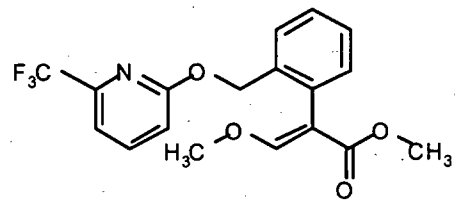
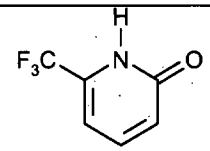
2.0 INTRODUCTION

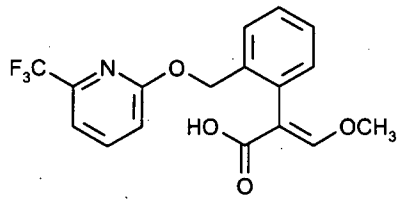
Picoxystrobin (DPX-YT669) is a strobilurin fungicide which has been shown to be effective in the control of plant diseases. In soil, picoxystrobin degrades aerobically into minor metabolites of IN-QDK50, IN-QDY62, and IN-QDY63. The chemical structures and pertinent information of the test substances are shown below:



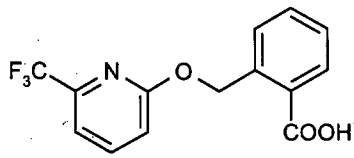
DuPont Code: DPX-YT669
Trivial Name: Picoxystrobin
IUPAC Name: Methyl (E)-2-{2-[6-(trifluoromethyl)pyridin-2-yloxymethyl]phenyl}-3-methoxyacrylate
Chemical Abstracts Name: methyl (E)-α-(methoxymethylene)-2-[[[6-(trifluoromethyl)-2-pyridinyl]oxy]methyl]-benzeneacetate
***CAS* Registry Number**: 117428-22-5
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.



DuPont Code: IN-QDK50
IUPAC Name: 6-(trifluoromethyl)pyridin-2-one
Chemical Abstracts Name: 6-(trifluoromethyl)pyridin-1H-2-one
***CAS* Registry Number**: 34486-06-1
Molecular Formula: $C_6H_4NOF_3$
Molecular Weight: Average, 163.10; Monoisotopic, 163.02



DuPont Code: IN-QDY62
Chemical Abstracts Name: (E)-3-methoxy-2-{2-[6-(trifluoromethyl)pyridin-2-yloxymethyl]phenyl}acrylic acid
***CAS* Registry Number**: N/A
Molecular Formula: $C_{17}H_{14}NO_4F_3$
Molecular Weight: Average, 353.29; Monoisotopic, 353.09



DuPont Code: IN-QDY63
Chemical Abstracts Name: 2-[6-(trifluoromethyl)pyridin-2-yloxymethyl]-benzoic acid
***CAS* Registry Number**: N/A
Molecular Formula: $C_{14}H_{10}NO_3F_3$
Molecular Weight: Average 297.23; Monoisotopic, 297.06

This analytical method for picoxystrobin, IN-QDK50, IN-QDY62, and IN-QDY63 in soil, at an LOQ of approximately 0.010 mg/kg (ppm), was developed to satisfy the requirements of the U.S. EPA Pesticide Assessment Guidelines Subdivision N and the EU Annex II 4.2.2.

Picoxystrobin (DPX-YT699) and its metabolites were extracted from soil with 75/25 acetone/1M HCl and acetone at ambient temperature using a wrist-action shaker. Following centrifugation, an aliquot of the extract was purified by solid-phase extraction using an Oasis™ HLB cartridge. The purified extract was filtered and analyzed by reversed-phase HPLC/ESI-MS/MS.

When using a sensitive LC/MS/MS instrument, the soil extract aliquots were filtered through a 0.45-µm PTFE disk, diluted ten-fold 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 and Model AE160 for weighing solid standards (Mettler Instrument Corporation, Hightstown, NJ)

Mettler Top-Loading Balance, Model PM600 and Model XS1003S for weighing soil samples (Mettler Instrument Corporation, Hightstown, NJ)

Centrifuges - Sorvall GLC-2B L-995 and RC 5C Plus (VWR International, West Chester, PA)

Extractor
Wrist Action Shaker, Model 75 (Burrell, Pittsburgh, PA)

VWR Disposable Skirted Centrifuge Tube, 50-mL, Polypropylene, cs. of 500, Part # 21008-480 (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 # 922975-902, 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.

Applied Biosystem/MDS Sciex API3200 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 3/10 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)

Nitrogen Evaporator - N-Evap Model 111 (Organomation Assoc., Berlin, MA)

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 Collection Vials
Disposable Centrifuge Tube, 50-mL, pkg. of 72, Part # 73785 (VWR International, West Chester, PA)

Glass Centrifuge Tubes – Pyrex® Conical Centrifuge Tubes, graduated, 50-mL capacity, Catalog No. 21048-050 (VWR International, West Chester, PA)

Solid-Phase Extraction Apparatus
Visiprep™ SPE Manifold, Catalog No. 5-7030M (Supelco, Inc., Bellefonte, PA)

Solid-Phase Extraction Disposable Flow Control Valve Liners - for the Visiprep™, Catalog No. 57059 (Supelco, Inc., Bellefonte, PA)

Solid-Phase Extraction Adapters, Catalog No. JTB 7122-00 (Mallinkrodt Baker, Inc., Phillipsburg, NJ)

Solid-Phase Extraction Cartridges
Oasis™ HLB cartridge, 0.5-g/6-mL, Part No. 186000115, (Waters, Milford, MA)

Solid-Phase Extraction Reservoirs – 25-mL (75-mL) size, Catalog No. 1213-1011 1213-1012 (Varian Inc., Palo Alto, CA)

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)

Concentrated Hydrochloric Acid, 36%, reagent grade, (EMD Chemicals)

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

Hexanes - HPLC grade (EMD Chemicals)

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

Reference standards (DuPont Crop Protection, Newark, DE):
DPX-YT669, IN-QDK50-003, IN-QDY62, and IN-QDY63

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*

Picoxystrobin and metabolites were extracted from a 5.0-g soil sample with 75/25 acetone/1M HCl and acetone solution at ambient temperature using a wrist-action shaker. Following centrifugation, an aliquot of the extract was purified by solid-phase extraction using an Oasis™ HLB cartridge. The analytes were retained in the cartridge and eluted with methanol and ethyl acetate. Exactly 1.0 mL of water was added to the eluate and it was evaporated until aqueous (1.0 mL) in an N_2-vap at 30°C. The extract was diluted with 1.0 mL of methanol, 0.8 mL of acetonitrile and appropriate amount of water to bring the volume to 5.0 mL and filtered through a 0.45-µm PTFE disk. The purified extract was analyzed by reversed-phase HPLC using a Zorbax® XDB C18 (4.6 x 50 mm, 1.8-µm particle) column and a mobile phase of 0.1% formic acid-0.1 mM ammonium formate (aq) and methanol. Detection of the analytes 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.

Prior to the blowing down of the eluate, water was added in order to avoid the complete drying of the eluate which will result to losses of the volatile pyridine metabolite IN-QDK50.

The soil method LOQ for all analytes was 0.010 mg/kg, which was equivalent to a 1.4-ng/mL calibration standard.

When using a sensitive LC/MS/MS instrument, purification of the soil extract aliquot by SPE on an Oasis™ HLB cartridge was shown to be not necessary. Instead, the soil extract aliquot was filtered through a 0.45-µm PTFE disk, diluted ten-fold with 20/16/64 methanol/acetonitrile/water and analyzed by reversed HPLC/ESI-MS/MS. Acceptable LC/MS/MS responses were obtained for picoxystrobin and metabolites in the calibration standards and fortified soil samples at about 10-fold decrease in concentration (i.e., 0.05 ng/mL as the lowest calibration standard and 0.14 ng/mL as the equivalent standard concentration of the LOQ fortified sample.)

With this extract-dilute-analyze (without SPE cleanup) procedure, there was a slight difference in composition and % of organic in the standards' diluent and samples'diluent, i.e., 36% acetonitrile and methanol and 41% acetonitrile, methanol, and acetone respectively. Acceptable recoveries were obtained and there was only a minimal influence of matrix effect on % recoveries of post-fortified sample extracts. For future work, however, it is recommended to use the same solvent/diluent for standards and investigative samples.

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 soil type to determine if matrix effect, suppression or enhancement, influenced percent recovery of picoxystrobin and its metabolites. The post-fortified samples, in this study, were extracts of control soil samples that were purified and prepared in the same manner as with the other samples, but fortified with the analytes 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

75/25 Acetone/1M Hydrochloric Acid (Extraction Solution)

Into a 1-L glass storage bottle, add 750 ml of acetone and 250 mL of 1M hydrochloric acid. Cap and shake to homogeneity. This solution should be prepared monthly.

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.

10 mM Ammonium Formate (SPE Rinse 1)
Mix 5 mL of 1.0 M ammonium formate with 495 mL of ultrapure water in a 500-mL glass storage bottle. 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

1.0 M Hydrochloric Acid (aq)

Add 85.9 mL of concentrated hydrochloric acid (36%, w/w) into a 1-L 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.

15/85 Methanol/Water (SPE Rinse)

Mix 75 mL of methanol with 425 mL of ultrapure water in a 500-mL glass storage bottle. This solution is stored capped at room temperature and should be stable for 3 months.

0.1% Formic Acid in Ethyl Acetate (Eluent)

Add 0.50 mL of concentrated formic acid to 500 mL of ethyl acetate in a 500-mL glass storage bottle. Cap and shake to homogeneity. This eluent is stable for at least a month.

Standard Diluent – 20/16/64 Methanol/ /Acetonitrile/ Water

Into a 500-mL glass storage bottle, mix 100 mL of methanol, add 80 ml of acetonitrile and 320 mL of ultrapure water. Cap and shake to homogeneity. This solution should be prepared monthly.

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

To a 1-L volumetric flask that is partially filled with ultrapure water, add 0.10 mL of 1.0 M ammonium formate and 1.0 mL of concentrated formic acid (98%, w/w). 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 <u>*Stock and Intermediate Standards Preparation and Stability*</u>

Weigh 10.00 mg ± 0.50 mg (recorded to the nearest 0.01 mg) each of picoxystrobin, IN-QDK50, IN-QDY62, and IN-QDY63 analytical standards into separate 100-mL volumetric flasks. Dissolve and dilute to the mark with acetonitrile to make stock standard solutions of approximately 100 µg/mL.

The stock standard solutions are stable for at least four months when stored capped at ≤-10°C (Reference 1).

4.2.4 *Fortification Standard Preparation and Stability*

Prepare a 20.0-µg/mL fortification solution in acetonitrile by adding 2000 µL each of the 100.0-µg/mL DPX-YT669, IN-QDY62, IN-QDK50 and IN-QDY63 stock standards into a 10-mL volumetric flask. Dilute to the mark with acetonitrile and mix to homogeneity.

Prepare a 5.0-µg/mL fortification solution in acetonitrile by adding 500 µL each of the 100.0-µg/mL DPX-YT669, IN-QDY62, IN-QDK50 and IN-QDY63 stock standards into a 10-mL volumetric flask. Dilute to the mark with acetonitrile and mix to homogeneity.

Prepare a 0.50 µg/mL fortification standard in acetonitrile by adding 1 mL of the 5.0-µg/mL fortification solution of DPX-YT669, IN-QDY62, IN-QDK50 and IN-QDY63 into a 10-mL volumetric flask. Dilute to the mark with acetonitrile and mix to homogeneity.

These solutions are stable for at least four months when stored capped at ≤-10°C (Reference 1).

4.2.5 *Chromatographic Standard Preparation and Stability*

Prepare calibration standards ranging from 0.50 to 50.0 ng/mL (or in concentrations expected to cover the range of DPX-YT669, IN-QDY62, IN-QDK50 and IN-QDY63 in the investigative samples) from the fortification mix standards diluted with 20/16/64 methanol/acetonitrile/water. 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)
100**	200	5.0 µg/mL Fortification Solution	10.0
50.0***	100	5.0 µg/mL Fortification Solution	10.0
30.0	60	5.0 µg/mL Fortification Solution	10.0
10.0	20	5.0 µg/mL Fortification Solution	10.0
5.0	500	100 ng/mL	10.0
2.0	200	100 ng/mL	10.0
1.0	100	100 ng/mL	10.0
0.50	50	100 ng/mL	10.0

* Diluent: 20/16/64 methanol/acetonitrile/water

** An intermediate calibration standard

*** Optional

For the extract-dilute-analyze procedure, prepare calibration standards ranging from 0.050 ng/mL to 10.0 ng/mL. The table below describes how standards were prepared for the validation work presented in this report:

STANDARD CONC. (NG/ML)	µL ADDED	FORTIFICATION STANDARD USED	FINAL VOLUME (ML)
30**	600	0.50 µg/mL Fortification Solution	10.0
10.0	200	0.50 µg/mL Fortification Solution	10.0
3.0	1000	30 ng/mL	10.0
1.0	1000	10 ng/mL	10.0
0.50	500	10 ng/mL	10.0
0.050	1000	0.5 ng/mL	10.0

* Diluent: 20/16/64 methanol/acetonitrile/water

** An intermediate calibration standard

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

4.2.6 *Source (& Characterization) of Samples*

Preprocessed soil samples from four different sources were used for the method validation. The source of the soil samples and the pertinent physical characteristics are summarized in the following table. Soil samples were characterized at Harris Environmental Technologies (Lincoln, NE) and Agvise Laboratories (Northwood, ND). Characterization records are maintained at DuPont Agricultural Products.

Soil Name (Location, DuPont Study No./Notebook No.)	Type	pH_w	Sand (%)	Silt (%)	Clay (%)	OM_{ash} (%)
45.PEI.BA1 (PEI*, Canada, DuPont-25345)	Sandy Loam	6.2	66.0	24.0	10.0	3.6
18.WI.BA (Wisconsin, U.SA., DuPont-26418)	Sandy Loam	6.0	74.0	14.0	12.0	2.7
Tama (Toulon, Ohio, U.S.A., 2007-018)	Silty Clay	6.5	5.2	52.0	42.8	4.2
Drummer (Rochelle, Illinois, 2008-005)	Clay Loam	6.0	27.0	39.0	34.0	5.9

*Prince Edward Island, Canada

4.2.7 *Storage & Preparation of Samples*

Preprocessed soil samples were received frozen and stored in a freezer maintained at ≤-10°C prior to preparation for analysis.

4.2.8 *Sample Fortification Procedure*

For the LOQ (0.010-mg/kg)), 10xLOQ (0.10-mg/kg) and 40xLOQ (0.40-mg/kg) fortification levels, spike 5.0-g of soil samples with 100 µL of the 0.50-, 5.0-, and 20.0-µg/mL DPX-YT669, IN-QDK50, IN-QDY62, and IN-QDY63) fortification solutions in acetonitrile, respectively. Let the fortified samples sit in the hood for 10-15 minutes before adding the extraction solvent.

4.2.9 *Analyte Extraction Procedure*

1. Weigh 5.00 g ± 0.50 g of soil sample into a 50-ml polypropylene centrifuge tube. Fortify the sample, if necessary.
2. Add 25 mL of the extraction solution (75/25 acetone/1M HCl) to the sample.
3. Shake in a wrist-action shaker for 30 minutes.
4. Centrifuge for at least 15 minutes at 3,000 rpm.
5. Decant the supernatant/extract into a clean 50-ml polypropylene centrifuge tube.
6. Repeat steps 2-4, but with only 10 mL of acetone as the extraction solvent.
7. Decant the supernatant/extract into the same 50-ml polypropylene centrifuge tube.
8. Bring the final volume to 35 mL with acetone. Cap and shake to homogeneity.
9. Transfer 5 mL of extract into a 15-mL (or 50-mL) glass graduated centrifuge tube.
10. Evaporate until 2.0 mL ± 0.1 mL in a nitrogen evaporator. (Set the N_2 evaporator with moderate nitrogen flow and the water bath at 25°C ± 5°C.) If the evaporated extract is below 2 mL, add acetone to bring it to 2 mL. Vortex mix for at least 30 seconds.

    Note: It is essential that the extract be not evaporated to dryness since this will result to the loss of the volatile pyridone metabolite IN-QDK50.

11. Dilute the sample extract to 15 mL with water, vortex mix, ultrasonicate for at least 10 minutes, and vortex mix again.
12. Proceed to the next section for sample cleanup procedure. The sample extract may be stored in the freezer (≤-10°C) for at least 3 days, if the sample cleanup procedure is not to be performed at this time.
13. For LC/MS/MS analysis without SPE cleanup:

    Transfer about 2.0 mL of sample extract from Step 8 into a 5-mL disposable syringe fitted with a disposable 13-mm, 0.45-µm PFTE disk. Filter the extract into a 20-mL glass scintillation vial.

    Transfer 100 µL of the filtered extract into a 2-mL HPLC vial and add 900 µL of 20/16/64 methanol/acetonitrile/water. Cap the vial and vortex mix.

Prepare post-fortified samples (optional):

LOQ equivalent (0.14 ng/mL): Into a 2-mL HPLC vial, add 100 µL of the filtered control extract, 900 µL of 20/16/64 methanol/acetonitrile/water, and 50 µL of the 3-ng/mL calibration standard. Cap the vial and vortex mix the sample.

10xLOQ equivalent (1.4 ng/mL): Into a 2-mL HPLC vial, add 100 µL of the filtered control extract, 900 µL of 20/16/64 methanol/acetonitrile/water, and 50 µL of the 30-ng/mL intermediate calibration standard. Cap the vial and vortex mix the sample.

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

4.2.10 *Analyte Purification/Concentration Procedure*

1. Attach a 500-mg/6cc Oasis® HLB cartridge onto a vacuum manifold port. Condition the cartridge with 5 mL of methanol, followed by 5 mL of water (use gravity flow) and discard eluate. (Use light vacuum as needed to obtain a flow of 1-2 drops/second.) Do not let the cartridge to go to dryness.

   Attach a 25-mL SPE reservoir onto the conditioned cartridge using an adaptor.

   Note: The cartridge will not go to dryness if sample loading or elution is by gravity flow. If a light vacuum is used, stop vacuum when only a thin film of liquid is left on top of the sorbent.

2. Pass the sample extract through the conditioned cartridge by gravity flow. (Use light vacuum as needed to obtain a flow of 1-2 drops/second. Do not let the cartridge to go to dryness.) Discard eluate.

3. Rinse the sample tube with 5 mL of 10 mM ammonium formate and pass the rinsate through the cartridge. Do not let the cartridge to go to dryness. Discard eluate.

4. Rinse the sample tube with 5 mL of 15/85 methanol/water and pass rinsate through the cartridge. Discard eluate.

5. Dry the cartridge using a full vacuum setting for at least a minute.

6. Place a graduated 50-mL glass centrifuge tube under the cartridge. Pass 2 mL of methanol, followed by 20 mL of 0.1% formic acid in ethyl acetate to elute the analytes from the cartridge. (Use light vacuum as needed to obtain a flow of 1-2 drops/second.) After all the elution solvent has passed through, vacuum (maximum) dry the cartridge for 20-30 seconds.

7. Add 1 mL of ultrapure water to the eluate/sample extract (from Step 6).

8. Evaporate the extract until 1.0 mL (aqueous) using a N2 evaporator with a moderate nitrogen flow and the water bath set at 25°C ± 5°C (this takes ~1hr). Do not let the extract to go to dryness! If the evaporated extract is at or below 1.0 mL, proceed to the next step.

9. Add 1 mL of methanol and 0.8 mL of acetonitrile.
10. Dilute the sample extract to 5 mL with ultrapure water, vortex mix, ultrasonicate for at least 10 minutes, and vortex mix again.
11. Transfer the sample extract into a 5-mL disposable syringe fitted with a disposable 13-mm, 0.45-µm PFTE disk. Filter the extract back into the sample tube. Vortex mix the sample.
12. Transfer 1 mL of the filtered sample extract into a 2-mL HPLC sample vial for HPLC/ESI-MS/MS analysis.

    Prepare post-fortified samples (optional):

    ~LOQ equivalent (1.4 ng/mL): Into a 2-mL HPLC vial, add 1000 µL of the filtered control extract from Step 11 and 50 µL of the 30-ng/mL calibration standard. Cap the vial and vortex mix the sample.

    ~10xLOQ equivalent (14 ng/mL): Into a 2-mL HPLC vial, add 1000 µL of the filtered control extract from Step 11 and 30 µL of the 0.50-µg/mL fortification standard. Cap the vial and vortex mix the sample.

    If there is a delay in the analysis, store the sample extracts frozen at ≤-10°C. Samples are stable for 3-4 days.
13. With the 40xLOQ fortified sample, transfer 200 µL of the filtered sample extract from Step 11 into a 2-mL HPLC vial. Add 800 µL 20/16/64 methanol/acetonitrile/water, cap the vial and vortex mix the sample.

    Prepare post-fortified samples (optional):

    ~40xLOQ equivalent (12 ng/mL): Into a 2-mL HPLC vial, add 1000 µL of the filtered control extract from Step 11 and 25 µL of the 0.5-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 (with SPE Cleanup)

System:	Shimadzu LC-20 AD System
Column:	Zorbax® XDB C18, 4.6 mm × 50 mm, 1.8-µm dp
Column Temperature:	40°C
Injection Volume:	25 µL
Autosampler Temperature	4°C
Flow Rate:	1.0 mL/min (post-column split, 0.3 mL/min into MS source)
Conditions:	Time %A %B Flow 0.00 80 20 1.0 2.00 80 20 1.0 2.10 40 60 1.0 7.10 23 77 1.0 7.20 5 95 1.0 9.20 5 95 1.0 9.30 80 20 1.0 11.50 80 20 1.0 A: 0.1 mM ammonium formate - 0.1% Formic acid. B: Methanol Flow in mL/min.
Analyte	Retention Times (minutes)
DPX-YT669	~7.4
IN-QDY62	~6.2
IN-QDK50	~3.8
IN-QDY63	~6.7
Total Run Time:	11.5

MS Conditions (with SPE Cleanup)

MS System:		Applied Biosystems SCIEX API 3200 LC/MS/MS						
Analyte Monitored	Ions Monitored (amu)	DP[A] (V)	EP[B] (V)	CEP[C] (V)	CE[D] (V)	CXP[E] (V)	Dwell Time (ms)	Acquisition Time (min)
DPX-YT669	368 → 145	20	10	20.85	32	3	150	0.00 – 11.5
	368 → 205				14	2		
IN-QDK50	164 → 116	16	10	14.00	35	4	150	0.00 – 11.5
	164 → 144				25	4		
IN-QDY62	354 → 131	16	10	16.00	31	4	150	0.00 – 11.5
	354 → 145				27	4		
IN-QDY63	298 → 135	16	10	14.00	29	10	150	0.00 – 11.5
	298 → 164				17	4		

Scan Type/Polarity:	MRM/Positive
Ion Source Voltage:	ESI, 5500
Collision Gas (CAD):	5 psig
Curtain Gas (CUR):	30 psig
Nebulizer Gas (GS1):	45 psig
Heater Gas (GS2):	70 psig
Source Heater (TEM):	600°C
Interface Heater (ihe):	ON
Resolution Q1	Low
Resolution Q2	Low

MS Flow Rate (Post-column split): 300-µL/min (approximately 10:3 split)

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

HPLC Conditions (without SPE Cleanup)

System:	Agilent HP1100 HPLC				
Column:	Zorbax® XDB C18, 4.6 mm × 50 mm, 1.8-µm dp				
Column Temperature:	40°C				
Injection Volume:	5 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% Formic acid in 0.1 mM ammonium formate. B: Methanol. Flow in mL/min
	0.00	70	30	1.0	
	1.00	70	30	1.0	
	1.10	70	30	1.0	
	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				
IN-QDY62	~6.0				
IN-QDK50	~2.5				
IN-QDY63	~6.5				
Total Run Time:	10.00				

MS Conditions

MS System:	Applied Biosystems 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 – 9.0
	368.2 → 205.0 ± 0.1			13	22		
IN-QDK50	164.1 → 116.1 ± 0.1	70	10	30	6	200	0.40 – 9.0
	164.1 → 143.9 ± 0.1			25	8		
IN-QDY62	354.2 → 191.2 ± 0.1	46	10	13	14	200	0.40 – 9.0
	354.2 → 145.0 ± 0.1			27	8		
IN-QDY63	298.2 → 135.1 ± 0.1	41	10	15	10	200	0.40 –9.0
	298.2 → 164.1 ± 0.1			33	6		
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, IN-QDY62, IN-QDK50 and IN-QDY63. The response of each analyte was optimized initially by infusing the analyte into the ionization source. The flow rate and mobile phase were adjusted to the elution conditions of the analyte from the HPLC column. Each of the protonated molecule of DPX-YT669, IN-QDY62, IN-QDK50 or IN-QDY63 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 for each analyte.

Residues of DPX-YT669, IN-QDY62, IN-QDK50 and IN-QDY63 were each identified in soil 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, the peak area from the total ion current (TIC) was used for each analyte.

A six-port electronically activated switching valve was used to direct the HPLC column effluent to waste prior to and following the elution of analytes. For the LC/MS/MS analysis (without SPE cleanup), the retention times of the analytes were within 3.0 - 8.0 minutes. The chromatographic run time is 10 minutes, but the MS sample collection time is 0.40 - 9.0 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 and 300-µL/min actually passed through the interface, the remainder going to waste.

4.3.3 *Calibration Procedures*

Prepare calibration standards that bracket the levels of DPX-YT669, IN-QDY62, IN-QDK50 and IN-QDY63 found in the soil 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, IN-QDY62, IN-QDK50 and IN-QDY63 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 0.50 ng/mL standard, control(s), LOQ fortifications, 1.0 ng/mL standard, LOQ fortifications, 2.0 ng/mL standard, ... 10 × LOQ fortifications, 30.0 ng/mL standard and 50 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-QDY62, IN-QDK50 and IN-QDY63 in the fortified samples (mg/kg found) was then calculated using the equation below:

Analyte Found, mg/kg (ppm) =

$$\frac{A \times Rf_{ave}\text{ (ng/mL/area counts)} \times \text{Extract Volume (mL)} \times \text{Final Volume (mL)} \times \text{Dilution Factor}}{\text{Sample Weight (g)} \times \text{Aliquot Volume(mL)} \times 1000}$$

where:

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

Rf_{ave} = Average Response Factor

Percent Recovery was calculated as:

$$\%\ \text{Recovery} = \frac{\text{Analyte Found (mg/kg)}}{\text{Fortification Level (mg/kg)}} \times 100$$

4.4.2 *Examples*

Calculation for the percent recovery of picoxystrobin (DPX-YT669) in Wisconsin, U.S.A. soil fortified at 0.010 mg/kg (Appendix 1, Data Sheet No. SETMV-WIS-FEB09, LOQ 1), which was prepared and analyzed on February 7-9, 2009, is shown below.

Rf_{avg} of six DPX-YT669 standards = 5.9844 x 10-5 ng/mL/area counts
Peak Area Counts (ac) for DPX-YT669, LOQ 1sample = 27005.00
(Average) Peak Area Counts (ac) for DPX-YT669, control = 1060.02
Sample Weight = 5.09 grams
Final Volume = 5.0 mL
Fortification Level = 0.010 mg/kg

DPX-YT669 Found =

$$\frac{(27005.00 - 1060.02)\ \text{ac} \times 5.9844 \times 10^{-5}\ \text{ng/mL/ac} \times 35\ \text{mL} \times 5.0\ \text{mL} \times 1}{5.09\ \text{g} \times 5\ \text{mL} \times 1000}$$

$$= 0.0107 = 0.011\ \text{mg/kg (ppm)}$$

$$\text{DPX-YT669 \%Recovery} = \frac{0.0107\ \text{mg/Kg}}{0.010\ \text{mg/Kg}} \times 100 = 104\%$$