APPENDIX A – ANALYTICAL METHOD

Golden Pacific LABORATORIES

Analytical Procedure for the Determination of Fluazinam and Five Metabolites (AMPA, DAPA, CAPA, DCPA, HYPA) in Water

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1.0 INTRODUCTION

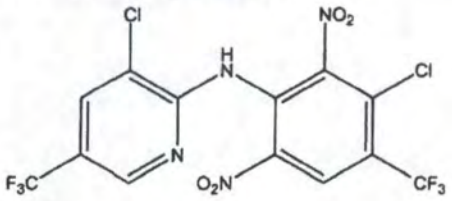
Fluazinam is a broad spectrum fungicide. An analytical procedure is required for the determination of residues of Fluazinam and five metabolites in water. This method is concerned with the measurement of Fluazinam, AMPA, DAPA, CAPA, DCPA and HYPA residues in water.

The target limit of quantitation (LOQ) for each analyte in water is 0.1 ng/mL.

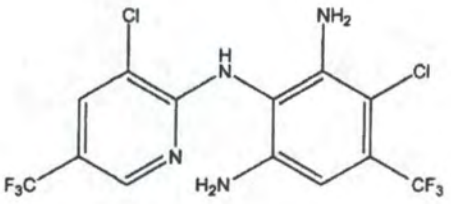
2.0 REFERENCE SUBSTANCES

Ishihara Sangyo Kaisha, Ltd. provided the following reference substances from their repository at MRI Global, Kansas City, Missouri: Fluazinam Technical (IKF-1216), DAPA, AMPA, CAPA, DCPA, and HYPA. The reference substances are used to prepare calibration and fortification solutions, and to determine procedural recoveries.

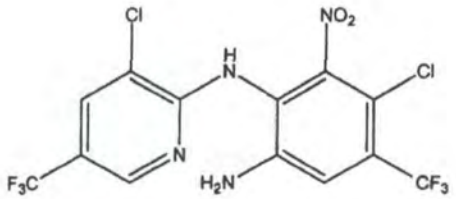
2.1 REFERENCE SUBSTANCES



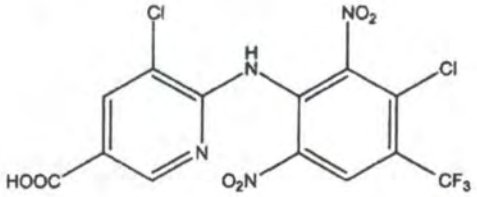
Common Name: Fluazinam Technical (IKF-1216)
IUPAC Name: 3-Chloro-*N*-(3-chloro-5-trifluoromethyl-2-pyridyl)-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine
CAS No.: 79622-59-6



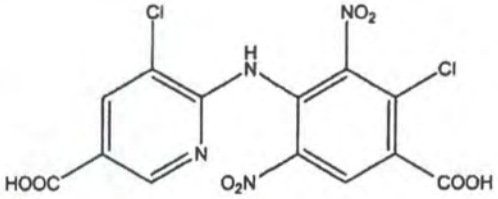
Common Name: DAPA
IUPAC Name: 3-chloro-2-(2,6-diamino-3-chloro-α,α,α-trifluoro-*p*-toluidino)-5-(trifluoromethyl)pyridine
CAS No.: NA



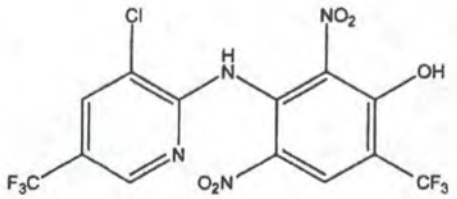
Common Name: AMPA
IUPAC Name: 2-(6-amino-3-chloro-α,α,α,-trifluoro-2-nitro-*p*-toluidino)-3-chloro-5-(trifluoromethyl)pyridine
CAS No.: NA



Common Name: CAPA
IUPAC Name: 5-chloro-6-(3-chloro-alpha,alpha,alpha-trifluoro-2,6-dinitro-*p*-toluidino)-nicotinic acid
CAS No.: NA



Common Name: DCPA
IUPAC Name: 6-(4-carboxy-3-chloro-2,6-dinitroanilino)-5-chloronicotinic acid
CAS No.: NA



Common Name: HYPA
IUPAC Name: 5-[[3-chloro-5-(trifluoromethyl)-2-pyridyl]amino]-alpha, alpha, alpha-trifluoro-4,6-dinitro-o-cresol
CAS No.: NA

All reference substances were stored frozen (< -10 °C). A copy of the certificate of analysis of the reference substances will be kept in the archives at GPL.

3.0 PRINCIPLE OF THE METHOD

An aliquot of a representative water sample is combined with an aliquot of 0.2% formic acid in acetonitrile. It is analyzed for Fluazinam, DAPA, AMPA, CAPA, DCPA and HYPA residues using LC-MS/MS.

Six concentrations of the combined reference substances in 10% acetonitrile: 90% water: 0.02% formic acid are used for standards. A calibration plot is drawn for each analyte and used for quantitation purposes.

4.0 EQUIPMENT

Unless otherwise indicated, the equipment listed below may be substituted with functionally equivalent equipment.

- Balance, Analytical: Mettler model AB 204-S, precision ± 0.1-mg, for standards
- Disposable Pasteur pipettes, glass
- Jar, amber glass: with Teflon lined cap, 30 mL, 60 mL, 120 mL
- Test tube with Teflon lined cap, glass: 8 and 16 mL
- Wiretrol pipette: 100 µL
- Volumetric flask, glass: 25 and 50 mL
- Volumetric pipette: various sizes
- HPLC vials, clear glass: 1.8 mL
- Glass syringes, 5 mL
- Pall Acrodisc Syringe Filters (1 µm) with Glass Fiber 25 mm
- AB Sciex API5000 LC-MS/MS with Shimadzu LC-20AD XR HPLC pumps, cbm-20A controller, SIL-20AC XR autosampler and Analyst Software ver. 1.5.2

5.0 CHEMICALS/REAGENTS

Alternate suppliers of reagents having comparable specifications may be used.

- Acetonitrile, Optima Grade, Fisher #A996-4
- Water, HPLC Grade, VWR# MK679510
- Formic Acid, 88%, Fisher #A118P-500

6.0 STANDARD SOLUTIONS

The reference substances are used in the preparation of the fortification and calibration solutions.

6.1 Fluazinam, DAPA, AMPA, CAPA, DCPA and HYPA Fortification Solutions

The Fluazinam, DAPA and AMPA reference substances are individually weighed directly into three 50-mL volumetric flasks and are made up to volume with acetone to give three solutions with concentrations of approximately 1.0 mg/mL Fluazinam (Solution A), 1.0 mg/mL DAPA (Solution B) and 1.0 mg/mL AMPA (Solution C) respectively after being corrected for purity.

The CAPA, DCPA and HYPA reference substances are individually weighed directly into three 25-mL volumetric flasks and are made up to volume with acetonitrile to give three solutions with concentrations of approximately 1.0 mg/mL CAPA (Solution D), 1.0 mg/mL DCPA (Solution E) and 1.0 mg/mL HYPA (Solution F) respectively after being corrected for purity.

Aliquots of Solutions A through F (0.1 mL) are combined and diluted with acetonitrile to a final volume of 100 mL to prepare a fortification solution of approximately 1.0 µg/mL Fluazinam, DAPA, AMPA, CAPA, DCPA and HYPA (Solution G). A 100 ng/mL fortification solution of the six analytes (Solution H) is prepared by taking a 5.0-mL aliquot of Solution G (1.0 µg/mL) and diluting it with acetonitrile to a final volume of 50 mL.

The fortification solutions are stored in a freezer set to maintain < -10 °C (frozen) in amber bottles and are renewed every 3 months or as needed.

6.2 Calibration Solutions

An aliquot (5.0 mL) of Solution H (100 ng/mL) is diluted to a final volume of 50 mL to prepare an intermediate solution containing 10 ng/mL Fluazinam, DAPA, AMPA, CAPA, DCPA and HYPA (Solution I). Solution I will be used to make calibration standards at appropriate concentrations. Calibration standards are diluted with 10% acetonitrile: 90% water: 0.02% formic acid. Typical concentrations are 2.0 ng/mL, 1.0 ng/mL, 0.5 ng/mL 0.2 ng/mL, 0.09 ng/mL, and 0.05 ng/mL.

All calibration solutions are stored frozen in amber bottles. Calibration solutions are prepared every three months or as required.

7.0 ANALYTICAL PROCEDURE

A 100-mL sample of water is measured into a 250-mL amber glass bottle and is fortified at the appropriate concentration.

Note: Samples should not be collected or analyzed in plastic bottles because more than one analyte of interest may adhere to the plastic. (More specifically: Fluazinam recovery loss of approximately 40%, and AMPA recovery loss of approximately 20% when fortified at approximately 1 ng/mL.)

The targeted levels for each analyte are as follows:

	Fortification Levels
LOQ	0.100 ng/mL
10 x LOQ	1.00 ng/mL

Laboratory fortifications are prepared using a syringe or Wiretrol pipette. Each sample is hand shaken for approximately 30 seconds to ensure homogeneity.

A 9-mL aliquot of the sample is combined with a 1-mL aliquot of 0.2% formic acid in acetonitrile in a glass tube. The sample is hand shaken for approximately 30 seconds to ensure homogeneity.

Note: If necessary, filter an aliquot of the sample (post acetonitrile addition) through a glass fiber syringe filter using a glass syringe to remove particulates. Glass fiber syringe filters with a 1-µm pore size have been confirmed to work. Additionally, filter QC samples to provide comparison data for analyte hold-up in the filter.

An aliquot is transferred to a chromatography vial and analyzed by LC-MS/MS. Samples having higher residue levels are diluted to an appropriate final volume using 10% acetonitrile: 90% water: 0.02% formic acid so that the response falls within the calibration range of the standards.

8.0 QUANTITATION

8.1 Instrumentation

An AB Sciex API5000 LC-MS/MS system is used as described in Section 4.

8.2 HPLC Conditions

Analytical Column: Phenomenex, Synergi Polar- RP 4 µ 80A
50 x 2.00 mm

Column Temperature: Ambient

Mobile Phase: Gradient:
A% = 0.1% formic acid in acetonitrile B% = 0.1% formic acid in water

Time (min)	A (%)	B (%)
0.0	10.0	90.0
4.0	80.0	20.0
5.0	80.0	20.0
5.5	10.0	90.0
6.5	10.0	90.0

Flow Rate: 500 µL/min
Injector: autosampler
Injection Volume: 20 µL

Approximate Retention Times:

DCPA	~2.4 min
HYPA	~3.4 min
CAPA	~3.6 min
DAPA	~3.8 min
AMPA	~4.0 min
Fluazinam	~4.3 min

8.3 Mass Spectrometer Parameters

Interface: Turbo Spray (ESI)
Polarity: Positive
Scan Type: Scheduled MRM Monitoring with Low/Unit resolution
Curtain Gas (CUR): 30.0
(GS1): 40.0
(GS2): 40.0
Ionspray Voltage (IS): 5500
Temperature (TEM): 450 °C
Collision Gas (CAD): 6.00
Entrance Potential (EP): 10.00
Collision Cell Exit Potential (CXP): 11.0
MRM Detection Window: 20 sec

Target Scan Time: 7.5 sec

Note: Due to the fact that the Scan Type is Scheduled MRM Monitoring, the retention times, MRM detection window and target scan time settings will have to be checked/optimized for each individual LC-MS/MS system and entered into the acquisition method prior to the start of the chromatographic run.

Ions Monitored/Declustering Potential (DP)/Collision Energy (CE):

Fluazinam (Quanitation)	Q1 465.0 m/z
	Q3 373.0 m/z
DP:	110.0
CE:	38.0
Fluazinam (Confirmation)	Q1 465.0 m/z
	Q3 338.0 m/z
DP:	110.0
CE:	67.0
DAPA (Quanitation)	Q1 405.0 m/z
	Q3 333.0 m/z
DP:	110.0
CE:	38.0
DAPA (Confirmation)	Q1 405.0 m/z
	Q3 353.0 m/z
DP:	110.0
CE:	47.0
AMPA (Quanitation)	Q1 435.0 m/z
	Q3 373.0 m/z
DP:	110.0
CE:	35.0
AMPA (Confirmation)	Q1 435.0 m/z
	Q3 354.0 m/z
DP:	110.0
CE:	38.0
CAPA (Quanitation)	Q1 441.0 m/z
	Q3 349.0 m/z
DP:	86.0
CE:	35.0

CAPA (Confirmation)	Q1 441.0 m/z
	Q3 303.0 m/z
DP:	86.0
CE:	60.0
DCPA (Quanitation)	Q1 417.0 m/z
	Q3 325.1 m/z
DP:	75.0
CE:	31.0
DCPA (Confirmation)	Q1 417.0 m/z
	Q3 279.0 m/z
DP:	75.0
CE:	53.0
HYPA (Quanitation)	Q1 447.1 m/z
	Q3 382.9 m/z
DP:	70.0
CE:	30.0
HYPA (Confirmation)	Q1 447.1 m/z
	Q3 355.1 m/z
DP:	70.0
CE:	30.0

Note: Confirmation ions are monitored to confirm the specificity of the method.

8.4 LC-MS/MS Detector Response Calibration

The LC-MS/MS responses (peak areas) are determined for a series of calibration standards. Through the Analyst Software, the concentrations of the standards injected and their corresponding peak responses are compiled. Analyst calculates a standard calibration curve using linear regression (see Equation 1) and a correlation coefficient (r) based on the standard concentrations and their respective peak responses.

For each analytical set, the calibration data is used to perform a linear regression analysis. The concentration of the sample that is injected (ng/mL) is taken as the X-axis and the detector response (peak area) is taken as the Y-axis to give Equation 2.

$$y = mx + b \qquad \textit{[Eq. 1]}$$

where: y = peak area response for analyte injected (sample/standard)
m = slope of the regression line
x = amount (ng/mL) of analyte found in the sample/standard
b = intercept of the regression line

$$\textit{peak area} = m(\textit{ng/mL in the sample/standard}) + b \qquad \textit{[Eq. 2]}$$

8.5 Sample Analysis

The peak area responses for Fluazinam, DAPA, AMPA, CAPA, DCPA and HYPA are computed using the Analyst software. The amount of material is determined from the corresponding calibration plot for each analyte. For samples, the amount found (ng/mL) of each analyte may be calculated from the observed peak area, using Equation 3.

$$x\ (ng/mL)\ in\ sample = \frac{peak\ area - b}{m} \qquad [Eq.\ 3]$$

Any apparent residues found in the control samples, greater than 30% of the lowest standard injected, may be subtracted as raw peak area from the fortified samples.

Both samples and standards must be analyzed under the same LC-MS/MS conditions and within the same analytical sequence.

9.0 CALCULATION OF RESIDUES

From the standard calibration curves, Fluazinam, DAPA, AMPA, CAPA, DCPA and HYPA concentrations (ng/mL) in unknown samples are determined using the following equation:

$$ng/mL = \frac{(ng/ml\ from\ curve)(final\ volume\ in\ mL)(aliquot\ factor)}{(sample\ amount\ (mL))} \qquad [Eq.\ 4]$$

$$\%\ Recovery = \frac{measured\ residues\ (ng/mL)}{fortification\ amount\ (ng/mL)} \times 100 \qquad [Eq.\ 5]$$

10.0 QUALITY CONTROL PROCEDURES

10.1 LC-MS/MS Analysis

The calibration standards from the analytical set will be plotted and the standard linear regression, slope and y-intercept values will be calculated using a 1/x weighted curve. These values will be used to establish the fortification recoveries. The calibration plot will not be forced through zero.

There will be a minimum of five calibration standards that bracket the concentration range of interest with the lowest standard corresponding to 70% or less of the lowest level of interest. Sample extracts containing analyte levels outside this range will be diluted accordingly to fit the calibrated range. Calibration standards are injected at the beginning and end of an analytical set. Standards will also be injected periodically throughout the set. No more than six sample injections will be made without a standard injection.

10.1.1 Acceptance Criteria

The acceptance criteria for the correlation coefficient (r) of the calibration curve must be ≥0.990 (or consequently, the coefficient of determination (r^2) must be ≥0.980).

11.0 QUANTITATION LIMIT

The limit of quantitation (LOQ) for this method is 0.100 ng/mL. The limit of detection (LOD) for this method is 0.0556 ng/mL. The LOD is based on the concentration of the lowest standard in the calibration curve.

12.0 TIME REQUIREMENT

Two hours are required for one person to prepare an analysis set from the time samples are prepared to LC-MS/MS analysis. Automated LC-MS/MS can be performed overnight or can be accomplished in approximately three hours. An additional one and a half hours is needed to calculate and tabulate the data. Therefore, at a minimum, six and a half hours are required to complete this analysis.

13.0 METHOD FLOW CHART

Analysis of Fluazinam and 5 Metabolites in Water by LC-MS/MS

Measure 100 mL of the sample water into a 250-mL glass amber bottle

↓

Fortify the matrix of interest at appropriate levels
Hand shake for approximately 30 seconds

↓

Combine a 9-mL aliquot of the sample with 1 mL of 0.2% formic acid in acetonitrile

↓

Hand shake for approximately 30 seconds

↓

If necessary: Filter an aliquot of the sample using a 1-µm glass fiber syringe filter

↓

Further dilute samples if necessary using 10% acetonitrile: 90% water: 0.02% formic acid

↓

Vial an aliquot and analyze by LC-MS/MS