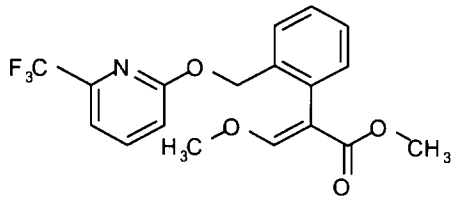
2.0 INTRODUCTION

Picoxystrobin (DPX-YT669) is a strobilurin fungicide which has been shown to be effective in the control of plant diseases. The chemical structure and pertinent information of the test substance are shown below:



DuPont Code:	DPX-YT669
Trivial Name:	Picoxystrobin
IUPAC Name:	Methyl (E)-2-{2-[6-(trifluoromethyl)pyridin-2-yloxymethyl]phenyl} -3-methoxyacrylate
Chemical Abstracts Name:	methyl (E)-α-(methoxymethylene)-2-[[[6-(trifluoromethyl)-2-pyridinyl]oxy]methyl]-benzeneacetate (9CI)
***CAS* Registry Number:**	117428-22-5
Molecular Formula:	$C_{18}H_{16}F_3NO_4$
Molecular Weight:	Average, 367.32; Monoisotopic, 367.10
Solubility, 20°C:	Water 3.1 mg/L. Organic (mg/mL): methanol 96;ethyl acetate, acetone, 1,2-dichloroethane, xylene > 250; n-heptane 4.
Stability:	Relatively stable at pH5 and pH 7. Hydrolysis DT50 at 50 °C and pH 9, ~15 days.

This analytical method for picoxystrobin in water, at an LOQ of approximately 0.10 µg/L (ppb), was developed to satisfy the requirements of the U.S. EPA Pesticide Assessment Guidelines Subdivision N and the EU Annex II 4.2.3.

Surface and drinking water samples fortified with picoxystrobin (DPX-YT669) were diluted and analyzed by reversed HPLC/ESI-MS/MS.

The confirmatory method was based on detection and the relative ratios of the two MS/MS parent-to-daughter ion transitions monitored during the validation.

3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified; note any specifications in the following descriptions before making substitutions. Substitutions should only be made if equivalency/suitability has been verified with acceptable control and fortification recovery data.

3.1 Equipment

Balances
Mettler Analytical Balance, Model AE240 for weighing solid standards (Mettler Instrument Corporation, Hightstown, NJ)

Mettler Top-Loading Balance, Model PM600 for weighing water samples (Mettler Instrument Corporation, Hightstown, NJ)

Centrifuge - Sorvall GLC-2B L-995 (VWR International, West Chester, PA)

HPLC/MS System
HP Series 1100 Liquid Chromatograph with G1332A degasser, G1312A binary pump, G1313A chilled autosampler, G1316A column compartment (Agilent, Little Falls, DE)

Zorbax® XDB C18 analytical column, 4.6 mm × 50 mm, 1.8-µm diameter packing, Part # 927975-902, DO NOT SUBSTITUTE (Agilent, Little Falls, DE)

Applied Biosystem/MDS Sciex API4000 LC/MS/MS (triple quadrupole mass spectrometer) with an electrospray interface (ESI) and Analyst Version 1.4.2 Software (Applied Biosystems, Framingham, MA) with Valco zero-dead volume 3-port connector for 1/10 splitflow to mass spectrometer.

HPLC Vials – Hewlett Packard Target Dual-Purpose Vials with Teflon/Silicone/Teflon Septa, amber, 2-mL, Catalog No. 5182-0056 (Agilent, Little Falls, DE)

Pipettes
Biohit Proline® Electronic Pipettors, variable volume, with tip ejector, 10-250 L and 50-1000 L, Catalog No. 53495-210 and 53496-205 (VWR International, West Chester, PA)

Disposable Pasteur Pipets, Borosilicate glass, 9-inch length, Catalog No. 14673-043 (VWR International, West Chester, PA)

EDP Electronic Digital Pipette, Catalog No. EP-10 ML (Rainin Instrument, Co., Inc., Woburn, MA)

Pipette Tips
Sorenson™ Multifit Research Pipet Tips, 5-200 µL and 100-1000 µL, Catalog No. 53550-076 and 53503-076 (VWR International, West Chester, PA)

Rainin Certified Disposable Pipette Tip, 10 mL, Catalog No. RC-10 ML (Rainin Instrument Co.)

Sample Containers
VWR Disposable Graduated Centrifuge Tube, 15-mL, Polypropylene, Part # 89039-666 (VWR International, West Chester, PA)

Syringes - 3cc Disposable plastic syringes, Catalog No. 309585 (Becton Dickinson, Franklin Lakes, NJ)

Syringe Filters
Acrodisc® CR PTFE disposable filter, 0.2-45-µm pore size, 25-mm diameter
Catalog No. 4225T and 4219 (VWR International, West Chester, PA)

Ultrasonicators -Bransonic® Ultrasonic Cleaner, 0.75-gallon capacity, Model 2200, Catalog No. 952-214 (Branson Ultrasonics Corp., Danbury, CT); S.V. Scientific, Model 9L250H

Vortex mixer - Fisher Vortex Genie®, Catalog No. 12-812
(Fisher Scientific Co., Pittsburgh, PA)

3.2 Reagents and Standards

Acetonitrile - OmniSolv® #AX0142-1, HPLC grade (EMD Chemicals, Gibbstown, NJ)

Formic Acid – Suprapur®, 98-100%, #11670-1 (EMD Chemicals)

Formic Acid, Ammonium Salt – A.C.S. reagent, #M530-08
(Mallinckrodt Baker, Inc., Phillipsburg NJ)

Methanol - OmniSolv® #MX0488-1, HPLC grade (EMD Chemicals)

Ultrapure Water – OmniSolv® #WX0004-1, HPLC grade, (EMD Chemicals); Milli-Q water

Reference standard DPX-YT669 (DuPont Crop Protection, Newark, DE)

3.3 Safety and Health

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 Principle of the Analytical Method

Surface and drinking water samples fortified with picoxystrobin (DPX-YT669) were diluted ten-fold with acetonitrile and water until percent acetonitrile was 90%. Diluted surface water samples were centrifuged at 3500 rpm for 5 minutes or at a speed that visibly separated the supernatant from the sample debris prior to analysis.

The diluted surface and drinking water samples were analyzed for picoxystrobin residues by reversed-phase HPLC using a Zorbax® XDB C18 (4.6 × 50 mm, 1.8-µm particle) column and a mobile phase of 0.1 mM formic acid - 0.1 mM ammonium formate (aq) and methanol. Detection of picoxystrobin was by electrospray mass spectrometry/mass spectrometry (ESI-MS/MS) in the positive ion mode. Two parent-to-daughter ion transitions per analyte were monitored during analysis.

The water method LOQ for picoxystrobin was 0.10 µg/L, which was equivalent to a 0.0080-ng/mL calibration standard.

The confirmatory method for the HPLC/ESI-MS/MS method was based on detection and the relative ratios of the two MS/MS parent-to-daughter ion transitions monitored during the validation.

During method validation, post-fortified samples were analyzed for each water type to determine if matrix effect, suppression or enhancement, influenced percent recovery of picoxystrobin. The post-fortified samples, in this study, were extracts of control water samples that were prepared in the same manner as with the other samples, but fortified with the analyte prior to HPLC/ESI-MS/MS analysis.

4.2 Analytical Procedure

4.2.1 Glassware & Equipment Cleaning Procedures

The effectiveness of any cleaning procedure used should be demonstrated by preparation and analysis of reagent blanks. In general, all reusable glassware and plasticware should be washed in hot tap water with laboratory grade, non-phosphate detergent, rinsed several times with tap water, rinsed several times with deionized water, rinsed once with acetone, and allowed to fully dry before use. Care should be taken to avoid working with high levels of the analyte being monitored in the same laboratory where samples are being extracted and analyzed.

4.2.2 Preparation & Stability of Reagent Solutions

1.0 M Ammonium Formate

Dissolve 6.3 grams of ammonium formate with approximately 50-mL of ultrapure water in a 100-mL volumetric flask. Bring final volume to the mark with ultrapure water and shake to homogeneity. This solution is stored capped at room temperature and should be stable for 3 months.

1.0 M Formic Acid

Add 3.85 mL of concentrated formic acid (98%, w/w) into a 100-mL volumetric flask that is partially filled with ultrapure water. Bring to the mark with ultrapure water and mix to homogeneity. This solution is stored capped at room temperature and should be stable for 3 months

Standard Diluent – 90/10 Acetonitrile/Water

Into a 500-mL glass storage bottle, mix 50 mL of ultrapure water and 450 ml of acetonitrile. Cap and shake to homogeneity. This solution should be prepared monthly.

Sample Diluent – ~ 98/2 Acetonitrile: Water

Into a 500-mL glass storage bottle, mix 10 mL of ultrapure water and 440 ml of acetonitrile. Cap and shake to homogeneity. This solution should be prepared monthly.

Mobile Phase A (0.1mM Formic Acid - 0. 1 mM Ammonium Formate)

To a 1-L volumetric flask that is partially filled with ultrapure water, add 100 µL of 1.0 M ammonium formate and 100 µL of 1 M formic acid. Dilute to the mark with ultrapure water and mix to homogeneity. This solution is stored cap at room temperature and should be prepared monthly.

4.2.3 *Stock and Intermediate Standards Preparation and Stability*

Weigh 10.00 mg ± 0.50 mg (recorded to the nearest 0.01 mg) of the picoxystrobin analytical standard into a 100-mL volumetric flask. Add about 80 mL of acetonitrile and ultrasonicate until completely dissolved and dilute to the mark with acetonitrile to make a stock standard solution of approximately 100 µg/mL.

Prepare a 0.50-µg/mL intermediate standard by adding 50 µL of the stock solution into a 10-mL volumetric flask, diluting it to the mark with acetonitrile and mixing to homogeneity.

The stock and intermediate standard solutions are stable for at least 6 months when stored capped at ≤-10°C.

4.2.4 *Fortification Standard Preparation and Stability*

Prepare a 0.10-µg/mL fortification solution in acetonitrile by adding 2000 µL of the 0.50-µg/mL DPX-YT669 stock standard into a 10-mL volumetric flask. Dilute to the mark with acetonitrile, cap and mix to homogeneity.

Prepare a 0.01-µg/mL fortification solution in acetonitrile by adding 1000 µL each of the 0.10-µg/mL DPX-YT669 stock standards into a 10-mL volumetric flask. Dilute to the mark with acetonitrile, cap and mix to homogeneity.

These solutions are stable for at least 6 months when stored capped at ≤-10°C.

4.2.5 *Chromatographic Standard Preparation and Stability*

Prepare a 20- ng/mL intermediate standard and calibration standards ranging from 0.0050 to 0.50 ng/mL (or in concentrations expected to cover the range of DPX-YT669 in the investigative samples) using a 9:1 acetonitrile:ultrapure water solution as diluent . The table below describes how standards were prepared for the validation work presented in this report:

Standard Conc. (ng/mL)	µL Added	Intermediate Standard Used	Final* Volume (mL)
20**	2000	0.10 µg/mL Fortification Solution	10.0
0.50	250	20.0 ng/mL Intermediate Standard	10.0
0.10	50	20.0 ng/mL Intermediate Standard	10.0
0.050	1000	0.50 ng/mL	10.0
0.010	200	0.50 ng/mL	10.0
0.0050	100	0.50 ng/mL	10.0

* Diluent: 9:1 acetonitrile/ultrapure water

** An intermediate calibration standard

Keep all chromatographic standards at or below 4°C after preparation. The standards are stable for at least two weeks when stored capped and frozen at ≤-10°C (Reference 1).

4.2.6 *Source (& Characterization) of Samples*

Water samples from four different sources were used for the method validation. The sources of the water samples and the pertinent physical characteristics are summarized in the following table. Water samples were characterized at Agvise Laboratories (Northwood, ND). Characterization records are maintained at DuPont Agricultural Products.

Measurement	White Clay Creek Water	Lums Pond Water	Newark Drinking Water	Kemblesville Well Water
pH	7.3	6.8	7.7	7.4
Calcium (ppm)	31	8.1	41	10
Magnesium (ppm)	12	5.2	12	7.5
Sodium (ppm)	13	14	18	11
Hardness (mg equivalent $CaCO_3$/L)	126	42	153	57
Conductivity (mmhos/cm)	0.31	0.17	0.39	0.17
Sodium Adsorption Ratio (SAR)	0.49	0.93	0.62	0.62
Total Dissolved Solids (ppm)	322	340	456	210
Turbidity (NTU)	1.35	6.80	0.23	2.98

4.2.7 *Storage & Preparation of Samples*

Control/unfortified surface and drinking water samples were received frozen and stored in a freezer maintained at ≤-10°C. Water samples were allowed to thaw and stored at approximately 4°C prior to sample preparation and analysis. The water samples were shaken vigorously by hand prior to subsampling to ensure homogeneity.

For residue incurred water samples with DPX-YT669, no additional filtration or purification should be performed prior to sample processing. Since DPX-YT669 has a tendency to adhere to plastic and glass surfaces when in water, it is recommended that the samples must be diluted with acetonitrile prior to transferring from one container to a second container. The samples should be diluted until the percent acetonitrile is 20% (e.g., 100-mL or 100-g water sample will require 25 mL of acetonitrile). If the quantity of water is unknown it may be determined from the mass of the sample. This can be accomplished by weighing an empty identical container and weighing the container containing the sample. The difference in grams will be equal to the weight of the sample (water density is 1.0 g/mL). *If acetonitrile was added to the sample, the initial water volume and the amount of acetonitrile added must be clearly marked on the container. This is to assure proper sample processing and disposal.*

4.2.8 *Sample Fortification Procedure*

For the LOQ (0.10-µg/L) and 10 × LOQ (1.0-µg/L) fortifications, fortify 10.0 mL (10.0-g) of water samples with 100 µL of the 0.010- and 0.10-µg/mL DPX-YT669 fortification solutions in acetonitrile, respectively. Cap and shake the samples vigorously.

4.2.9 *Analyte Extraction Procedure*

1. Measure **accurately** 10.0 mL (± 1%) of drinking or surface water samples using 15-ml polypropylene centrifuge tubes. Fortify the samples, if necessary. Cap and shake the samples vigorously.
2. Add exactly 2.5 mL of acetonitrile to each of the samples if the acetonitrile content has not been adjusted yet (see section 4.2.7).
3. Cap and shake vigorously for about 1-2 minutes by hand or by a wrist-action shaker (set a full angle deflection).
4. For drinking (tap and well) water samples, transfer 100 µL of each sample from Step 3 into a 2-mL HPLC vial. Dilute each sample with 900 µL of the sample diluent, cap and mix well.

   The samples are ready for HPLC/ESI-MS/MS analysis. If there is a delay in the analysis, store the samples frozen at ≤-10°C. Samples are stable for 3-4 days.

5. For surface (creek and pond) water samples, using a pipette, transfer immediately 1 mL of each sample from Step 3 into a 15-mL polypropylene centrifuge tube. Dilute each sample to exactly 10.0 mL with the sample diluent, cap and mix well. Centrifuge for 5-15 minutes at 3,500 rpm or at a speed that visibly separates the supernatant from the sample debris. Transfer immediately about 1mL of each supernatant to a 2-mL HPLC vial.

   The samples are ready for HPLC/ESI-MS/MS analysis. If there is a delay in the analysis, store the samples frozen at ≤-10°C. Samples are stable for 3-4 days.

6. Prepare post-fortified samples (optional):

   Drinking (tap and well) water: ~LOQ equivalent (0.0098 ng/mL) and ~10 × LOQ equivalent (0.099 ng/mL):

   Into a 2-mL HPLC vial, add 100 µL of the control sample from Step 3, 900 µL of the sample diluent, and 20 µL of the 0.50-ng/mL calibration standard. Cap the vial and vortex mix the sample.

   Into a 2-mL HPLC vial, add 100 µL of the control sample from Step 3, 900 µL of the sample diluent, and 10 µL of the 0.010-µg/mL fortification standard. Cap the vial and vortex mix the sample.

   Surface (pond and creek) water: ~LOQ equivalent (0.0098 ng/mL) and ~10 × LOQ equivalent (0.099 ng/mL):

   Into a 2-mL HPLC vial, add 1000 µL of the supernatant from Step 5 and 20 µL of the 0.50-ng/mL calibration standard. Cap the vial and vortex mix the sample.

   Into a 2-mL HPLC vial, add 1000 µL of the supernatant from Step 5 and 10 µL of the 0.010-µg/mL fortification standard. Cap the vial and vortex mix the sample.

4.3 Instrumentation

4.3.1 Description

Method validation data in this study were generated using an Agilent HP Series 1100 HPLC coupled to Applied Biosystems MDS SCIEX API 4000 (a triple quadrupole MS) with an electrospray ion source.

4.3.2 Operating Conditions

The HPLC and MS operating conditions used during method validations are summarized in the following tables:

HPLC Conditions

System:	Agilent HP1100 HPLC
Column:	Zorbax® XDB C18, 4.6 mm × 50 mm, 1.8-µm dp
Column Temperature:	40°C
Injection Volume:	10-15 µL
Autosampler Temperature	4°C
Flow Rate:	1.0 mL/min (post-column split, 100 µL/min into MS source)
Conditions:	Time %A %B Flow A: 0.1 mM Formic acid - 0.1 mM ammonium formate. 0.00 70 30 1.0 1.00 70 30 1.0 B: Methanol. 1.10 70 30 1.0 Flow in mL/min 2.00 60 40 1.0 2.10 40 60 1.0 7.50 23 77 1.0 7.60 5 95 1.0 8.60 5 95 1.0 8.70 70 30 1.0 10.00 70 30 1.0
Analyte	Retention Times (minutes)
DPX-YT669	~7.0
Total Run Time:	10.00

MS Conditions

MS SYSTEM:	**APPLIED BIOSYSTEM/MDS SCIEX API4000 LC/MS/MS**						
ANALYTE MONITORED	**IONS MONITORED (AMU)**	**DP[A] (V)**	**EP[B] (V)**	**CE[C] (V)**	**CXP[D] (V)**	**DWELL TIME (MS)**	**ACQUISITION TIME (MIN)**
DPX-YT669	368.2 → 145.0 ± 0.1	41	10	31	8	200	0.40 – 8.0
	368.2 → 205.0 ± 0.1			13	22		
Scan type/Polarity:	MRM/Positive						
Ion Source Voltage:	ESI, 4500						
Collision Gas (CAD):	10 psig						
Curtain Gas (CUR):	20 psig						
Nebulizer Gas (GS1):	45 psig						
Heater Gas (GS2):	45 psig						
Source Heater (TEM):	400°C						
Interface Heater (ihe):	ON						
Resolution Q1	Unit						
Resolution Q2	Unit						
MS Flow Rate:	(Post-column split): 100-µL/min (approximately 10:1 split)						

[a] Declustering Potential [b] Entrance Potential [c] Collision Energy [d] Collision Exit Potential

A triple quadrupole MS instrument with an electrospray ionization (ESI) source was used for the detection of DPX-YT669. The MS response was optimized initially by infusing the analyte into the ionization source. The flow rate and mobile phase were adjusted to the elution condition of the analyte from the HPLC column. The protonated molecule of DPX-YT669 detected was fragmented in the MS/MS collision cell. The tune file created was adjusted to maximize the response of the fragmented ions detected. Two parent-daughter ion transitions were monitored.

Residues of DPX-YT669 were each identified in water samples by its retention time, the presence of two parent-daughter ion transitions with a signal-to-noise ratio greater than 5, and the ratio of the two ion transitions within an acceptable range as determined during the method validation. For quantification of the peak area from the total ion current (TIC, sum of multiple ions) was used DPX-YT669.

A six-port electronically activated switching valve was used to direct the HPLC column effluent to waste prior to and following the elution of DPX-YT669. The retention time was within 3.0 – 8.0 minutes. The chromatographic run time was 10 minutes, but the MS sample collection time was 0.40 – 8.0 minutes (could be shortened to 5-8 minutes). Outside of this sample collection time, the column effluent was directed to waste. This process reduced the ionization source contamination and allowed more samples to be analyzed prior to source cleaning.

Since the electrospray interface is optimal at low flow rates, the column effluent flow was split such that only 100-µL/min actually passed through the interface (approximately 10:1 split), the remainder going to waste.

4.3.3 *Calibration Procedures*

Prepare calibration standards that bracket the levels of DPX-YT669 found in the water samples to be analyzed. Preparation of standards is described in Section 4.2.5 of this report.

4.3.4 *Sample Analysis*

Each set of analytical samples should consist of at least 5 calibration standards, at least one control (a sample without the analyte of interest and matches the analytical samples as closely as possible), and the investigative (treated/fortified) samples. In addition, at least one post-fortified sample of the control with DPX-YT669 at a known level should be included to assess if matrix affect influences the residue levels found or percent recovery.

The calibration solvent should be injected prior to the chromatographic runs of standards and samples in an analytical set. This helps assess the source of interference peak(s). Then a standard can be analyzed, followed by a maximum of 4 samples (controls, fortified controls, or treated samples), followed by another standard, *etc.* The last injection should be a standard.

To minimize carry over, standards and samples MUST be injected in the order from low to high concentrations of DPX-YT669 (e.g., solvent blank first, then 0.0050 ng/mL standard, control, LOQ fortifications, 0.010 ng/mL standard,

10 × LOQ fortifications, 0.10 ng/mL standard and 0.5 ng/mL standard last followed by three blank injections).

It is recommended to inject the calibration solvent at least three times at the end of any analytical set to ensure that the column is flushed completely.

4.4 Calculations

4.4.1 Methods

The average response factor was calculated as follows:

$$\text{Response} = \frac{\text{Concentration (ng/mL) of Standard}}{\text{Peak Area Counts}}$$

$$Rf_{avg} = \frac{\sum \text{Standard Response}}{n}$$

where:

Rf_{ave} = Average Response Factor
n = total number of standards analyzed in a sample set

Concentration of DPX-YT669 in the fortified samples (µg/L found) was then calculated using the equation below:

Analyte Found, µg/L (ppb) =

$$= \frac{\text{A (area counts) x Rfave (ng/mL/area counts) x Final Volume (mL) x Dilution Factor}}{\text{Sample Volume (mL)}}$$

$$= 1\frac{ng}{mL} \times \frac{1\,\mu g}{1000\,ng} \times \frac{1000\,mL}{1\,L} = \mu g/L$$

where:

A = Corrected Peak Area Counts

= Peak Area Counts in sample – Peak Area Counts in control

Rf_{ave} = Average Response Factor

Sample Volume, mL = Sample Weight, g (water density = 1.0 g/mL)

Final Volume = 12.5 mL

Dilution Factor = 10 (100 µL diluted to 1 mL)

Percent Recovery was calculated as:

$$\%\ \text{Recovery} = \frac{\text{Analyte Found}\,(\mu g/L)}{\text{Fortification Level}\,(\mu g/L)} \times 100$$

4.4.2 *Examples*

Calculation for the percent recovery of picoxystrobin (DPX-YT669) from a drinking water sample fortified at 0.10 µg/L (Appendix 1, YT669M_020410, DPX-YT669, Tap LOQ 1), which was prepared and analyzed on Feb. 4, 2010, is shown below.

Rf_{avg} of six DPX-YT669 standards = 3.6589 x 10-6 ng/mL/area counts

Peak Area Counts (ac) for DPX-YT669, LOQ 1sample = 2240

(Average) Peak Area Counts (ac) for DPX- YT669, control = 0

Sample Weight = Sample Volume = 10.00 grams = 10.0 mL

Total Extract Volume = 12.5 mL

Dilution Factor = 10 (100 µL of sample diluted to 1 mL)

Fortification Level = 0.11 ng/mL = 0.11 µg/L

$$\text{DPX-YT669 Found} = \frac{(2240 - 0)\text{ ac} \times 3.6589 \times 10^{-6}\text{ ng/mL/ac} \times 12.5\text{ mL} \times 10}{10.0\text{ mL}}$$

$$= 0.102 = 0.10\text{ ng/mL} = 0.10\ \mu\text{g/L (ppb)}$$

$$\text{DPX-YT669 \%Recovery} = \frac{0.102\ \mu\text{g/L}}{0.11\ \mu\text{g/L}} \times 100 = 94\%$$