Four analytes, including famoxadone (DPX-JE874), and its metabolites (IN-JS940, IN-KF015 and IN-KZ007) were analyzed on an LC-ESI-MS/MS system using the negative ion mode, while the metabolite IN-H3310 was analyzed using the positive ion mode. All interferences were negligible at <30% of the LOQ. In all cases, the average recoveries at each fortification level for both primary (Q) and confirmation (C) ESI-MS/MS transitions were within the acceptance range of 70%–120%, with RSDs ≤20%. Thus, the residue analytical method as described in DuPont-43971 was demonstrated as valid for the determination of famoxadone and its metabolites in soil and water.

2.0 INTRODUCTION

To satisfy US and EU regulatory ILV requirements, a residue analytical method must be validated at an independent laboratory prior to its submission to the appropriate regulatory authority. This study was conducted to fulfill those requirements.

The analytical method DuPont-43971 entitled "Analytical Method for the Determination of Famoxadone (DPX-JE874) and its Metabolites in Soil and Water by LC-ESI-MS/MS" was proven valid for the quantitation of famoxadone (DPX-JE874) and its metabolites, IN-JS940, IN-KF015, IN-KZ007, and IN-H3310, in soil and water.

Famoxadone and its metabolites were extracted from control soil and water, fortified with the analytes at the LOQ (10 ppb in soil and 0.1 ppb in water) and at 10×LOQ. In soil, the samples were subjected to liquid extraction, by methanol/buffered, acidic water solution. In water, the samples were processed by solid phase extraction. At least two transitions were monitored for each analyte. All transitions of famoxadone and its metabolites, IN-JS940, IN-KF015, and IN-KZ007, were detected by negative ion mode ESI-MS/MS. Transitions of the metabolite IN-H3310 were detected by positive ion mode ESI-MS/MS.

For soil, the analytical method was designed to achieve an LOQ of 10 ppb and the limit of detection (LOD) was estimated to be 3 ppb. For water, the analytical method was designed to achieve an LOQ of 0.1 ppb and the LOD was estimated to be 0.03 ppb. The independent validation evaluated recoveries of famoxadone and its metabolites in samples fortified at the LOQ and 10× LOQ level. The method was performed as written.

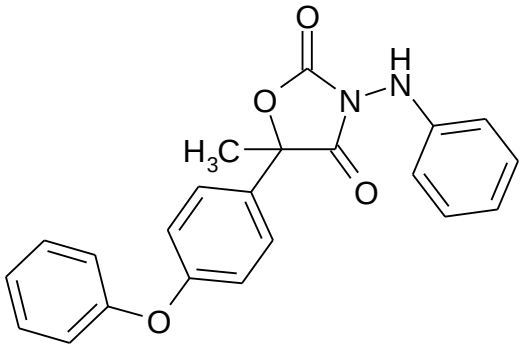
3.0 MATERIALS AND METHODS

3.1 Test Substances

The reference analytical standards (test substances) used for this study are described below:

DuPont code: DPX-JE874 (Famoxadone)

Chemical Structure:

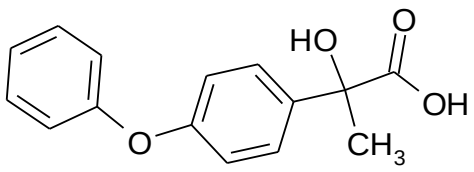


DPX-JE874

CAS Name:	5-methyl-5-(4-phenoxyphenyl)-3-(phenylamino)-2, 4-oxazolidinedione
Common Name:	Famoxadone
Molecular weight:	374.40 g/mole
Formula:	$C_{22}H_{18}N_2O_4$
Source:	DuPont
CAS Number:	131807-57-3
Batch/Lot Number:	D103511-108-4
Purity:	99.4%
Receipt date:	11 August 2015
Expiration date:	13 August 2017
Storage:	Ambient

DuPont code: IN-JS940

Chemical Structure:

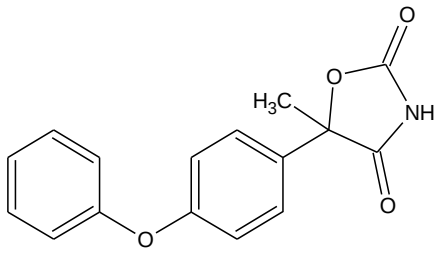


IN-JS940

CAS Name:	α-hydroxy- α -methyl-4-phenoxybenzeneacetic acid
Molecular weight:	258.28 g/mole
Formula:	$C_{15}H_{14}O_4$
Source:	DuPont
CAS Number:	Not Available
Batch/Lot Number:	GVK-DU-P554-1
Purity:	95.9%
Receipt date:	11 August 2015
Expiration date:	14 September 2015
Storage:	Desiccator

DuPont code: IN-KF015

Chemical Structure:

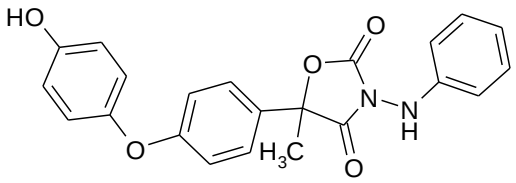


IN-KF015

IUPAC Name:	5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione
Molecular weight:	283.28 g/mole
Formula:	$C_{16}H_{13}NO_4$
Source:	DuPont
CAS Number:	Not Available
Batch/Lot Number:	GVK-DU-P555-1
Purity:	99.7%
Receipt date:	11 August 2015
Expiration date:	17 December 2019
Storage:	Ambient

DuPont code: IN-KZ007

Chemical Structure:

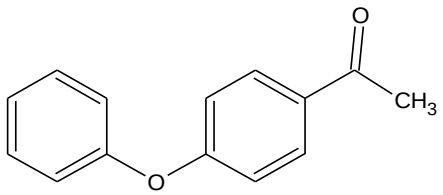


IN-KZ007

CAS Name:	5-[4-(4-hydroxyphenoxy)phenyl]-5-methyl-3-(phenylamino)-2,4,-oxazolidinedione
Molecular weight:	390.40 g/mole
Formula:	$C_{22}H_{18}N_2O_5$
Source:	DuPont
CAS Number:	Not Available
Batch/Lot Number:	GVK-DU-P556-1 Lot 2
Purity:	95.7%
Receipt date:	11 August 2015
Expiration date:	02 July 2020
Storage:	Ambient

DuPont code: IN-H3310

Chemical Structure:



IN-H3310

IUPAC Name:	1-(4-phenoxyphenyl)ethanone
Molecular weight:	212.24 g/mole
Formula:	$C_{14}H_{12}O_2$
Source:	DuPont
CAS Number:	Not Available
Batch/Lot Number:	E87531-42
Purity:	98.8%
Receipt date:	11 August 2015
Expiration date:	15 February 2020
Storage:	Ambient

Famoxadone (DPX-JE874), IN-JS940, IN-KF015, IN-KZ007 and IN-H3310, were supplied by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Newark, DE. Information pertaining to the characterization and stability of the test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware. Certificates of analysis for the compounds listed above are presented in **Appendix 1**.

3.2 Test System

In this study, the analytical method was validated for soil and water samples. The samples were shipped from DuPont on 19 August 2015. Sassafras soil and surface water from Pike Creek in Newark, Delaware were provided as blank matrices. Soil fortification samples were made using 10.0 ± 0.10 g of blank soil. Water fortification samples were made using 200 mL of blank water. Water samples were adjusted to a final concentration of approximately 0.01% formic acid and 5% acetonitrile. The samples were assigned unique identification (ID) numbers by the laboratory, each consisting of an alpha-numeric sample ID along with additional designations such as "Control" or "LOQ", as appropriate. Matrix characterization data are available in **Appendix 2**.

3.3 Equipment

The equipment used in this validation were either the same as that specified in the analytical method or the equivalent. A Shimadzu LC-30AD HPLC was used instead of an Agilent 1200 Series Infinity HPLC system. An AB SCIEX Triple Quad 5500 was used instead of an AB SCIEX API 4000 Triple Quad MS/MS system. These differences were demonstrated as equivalent to that specified in the method.

3.4 Reagents

The reagents used were either the same as those specified in the analytical method or of equivalent grade.

3.5 Principles of the Analytical Method

The analyses followed the analytical method for famoxadone and its metabolites, as described in the method DuPont-43971. The following is a summary of the method conducted at Alliance Pharma.

In Soil

Test samples in soil were prepared by spiking famoxadone and its metabolites into 10.00 ± 0.10 g of blank soil, fortified at either the LOQ (10 ppb) or 10× LOQ (100 ppb). After fortification, 20 mL of methanol/30mM sodium acetate (4:1, *v/v*, adjusted to pH 3) was added to each sample. The samples were vortex mixed for 1.5 minutes, sonicated for 5 minutes, and briefly vortex mixed again. The samples were centrifuged at a rate of approximately 2500 rpm for 5 minutes to drive particulates to the bottom of the tube. The supernatants were decanted into clean 50-mL polypropylene centrifuge tubes. Then, 15 mL of methanol/30mM sodium acetate (4:1, *v/v*, adjusted to pH 3) was added to each soil pellet and the procedure was repeated. The supernatants were combined and diluted to 50 mL with 0.01 M aqueous formic acid and mixed thoroughly. The sample extracts were transferred to HPLC sample vials for analysis via LC-ESI-MS/MS

In Water

Test samples in water were prepared by first adjusting the blank water matrix to a final concentration of approximately 0.01% formic acid and 5% acetonitrile by volume (i.e., 20 µL of formic acid and 10 mL of acetonitrile were added to a 250 mL centrifuge tube and diluted to a final volume of 200 mL with the blank water matrix). Then, the 200 mL adjusted water samples were spiked with famoxadone and its metabolites at either the LOQ (0.10 ppb) or 10× LOQ (1.0 ppb). The samples were mixed well.

Next, the water samples were extracted via a C_{18} SPE (3-cc/500-mg) cartridge. The cartridge was attached to a vacuum manifold port and conditioned using 5 mL of methanol and 5 mL of deionized water. The eluate was discarded. The samples were added to the conditioned cartridge and the drip rate was adjusted to 2-3 mL per minute, assisted by vacuum. The cartridge was dried using full vacuum and the eluate was discarded. The cartridge was treated with 2.5 mL of 0.01% formic acid in acetonitrile and the eluate was passed via gravity flow and collected. The cartridge was then treated with 2.5 mL of 0.01% formic acid in methanol and the eluate was passed via gravity flow and collected.

The combined eluates were vortex mixed and diluted for analysis by combining 0.60 mL of the final extract and 0.40 mL of 0.01 M formic acid in water. The diluted sample extracts were transferred to HPLC sample vials for analysis via LC-ESI-MS/MS.

For both soil and water samples, analytes famoxadone (DPX-JE874), IN-JS940, IN-KF015, and IN-KZ007 were detected in the negative ion mode and IN-H3310 was detected in the positive ion mode. Two parent-to-daughter ion transitions of each analyte were monitored. The primary ion transitions were used for quantitative analysis and the secondary ion transitions were used for confirmation.

Method validation was accomplished by evaluating the analytes in validation set which consisted of 2 blank control samples, 5 replicate samples fortified at the LOQ, and 5 replicate samples fortified at 10× LOQ.

3.6 Modifications, Interpretations, and Critical Steps

The analytical method was performed exactly as written except for the following:

A Shimadzu LC-30AD HPLC system was used instead of an Agilent 1200 Series Infinity HPLC system. An AB SCIEX Triple Quad 5500 MS/MS system was used instead of an AB SCIEX API 4000 Triple Quad MS/MS system.

The equipment substituted were demonstrated to be equivalent to the equipment specified in the method. The substitutions did not impact the analytical results.

3.7 Instrumentation

HPLC Conditions

System:	Shimadzu LC-30AD / Sil-30AC Autosampler				
Column:	Agilent XDB-C18, 50 × 4.6 mm, 1.8µm				
Column Temperature:	40°C				
Injection Volume:	30 µL				
Autosampler Temperature:	4°C				
Conditions:	A: 0.01 M Formic Acid in Water				
	B: 0.01 M Formic Acid in Methanol				
	Step	Time (min)	Event	Parameter	Flow (mL/min)
	1	0.00	B. Conc.	65%	0.5
	2	1.00	B. Conc.	65%	0.5
	3	10.0	B. Conc.	87.5%	0.5
	4	10.4	B. Conc.	65%	0.5
	5	14.0	B. Conc.	65%	0.5
Analyte Retention Time (minutes)					
DPX-JE874	~7.2				
IN-JS940	~3.2				
IN-KF015	~4.7				
IN-KZ007	~3.7				
IN-H3310	~5.2				

The detection method utilized was LC-ESI-MS/MS employing atmospheric pressure electrospray ionization interface in both negative and positive mode on a triple quadrupole instrument. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for famoxadone and its metabolites are shown in the tables below. Quantification transitions are listed in bold.

Negative Mode ESI

SYSTEM:		AB SCIEX 5500					
Compounds	**Parent Ion (m/z)**	**Product Ion (m/z)**	**Dwell Time (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
DPX-JE874	373.1	**282.1**	150	-65	-10	-28	-19
		133.0*				-22	-9
		329.1				-26	-9
IN-JS940	256.9	**211.0**	150	-50	-10	-15	-3
		93.0				-20	-3
IN-KF015	282.2	**195.0**	150	-55	-10	-24	-11
		239.2				-21	-11
IN-KZ007	389.1	**345.0**	150	-65	-10	-7	-9
		132.7				-30	-23
Ion Mode :		Negative					
Turbo Spray Voltage :		-4500V					
Source Temperatures :		650°C					
CUR :		15 psig					
CAD :		6 psig					
GS1 :		65 psig					
GS2 :		65 psig					

Positive Mode ESI

SYSTEM:		AB SCIEX 5500					
Compounds	**Parent Ion (m/z)**	**Product Ion (m/z)**	**Dwell Time (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
IN-H3310	213.2	**170.9**	150	65	10	21	10
		153.1*				27	8
		153.0				35	8
Ion Mode :		Positive					
Turbo Spray Voltage :		4500V					
Source Temperatures:		650°C					
CUR:		15 psig					
CAD:		6 psig					
GS1:		65 psig					
GS2:		65 psig					

*Confirmatory transition was used for soil analysis.

The instrument was operated in the ESI-MS/MS (MRM) negative ion mode for quantitative analysis of famoxadone and metabolites IN-JS940, IN-KF015, and IN-KZ007. The instrument was operated in the positive mode for the quantitative analysis of metabolite IN-H3310. The ion chromatograms were integrated and the peak areas were used for quantitation.

For each analytical run, a five-point standard curve was prepared by injecting constant volumes of mixed standard solutions composed of each analyte. Constant volume injections were also used for sample extracts.

3.8 Calculations

Residues of famoxadone and its metabolites were quantitated by external standards. A calibration curve for each analyte was generated by plotting the detector's response in peak area versus the concentration (ng/mL) of standard injected. The data system derived an equation for the fit of the standard curve with a weighted [$(1/x^2)$ where x = concentration] linear regression, and this equation was used to calculate the intercept and the slope of the linear regression curve.

The calibration curve was obtained by direct injection of 30 µL of standard into the LC-ESI-MS/MS for each analyte. The standard curve range for soil was 1.5 ng/mL to 25 ng/mL, and the standard curve range for water was 1.8 ng/mL to 30 ng/mL. In a given injection run, the same injection volume was used for all samples and standards.

Peak integration and quantitation were performed using Analyst software, version 1.6, from Applied Biosystems. Recovery results were calculated for each set of samples using Microsoft Excel software.

The equations used for quantitation are shown below:

$$R = \frac{C_{End} \times V_F \times A_F}{S \times C_{convert}}$$

where,

R represents the analyte residue in µg/kg or µg/L (ppb)

C_{End} represents the final concentration of analyte derived from the calibration curve (ng/mL)

$$C_{End} = \frac{Analyte\ Peak\ Area - Intercept}{Slope}$$

V_F represents the final volume (mL)

A_F represents the aliquot factor

$$A_F = \frac{Total\ Extraction\ Volume (V_{Total\ Ex})}{Aliquot\ Extraction\ Volume\ (V_{Aliq\ Ex})}$$

S represents the amount of soil (kg) or water (L) analyzed

$C_{convert}$ represents the conversion factor: 1000 ng/µg

$$Recovery(\%) = \frac{R}{R_{fortified}} \times 100\%$$

where,

$R_{fortified}$ represents the fortification level (µg/kg or µg/L [ppb])