1.0 PRINCIPLE

This method report documents a procedure for use to determine the residues of fenazaquin, 2-oxy-fenazaquin and 4-OHQ in soil. The method developed at Ricerca was validated in soil from 4 separate test sites.

The samples (50 g) were extracted with acetonitrile followed by a second extraction with acetonitrile:0.1N NaOH, (1:1, v:v). The extracts were pooled and gravity filtered through filter paper. Determination and quantitation of fenazaquin, 2-oxy-fenazaquin and 4-OHQ residues were conducted using HPLC employing mass spectrometric detection (LC/MS/MS). The limit of quantitation (LOQ) was 0.01 ppm. The sample extract for the analysis of fenazaquin was either analyzed directly by LC/MS/MS or diluted as needed. The sample extract for the analysis of 2-oxy-fenazaquin was analyzed by LC/MS/MS without further processing. For the analysis of 4-OHQ, a 160-mL aliquot (equivalent to 20 g soil) of the pooled filtered extract was concentrated to the aqueous layer (~40 mL) using rotary evaporation. The aqueous layer was adjusted to pH 5-6 with concentrated HCl. The pH-adjusted sample had 5 mL of a pH 7.0 K-PO_4 buffer solution added to achieve a pH of approximately 7.0. The sample was transferred to a 250-mL separatory funnel containing 2.5 g NaCl. The sample was shaken to dissolve the NaCl and then partitioned once with 100 mL of methylene chloride (DCM) followed by a second partitioning with 50 mL DCM. The DCM layers were pooled then dried over Na_2SO_4. The combined dry DCM extract was concentrated to dryness by rotary evaporation. The sample residue was dissolved in 2 mL of acetonitrile then analyzed by LC/MS/MS.

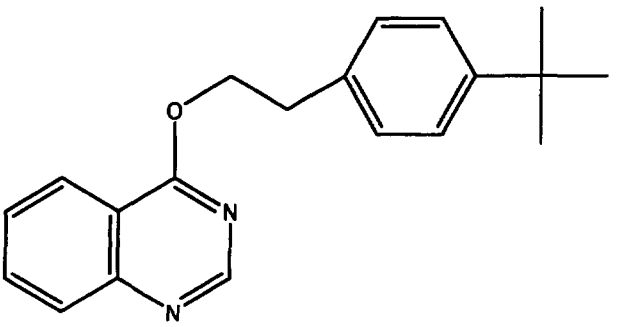
2.0 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques (such as sample extract evaporation) may be substituted for those described in this method, except where specifically noted otherwise. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

3.0 TEST/REFERENCE SUBSTANCE

A summary of the compound information is listed below which includes structure, chemical names, purity and expiration date.

- **Fenazaquin**

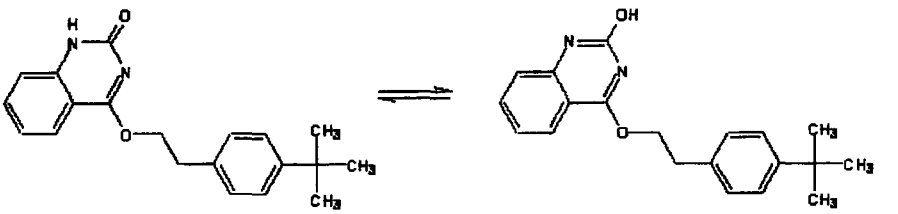


Fenazaquin

Common name: Fenazaquin

Chemical Name (IUPAC): 4-[[4-(1,1-dimethylethyl)phenyl]ethoxy]quinazoline)
CAS No.: 120928-09-8
Lot No.: H29803 0416
Purity: 99.92%
Expiration Date (per Sponsor): May, 2017
Molecular Weight: 306
Storage: Ambient

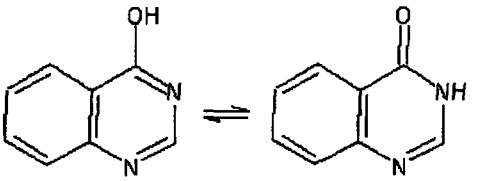
- **2-Oxy-fenazaquin (2-OXY)**



2-Oxy-fenazaquin

Common name: 2-Oxy-fenazaquin
CAS Number: NA
Lot Number: 092209
Molecular Weight: 322
Source: Gowan Company
Expiration Date: September 23, 2010
Purity: 100%
Storage: Frozen (-20°C)

- **4-Hydroxyquinazoline (4-OHQ)**



4-Hydroxyquinazoline

Common name: 4-Hydroxyquinazoline
CAS Number: NA
Lot Number: 10116012
Molecular Weight: 146
Source: Gowan Company
Expiration Date: September 23, 2011
Purity: 100%
Storage: Ambient

All preparations of the test substances for analysis were uniquely identified.

4.0 MATERIALS AND METHODS

4.1 PREPARATION OF STANDARD SOLUTIONS

All standard solutions prepared in this section were stored in the dark typically at <-5°C when not in use.

4.1.1 Stock Standard Solution

Typically, 50 mg of each analytical standard, fenazaquin and 4-hydroxyquinazoline, were accurately weighed into separate 50-mL volumetric flasks and brought to volume with acetonitrile to obtain a concentration of 1000 µg/mL. Likewise, 25 mg of 2-oxy-fenazaquine were weighed into a 50-mL volumetric flask and brought to volume with acetonitrile for a final concentration of 500 µg/mL.

4.1.2 Intermediate/Fortification Standard Solutions

Typically the following concentrations of intermediate/fortification standard solutions were prepared as individual standards of fenazaquin, 4-hydroxyquinazoline (4-OHQ) or 2-oxy-fenazaquin (2-OXY).

For fenazaquin and 4-OHQ standards:

10 µg/mL: 1 mL of the1000 µg/mL stock solution was transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

50 µg/mL: 5 mL of the1000 µg/mL stock solution was transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

100 ng/mL: 1 mL of the10 µg/mL standard solution was transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile. (This preparation was performed for fenazaquin only.)

500 ng/mL: 5 mL of the 10 µg/mL standard solution were transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

For 2-oxy-fenazaquin standard:

10 µg/mL: 2 mL of the 500 µg/mL stock solution was transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

50 µg/mL: 10 mL of the 500 µg/mL stock solution was transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

100 ng/mL: 1 mL of the10 µg/mL standard solution was transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

500 ng/mL: 5 mL of the 10 µg/mL standard solution were transferred to a 100-mL volumetric flask. The contents were brought to volume with acetonitrile.

4.1.3 (Calibration) Standard Solutions

The following concentrations of calibration standard solutions were prepared as individual standards of fenazaquin, 2-oxy-fenazaquin and 4-hydroxyquinazoline:

For fenazaquin and 2-oxy-fenazaquin standards:

10 ng/mL: 20.0 mL of the 100 ng/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

5 ng/mL: 10.0 mL of the 100 ng/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

2 ng/mL: 4.0 mL of the 100 ng/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

1 ng/mL: 2.0 mL of the 100 ng/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

0.5 ng/mL: 1.0 mL of the 100 ng/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

For 4-hydroxyquinazoline standards:

1000 ng/mL: 20.0 mL of the 10 µg/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

500 ng/mL: 10.0 mL of the 10 µg/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

200 ng/mL: 4.0 mL of the 10 µg/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

100 ng/mL: 2.0 mL of the 10 µg/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

50 ng/mL: 10 mL of the 10 µg/mL standard solution was transferred to a 200-mL volumetric flask. The contents were brought to volume with acetonitrile.

4.2 ANALYTICAL PROCEDURE

4.2.1. Equipment

Applied Biosystems API 3000 or 3200Q LC/MS/MS with Synergi Hyrdo PR 80 Angstroms, 4 µm, 2.0 × 50 mm column (Phenomenex®, Torrance, California), Phenomenex C18 guard column, and autosampler were used for mass spectral analysis. General laboratory equipment was used (balance, pH meter, centrifuge, vortex mixer, evaporator, beakers, flasks, separatory funnels, pipettes, assorted glassware, etc.).

4.2.2. Reagents

- Acetonitrile (Fisher Scientific, Optima grade)
- 1N NaOH solution (Fisher Scientific, Certified)
- Sodium Sulfate (Fisher Scientific, ACS grade)
- Sodium Chloride (Fisher Scientific, ACS grade)
- Methylene Chloride (Fisher Scientific, Certified ACS, Plus)
- Potassium Phosphate Monobasic (KH_2PO_4) (Fisher Scientific, ACS grade)
- Potassium Phosphate Dibasic (K_2HPO_4) (Fisher Scientific, ACS grade)
- Hydrochloric Acid (Fisher Scientific, HPLC grade)
- Water (Fisher Scientific, HPLC grade)
- Acetic Acid (Sigma-Aldrich ACS Reagent grade)
- Formic Acid (Sigma-Aldrich ACS ≥96%)

4.2.3. Preparation of Solutions

Preparation of 1M Potassium Phosphate Monobasic (KH_2PO_4) Solution: 13.6 g of KH_2PO_4 was dissolved in 100 mL deionized water.

Preparation of 1M Potassium Phosphate Dibasic (K_2HPO_4) Solution: 17.4 g of K_2HPO_4 was dissolved in 100 mL deionized water.

Preparation of pH 7.0 K-PO_4 Buffer Solution: 60 mL of 1M potassium phosphate dibasic solution was mixed with 40 mL of the 1M potassium phosphate monobasic solution. The pH was adjusted to pH 7.0 with the appropriate solution (up using dibasic solution and down using monobasic solution).

4.2.4. Assay Method

- Typically 50 g of sample was weighed for analysis.
- The sample was extracted with 200 mL acetonitrile. Samples were sonicated for 10 minutes then agitated using a wrist-action shaker for 10 minutes.
- The sample and extract were centrifuged to separate the solids. The liquid was decanted.
- The extraction procedure was repeated a second time with 200 mL of acetonitrile:0.1N NaOH, (1:1, v:v).
- The extracts were pooled and mixed well.
- The pooled extract was gravity filtered through filter paper to remove any particulates.

2-Oxy-Fenazaquin:

- The sample extract for the analysis of 2-oxy-fenazaquin was transferred to autosampler vials for LC/MS/MS analysis without further processing.
- The extract for the low-level concurrent recovery (0.01 ppm) and samples were assayed directly (target conc. 1.25 ng/mL). The extract for the high-level concurrent recovery(1.0 ppm) was diluted 100 fold by diluting 1 mL of the extract in 9 mL of acetonitrile followed by a second dilution of 1 mL of the diluted extract in 9 mL acetonitrile (target conc. 1.25 ng/mL). Samples were then analyzed by LC/MS/MS.

Fenazaquin:

- The sample extract for the analysis of fenazaquin was either analyzed directly by LC/MS/MS or diluted as needed. Typically the sample extract was diluted 500 fold by diluting 1 mL of the extract in 9 mL acetonitrile followed by a second dilution of 1 mL of the diluted sample in 9 mL acetonitrile. This diluted sample was further diluted 5 fold (1 mL in 4 mL acetonitrile). The final diluted sample was transferred to autosampler vials and assayed by LC/MS/MS.
- The extract for the low-level concurrent recovery samples (0.01 ppm) was assayed directly (target conc. 1.25 ng/mL). The extract for the high-level concurrent recovery(1.0 ppm) was diluted 100 fold by diluting 1 mL of the extract in 9 mL of acetonitrile followed by a second dilution of 1 mL of the diluted extract in 9 mL acetonitrile (target conc. 1.25 ng/mL). Samples were then analyzed by LC/MS/MS.

4-OHQ:

- For each sample, a 160-mL aliquot (equivalent to 20 g soil) of the pooled filtered extract was transferred to a 500-mL boiling flask and concentrated to the aqueous layer (~40 mL) using rotary evaporation. The aqueous layer was adjusted to pH 5-6 with concentrated HCl. The pH-adjusted sample had 5 mL of a pH 7.0 K-PO_4 buffer solution added to achieve a pH of approximately 7.0. The sample was transferred to a 250-mL separatory funnel containing 2.5 g NaCl. The sample was shaken to dissolve the NaCl and then partitioned once with 100 mL of methylene chloride (DCM) followed by a second partitioning with 50 mL DCM. The DCM layers were pooled then dried over Na_2SO_4. The combined dry DCM extract was transferred to a 250-mL boiling flask and concentrated to dryness by rotary evaporation. The sample residue was dissolved in 2 mL of acetonitrile. Approximately 1 mL was transferred to autosampler vial for analysis by LC/MS/MS.
- The residue for the extract of the low-level concurrent recovery sample (0.01 ppm) for the analysis of 4-OHQ was dissolved in 2 mL of acetonitrile. Approximately 1 mL was transferred to autosampler vial for analysis by LC/MS/MS without further processing (target conc. 100 ng/mL). The residue for the high-level concurrent recovery sample (1.0 ppm) was dissolved in 2 mL acetonitrile then further diluted 100 fold by diluting 1 mL of the extract in 9 mL acetonitrile followed by a second dilution of 1 mL of the diluted extract in 9 mL acetonitrile. Approximately 1 mL of the final diluted sample was transferred to autosampler vial for analysis by LC/MS/MS (target conc. 100ng/mL).

4.2.5. LC/MS/MS Analysis

Applied Biosystems API 3000 or 3200Q LC/MS/MS

Analysis for fenazaquin and 2-oxy-fenazaquin:
Column: Synergi Hyrdo PR 80 Angstroms, 4 µm, 2.0 × 50 mm
Guard Column: Phenomenex C_{18} (Phenomenex®, Torrance, California)

Inlet Conditions:	Turbo Ion Spray					
Mobile Phase:	A: Water with 0.1% formic acid					
	B: Acetonitrile with 0.1% formic acid					
Gradient:	Time (min.) -	0	0.6	5.0	5.1	7.0
	%B -	10	95	95	10	Stop
Flow Rate:	0.35 mL/min					
Injection Volume:	10 µL					
Positive MRM:	Fenazaquin: 307.2/161.0; 2-oxy-fenazaquin: 323.0/161.0					

Analysis for 4-OHQ:
Column: Synergi Hyrdo PR 80 Angstroms, 4 µm, 2.0 x 50 mm
Guard Column: Phenomenex C_{18} (Phenomenex®, Torrance, California)

Inlet Conditions:	Turbo Ion Spray					
Mobile Phase:	A: Water with 0.025% acetic acid					
	B: Acetonitrile with 0.025% acetic acid					
Gradient:	Time (min.)	0	1.1	4.0	4.1	7.5
	%B -	10	95	95	10	Stop
Flow Rate:	0.35 mL/min					
Injection Volume:	1 µL					
Negative MRM:	4-OHQ: 145.0/102.0					

4.2.6. Calibration and Linearity

A series of calibration standards for fenazaquin, 2-oxy-fenazaquin or 4-OHQ at five separate levels were injected with each set to quantify residues in the samples. These calibration standards generated a linear plot of the concentration of each standard versus area with 1/x weighting. The resulting linear plot of the calibration standards had to yield a correlation coefficient (r^2) of at least 0.99 for the analytical set to be acceptable. In addition, standards were injected typically every 3 to 20 injections to ensure that sensitivity was not changing through the sequence.

In order for the peak areas of the samples not to exceed the peak area of the highest standard in the set, samples were diluted and re-injected as necessary so that the peak area was within the range of standards used for the analysis.

4.2.7. Limit of Quantitation (LOQ)

An LOQ of 0.01 ppm was assigned based upon the lowest fortification level at which method performance was successfully verified prior to sample analyses. The limit of detection for each analyte was estimated to be 0.003 ppm or approximately one-third of the LOQ.

5.0 RESIDUE SAMPLE CALCULATIONS

Quantitation of analyte residues was made by injecting a series of calibration standards with the samples. The response of the standards by area count was plotted against concentration. The sample concentration in ng/mL was determined from the first order 1/x weighted curve generated from the calibration standards.

The final concentration of **fenazaquin and 2-oxy fenazaquin** in the sample in ppm, calculated based on both the wet weight and dry weight of the soil, was calculated using the following formulas:

Total ng = extract volume (mL) x sample concentration (ng/mL) x dilution factor

µg/g (wet weight ppm) = Total ng/ wet weight (g) of soil extracted x 1µg/1000 ng

µg/g (dry weight ppm) = Total ng/ (wet weight (g) of soil extracted – (wet weight (g) of soil extracted x % moisture)) x 1µg/1000 ng

The final concentration of **4-OHQ** in the sample in ppm, calculated based on both the wet weight and dry weight of the soil, was calculated using the following formulas:

Equivalent weight (g) of soil partitioned
= (volume (mL) of extract partitioned/ total extract volume (mL) x Wet Weight of soil extracted (g)

Total ng = sample concentration (ng/mL) x final volume of partitioned extract after drying (mL)

µg/g (wet weight ppm) = Total ng/ Equivalent weight (g) of soil partitioned x 1µg/1000 ng

µg/g (dry weight ppm) = Total ng/ (Equivalent weight (g) of soil partitioned – (Equivalent weight (g) of soil partitioned x % moisture)) x 1µg/1000 ng

As an example, calculation of **fenazaquin** concentration for sample TCI-08-211-03-43 (Site 3, sample 43) was as follows:

Residue in ng/mL from the best-fit line = 1.99 ng/mL

Sample Wet Weight = 50.03 g

% Moisture = 3.3%

Extraction volume = 400 mL

Dilution factor = 500

Total ng = 400 mL x 1.99 ng/mL x 500 dilution factor
= 398,000 ng

µg/g (wet weight ppm) = 398,000 ng/ 50.03 g x 1µg/1000 ng
= 7.955 µg/g

µg/g (dry weight ppm) = 398,000 ng/ (50.03 g – (50.03 g x 3.3/100)) x 1µg/1000 ng
= 8.230 µg/g

As an example, calculation of **2-oxy-fenazaquin** concentration for sample TCI-08-211-03-43 (Site 03, sample 43) was as follows:

Residue in ng/mL from the best-fit line = 0.0664 ng/mL

Sample Wet Weight = 50.03 g

% Moisture = 3.3%

Extraction volume = 400 mL

Dilution factor = 500

Total ng = 400 mL x 0.0664 ng/mL x 1 dilution factor
= 26.56 ng

µg/g (wet weight ppm) = 26.56 ng/ 50.03 g x 1µg/1000 ng
= 0.001 µg/g

µg/g (dry weight ppm) = 26.56 ng/ (50.03 g – (50.03 g x 3.3/100)) x 1µg/1000 ng
= 0.001 µg/g

As an example, calculation of **4-OHQ** concentration for sample TCI-08-211-03-43 (Site 03, sample 43) was as follows:

Residue in ng/mL from the best-fit line = 168 ng/mL

Sample Wet Weight = 50.03 g

% Moisture = 3.3%

Extraction volume = 400 mL

Volume of extract partitioned = 160 mL

Final volume of partitioned extract after drying (mL) = 2 mL

Equivalent weight (g) of soil partitioned
= (160 mL/ 400 mL x 50.03 g
= 20.01 g

Total ng = 168 ng/mL x 2 mL
= 336 ng

µg/g (wet weight ppm) = 336 ng / 20.01 g x 1µg/1000 ng
= 0.017 µg/g

µg/g (dry weight ppm) = 336 ng / (20.01 g – (20.01 g x 3.3/100)) x 1µg/1000 ng
= 0.017 µg/g

Calculation of percent recovery was done by the following calculation:

$$\frac{Residue\,(ppm) \times 100}{Fortified\ Amount\,(ppm)}$$

As an example, calculation of **fenazaquin** recovery for sample (Concurrent recovery 0.01 ppm, Site 3, set 1) was as follows:

Weight of the soil (g) = 50.00 g

Fortified with 1.0 mL of 500 ng/mL fenazaquin spiking solution = 500 ng fenazaquin

Total extraction volume (mL) = 400 mL

Fortified Concentration ng/mL = 500 ng fenazaquin / 400 mL extraction volume
= 1.25 ng/mL

Residue in ng/mL from the best-fit line = 1.400 ng/mL

% Recovery = 1.400 ng/mL/ 1.25 ng/mL x 100 = 112.0%

The quantitation of 2-oxy-fenazaquin was done in the same manner.

As an example, calculation of **4-OHQ** recovery for sample (Concurrent recovery 0.01 ppm, Site 3, set 1) was as follows:

Weight of the soil (g) = 50.00 g

Fortified with 1.0 mL of 500 ng/mL 4-OHQ spiking solution = 500 ng 4-OHQ

Total extraction volume (mL) = 400 mL

Volume of extract partitioned = 160 mL

Final volume of partitioned extract after drying (mL) = 2 mL

Fortified Concentration ng/mL = (500 ng 4-OHQ / 400 mL extraction volume x 160 mL volume partitioned)/2 mL final volume after drying
= 100 ng/mL

Residue in ng/mL from the best-fit line = 85.8 ng/mL

% Recovery = 85.8 ng/mL/ 100 ng/mL x 100 = 85.8%