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SYN545377 – Residue Method GRM073.01A for the Determination of the Photolysis Products IN-NXX69, IN-NXX70, IN-QKV54, IN-RNU71 in Surface Water. Final Determination by LC-MS/MS

Analytical Method

DATA REQUIREMENT(S): EPA 850.6100

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Abbreviations and Symbols

Abbreviation	Definition
^{o}C	degrees Celsius or Centigrade
aq	aqueous
CAS	Chemical Abstract Services
DMSO	dimethyl sulfoxide
EPA	Environmental Protection Agency (U.S.)
ESI	electrospray interface
g	gram
GRM	Global Residue Method
HPLC	high performance liquid chromatography
i.d.	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
L	litre
LC	Liquid chromatography
LC-MS/MS	tandem liquid chromatography/mass spectrometry/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
ACN	acetonitrile
M	molar
MeOH	methanol
mM	millimolar
µg	microgram
µL	microlitre
µm	micrometre
mg	milligram
mL	millilitre
mm	millimetre
min	minute
mol	mole
MRM	multiple reaction monitoring
ms	millisecond
MS/MS	tandem mass spectrometry/mass spectrometry
MW	molecular weight
m/z	mass to charge ratio

Abbreviations and Symbols (continued)

Abbreviation	Definition
N/A (or n/a)	not applicable
ND (or nd)	not detectable (below limit of detection)
ng	nanogram
No.	number
OCSPP	Office of Chemical Safety and Pesticide Pollution
OES	Occupational Exposure Standards
ppb	parts per billion or micrograms per kilogram or micrograms per litre
PTFE	polytetraflouroethylene
R (or r)	correlation coefficient
R^2 (or r^2)	coefficient of determination or square of correlation coefficient
RSD	relative standard deviation
RT	retention time
SD	standard deviation
SDS	safety data sheet
sec	second
SPE	solid phase extraction
UPLC	ultra performance liquid chromatography
V	volt
Vol (or vol)	volume

1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM073.01A is suitable for the determination of photolysis products IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 (Figures 1 through 4) in surface water. The limit of quantification (LOQ) of the method has been established at 0.10 µg/L (or 0.10 ppb).

This method satisfies US EPA guideline EPA OCSPP 850.6100.

1.2 Method Summary

IN-NXX69 is analysed separately from IN-NXX70, IN-QKV54, and IN-RNU71 in order to prevent the breakdown of IN-NXX69.

For IN-NXX69 analysis, water samples are acidified to approximately pH 4 prior to fortification. Acidified samples are passed through a 6-mL/500-mg ENV solid phase extraction (SPE) cartridge where IN-NXX69 is retained. The cartridge is washed with water prior to eluting the compound with 0.1% formic acid in acetonitrile. The column eluates are evaporated to dryness and then redissolved in equal portions of methanol and 0.02 M formic acid (aq) with sonication and vortex-mixing.

For IN-NXX70, IN-QKV54, and IN-RNU71 analysis, water samples are passed through a 6-mL/500-mg ENV solid phase extraction (SPE) cartridge where the analytes are retained. The cartridge is washed with water prior to eluting the compound with 0.02 M ammonium hydroxide in acetonitrile. The column eluates are evaporated to dryness and then redissolved in equal portions of methanol and 0.02 M formic acid (aq) with sonication and vortex-mixing.

All samples are analyzed by liquid chromatography/mass spectrometry (LC-MS/MS).

The limit of quantification of the method is 0.10 ppb (or 0.10 µg/L) for all compounds.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with mouth.
4. Wash any contaminated area immediately.

2.3.1 Stock Solutions

Prepare individual 100 µg/mL stock solutions for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 by one of the following methods.

Note: the amount weighed out must be corrected for the purity of the analytical standard as indicated on the certificate of analysis.

Weigh out accurately, using a five figure balance, sufficient IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 analytical standard (e.g. 0.01002 g for a 99.9% pure substance). Carefully transfer into separate "Class A" volumetric flasks (e.g. 100 mL size) using acetonitrile. For IN-NXX69 and IN-NXX70, dilute to the mark with acetonitrile and mix well to give individual 100 µg/mL stock solutions. IN-QKV54 and IN-RNU71 may require the addition of dimethyl sulfoxide to aid is dissolving the sompounds. Add as little dimethyl sulfoxide as possible (e.g. 5-10%) using "Class A" volumetric pipettes to the "Class A" volumetric flask before bringing to volume with acetonitrile. Mix well to give individual 100 µg/mL stock solutions. Note: Sonication may be required to fully dissolve some compounds. If sonication is required, allow the solutions to come to room temperature before bringing to volume.

Alternatively, the appropriate volume of acetonitrile or acetonitrile/dimethyl sulfoxide to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity.

$$V = \frac{W \times P}{C} \times 1000$$

P	=	Standard purity (P(%)/100)
V	=	Volume of acetonitrile or acetonitrile/dimethyl sulfoxide required
W	=	Weight, in mg, of the solid analytical standard
C	=	Desired concentration of the final solution, (µg/mL)
1000	=	Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.3.2 Fortification Solutions

Sample fortification solutions containing IN-NXX69, IN-NXX70, IN-QKV54, or IN-RNU71 should be prepared by serial dilution in acetonitrile. It is recommended that the following solutions are prepared: 1.0 µg/mL, 0.10 µg/mL and 0.010 µg/mL. Mixed standards of IN-NXX70, IN-QKV54, and IN-RNU71 may be prepared if desired. IN-NXX69 must be prepared separately to avoid breakdown during the analysis for IN-NXX70, IN-QKV54, and IN-RNU71 which may result in elevated recoveries due to conversion of IN-NXX69 to either IN-NXX70 or IN-QKV54.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

No significant matrix effects of the instrument response for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 were observed in the water types tested using the procedures described in Section 3 during method validation. Therefore non matrix-matched calibration standards should normally be used for quantification. However, during method validation IN-NXX70, IN-QKV54, and IN-RNU71 were analysed along with parent SYN545377 and other metabolites, and significat matrix effects were observed for some of the metabolites. Matrix-matched standards were used for all compounds, excluding IN-NXX69. A description of both non matrix-matched as well as matrix-matched standards is presented.

Non matrix-matched calibration standards for IN-NXX69 in surface water are prepared by first preparing a 10 ng/mL standard in methanol and 0.02 M formic acid (aq). This standard is serially diluted in methanol/0.02 M formic acid (aq) 50/50 v/v to achieve additional curve standards. Non-matrix standards for IN-NXX70, IN-QKV54, and IN-RNU71 are prepared in the same fashion, though IN-NXX69 must remain separate from the other analytes.

Mixed Sub-Stock Concentration (µg/mL)	**Initial Standard Volume (mL)**	**Methanol Volume (mL)**	**0.02 M Formic Acid (aq) Volume (mL)**	**Calibration Standard Concentration (ng/L)**
1.0	0.10	4.90	5.00	10
Initial Standard Concentration (ng/mL)	**Initial Standard Volume (mL)**	**Methanol/0.02 M Formic Acid (aq) 50/50 v/v Volume (mL)**		**Calibration Standard Concentration (ng/L)**
10	2.00	2.00		5.0
	1.50	3.50		3.0
	1.00	4.00		2.0
	0.50	4.50		1.0
	0.40	4.60		0.80
	0.30	4.70		0.60
	0.25	4.75		0.50

Matrix-matched calibration standards for IN-NXX70, IN-QKV54, and IN-RNU71 are prepared by taking six additional aliquots of control sample through the SPE clean-up and evaporation steps (step 3.3 r) and reconstituting the matrix in standard, methanol, and 0.02 M formic acid (aq) using sonication and vortex-mixing.

Mixed Sub-Stock Concentration (µg/mL)	Initial Standard Volume (mL)	Methanol Volume (mL)	0.02 M Formic Acid (aq) Volume (mL)	Calibration Standard Concentration (ng/L)
1.0	0.020	0.98	1.0	10
	0.010	0.99	1.0	5.0
0.10	0.040	0.96	1.0	2.0
	0.020	0.98	1.0	1.0
	0.015	0.985	1.0	0.75
	0.010	0.99	1.0	0.50

A calibration curve may be generated to quantify IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 residues. Standards over an appropriate concentration range should be prepared as described above, using the requisite volume of IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 standards in acetonitrile. Additional or different concentrations may also be prepared at the discretion of the study director.

Any matrix effects observed may be compensated for by use of matrix matched standards at the discretion of the study director, or by dilution of the final sample with methanol/0.02 M formic acid (aq) 50/50 v/v should instrument sensitivity permit.

2.3.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of 1 year for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 in acetonitrile or acetonitrile/dimethyl sulfoxide is recommended unless additional data are generated to support a longer expiration date. IN-NXX69 may require the addition of formic acid to ensure a pH of approximately 4 is maintained for extended periods of storage. Further testing is required.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as ‘Hazards in the Chemical Laboratory’, edited by S G Luxon, The Chemical Society, London (Reference 1).

Solvent and Reagent hazards

	Acetonitrile	Methanol	Formic Acid	Ammonium Hydroxide	Dimethyl Sulfoxide
Harmful Vapour	✓	✓	✓	✓	✓
Highly Flammable	✓	✓	✗	✗	✗
Harmful by Skin Absorption	✓	✓	✓	✓	✓
Irritant to respiratory system and eyes	✓	✓	✓	✓	✓
Causes severe burns	✗	✗	✓	✓	✗
OES Short Term (mg/m^3)	105	310	N/A	N/A	N/A
OES Long Term (mg/m^3)	70	260	9	N/A	5

Suitable personal protective equipment should be worn when handling chemicals and reagents. The appropriate SDS should be consulted for each reagent and a local risk assessment should be carried out. In all cases avoid breathing vapour. Avoid contact with eyes and skin.

3.0 ANALYTICAL PROCEDURE

A summary of the methods are included in flow-chart form in Appendices 4 and 5.

3.1 Sample Preparation

a) If water samples are received deep frozen they should be allowed to completely defrost. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to analysis.

3.2 Solid Phase Extraction Procedure for IN-NXX69

b) Transfer 10 mL of the water sample to be analysed into an appropriate polypropylene centrifuge tube (e.g. 50 mL size). Add concentrated formic acid dropwise to each sample to reach a pH of approximately 4. Mix the samples well.

c) Sample fortification, if required, is to be carried out at this time. At least one untreated control sample and two control samples fortified with a known amount of IN-NXX69 should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

d) Take one ENV cartridge (size 500 mg, 6 mL) for each sample to be analysed and place on a suitable vacuum manifold. Condition the cartrdiges with one colum volume (~6 mL) of methanol followed by one column volume (~6 mL) of 1 mM formic acid (aq), allowing the eluate to go to waste. Attach a suitable reservoir (e.g. 20-mL) to each ENV cartridge if desired, and add 5 mL of 1 mM formic acid (aq) allowing the eluate to go to waste. Do not allow the cartridges to become dry.

e) Load the water samples onto the SPE cartridges allowing the samples to pull through under gravity flow. Residues of IN-NXX69 are retained on the cartridge.

f) On completion of loading, wash the reservoir and ENV with 5 mL of water. Allow to percolate through under gravity flow, again discarding the column eluate.

g) Remove the column reservoir and column connector from the SPE cartridge if used. Apply a high vacuum for approximately 20 - 30 seconds to remove excess water from the cartridges.

h) Place suitable collection tubes (e.g. 40 mL glass test tubes) under each port, as required, in the manifold rack. Elute the cartridges with 2 x 5 mL (or 10 mL total) of 0.1% formic acid in acetonitrile, under gravity flow collecting the column eluate. Vacuum may be applied for 5 seconds to collect the excess solvent from the SPE cartridges. IN-NXX69 is eluted in this step.

i) Evaporate the samples to dryness under a stream of nitrogen in an N-Evap with a heated water bath (recommended temperature around 40 °C).

j) Reconstitute the extracts in 0.50 mL methanol. Mix the samples thoroughly by vortex-mixing for approximately 20 seconds followed by ultrsonication for approximately 2 min. Add 0.50 mL 0.02 M formic acid (aq). Mix the samples thoroughly by vortex-mixing for approximately 20 seconds followed by ultrsonication for approximately 2 min.

k) Filter the samples through 0.2-μm PTFE filter. Transfer an aliquot to a suitable autosampler vial ready for final determination by LC-MS/MS. The final sample concentration is 10 mL/mL (or 0.01 L/mL). Solid Phase

3.3 Extraction Procedure for IN-NXX70, IN-QKV54, and IN-RNU71

l) Transfer 20 mL of the water sample to be analysed into an appropriate polypropylene centrifuge tube (e.g. 50 mL size). Sample fortification, if required, is to be carried out at this time. At least one untreated control sample and two control samples fortified with a known amount of IN-NXX70, IN-QKV54, and IN-RNU71 should be analysed alongside each batch of samples to demonstrate acceptable performance of the method and allow recovery corrections to be made if desired.

m) Take one ENV cartridge (size 500 mg, 6 mL) for each sample to be analysed and place on a suitable vacuum manifold. Condition the cartrdiges with one colum volume (~6 mL) of methanol followed by one column volume (~6 mL) of 1 mM formic acid (aq), allowing the eluate to go to waste. Attach a suitable reservoir (e.g. 30-mL) to each ENV cartridge if desired, and add 5 mL of 1 mM formic acid (aq) allowing the eluate to go to waste. Do not allow the cartridges to become dry.

n) Load the water samples onto the SPE cartridges allowing the samples to pull through under gravity flow. Residues of IN-NXX70, IN-QKV54, and IN-RNU71 are retained on the cartridge.

o) On completion of loading, wash the reservoir and ENV with 5 mL of water. Allow to percolate through under gravity flow, again discarding the column eluate.

p) Remove the column reservoir and column connector from the SPE cartridge if used. Apply a high vacuum for approximately 20 - 30 seconds to remove excess water from the cartridges.

q) Place suitable collection tubes (e.g. 50 mL glass test tubes) under each port, as required, in the manifold rack. Elute the cartridges with 3 x 5 mL (or 15 mL total) of 0.02 M ammoinium hydroxide in acetonitrile, under gravity flow collecting the column eluate. Vacuum may be applied for 5 seconds to collect the excess solvent from the SPE cartridges. IN-NXX70, IN-QKV54, and IN-RNU71 are eluted in this step.

r) Evaporate the samples to dryness under a stream of nitrogen in an N-Evap with a heated water bath (recommended temperature around 40 °C).

s) Reconstitute the extracts in 1.0 mL methanol. Mix the samples thoroughly by vortex-mixing for approximately 20 seconds followed by ultrsonication for approximately 2 min. Add 1.0 mL 0.02 M formic acid (aq). Mix the samples thoroughly by vortex-mixing for approximately 20 seconds followed by ultrsonication for approximately 2 min.

t) Filter the samples through 0.2-µm PTFE filter. Transfer an aliquot to a suitable autosampler vial ready for final determination by LC-MS/MS. The final sample concentration is 10 mL/mL (or 0.01 L/mL).

3.4 Experimental Precautions

a) The SPE procedure has been developed using cartridges from the stated manufacturer. Similar cartridges from other manufacturers may be used. In all cases however, it is strongly recommended that the elution profile of the chosen batch of cartridges is checked prior to commencing analysis to assess any variation in manufacturers' products and between batches.

b) Bottled HPLC grade ultra pure water is used to prepare the LC mobile phase, which produces a lower background noise in the MS/MS chromatograms than water taken from a laboratory water purification system.

c) It is critical that the pH of samples for IN-NXX69 analysis be adjusted to approximately 4 prior to fortification in order to stabilize IN-NXX69 through the method. The fortified samples will demonstrate that IN-NXX69 can be successfully recovered through the method for residue determination in treated samples that may not have had an initial pH of 4.

d) If dilutions are required for the analysis of IN-NXX70, IN-QKV54, or IN-RNU71, additional control aliquots may be required to take through the SPE procedures. Diltuions should be made in control matrix when quantitating against a matrix curve.

3.5 Time Required for Analysis

The methodology is normally performed with a batch of 15 samples. One person can complete the analysis of 15 samples in 2 days (8 hour working period), approximately 1 day for each SPE procedure.

3.6 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any work flow interruptions. Samples should be stored frozen in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an Applied Biosystems/Sciex API5000 UPLC system. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description

Pump	:	Waters Acquity Binary Solvent Manager UPB
Column Oven	:	Waters Acquity Column Manager UPM
Detector	:	Applied Biosystems/Sciex API 5000 triple quadrupole mass spectrometer with Analyst™ software version 1.5.1
Autosampler	:	Waters Acquity Sample Organizer
Gas Supply	:	House Nitrogen

4.2 Chromatography Conditions

Column	:	Phenomenex Synergi Polar RP, 2.5 µm, 50 mm x 3.0 mm i.d.
Column Oven Temperature	:	40 °C
Injection volume	:	10 µL
Stop Time	:	10 minutes
Injection protocol	:	Analyse calibration standard after up to 5 sample injections
Mobile phase	:	solvent 1 = 0.1% formic acid (aq) solvent 2 = 0.1% formic acid in methanol

Mobile Phase Composition

Time (min)	% Solvent 1	% Solvent 2	Flow, mL/min
0.00	50	50	0.50
3.00	30	70	0.50
6.00	30	70	0.50
7.00	5	95	0.50
8.00	5	95	0.50
8.10	50	50	0.50
10.00	50	50	0.50

Typically, IN-NXX69 is injected in a separate run from IN-NXX70, IN-QKV54, and IN-RNU71, particularly when matrix standards are used for the latter three compounds. The same instrument conditions though are used for all compounds.

Under these conditions the retention time for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 is approximately 3.49 minutes, 3.97 minutes, 4.17 minutes, and 2.49 minutes, respectively.

4.3 Mass Spectrometer Conditions for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71

Interface	:	Turbo Ion Spray (ESI)
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 15 (arbitrary units)
Temperature (TEM)	:	600 °C
Ionspray voltage	:	5500 V
Collision gas setting (CAD)	:	Nitrogen set at 10 (arbitrary units)
Gas 1 (GS1)	:	Air set at 60 (arbitrary units)
Gas 2 (GS2)	:	Air set at 40 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**IN-NXX69 primary transition**	**IN-NXX69 confirmatory transition**	**IN-NXX70 primary transition**	**IN-NXX70 confirmatory transition**	**IN-QKV54 primary transition**	**IN-QKV54 confirmatory transition**	**IN-RNU71 primary transition**	**IN-RNU71 confirmatory transition**
Q1 *m/z*	:	437.0	437.0	437.0	439.0	344.0	344.0	437.0	437.0
Q3 *m/z*	:	406.1	343.9	344.0	346.0	236.0	186.0	406.0	300.0
Scheduled MRM time (min)[1]	:	3.40	3.40	3.90	3.90	4.10	4.10	2.40	2.40
Resolution Q1	:	Unit	Unit	Unit	Unit	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	70 V	70 V	110 V	110 V	114 V	114 V	150 V	150 V
Entrance potential (EP)	:	10 V	10 V	10 V	10 V	10 V	10 V	10 V	10 V
Collision energy (CE)	:	50 V	50 V	45 V	45 V	44 V	49 V	38 V	52 V
Collision cell exit potential (CXP)	:	30 V	30 V	23 V	23 V	15 V	15 V	14 V	14 V

[1] Window of ±45 seconds with a target scan time of 1.0000 second

Typical chromatograms are shown in the Figures Section.

4.4 Confirmatory Procedures for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 residues may be calculated in µg/L for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (50% LOQ to at least 10xLOQ was used in method validation). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of five injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package. 1/x weighting was used for the method validation.

d) The following equation can be rearranged and used to calculate residues, expressed as ng/mL, as follows:

$$y = mx + b$$

Where y is the instrument response value, x is the standard or sample concentration in ng/mL, m is the gradient of the line of best fit ("X-variable 1" in MS Excel) and b is the intercept value. An example of this equation generated using the experimental values of m and b should be included in the raw data, as should the "R" or "R-Squared" value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - b}{m}$$

e) Calculate the IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 residues in the sample, expressed as ppb, as follows:

$$\text{Residue (ppb)} = \frac{\text{Sample ng/mL Found x Final Sample Volume (mL) x Dilution Factor}}{\text{Initial Sample Volume (mL)}}$$

f) Alternatively residues in the sample, expressed as µg/L, may be calculated as follows:

$$\text{Residue}\ (\mu g/L) = \frac{\text{Analyte found}\ (\mu g/mL)}{\text{Sample conc.}\ (L/mL)}$$

Where analyte found is calculated from the standard calibration curve (ng/mL calculated is converted to µg/mL by the conversion factor 1 µg/1000 ng) and sample conc. is the final sample concentration in L/mL, accounting for any concentration in the SPE step where used.

If residues need to be corrected for average percentage recovery, e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}}\ (\text{ppb or } \mu g/L)$$

5.2 Single Point Calibration Procedure

IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 residues may be calculated in µg/L for each sample using a mean standard response from each of the injections bracketing the sample as follows.

a) Make repeated injections of a standard containing IN-NXX69, IN-NXX70, IN-QKV54, or IN-RNU71 at an appropriate concentration into the LC-MS/MS operated under conditions as described in Section 4. When a consistent response is obtained, measure the peak areas obtained for IN-NXX69, IN-NXX70, IN-QKV54, or IN-RNU71.

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to IN-NXX69, IN-NXX70, IN-QKV54, or IN-RNU71.

c) Re-inject the standard solution after a maximum of four injections of sample solutions.

d) Calculate the IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 residues in the sample, expressed as µg/L using a mean standard response from each of the injections bracketing the sample as follows.

$$\text{Residue}\ (\mu\text{g/L}) = \frac{\text{PK area (SA)}}{\text{PK area (STD)}} \times \frac{\text{Standard Conc.}}{\text{Sample Conc.}}$$

Peak response for sample

Average peak response for bracketing standards

Concentration of standard (µg/mL)

Sample concentration (L/mL)

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}}\ (\mu\text{g/L})$$

Although single point calibration may be used to quantify residues it is recommended that a calibration curve is generated with each analytical run to demonstrate the linearity of instrument response (Reference 4).

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analysed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analysed with each batch of samples.

Recovery data must be generated to verify method performance and allow recovery corrections to be made (if appropriate). Fortified control samples should be included with each sample set therefore.

At least two recovery samples (control samples accurately fortified with known amounts of IN-NXX69, IN-NXX70, IN-QKV54, and/or IN-RNU71 in acetonitrile) should also be analysed alongside each set of samples. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of $\leq$20%.

Where the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

LC-MS/MS is a highly specific detection technique. Interference arising from the matrices tested has not been observed.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware Interference

This method uses mainly disposable labware. All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

8.0 METHOD VALIDATION

8.3 Limit of Quantification (LOQ)

The limit of quantification of the method is defined as the lowest analyte concentration in a sample at which the methodology has been validated and a mean recovery of 70-110% with a relative standard deviation of ≤ 20% has been obtained. Generally, for accurate quantification, the response for an analyte peak should be no lower than four times the mean amplitude of the background noise in an untreated sample at the corresponding retention time.

The limit of quantification has been set at 0.10 ppb (0.10 μg/L).

8.4 Limit of Detection (LOD)

The limit of detection of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. An estimate of the LOD can be taken as three times background noise. Note that the LOD may vary between runs and from instrument to instrument.

The limit of detection for this procedure in the water type tested during method validation was estimated at 0.03 ppb for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71, using the primary and confirmatory transitions, based on 30% of the LOQ.

8.5 Matrix Effects

Specific matrix effects testing was not performed to determine the magnitude of any possible matrix effects. The suppressive effects of the surface water matrix were observed during method validation for a few metabolites by the increased recoveries at higher fortification levels when diluted in solvent. This was further confirmed by the acceptable recoveries obtained when quantitated against a matrix-matched curve.

IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 did not demonstrate this matrix suppression. IN-NXX70, IN-QKV54, and IN-RNU71 were quantitated using matrix-matched standards for ease during the analysis since they were analysed along with 8 other compounds.

Matrix matched standards may be used to compensate for any significant effects, at the discretion of the study director. Alternatively, where instrument sensitivity permits, samples may be further diluted in 50:50 MeOH:0.02 M formic acid (aq) to reduce or eliminate these effects.

The method includes procedures to reduce matrix effects as far as practically possible, however matrix effects in some matrices may be still observed. In these instances matrix matched standards may be used to compensate for the matrix effects at the discretion of the study director.

8.6 Detector Linearity

For accurate quantification of residue concentrations, analyses should be carried out within the linear range of the detector. For multi point calibration, detector range and linearity will be demonstrated within each sample set.

The linearity of the LC-MS/MS detector response for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 was tested in the range from 0.0050 ng to 0.10 ng injected on column (equivalent to 0.50 ng/mL to 10 ng/mL standards when using a 10 µL injection volume) and was found to be linear. This is equivalent to 50% of the LOQ to 10 x LOQ.

If a residue beyond the tested concentration range is expected, dilute the sample appropriately to bring it within the tested linear range prior to quantification.

Standards of different concentration levels were injected and the response plotted against the concentration (ng/mL) injected, using Analyst, for both primary and confirmatory transitions for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71.

Detector linearity graphs are presented in Figures 45 - 52.

8.7 Extract Stability

Extract stability was not assessed during this method validation and should be assessed with concurrent recovery data if samples are stored for extended periods of time.

9.0 LIMITATIONS

The method has only been tested on surface water. It can reasonably be assumed that the method can be applied to other water matrices not tested in this method provided successful recovery tests at the relevant levels validate the suitability of the method.

10.0 CONCLUSIONS

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 residues in surface water. Only commercially available laboratory equipment and reagents are required. The analysis of approximately 15 water samples for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 can be completed by one person in 2 days (8 hour working period), approximately 1 day for each SPE procedure). Untreated and fortified samples should be analysed with each set of samples to demonstrate absence of any interference and adequate recovery, if possible. The limit of quantification of the method is 0.10 ppb (0.10 µg/L).

This method satisfies US EPA guideline EPA OCSPP 850.6100.

TABLE 1 Characterisation Data of Water Samples used for Method Validation.

Matrix	Source	pH	Calcium (ppm)	Magnesium (ppm)	Sodium (ppm)	Hardness As CaCO3 mg/L	Conductivity (mmhos/cm)	Sodium Adsorption Ration (SAR)	Total Dissolved Solids (ppm)	Turbidity (NTU)	Alkalinity As CaCO3 mg/L	Dissolved Oxygen (mg/L0
Surface Water	Rice Paddy Water from Rapides Parish, Louisiana	7.8	89	37	26	379	0.75	0.59	450	0.66	350	5.2

CHEMICAL STRUCTURES

FIGURE 1 IN-NXX69

IUPAC Name : None given
Molecular Formula : $C_{19}H_{13}BrN_6O_2$
Molecular Weight : 437.25

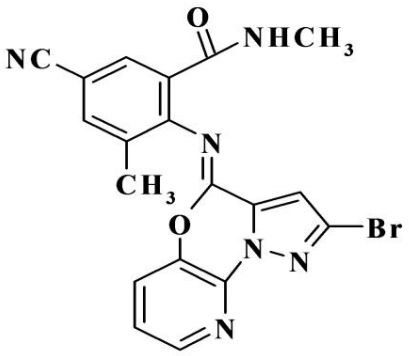


FIGURE 2 IN-NXX70

IUPAC Name : 2-[3-Bromo-1-(3-hydroxypyridin-2-yl)-1H-pyrazol-5-yl]-3,8-dimethyl-4-oxo-3,4-dihydroquinazoline-6-carbonitrile
Molecular Formula : $C_{19}H_{13}BrN_6O_2$
Molecular Weight : 437.26

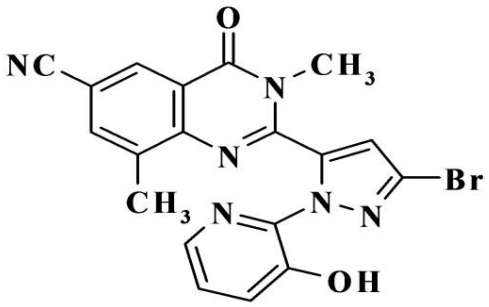


FIGURE 3 IN-QKV54

IUPAC Name : 2-(5-Bromo-1H-pyrazol-3-yl)-3,4-dihydro-3,8-dimethyl-4-oxo-6-quinazolinecarbonitrile

Molecular Formula : $C_{14}H_{10}BrN_5O$

Molecular Weight : 344.17

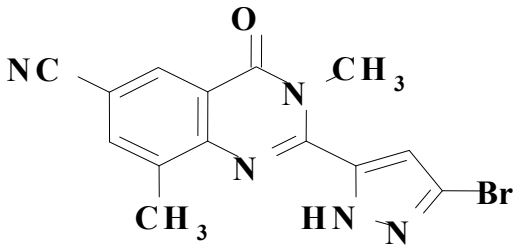
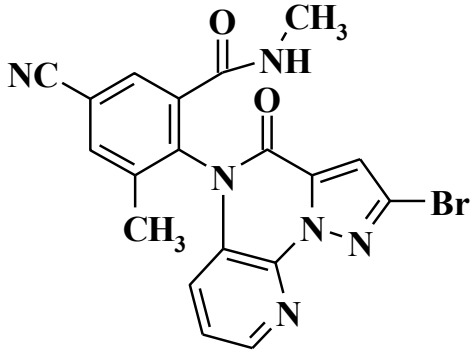


FIGURE 4 IN-RNU71

IUPAC Name : 2-(2-Bromo-4-oxopyrazolo[1,5-a]pyrido[3,2-e]pyrazin-5(4H)-yl)-5-cyano-N,3-dimethylbenzamide

Molecular Formula : $C_{19}H_{13}BrN_6O_2$

Molecular Weight : 437.26



APPENDIX 1 Apparatus

Recommended Suppliers

Equipment	Description	Supplier
25-mL Serological pipettes	Part no. 13-678-36D	https://www.fishersci.com/us/en/home.html
50-mL polypropylene centrifuge tubes	Part no. 14-959-49A	https://www.fishersci.com/us/en/home.html
250-µL Microman® positive displacement pipettor	Model no. M250 Part no. F148505	http://www.gilson.com/
Maxi-Mix I vortex mixer	Thermo Scientific Model no. M16715 Part no.12-815-50	https://www.fishersci.com/us/en/home.html
Visiprep™ SPE vacuum manifold	Supelco 24-port model Part no. 57250-U	http://www.sigmaaldrich.com/united-states.html
Bond Elut ENV cartridges (6 mL/500 mg)	Part no. 12255011	http://www.agilent.com/home
30-mL syringe with slip tip (used as reservoir)	Part no. 14-823-16G	https://www.fishersci.com/us/en/home.html
5-mL Serological pipettes	Part no. 13-678-25D	https://www.fishersci.com/us/en/home.html
Vacuum pump	Gast Model no. DOA=P704-AA Part no. EW-07061-40	http://www.coleparmer.com/
50-mL glass centrifuge tubes	Pat no. 0553841A	https://www.fishersci.com/us/en/home.html
N-Evap™	Model no. 112	https://www.organomation.com/
1000-µL Microman® positive displacement pipettor	Model no. M250 Part no. F148506	http://www.gilson.com/
3-mL disposable syringes with luer-lok™ tips	Part no. 14-823-435	https://www.fishersci.com/us/en/home.html
4 mm 0.2 µm PTFE syringe filters	Part no. 09-911-5	https://www.fishersci.com/us/en/home.html
13 mm 0.2 µm PTFE syringe filters	Part no. 9445601	http://cobertassociates.com/
National™ Target™ Snap Cap Wide-Opening Vials	Part no. 03-395C	https://www.fishersci.com/us/en/home.html
National™ Target™ Kim-Snap™ Closures	Part no. 03-396S	https://www.fishersci.com/us/en/home.html
LC-MS/MS system	AB Sciex API 5000	http://sciex.com/
Synergi Polar RP, 2.5 µm; 50 mm x 3.0 mm	Part no. 00B-4371-Y0	http://phenomenex.com/
Binary Pump	Waters Acquity Binary Solvent Manager UPB	http://www.waters.com/waters/home.htm?locale=en_US
Autosampler	Waters Acquity Sample Organizer	http://www.waters.com/waters/home.htm?locale=en_US
Colum Oven	Waters Acquity Column Manager UPM	http://www.waters.com/waters/home.htm?locale=en_US

APPENDIX 2 Reagents

Recommended Suppliers

Reagent	Description	Supplier
Acetonitrile	Optima® grade	https://www.fishersci.com/us/en/home.html
Dimethyl Sulfoxide	ACS Reagent Grade	https://www.fishersci.com/us/en/home.html
Methanol	Optima® grade	https://www.fishersci.com/us/en/home.html
Ultra pure water	HPLC grade	https://www.fishersci.com/us/en/home.html
Formic Acid	98+%	https://www.fishersci.com/us/en/home.html
Ammonium Hydroxide	28%; ACS?	https://www.fishersci.com/us/en/home.html
IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 analytical standards	GLP certified	IN-NXX69 supplied and characterized by ABC Laboratories, Inc. 7200 E. ABC Lane, Columbia, MO, 65202. IN-NXX70, IN-QKV54, and IN-RNU71 supplied and characterized by E. I. du Pont de Nemours and Company, DuPont Crop Protection, Global Technology Division 1090 Elkton Road, Stine-Haskell Research Center, Newark, Delaware 19714

Preparation of Reagents

a) 1 M Formic Acid (aq) was typically prepared by adding 45 mL of formic acid (98+%) to approximately 800 mL of ultra pure water and thoroughly mixing. The solution was then brought to 1000 mL with ultra pure water and mixed well. Alternatively, the 45 mL of formic acid can be added directly to 955 mL of ultra pure water and mixed well.

b) 1 mM Formic Acid (aq) was typically prepared by adding 1.0 mL of 1 M formic acid (aq) to 1000 mL of ultra pure water and mixing well.

c) 0.02 M Formic Acid (aq) was typically prepared by diluting 20 mL of 1 M formic acid (aq) to 1000 mL with ultra pure water and mixing well.

d) 1 M Ammonium Hydroxide (aq) was typically prepared by adding 70 mL of ammonium hydroxide (28%) to 930 mL of ultra pure water and mixing well.

e) 0.02 M Ammonium Hydroxide in ACN was typically prepared by diluting 20 mL of 1 M ammonium hydroxide (aq) to 1000 mL with acetonitrile and mixing well.

f) 0.1% Formic Acid in ACN was typically prepared by adding 1.0 mL of formic acid (98+%) to 1000 mL of acetonitrile and mixing well.

g) 50:50 MeOH:0.02 M Formic Acid (v:v) was typically prepared by adding 500 mL of methanol to 500 mL of 0.02 M formic acid (aq) and mixing well.

h) Mobile phase solvent 1 (0.1% formic acid (aq)) was typically prepared by adding 20 mL of formic acid (98+%) to 20 L of HPLC-grade water and mixing well.

i) Mobile phase solvent 2 (0.1% formic acid in methanol) was typically prepared by adding 20 mL of formic acid (98+%) to 20 L of methanol and mixing well.

APPENDIX 3 LC-MS/MS Tuning Procedure

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polypropylene glycol (PPG) solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71

Infuse standard solutions of IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71 (0.1 to 1.0 μg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 10-20 μL/min. Roughly adjust interface parameters (sprayer position, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high parent ion signal at *m/z* 437.0 for IN-NXX69, *m/z* 437.0 for IN-NXX70, *m/z* 344.0 for IN-QKV54, and *m/z* 437.0 for IN-RNU71 in positive ionisation mode.

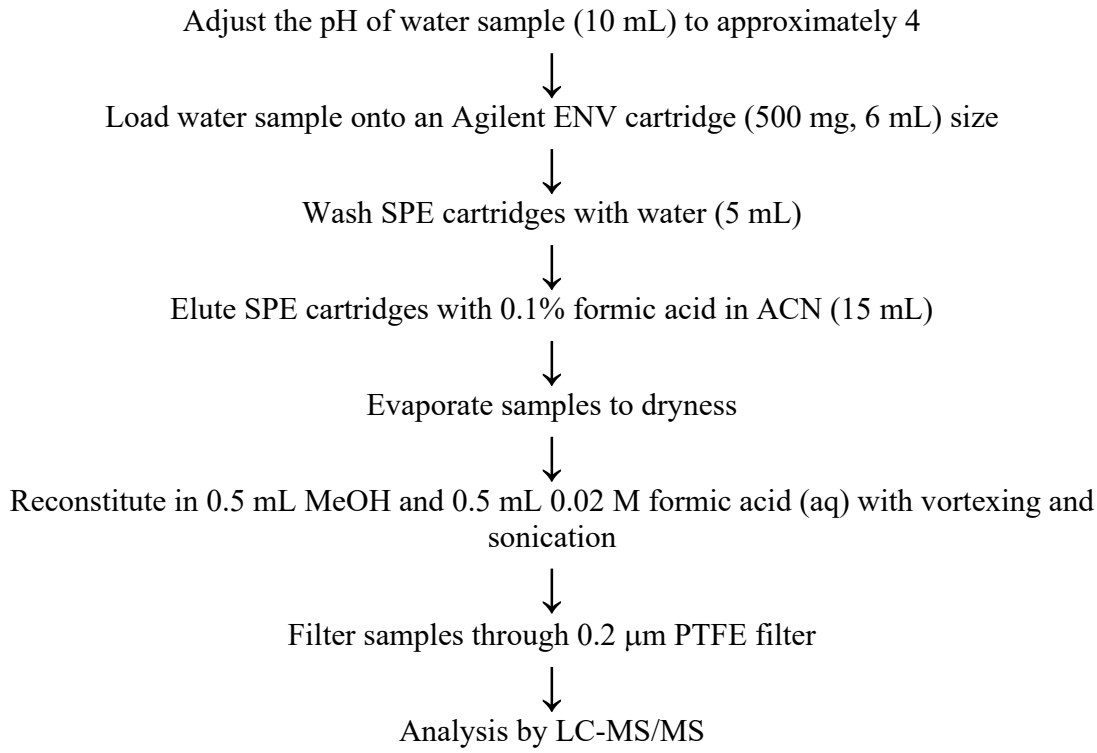
Using the Analyst software quantitative optimisation routine, tune the instrument for IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71, ensuring that the correct ion is selected. If desired, manual tuning of the ion optics and collision energy can be carried out to ensure maximum sensitivity.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection of a IN-NXX69, IN-NXX70, IN-QKV54, and IN-RNU71standard using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity.

The parent ion is subjected to further fragmentation by collisional activation. The two most sensitive daughter ions are then selected and used for quantitative analysis.

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

APPENDIX 4 Method Flow Chart for IN-NXX69



APPENDIX 5 Method Flow Chart for IN-NXX70, IN-QKV54, and IN-RNU71

Load water sample (20 mL) onto an Agilent ENV cartridge (500 mg, 6 mL) size

↓

Wash SPE cartridges with water (5 mL)

↓

Elute SPE cartridges with 0.02 M ammonium hydroxide in ACN (15 mL)

↓

Evaporate samples to dryness

↓

Reconstitute in 1.0 mL MeOH and 1.0 mL 0.02 M formic acid (aq) with vortexing and sonication

↓

Filter samples through 0.2 µm PTFE filter

↓

Analysis by LC-MS/MS