Title: Determination of Methoxyfenozide and Tebufenozide in Surface Water by Ultra Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

1. Scope:

   This section method (SM) provides stepwise procedure for methoxyfenozide and tebufenozide analysis in surface water. It is followed by all authorized EA personnel.

2. Principle:

   The methoxyfenozide and tebufenozide are extracted from the surface water sample with methylene chloride. The extract is passed through sodium sulfate to remove residual water. The anhydrous extract is evaporated on a rotary evaporator and then a solvent exchange is performed with methanol. The extract is concentrated to a final volume of 1 mL and then vialed into an autosampler vial for analysis on Ultra Performance Liquid Chromatography (UPLC) coupled to a positive electrospray ionization triple quadrupole mass spectrometry (ES-LC/MS/MS).

3. Safety:

   3.1 All general laboratory safety rules for sample preparation and analysis shall be followed.

   3.2 Methylene chloride is a regulated and controlled carcinogenic hazardous substance. It must be stored and handled in accordance with California Code of Regulations, Title 8, Subchapter 7, Group 16, Article 110, Section 5202.

4. Interferences:

   There were no matrix interferences for methoxyfenozide and tebufenozide at the time of method development.

5. Apparatus and Equipment:

   5.1 Rotary Evaporator (Buchi/Brinkman or equivalent)
   5.2 Nitrogen Evaporator (Meyer N-EVAP Organomation Model #112 or equivalent)
   5.3 Balance (Mettler PC 4400 or equivalent)
   5.4 Vortex-vibrating mixer
   5.5 UPLC equipped with a triple quadrupole mass spectrometry and ES ion source.

6. Reagents and Supplies:

6.1 Methoxyfenozide CAS#161050-58-4
6.2 Tebufenozide CAS#112410-23-8
6.3 Methylene Chloride, nanograde or equivalent pesticide grade
6.4 Water, MS grade, Burdick & Jackson or equivalent
6.5 Methanol, MS grade, Burdick & Jackson or equivalent
6.6 Formic Acid, HPLC grade
6.7 Ammonium formate, reagent grade or equivalent
6.8 Separatory funnel, 2 L
6.9 Boiling flask, 500 mL
6.10 Sodium Sulfate, ACS grade
6.11 Funnels, long stem, 60°, 100 mm I.D.
6.12 Volumetric Pipette, 0.5 mL
6.13 Graduated conical tubes with glass stopper, 15 mL
6.14 Glass wool, Pyrex® fiber glass slivers 8 microns
6.15 Disposable Pasteur pipettes, and other laboratory ware as needed
6.16 Recommended analytical column:
Waters Acquity BEH C18 1.7µm, 2.1 x 100 mm
6:17 Aqueous Solution: For 500 mL, mix 470 ± 2mL water, 25 ± 0.5 mL methanol, 4.50 ± 0.25 mL 1 M ammonium formate and 0.5 ± 0.05 mL formic acid.
6.18 Organic Solution: For 500mL, mix 450 ± 2mL methanol and 45 ± 0.5 mL water with 4.50 ± 0.25 mL 1 M ammonium formate and 0.5 ± 0.05 mL formic acid.

7. Standards Preparation:

7.1 The individual stock standards of 1.0 mg/mL were obtained from the CDFA/CAC Standards Repository. The standards were diluted to10 µg/mL with methanol for identification purposes.

A combination standard of 1 µg/mL was prepared from the individual 10 µg/mL standards with methanol. The standard was also used to dilute the following concentrations: 0.025, 0.05, 0.1, 0.25 and 0.5 µg/mL in methanol for instrument calibration.

7.2 Keep all standards in the designated refrigerator for storage.

7.3 The expiration date of each standard is six months from the preparation date.

8. Sample Preservation and Storage:

Store all samples waiting for extraction in a separate refrigerator (4 ± 3 °C).

9. Test Sample Preparation:

9.1 Background Preparation

The Department of Pesticide Regulations (DPR) provides the background water for matrix blank and spikes.

9.2 Preparation of blank and spike

Matrix blank: Weigh out 500 g of background water and follow the test sample extraction procedure.

Matrix spike: Weigh out 500 g of background water. Spike a client requested amount of insecticides into the background water, mix well and let it stand for one minute. Follow the test sample extraction procedure.

9.3 Test Sample Extraction

9.3.1 Remove samples from the refrigerator and allow them to reach ambient temperature.

9.3.2 Mix sample well before weighting aliquot. Weight 500 ± 0.1 g of water samples by subtracting the weight of the sample container before and after water has been transferred into a separatory funnel.

9.3.3 Shake with 80 ± 5 mL of methylene chloride for 1 minute. Vent frequently to relieve pressure.

9.3.4 After phases have separated, drain the lower methylene chloride layer through 25 ± 4 g of anhydrous sodium sulfate and glass wool into a 500 mL boiling flask.

9.3.5 Repeat steps 9.3.3 & 9.3.4 two more times using 60 ± 5 mL of methylene chloride and shake for 1 minute each time. Combine the extracts in the same boiling flask.

9.3.6 After draining the final extraction, rinse the sodium sulfate with 25 ± 5 mL of methylene chloride.

9.3.7 Evaporate the sample extract to 2 - 4 mL on a rotary evaporator using a water bath at 35 ± 2 °C and 15 – 20 inch Hg vacuum. Transfer the extract to a calibrated 15 mL graduated test tube.

9.3.8 Rinse flask 3 more times with 2 - 4 mL of methylene chloride and transfer each rinse to the same test tube.

9.3.9 Evaporate the sample extract to dryness in a water bath at 40 ± 2 °C under a gentle stream of nitrogen. Then bring to a final volume of 0.5 mL with methanol, mix well and transfer to an autosampler vial. Submit extract for LC-MS analysis.

10. Instrument Calibration:

10.1 The calibration standard curve consists of a minimum of three levels. The lowest level must be at or below the corresponding reporting limit. The current working standard levels are 0.025, 0.05, 0.1, 0.25, and 0.5 µg/mL.

10.2 Calibration is obtained using a linear or quadratic regression with the correlation coefficient (r) equal to or greater than 0.995, with all levels weighted 1/x.

11. Analysis**:**

11.1 Injection Scheme

The LC-MS needs to be conditioned with standard or a sample extract 2 to 5 runs before running the following sequence: A set of calibration standards, a matrix blank, a matrix spike, a set of up to 12 test samples, then a set of standards, etc.

11.2 UPLC-MS/MS

11.2.1 UPLC Instrument: Waters Acquity Ultra Performance LC
Column: Waters Acquity BEH C18 1.7µm, 2.1 x 100 mm
Column Temperature: 60 °C
Mobile Phase: Gradient

Solvent 1: Aqueous Solution
Solvent 2: Organic Solution
Gradient:

Time(min)	Flow rate (mL/min)	Solvent 1	Solvent 2
0	0.60	90.0	10.0
0.5	0.60	90.0	10.0
7.00	0.60	10.0	90.0
7.80	0.60	10.0	90.0
8.00	0.60	90.0	10.0
8.50	0.60	90.0	10.0

Injection Volume: 1.0 µL

11.2.2 Mass Spectrometry and Operating Parameters
Model: Waters Xevo Triple Quadrupole
Ion ProbeType: Electrospray Ionization (ESI)
Ion Mode: Positive
Source Temp: 150 °C

Compound	Retention Time (min)	Precursor ion	Product Ion	Dwell (s)	Cone(V)	Collision Energy/-ev
Methoxyfenozide	5.80	369.24 369.24	**149.05** 313.13	0.128 0.128	12.0 12.0	16.0 6.00
Tebufenozide	6.33	353.25 353.25	**133.05** 297.15	0.128 0.128	14.0 14.0	16.0 6.00

Quantitation ions are in bold.

12. Quality Control:

12.1 Method Detection Limits (MDL)

Method Detection Limit (MDL) refers to the lowest concentration of the analyte that a method can detect reliably. To determine the MDL, 7 surface water samples are spiked at 0.1ppb and processed through the entire method along with a blank. The standard deviation derived from the spiked sample recoveries was used to calculate the MDL for each analyte using the following equation:

MDL = tS

Where t is the Student t test value for the 99% confidence level with n-1 degrees of freedom and S denotes the standard deviation obtained from n replicate analyses. For the n=7 replicates used to determine the MDL, t=3.143.

The results for the standard deviations and MDL are in Appendix 1.

12.2 Reporting Limit (RL)

Reporting limit (RL) refers to a level at which reliable quantitative results may be obtained. The MDL is used as a guide to determine the RL. The RL is chosen in a range 1-5 times the MDL, as per client agreement. The reporting limit for methoxyfenozide and tebufenozide is 0.05 ppb.

12.3 Method Validation

The method validation consisted of five sample sets. Each set included five levels of fortification and a method blank. All spikes and method blanks were processed through the entire analytical method. Spike levels and recoveries for the analytes are shown in Appendix 2.

12.4 Control Charts and Limits

Control charts were generated using the data from the method validation for each analyte. The upper and lower warning and control limits are set at $\pm$ 2 and 3 standard deviations of the percent recovery, respectively, shown in Appendix 2.

12.5 Acceptance Criteria

12.5.1 Each set of samples will have a matrix blank and a spiked matrix sample.

12.5.2 The retention time should be within $\pm$ 2 percent of that of the standards.

12.5.3 The recoveries of the matrix spikes shall be within the control limits.

12.5.4 The sample shall be diluted if results fall outside of the calibration curve.

13. Calculations**:**

Quantitation is based on an external standard (ESTD) calculation using either the peak area or height. The triple quadrupole LCMS software used a linear curve fit, with all levels weighted 1/x. Alternatively, at the chemist's discretion, sample results may be calculated using the response factor for the standard.

$$ppb = \frac{(\text{sample peak area or ht}) \times (\text{std conc.}) \times (\text{std vol. Injected}) \times (\text{final vol. of sample})(1000\ \mu L/mL)}{(\text{std peak area or ht}) \times (\text{sample vol. injected}) \times (\text{sample wt (g)})}$$

14. Reporting Procedure**:**

Sample results are reported out according to the client's analytical laboratory specification sheets.

15. Discussion and References**:**

15.1 A storage stability study was done with this project. The storage stability study consisted of a 1.0 ppb spike level and 3 replicates over a 28 day period. Twelve liters of background well water were spiked and then transferred to twelve one liter amber bottles. These spiked samples were stored in the refrigerator until analyzed on 0, 2, 4, 7, 15, 21 and 28 days. Along with the storage spikes, a blank and method control spike were also extracted. This storage study showed no significant degradation for these compounds within 28 days. Results for the storage study are shown in Appendix 3.

15.2 Solid phase extraction using an Oasis HLB 500mg cartridge was also tried. A 500 mL surface water sample was filtered through a glass fiber filter. The filtered sample was passed through a solid phase extraction HLB cartridge and methoxyfenozide and tebufenozide were eluted from the solid phase cartridge with acetonitrile. The extract was concentrated to just dryness with nitrogen in a heated water bath, and then adjusted to a 0.5 mL volume with methanol. Recoveries were good and in the 80-90% range. There were some concerns about filtering away the sediment that could be in more turbid samples and the possible loss of methoxyfenozide and tebufenozide that might occur during that process. It was decided to retain liquid /liquid extraction as the primary extraction process.

16. References:

16.1 Hall,Gregory; Engebretson, Jo; Hengel, Mathew J. and Shibamoto, Takayuki "Analysis of Methoxyfenozide Residues in Fruits, Vegetables, and Mint by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), J. Agric. Food Chem. 2004, 52, 672-676

16.2 "Crop Protection Handbook, 2010", MeisterPro Executive Office 27722 Euclid Ave., Willoughby, OH

Appendix 1

The Determination of Method Detection Limit (MDL) and Reporting Limit (RL)

Lab #	Spk\Analyte	Methoxyfenozide	Tebufenozide
2011-1806	blk	nd	nd
2011-1807	0.1ppb spk 1	0.086	0.088
2011-1808	0.1ppb spk 2	0.087	0.087
2011-1809	0.1ppb spk 3	0.086	0.087
2011-1810	0.1ppb spk 4	0.089	0.089
2011-1811	0.1ppb spk 5	0.089	0.088
2011-1812	0.1ppb spk 6	0.092	0.092
2011-1813	0.1ppb spk 7	0.088	0.0088
	SD	0.00204	0.001822
Reported	**MDL**	**0.00641**	**0.00573**
	RL	**0.05**	**0.05**

All concentrations are expressed in ppb.

Appendix 2

Method Validation Data:

Analyte	Spike ppb	Recovery (%) Set1	Set2	Set3	Set4	Set5		%
Methoxyfenozide	0.1	93.0	97.0	114	87.0	91.0	Mean:	93.3
	0.25	94.0	93.6	96.4	87.2	92.8	SD:	6.49
	0.5	94.8	96.2	95.6	100	90.6	UCL:	113
	1	90.2	86.6	91.8	90.4	83.7	UWL:	106
	5	94.6	94.6	99.4	98.0	79.6	LWL:	80.3
							LCL:	73.8
Tebufenozide	0.1	92.0	97.0	112	87.0	91.0	Mean:	93.0
	0.25	93.6	94.0	95.6	87.2	93.2	SD:	6.07
	0.5	94.9	96.2	94.6	99.8	90.8	UCL:	111
	1	90.5	86.6	91.1	90.6	84.7	UWL:	105
	5	93.4	92.4	98.6	97.6	79.6	LWL:	80.8
							LCL:	74.7

Appendix 3

Storage study Summary for Methoxyfenozide & Tebufenozide in Surface Water

Analyte \ Recovery %		Day 0	Day 2	Day 4	Day 7	Day 14	Day 21	Day 28
Methoxyfenozide	blank	ND	ND	ND	ND	ND	ND	ND
	QC spike		81.5%	85.9%	82.4%	84.7%	87.5%	84.7%
	Spike 1	85.5%	81.5%	81.3%	91.4%	87.2%	91.9%	86.7%
	Spike 2	91.5%	87.7%	88.3%	85.2%	82.5%	89.6%	87.8%
	Spike 3	86.6%	82.9%	87.8%	86.2%	82.5%	88.0%	83.4%
Tebufenozide	blank	ND	ND	ND	ND	ND	ND	ND
	QC spike		80.5%	85.2%	81.6%	84.2%	88.6%	84.2%
	Spike 1	86.1%	81.8%	81.5%	90.0%	87.1%	91.1%	87.7%
	Spike 2	90.5%	88.4%	85.8%	86.4%	82.6%	87.4%	85.3%
	Spike 3	85.7%	82.5%	87.9%	86.0%	81.3%	87.7%	79.4%

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