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Method of Test for Pesticide Residues in Poultry and Livestock Products - Test of Amitraz and its Metabolite

1. Scope

This method is applicable for the determination of amitraz and its metabolites, N'-(2,4-dimethylphenyl)-N-methylformamidine (DMPF), in muscle, viscera, fat, eggs and milk of poultry and livestock products.

2. Method

After preparation of the sample solution by the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, Safe), pesticides are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: CORTECS UPLC, C18, 1.6 μm, 2.1 mm i.d. × 10 cm, or an equivalent product.

2.1.1.3. Guard column: CORTECS UPLC, C18, 1.6 μm, 2.1 mm i.d. × 5 mm, or an equivalent product.

2.1.2. Blender.

2.1.3. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, > 1000 rpm, or an other mechanical shaker.

2.1.4. Centrifuge: centrifugal force ≥ 4000 ×g, temperature control < 15°C.

2.1.5. Nitrogen evaporator.

2.1.6. Vortex mixer.

2.2. Chemicals

Formic acid, reagent grade;
Ammonium acetate, reagent grade;
Acetonitrile, HPLC grade;
Methanol, HPLC grade;
Sodium citrate, residue grade;
Disodium hydrogen citrate, residue grade;
Sodium chloride, residue grade;
Magnesium sulfate anhydrous, residue grade;
Octadecylsilane end-capped (C18 EC), analytical grade;

Deionized water, resistivity ≥ 18 MΩ·cm (at 25°C);

Amitraz and DMPF, reference standards.

2.3. Apparatus

2.3.1. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.2. Membrane filter: 0.2 μm, PTFE.

2.3.3. Volumetric flask: 10 mL, amber.

2.3.4. Ceramic homogenizer(note 1): Bond Elut QuEChERS P/N 5982-9313, or an equivalent product.

2.3.5. Extraction powder(note 2): containing 4 g of magnesium sulfate anhydrous, 1 g of sodium citrate, 0.5 g of disodium hydrogen citrate, and 1 g of sodium chloride.

2.3.6. Clean-up centrifuge tube(note 2): containing 750 mg of magnesium sulfate anhydrous and 250 mg of C18 EC, 5 mL.

Note 1: Ceramic homogenizer can be used depending on the viscosity of the sample.

Note 2: Commercial extraction/clean-up kit can be used as needed.

2.4. Mobile phase

2.4.1. Solvent A

Dissolve and dilute 0.4 g of ammonium acetate with deionized water to 1000 mL. Add 1 mL of formic acid, and filter with a membrane filter.

2.4.2. Solvent B

Dissolve and dilute 0.4 g of ammonium acetate with methanol to 1000 mL, and filter with a membrane filter.

2.5. Standard solution preparation

Transfer about 10 mg of amitraz and DMPF accurately weighed to each 10-mL volumetric flask, dissolve and dilute to volume with acetonitrile as the standard stock solutions. Store under freezing in the dark. When to use, mix appropriate volume of each standard stock solution, and dilute with acetonitrile to 1 μg/mL as the standard solution.

2.6. Sample solution preparation

Transfer about 10 g of the homogenized muscle, visceral or milk

sample accurately weighed; transfer about 2 g of the homogenized fat sample accurately weighed; remove eggs' shell and transfer about 10 g of the mixed egg white and yolk sample accurately weighed into a 50-mL centrifuge tube. Add 10 mL of acetonitrile, 1 granule of a ceramic homogenizer and the extraction powder, cap the centrifuge tube, shake vigorously several times by hands to prevent aggregation of salt, and then shake at 1000 rpm by the high speed dispersing device or shake vigorously by hands for 3 min. Centrifuge at 4000 ×g for 5 min at 15°C, and transfer 5 mL of the supernatant (avoiding taking the fat layer) to a clean-up centrifuge tube. Shake at 1000 rpm by the high speed dispersing device or shake vigorously by hands for 2 min, and centrifuge at 4000 ×g for 5 min at 15°C. Take 1 mL of the supernatant, and filter with a membrane filter as the sample solution.

2.7. Matrix-matched calibration curve

Take a blank sample, follow the procedure described in section 2.6 to obtain the supernatant after the clean-up procedure. Take several 1 mL of the supernatant, and evaporate to near dryness by gently flushing with a stream of nitrogen. Separately add 5-200 μL of the standard solution (add 2-100 μL for fat)(note 3), dilute with acetonitrile to 1 mL, mix well, and filter with membrane filters as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curves of amitraz and DMPF by the peak areas vs. the added concentrations (0.002-0.1 μg/mL for fat, and 0.005-0.2 μg/mL for other matrices).

LC-MS/MS operating conditions(note 4)

Column: CORTECS UPLC, C18, 1.6 μm, 2.1 mm i.d. × 10 cm.

Guard column: CORTECS UPLC, C18, 1.6 μm, 2.1 mm i.d. × 5 mm.

Mobile phase: a gradient program of solvent A and solvent B is as follows:

Time (min)	A (%)	B (%)
0.0 → 1.0	99 → 10	1 → 90
1.0 → 2.0	10 → 1	90 → 99
2.0 → 3.2	1 → 1	99 → 99

3.2 → 3.5	1 → 99	99 → 1
3.5 → 5.0	1 → 99	1→ 1

Flow rate: 0.3 mL/min.

Injection volume: 2 μL.

Capillary voltage: 3 kV.

Ionization mode: ESI^+.

Ion source temperature: 150°C.

Desolvation temperature: 450°C.

Cone gas flow: 30 L/hr.

Desolvation flow rate: 900 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in the attached table.

Note 3: As amitraz can be easily metabolized to DMPF, the matrix-matched calibration curves of amitraz and DMPF should prepare separately.

Note 4: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.8. Identification and quantification

Accurately inject 2 μL of the sample solution and the matrix-matched standard solutions into LC-MS/MS separately, and operate according to the conditions in section 2.7. Identify amitraz and DMPF based on the retention time and the relative ion intensities(note 5). Calculate the amount of amitraz in the sample by the following formula(note 6):

$$\text{The amount of amitraz in the sample (ppm)} = \frac{\Sigma[C \times V \times F]}{M}$$

Where,

C: the concentration of amitraz or DMPF in the sample solution calculated by the matrix-matched calibration curve (μg/mL)

V: the volume of acetonitrile for sample extraction (10 mL)

M: the weight of the sample (g)

F: conversion factor of DMPF

Amitraz: 0.55

DMPF: 1

Note 5: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions (≤ 100%). Maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≤ 10	± 50

Note 6: According to CODEX, the residue definition of amitraz in animal products is sum of amitraz and DMPF calculated as DMPF.

Remark

1. Limits of quantification (LOQs) for amitraz and DMPF are listed in the attached table.
2. Further validation should be performed when interfering compounds are found in the samples.

Reference

1. EU Reference Laboratories for Residues on Pesticides. 2007. Analysis of amitraz and its main metabolite in pears via QuEChERS and LC-MS/MS. [eurl-pesticides.eu/library/docs/srm/meth_Amitraz_CrlSrm.pdf]
2. European Committee for Standardization. 2018. Foods of plant origin – Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE - Modular QuEChERS-method. NF EN 15662:2018 (English version).
3. Lin, S. K., Chuang, C. W. and Wang, Y. S. 2019. Development a method for pesticide residues analysis on animal products. Commissioned Research Report of Taiwan Food and Drug Administration.

Reference chromatogram

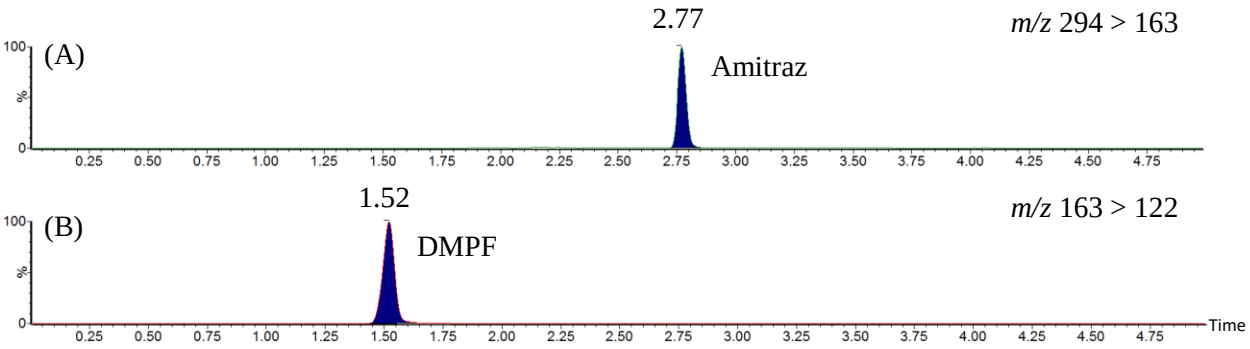

Figure. MRM chromatograms of amitraz and its metabolite, DMPF, standards analyzed by LC-MS/MS.

Table. MRM parameters and LOQs of amitraz and DMPF

No	Analyte	Quantitative ion pair			Qualitative ion pair			LOQ (ppm)				
		Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) > product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Muscle	Viscera	Fat	Eggs	Milk
1	Amitraz	294 > 163	20	16	294 > 122	20	34	0.01	0.01	0.01	0.01	0.005
2	DMPF	163 > 122	50	20	163 > 132	50	20	0.01	0.01	0.01	0.01	0.005