

Title: Determination of Azoxystrobin, Azoxystrobin Acid, Azoxystrobin Z-metabolite, Dicloran, Iprodione, Isoiprodione, Vinclozalin and 3,5-Dichloroaniline in Well Water

1. Scope:

This section method (SM) provides stepwise procedure for Azoxystrobin, Dicloran, Iprodione, Isoiprodione, Vinclozalin and their degradation products analysis in well water. It is followed by all authorized EA personnel.

2. Principle:

The Azoxystrobin, Dicloran, Iprodione, Isoiprodione, Vinclozalin and their degradation products are extracted from the acidified well water samples 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 vialled into 3 autosampler vials for analysis on GCMS-SIM (Gas Chromatography with Mass Spectrometer operated in the Selected Ion Monitoring mode) with a DB35ms column, GCMS-SIM with a HP5ms column and LCMS with APCI ion source.

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 was a matrix interference for 3, 5 dichloroaniline on the DB35ms column that made it necessary to analyze it using an HP-5 ms column.

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 Gas Chromatograph equipped with a mass selective detector (MSD) and DB35ms column
- 5.6 Gas Chromatograph equipped with a mass selective detector (MSD) and HP5ms column
- 5.7 Liquid Chromatograph equipped with an ion trap (LCMS) and APCI ion source.

6. Reagents and Supplies:

- 6.1 Azoxystrobin CAS#131860-33-8
- 6.2 Azoxystrobin Acid CAS#
- 6.3 Azoxystrobin Z-metabolite CAS#
- 6.4 Dicloran CAS#99-30-9
- 6.5 Iprodione CAS#36734-19-7
- 6.6 Isoiprodione CAS#63637-89-8
- 6.7 3, 5- Dicloroaniline CAS#626-43-7
- 6.8 Vinclozolin CAS#50471-44-8
- 6.9 Methylene Chloride, nanograde or equivalent pesticide grade
- 6.10 Sulfuric Acid,
- 6.11 Water, MS grade, Burdick & Jackson or equivalent
- 6.12 Methanol, MS grade, Burdick & Jackson or equivalent
- 6.13 Formic Acid, HPLC grade
- 6.14 Ammonium formate, reagent grade or equivalent
- 6.15 Separatory funnel, 2 L
- 6.16 Boiling flask, 500 mL
- 6.17 Sodium Sulfate, ACS grade
- 6.18 Funnels, long stem, 60°, 10 mm diameter
- 6.19 Volumetric Pipette, 0.5 mL
- 6.20 Graduated conical tubes with glass stopper, 15 mL
- 6.21 Glass wool, Pyrex® fiber glass slivers 8 microns
- 6.22 Disposable Pasteur pipettes, and other laboratory ware as needed
- 6.23 Recommended analytical columns:

For MSD - 5% (Phenyl)-methylpolysiloxane (HP-5MS or equivalent) fused silica column, 30 m x 0.25 mm id x 0.25 µm film thickness.

For MSD – 35% (Phenyl)-methylpolysiloxane (DB-35MS or equivalent) fused silica column, 30 m x 0.25 mm id x 0.25 µm film thickness.

For HPLC/MS – Waters SymmetryShieldRP₁₈ 5 µm, 3.9 x 150 mm cartridge
Guard column: Waters SymmetryShieldRP₁₈ 5 µm, 3.9 x 20 mm cartridge

Guard column holder: Waters Sentry guard holder universal.

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 to 10 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, 0.2 and 0.5 µg/mL in methanol for GC and LC 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 (0 - 5 °C).

9. **Test Sample Preparation:**

9.1 Background Preparation

The Department of Pesticide Regulation (DPR) provided the well water for background to be used in method validation and QC.

9.2 Preparation of blank and spike

Matrix blank: Weigh out 1000 g of background water and follow the test sample extraction procedure.

Matrix spike: Weigh out 1000 g of background water. Spike a client requested amount of herbicides into the background water and let it stand for 1 minute. Follow the test sample extraction procedure.

9.3 Test Sample Extraction

- 9.3.1 Record the weight of water samples to 0.1 g by subtracting the weight of the sample container before and after water has been transferred into a funnel.
- 9.3.2 Add 2.5 mL of sulfuric acid to each separatory funnel and mix well.
- 9.3.3 Shake with 100 ± 5 mL of methylene chloride for 2 minutes. 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 that has been pre-rinsed with 15 mL of methylene chloride, into a 500 mL boiling flask.
- 9.3.5 Repeat steps 9.3.3 & 9.3.4 two more times using 80 ± 5 mL of methylene chloride 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. Add 2-4 mL of methanol and rotoevaporate to 1-2 mL. Transfer the extract to a calibrated 15 mL graduated test tube.
- 9.3.8 Rinse flask 3 more times with 2 - 4 mL of methanol and transfer each rinse to the same test tube.
- 9.3.9 Evaporate the sample extract to a volume slightly less than 1 mL in a water bath at 38 ± 2 °C under a gentle stream of nitrogen. Then bring to a final volume of 1.0 mL with methanol, mix well and transfer to three autosampler vials with inserts. Submit extract for GCMS and LCMS 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 limits.
- 10.2 The calibration curves for the GCMS and LCMS instruments were obtained using quadratic fit.

11. Analysis:

11.1 HPLC-MS

11.1.1 HPLC Instrument: Waters model 2695 HPLC and auto-sampler with column heater and remote control through Thermo Finnigan Xcalibur system.

Guard Column: Waters SymmetryShieldRP₁₈ 5 µm, 3.9 x 20 mm cartridge

Column: Waters SymmetryShield RP₁₈ 5 µm, 3.9 x 150 mm column

Column Temperature: 40 °C

Mobile Phase: Gradient

Solvent 1: 3762 mL water, 200 mL methanol, 38 mL 1M ammonium formate and 4.0 mL formic acid.

Solvent 2: 3600 mL methanol, 360 mL water, 36 mL 1.0 M ammonium formate, 4 mL formic acid.

Gradient:

<u>Time(min)</u>	<u>Flow rate</u>	<u>Mobile Phase 1</u>	<u>Mobile Phase 2</u>
0	0.75	85.0	15.0
3.0	0.75	85.0	15.0
4.0	0.75	50.0	50.0
10.0	0.75	50.0	50.0
14.0	0.75	40.0	60.0
16.0	0.75	5.0	95.0
22.0	0.75	5.0	95.0
24.5	0.75	85.0	15.0
27.0	0.75	85.0	15.0

Injection Volume:20 µL

11.1.2 Liquid Chromatograph Mass spectrometer (LC-MS) and Operating Parameters

Model: Finnigan Model DECA ion trap MS
 Ion Source Type: Atmospheric pressure Ionization (APCI)
 Source Polarity: Positive
 APCI Vaporizer Temp: 450 °C
 Capillary Temperature: 220 °C
 Sheath Gas: 60
 Auxiliary Gas: 10
 Mode of operation: MS/MS

Compound Name	Retention Time (min.)	Molecular Weight	Mass Range	Product Ions
Azoxystrobin Acid	15.92	389	105-404	372.1
Azoxystrobin Z-metabolite	17.05	403	150-404	372.1
Azoxystrobin	17.85	403	150-404	372.1

Note: The column conditions, temperature, mobile phase, etc. may slightly shift retention time.

11.1.3 Operating parameter

Compound Name	Segment / Scan #	Segment Time	Parent Mass (m/z)	Isolation Width (m/z)	Normalized Collision Energy(%)	Activation Q
Azoxystrobin Acid	1/2		390	2.0	25	0.250
Azoxystrobin Z- metabolites	1/1		404	2.0	25	0.300
Azoxystrobin	1/1		404	2.0	25	0.300

11.2 GCMS Instrumentation:

11.2.1 Model: Agilent GCMS

Column: 35% (Phenyl)-methylpolysiloxane (DB-35MS or equivalent) fused silica column, 30 m x 0.25 mm id x 0.25 µm film thickness.

Temperature Program: initial column temperature 80 °C, hold 2 min., ramp at 10 °C/min. to temperature of 230 °C and hold for 0 min. ramp at 30 °C/min. to final temperature of 280°C and hold for 6 min.;

Injector Temperature: 250 °C
Transfer line Temperature: 280 °C

Injection volume: 2uL.

Compound	Retention Time (min.)	Selected ions	Starting time (min.)
3,5-Dichloroaniline	12.46	163,161,126	6.00
Iprodione/Isoprodione	15.98	174,219,187	
Dicloran	16.8	206,176,124	
Vinclozolin	17.65	212,285,198	

Quantitation ions are in bold.

11.3 GCMS Instrumentation:

11.3.1 Model: Agilent GCMS

Column: 5% (Phenyl)-methylpolysiloxane (HP-5MS or equivalent) fused silica column, 30 m x 0.25 mm id x 0.25 µm film thickness.

Temperature Program: initial column temperature 60 °C, hold 1 min., ramp at 20 °C/min. to temperature of 230 °C and hold for 0 min. ramp at 30°C/min. to final temperature of 280°C and hold for 6 min.;

Injector Temperature: 250 °C
Transfer line Temperature: 280 °C

Injection volume: 2uL.

Compound	Retention Time (min.)	Selected ions	Starting time (min.)
3,5-Dichloroaniline	12.46	163,161,126	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 well 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:

$$\text{MDL} = tS$$

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 the azoxystrobin, azoxystrobin-acid, azoxystrobin-Z metabolite, dicloran, ipodione/ isoprodione, vinclozolin is 0.05 ppb and 3,5-dicloroaniline is 0.1 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 ± 2 and 3 standard deviations of the % 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 ± 2 per cent 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 LCMS software used a quadratic curve fit, with all levels weighted $1/x$. GCMS uses quadratic curve fit, with all levels weighted equally. Alternatively, at the chemist's discretion, sample results may be calculated using the response factor for the standard.

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

14. Reporting Procedure:

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

15. Discussion and References:

15.1 At the beginning of this project the intent was to develop a method that would allow all the compounds to be extracted and analyzed together. The standards were prepared in methanol and infused into the LCMS. Only Azoxystrobin and its metabolites were sensitive enough to be screened using this instrument. Dicloran, 3, 5 dichloroaniline, iprodione/isoiprodione and vinclozolin were run using the GCMSD with DB-35ms column. The iprodione/isoprodione analyzed in this method is not truly iprodione or isoiprodione but a methanol/instrument conversion of them. Due to sample matrix interference the 3, 5 dichloroaniline was analyzed using a HP-5ms column.

15.2 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. Twenty-one liters of background well water were spiked and then transferred to twenty-

one of the one liter amber bottles. These spiked samples were stored in the refrigerator until analyzed on 0, 2, 4, 7, 14, 21 and 28 days. Along with the storage spikes a blank and method control spike were also extracted. This storage study showed degradation for the vinclozolin within two days while the rest of the compounds showed no significant degradation. Literature indicates that vinclozolin breaks down to 3, 5 dichloroaniline. To check this process a second storage study was repeated for vinclozolin and 3, 5 dichloroaniline in which each compound was spiked individually. This study consisted of a 1.0 ppb spike level and 3 replicates over a 7 day period for each compound. The results for the second short storage study showed some degradation of vinclozolin to 3,5 dichloroaniline and iprodione/isoiprodione. 3,5 dichloroaniline showed no degradation. Results for the storage studies are shown in Appendixes 3-5.

15.3 We have observed gradual losses in sensitivity and peak tailing caused by the sample matrix. We recommend changing the injector liner and trimming the column when this occurs.

15.4 References:

15.41 Anisuzzama, A.; Storehalder, T.; Williams, D.; Ogg, N.; Kilbourne, T.; John Samuel, J.; and Cottrell, C; *Effect of Alcohols on the Stability of Iprodione in Solution*, Pesticide Section, Consumer Analytical Laboratory, Ohio Department of Agriculture, and The Ohio State University

15.42

Hsu, J. and Feng, H.; *Determination of Organophosphate Pesticides in the surface water using Gas Chromatography*, 2004, Environmental monitoring method, Center for Analytical Chemistry, CDFA

Appendix 1

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

Results:	GCMS DB-35ms		GCMS HP-5ms	
Spk\Analyte	Iprodione/Isoiprodione	Dicloran	Vinclozolin	3,5 Dichloroaniline
	<u>Spike Level: 0.2 ppb</u>	<u>Spike Level: 0.1 ppb</u>	<u>Spike Level: 0.1 ppb</u>	<u>Spike Level: 0.1 ppb</u>
Spike 1	0.202	0.094	0.093	0.110
Spike 2	0.208	0.118	0.108	0.097
Spike 3	0.187	0.110	0.098	0.111
Spike 4	0.220	0.116	0.103	0.112
Spike 5	0.244	0.115	0.113	0.104
Spike 6	0.232	0.114	0.110	0.066
Spike 7	0.197	0.110	0.105	0.055
SD	0.2019	0.00810	0.00704	0.02351
MDL	0.634	0.0255	0.0221	0.0739
RL	0.1	0.05	0.05	0.1

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

Results:	LCMS DECA II		
Spk\Analyte	Azoxystrobin Acid	Azoxystrobin Z-metabolite	Azoxystrobin
0.1ppb spk 1	0.125	0.094	0.097
0.1ppb spk 2	0.125	0.098	0.096
0.1ppb spk 3	0.123	0.092	0.091
0.1ppb spk 4	0.130	0.098	0.097
0.1ppb spk 5	0.119	0.097	0.097
0.1ppb spk 6	0.105	0.082	0.083
0.1ppb spk 7	0.135	0.099	0.096
SD	0.00949	0.00596	0.00524
MDL	0.0298	0.0187	0.0165
RL	0.05	0.05	0.05

Appendix 2

Method Validation Data

Analyte	Spike ppb	Recovery (%)					Mean:	%
		Set 1	Set 2	Set 3	Set 4	Set 5		
3, 5-Dicloroaniline	0.2	94.5	59.5	86.5	70.5	46.4	Mean:	80.3
	0.25	78.4	94.8	105	92.8	74.8	SD:	15.8
	0.5	50.8	81.2	101	78.4	102	UCL:	128
	2.0	81.0	61	64.5	71.5	84.5	UWL:	112
	5.0	93.2	72.6	96.8	77.6	87.2	LWL	48.7
							LCL:	32.7
Iprodione Isoiprodione	0.1	115	75.6	77.3	84.1	48.6	Mean:	87.6
	0.25	88.4	85.2	67.6	72	72	SD:	18.2
	0.5	61	85.2	90.4	79	78.4	UCL:	142
	1.0	84	109	96.5	87.2	85	UWL:	124
	5.0	108	106	117	126	90.8	LWL	51.1
							LCL:	32.9
Dicloran	0.1	121	77	77.9	64.9	52.1	Mean:	76.4
	0.25	108	84.4	68.4	62.8	74.4	SD:	15.5
	0.5	68.2	80.4	84.4	63.8	79.6	UCL:	123
	1.0	101	80.6	69.4	63.1	66.6	UWL:	108
	5.0	80	58.2	66.8	84.2	74	LWL	45.4
							LCL:	29.8
Vinclozolin	0.1	106	82	91	78.6	68	Mean:	79.1
	0.25	99.6	82.8	66.8	63.2	74.4	SD:	12.4
	0.5	66.8	78.8	84.8	74.4	76.2	UCL:	116
	1.0	105	79.4	73.2	66.5	75.6	UWL:	104
	5.0	92.6	63.8	62.8	84.8	80.2	LWL	54.4
							LCL:	42.0

Appendix 3 Storage Stability Study

Analyte \ Recovery %		Day 0	Day 2	Day 4	Day 7	Day 14	Day 21	Day 28
Azoxystrobin	blank QC	nd	nd	nd	nd	nd	nd	nd
	spike		99.6%	95.2%	104%	101%	89.6%	95.6%
	Spke 1	86.6%	91.7%	89.9%	88.1%	99.7%	78.2%	87.9%
	Spke 2	104%	94.2%	79.9%	95.6%	102%	76.0%	90.1%
	Spke 3	99.4%	102%	95.1%	91.8%	102%	74.0%	89.1%
Azoxystrobin Z	blank QC	nd	nd	nd	nd	nd	nd	nd
	spike		98.0%	96.0%	106.0%	102%	86.8%	97.2%
	Spke 1	101%	91.1%	94.4%	90.8%	102%	79.6%	87.8%
	Spke 2	109%	95.6%	81.5%	97.7%	105%	75.8%	89.0%
	Spke 3	102%	102%	95.8%	92.9%	105%	75.7%	89.8%
Azoxystrobin Acid	blank QC	nd	nd	nd	nd	nd	nd	nd
	spike		99.2%	93.6%	104%	99.2%	92.0%	100%
	Spke 1	103%	92.0%	89.6%	88.8%	99.1%	84.0%	93.0%
	Spke 2	107%	93.7%	80.3%	96.1%	103%	80.6%	95.1%
	Spke 3	101%	95.7%	92.3%	92.9%	102%	80.0%	95.8%
3,5 Dichloroaniline	blank QC	nd	nd	nd	nd	nd	nd	nd
	spike		104%	65.6%	83.2%	81.2%	84.4%	85.2%
	Spke 1	101%	108%	56.6%	101%	102%	118%	78.2%
	Spke 2	107%	94.0%	73.5%	97.1%	89.8%	102%	87.2%
	Spke 3	82.9%	103%	99.5%	93.3%	98.0%	100%	86.5%
Iprodione/Isoiprodione	blank QC	nd	nd	nd	nd	nd	nd	nd
	spike		111%	103%	88.0%	68.4%	86.6%	99.0%
	Spke 1	97.1%	102%	84.7%	123%	112%	87.0%	119%
	Spke 2	98.0%	107%	80.9%	154%	132%	77.8%	113%
	Spke 3	94.3%	88.6%	112%	128%	96.6%	90.5%	129%

Appendix 3 continued
Storage Stability Study

Dicloran	blank QC spike	nd						
	Spke 1	99.7%	91.5%	81.1%	79.9%	97.8%	76.0%	101%
	Spke 2	96.3%	84.0%	65.6%	103%	115%	76.4%	96.1%
	Spke 3	88.8%	104%	88.6%	97.2%	78.0%	85.6%	114%
	blank QC spike	nd						
Vinclozolin	Spke 1	85.2%	18%	8.6%	4.4%	3.5%	2.1%	5.7%
	Spke 2	85.2%	15%	8.4%	8.3%	3.4%	1.7%	5.7%
	Spke 3	80.9%	22%	10.2%	6.1%	2.1%	1.6%	6.9%
	blank QC spike	nd						
	Spke 1	85.2%	18%	8.6%	4.4%	3.5%	2.1%	5.7%

Appendix 4

Short Storage Study For 3,5-Dichloroaniline(3,5-DCA)

Analyte \ Recovery %		Day 0	Day 2	Day 7
3,5-dichloroaniline	blank	nd	nd	nd
	QC spike		98.0%	79.6%
	3,5-DCA Spk 1, 1.0 ppb	88.4%	72.8%	100%
	3,5-DCA Spk 2, 1.0 ppb	82.3%	82.1%	65.9%
	3,5-DCA Spk 3, 1.0 ppb	79.8%	60.1%	73.1%

Appendix 5

Short Storage Study for Vinclozolin

Analyte \ Recovery %		Day 0	Day 2	Day 7
Vinclozolin	blank	nd	nd	nd
	QC spike		93.4%	118%
	Vinclozolin Spk 1, 1.0 ppb	100%	41.8%	26.3%
	Vinclozolin Spk 2, 1.0 ppb	74.9%	49.5%	24.8%
	Vinclozolin Spk 3, 1.0 ppb	103%	24.0%	12.3%
Iprodione/Isoiprodione	blank	nd	nd	nd
	QC spike		80.0%	108%
	Vinclozolin Spk 1, 1.0 ppb	8.65%	18.3%	38.6%
	Vinclozolin Spk 2, 1.0 ppb	3.85%	23.8%	38.4%
	Vinclozolin Spk 3, 1.0 ppb	5.25%	9.45%	19.9%
3,5-dichloroaniline	blank	nd	nd	nd
	QC spike		98.0%	79.6%
	Vinclozolin Spk 1, 1.0 ppb	nd	3.9%	5.7%
	Vinclozolin Spk 2, 1.0 ppb	nd	4.9%	6.5%
	Vinclozolin Spk 3, 1.0 ppb	nd	1.6%	4.3%

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