2.0 INTRODUCTION

Independent laboratory validation of enforcement methods are required by the U.S. EPA OPPTS 850.7100 (Reference 1) and EU Guidance document SANCO/825/00 rev. 7 (Reference 2).

The subject method is applicable for the quantitation of picoxystrobin in water, as described in DuPont-29617 (Reference 3). Pond water was chosen to validate the analytical method as a representative matrix.

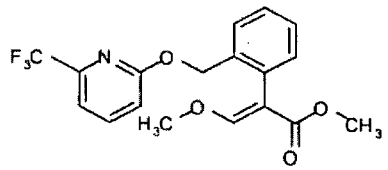
Fortification levels in this study were chosen to provide method performance data at the method LOQ and 10×LOQ for the matrix examined. The stated method LOQ was 0.10 µg/L in water.

The analytical method was performed without any significant modifications. The method was successfully validated for picoxystrobin in pond water in one trial. This independent laboratory validation study demonstrated that the analytical method DuPont-29617 is acceptable for the quantitation of picoxystrobin in water, according to guidelines set forth by US EPA Ecological Effects Guidelines, OPPTS 850.7100 "Data Reporting for Environmental Chemistry Methods" (Reference 1) and EU Guidance document SANCO/825/00 rev. 7 (Reference 2).

3.0 MATERIALS AND METHODS

3.1 Test and Reference Substance

The *Chemical Abstract* structure and chemical name of the analyte is shown below:



DuPont Code: DPX-YT669

Common Name: Picoxystrobin

***Chemical Abstracts* Name**: Methyl (E)-α-(methoxymethylene)-2-[[[6-(trifluoromethyl)-2-pyridinyl]oxy]methyl]-benzeneacetate

CAS Registry No.: 117428-22-5

Lot No.: ASJ10099-03

Purity: 99.9%

Storage: Refrigerated desiccator

The test substance was supplied by E. I. du Pont de Nemours and Company, DuPont Agricultural Products, Stine Haskell Research Center, Newark, DE. Information pertaining to the characterization and stability of the test substance is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, DE. Characterization data were provided by DuPont Agricultural Products, E.I. du Pont de Nemours and Company, Wilmington, DE. A Certificate of Analysis, including lot number and purity, is included with the study raw data file that will be archived by E. I. du Pont de Nemours and Company.

3.2 Test System

The subject method is applicable for the quantitation of picoxystrobin in water. Pond water was chosen to validate the analytical method because it is expected to be one of the more difficult water sources to analyze.

This control matrix was acquired from a local Colorado pond. The sample was stored frozen prior to being analyzed. The pertinent physical characteristics are summarized in the following table. Water samples were characterized at Agvise Laboratories (Northwood, ND). Characterization records are maintained at Pyxant Labs Inc.

MEASUREMENT	COLORADO POND WATER
pH	7.6
Calcium	77 ppm
Magnesium	17 ppm
Sodium	59 ppm
Hardness	261 mg equivalent CaCO3/L
Conductivity	0.76 mmhos/cm
Sodium Adsorption Ratio (SAR)	1.58
Total Dissolved Solids	466 ppm
Turbidity	37.3 NTU

3.3 Equipment

The following equipment items were used in the conduct of this independent laboratory validation.

3.3.1 Instrumentation/Chromatography

MDS Sciex API 4000 LC-MS/MS System, Serial No. V0560403 (Applied Biosystems Group, Foster City, CA), equipped with a TurboIonSpray interface and Analyst software version 1.4.2

HPLC Column: 4.6 mm i.d. x 15 mm, Agilent Zorbax® XDB C18, Serial No. USWDY11950, 1.8-µm diameter packing, Part No. 927975-902 (Agilent Technologies, Inc., Santa Clara, CA)

Shimadzu LC-10AD VP HPLC pumps, Serial Nos. C2096 41 53747US and C2096 41 53748US (Shimadzu US Manufacturing Inc., Columbia, MD)

Shimadzu SIL HTC Autosampler, Serial No. L2002 42 50137US (Shimadzu US Manufacturing Inc., Columbia, MD)

Shimadzu CTO-10A VP Column Oven, Serial No. C2102 41 50408US (Shimadzu US Manufacturing Inc., Columbia, MD)

Shimadzu DGU-14A Degasser, Serial No. SS132668 (Shimadzu US Manufacturing Inc., Columbia, MD)

3.3.2 *General Lab Equipment/Devices*

Cahn Microbalance, Model No. C-34/35, Serial No. C1066/C2251 (Orion Research Inc., Beverly, MA 01915)

Sartorius Top-Loading Balance, Model No. BA2100S, Serial No. 20303446 (Brinkmann Instruments Co., Westbury, NY 11590)

Fisher brand Vortex Geni 2 Mixer, Catalog No. 12-812 (Scientific Industries, Inc., Bohemia, NY 11716)

Beckman Centrifuge, Model No. X-12 R, Serial No. ALX04DE6 (Organomation, South Berlin, MA 01549)

Microman Positive Displacement Pipettes, various sizes (Gilson, Middleton, WI 53562)

Purelab Classic UV UHP Water System, Model No. PL5232, ELGA (Lowell, MA 01851)

Air Displacement Pipettes, various sizes (Eppendorf Research, Westbury, NY 11590)

3.3.3 *Labware*

15 mL Polypropylene Centrifuge Tubes, Part No. 20171-024 (VWR, West Chester, PA 19380)

Nunc U96 Deepwell plate and cover, 2.0 mL Polypropylene (Nalge Nunc International, Rochester, NY 14625)

Disposable Transfer Pipettes, 3 mL, Part No. 16001-176 (VWR, West Chester, PA 19380)

Glass Volumetric Flasks, 10 and 100 mL volume, Class A (VWR, West Chester, PA 19380)

3.4 ***Reagents***

Ammonium Formate –99.99+% pure (Sigma-Aldrich, St. Louis, MO 63103)

Formic Acid, 99.0 % pure, Fluka, Catalog No. 06440 (Sigma-Aldrich, St. Louis, MO 63103)

Acetonitrile – HPLC-Grade, Catalog No. 300000000 (Pharmco-AAPER, Brookfield, CT)

Methanol – HPLC-Grade, Catalog No. MX0475-1 (EMD Chemicals, Gibbstown, NJ)

Water – Ultra High Purity, obtained from Purelab Classic UV UHP Water System

Water – HPLC-Grade Catalog No. AH365-4 (Honeywell Burdick and Jackson, Muskegon, NI 49442)

3.5 Principles of the Analytical Method

Water sample aliquots (10 mL) were measured into 15-mL propylene centrifuge tubes, and 2.5 mL of acetonitrile was added into each tube. The samples were shaken to homogeneity and exactly 1 mL of each sample was placed into another 15-mL propylene centrifuge tube and diluted with 98/2 acetonitrile/water (v/v). After centrifuging, the supernatant was transferred to an autosampler vial and analyzed for picoxystrobin by HPLC/ESI-MS/MS.

3.6 Modifications, Interpretations, Critical Steps, and Deviations

Originally, it was intended to use an API 5000 instead of an API 4000 as specified in the method. However, inconsistent calibration standard average response factors and large interferences were observed in the controls on the API 5000 during the initial injection and the first reinjection; therefore an API 4000 was used for the second reinjection.

Some API 4000 LC/MS/MS instrumental parameters were modified to optimize sensitivity. Picoxystrobin was injected with standards embedded after every fourth sample. The first standard injection was used as a warm up and not used for quantitation.

One method deviation occurred during the course of this study. Section 4.2.9 of the method states that the final sample extracts are stable for three or four days if stored at $\leq$ -10°C. However, extracts were reinjected after 15 days of storage at -20°C. Since samples were within specifications of 70-120% recovery after quantitation against stable calibration standards, there is no negative impact on the study.

Section 4.2.5 of the method states that standards stored at frozen at $\leq$ -10°C are stable for at least two weeks. Standards were stored at -20°C and were injected within 16 days of preparation. No degradation was observed since the %RSD of the average response factor of the standard peak areas was < 20%.

3.7 Instrumentation

3.7.1 Chromatography

Reversed-phase liquid chromatography was used to separate the analytes from co-extractants. An Agilent Zorbax® XDB C18 column was used.

HPLC Conditions

System:	MDS Sciex API 4000 LC/MS/MS
Column:	Agilent Zorbax® XDB C18, 4.6 × 50 mm, 1.8 µm diameter packing
Column Temperature:	40°C
Injection Volume:	15 µL
Flow Rate:	1.0 mL/min
Split Flow:	Split to waste (post-column split, 100µL /min into MS source)
Conditions:	A: 0.1 mM formic acid and 0.1 mM ammonium formate B: methanol
Picoxystrobin Retention Time:	~ 6.9 min
Total Run Time:	10 min

Gradient:

Time	%A	%B
0.00	70	30
1.00	70	30
1.10	70	30
2.00	60	40
2.10	40	60
7.50	23	77
7.60	5	95
8.60	5	95
8.70	70	30
10.0	70	30

3.7.2 *HPLC/MS/MS Analysis*

Analysis of picoxystrobin was performed using a MDS Sciex API 4000 LC/MS/MS, equipped with a TurboIonSpray source, and operated in MRM, positive ion mode. Quantitation was based on an average response factor using peak areas supplied by Analyst software version 1.4.2. Calculations were performed using Microsoft Excel. A summary of representative experimental conditions is provided in the following table:

MDS Sciex API 4000 MS/MS Mass Spectrometer Conditions Positive Ion Mode

Analyte	Ions Monitored	CXP (Collision Cell Exit Potential)	DP (Declustering Potential)	Dwell Time (msec)	Collision Energy
Picoxystrobin	368 → 145 AMU	18V	66V	200	31
	368 → 205 AMU	22V	66V	200	15

NEB	CUR	TEM	CAD	IS	EP
45	20	400	10	4500	10

3.7.3 *Calibration Procedure*

Calibration standards were analyzed throughout the batch. The response factor of each calibration standard was calculated by dividing the analyte peak area of each standard by the analyte concentration for that standard. The average response was calculated for calibration standards injected with each batch. The first standard injection was used for warm up purposes only and not used for quantitation.

3.8 ***Calculations***

Calculations from Section 4.4.1 of the method were used as written.

APPENDIX 2 COMMUNICATION LOG

DATE	PERSONS INVOLVED	METHOD	REASON FOR CONTACT	RESULTING CHANGES TO METHOD	TIMING
23Feb2010	Elena Cabusas, Samuel Seal	Phone	Request column part number	None	Prior to Trial 1
02Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz, Elena Cabusas, Kristin Milby	Email	Discussion of Trial 1 results. Trial 1 to be re-injected following system flush; injection volume may be reduced depending on sensitivity. Post-fortified control samples will be analyzed with the re-injection.	None	After Trial 1
02Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz, Elena Cabusas, Kristin Milby, David Robaugh, Brian Graham	Email	Chromatograms from initial injection of Batch 1 were sent.	None	After Trial 1
02Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz, Elena Cabusas, Kristin Milby, David Robaugh, Brian Graham	Email	Discuss chromatograms from initial injection of Batch 1.	None	After Trial 1
02Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz	Email	Discuss diagnostics to be performed in order to eliminate background interferences, and the timing for the diagnostics and the re-injection.	None	After Trial 1
03Mar2010	Elena Cabusas, Samuel Seal, Melissa Gaubatz	Phone	Discuss extraction steps and standard diluents. Discussed assaying the post-fortified control samples with the re-injection.	None	After Trial 1
08Mar2010	Del Koch, Melissa Gaubatz, Samuel Seal, David Robaugh, Brian Graham	Email	Discuss re-injection results; interferences were eliminated but batch did not pass acceptance criteria. Discussed re-injecting a second time on the API 4000 LC/MS/MS rather than the API 5000 LC/MS/MS	None	After Trial 1
11Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz, Audrey Knobloch, Rachel Dillon	Email	Data from reinjection on the API 4000 were sent and discussed.	None	After Trial 1
15Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz, Elena Cabusas	Email	Discuss and confirm reprocessing of data following re-injection, including omitting first standard	None	After Trial 1

15Mar2010	Elena Cabusas, Samuel Seal,	Phone	Discuss omitting first standard since it was used as a warm up, which was approved.	None	After Trial 1
15Mar2010	Del Koch, Samuel Seal, Melissa Gaubatz, Elena Cabusas, Audrey Knobloch, Brian Graham, David Robaugh	Email	Trial 1 results accepted by Sponsor following reprocessing re-injection data and omitting first standard.	None	After Trial 1