standard at 0.01 µg/mL; c) solvent-based calibration standard at 0.01 µg/mL; d) sample extract; e) extract of recovery experiment at 0.01 mg/kg (Measured at ESI-pos. mode using Sciex API 4000 QTrap, 5µL injection)*

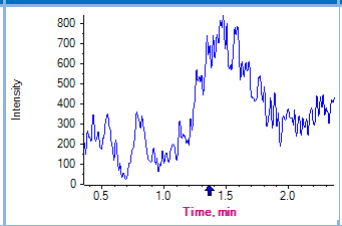
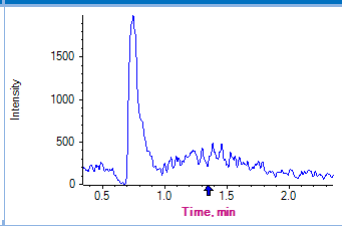
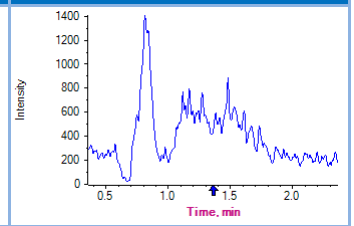
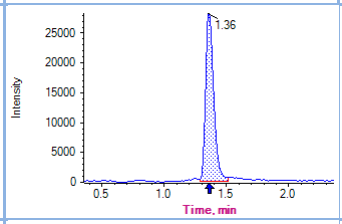
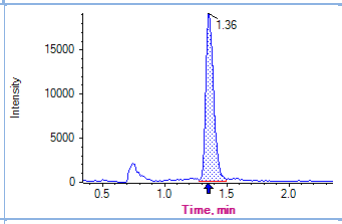
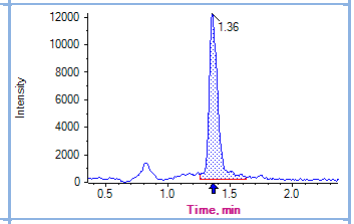
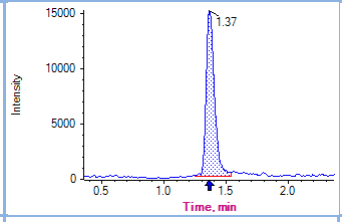
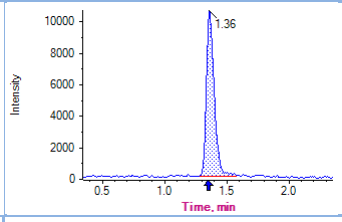
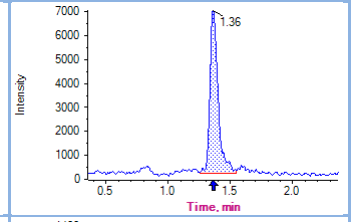
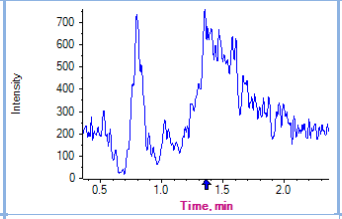
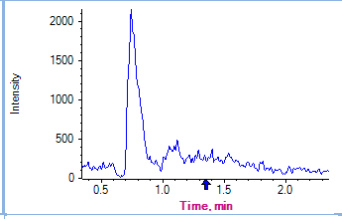
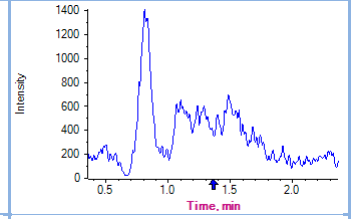
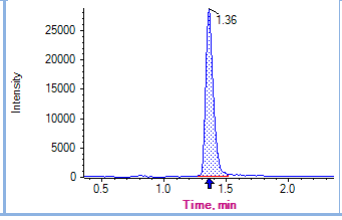
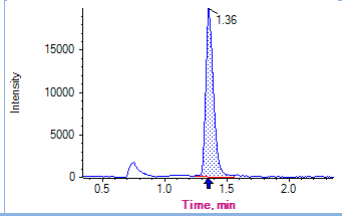
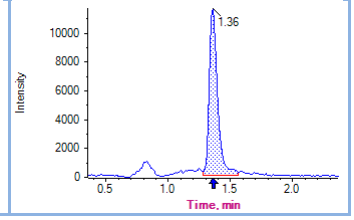
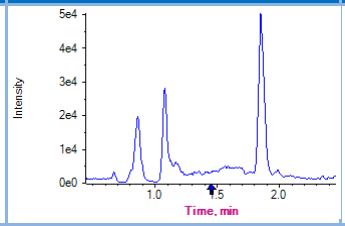
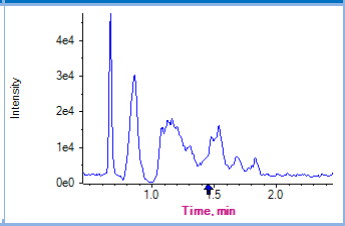
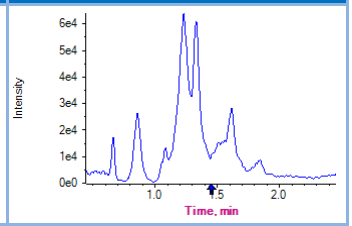
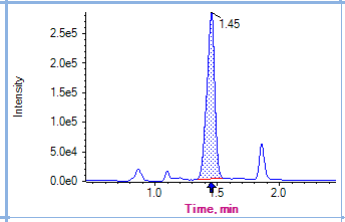
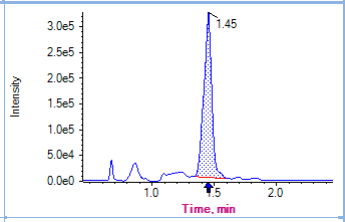
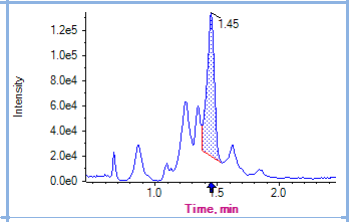
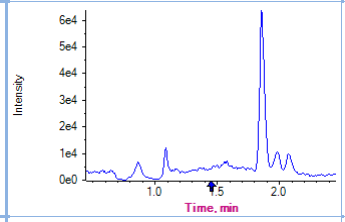
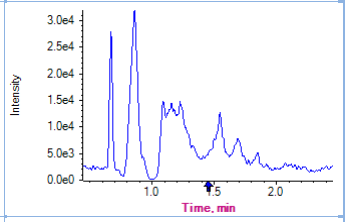
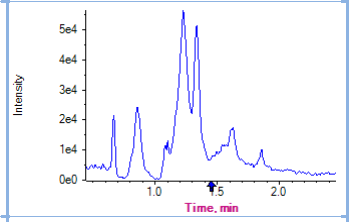
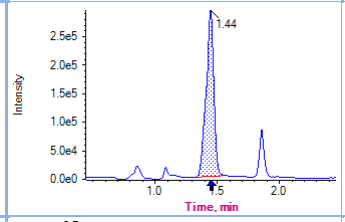
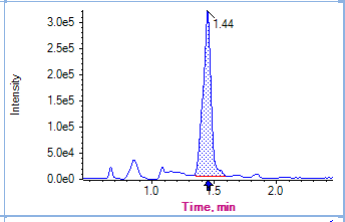
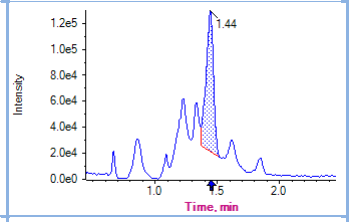
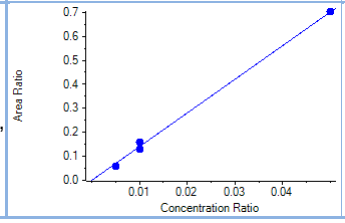
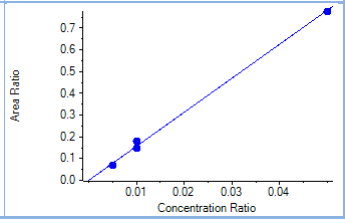
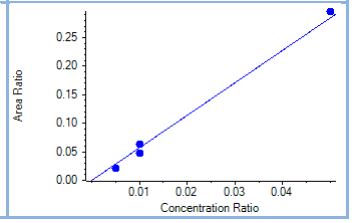
	m/z 151/108	m/z 151/93	m/z 151/134
a) Blank Egg 1g/mL			
b) Calibration egg 0.01 µg/mL			
c) Calibration solvent 0.01 µg/mL			
d) Egg sample extract 1g/mL			
e) Recovery egg 0.01 mg/kg			

Figure 3: *Chromatograms of 4-Amino-3 methylbenzoic acid obtained with from injections of extracts of the QuEChERS-based procedure involving alkaline hydrolysis as follows: a) blank extract; b) matrix-matched calibration standard at 0.01 µg/mL; c) sample extract; d) extract of recovery experiment at 0.01 mg/kg. In addition under e) the matrix-matched calibration curve at 0,005 µg/mL, 0.01 µg/mL, 0.05 µg/mL is shown; (Measured at ESI-pos. mode using Sciex API 5500 QTrap, 5µL injection)*

	m/z 151/108	m/z 151/93	m/z 151/134
a) Blank Egg 1g/mL			
b) Calibration egg 0.01 µg/mL			
c) Egg sample extract 1g/mL			
d) Recovery egg 0.01 mg/kg			
e) Matrix-matched calibration curve 0.005 µg/mL, 0.01 µg/mL, 0.05 µg/mL			

Conclusions

Based on information from metabolism studies 4-amino-3 methylbenzoic acid was identified as a potential marker for amitraz-use in chicken farms. Two CEN-QuEChERS-based methods for the analysis of 4-amino-3 methylbenzoic acid in eggs were developed, one concerning the analysis of free compound and one involving a hydrolysis step to release conjugated residues. 18 samples from farms suspected to potentially contain amitraz residues were tested by these two methods, however, none of these samples was found to contain any measurable residues of 4-amino-3 methylbenzoic acid.

Literature

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[4] EFSA Journal 2018;16(5):5164; Occurrence of residues of fipronil and other acaricides in chicken eggs and poultry muscle/fat https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2018.5164
[5] JMPR evaluations 1998, part II: http://www.inchem.org/documents/jmpr/jmpmono/v098pr02.htm
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Acknowledgement

We would like to thank Dr. André de Kok and Dr. Barbara Kiedrowska from the NVWA (NL-Wageningen) for providing samples egg homogenates from farms strongly suspected of having employed amitraz.

History

Action	When	Document Version
Initial Experiments	September 2017	
Further Validation Experiments	Jan.-Mar. 2018	
Observation document placed on-line	May 2019	V1