

## 1. Introduction

The objective of this study was to validate methods of analysis for the determination of folpet and the degradates phthalimide, phthalamic acid, phthalic acid, 2-cyanobenzoic acid and benzamide in water and sediment taken from a representative microcosm.

The protocol was signed by the Study Director and Huntingdon Life Sciences Management on 9 February 2009 and by the Sponsor on 14 February 2009.

The study was undertaken at Huntingdon Life Sciences, Eye between 9 March 2009 and 12 May 2009.

The study was designed and performed to support Cambridge Environmental Assessments study CCC2003, Folpet (Folpan 80 WDG): An Assessment of the Potential Biological Effects on Aquatic Ecosystems Following Ten Applications in Freshwater Microcosms.

The signed protocol, a copy of the final report and the primary data pertaining to the study has been retained in the archives of Huntingdon Life Sciences.

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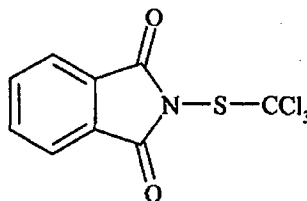
## 2. Materials

### 2.1 Analytical Standards

#### FOLPET

Common names: Folpet, Folpan  
Chemical name (IUPAC): 2-[(trichloromethyl)thio]-1*H*-isoindole-1,3(2*H*)-dione  
CAS registry number: 133-07-3

Structure:



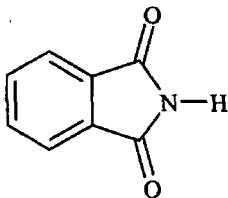
Molecular formula:  $C_9H_4Cl_3NO_2S$   
Molecular weight: 296.6  
Storage conditions: Ambient  
Supplier: Sponsor  
Batch number: 381-101-01  
Purity: 99.3%  
Expiry date: 19 November 2011  
Appearance: White crystalline solid

Certificate of Analysis is presented in Annex 1.

## PHTHALIMIDE

Common name: Phthalimide  
Chemical name (IUPAC): Isoindole-1,3-dione  
CAS registry number: 85-41-6

Structure:



Molecular formula:  $C_8H_5NO_2$   
Molecular weight: 147.1  
Storage conditions: Ambient  
Supplier: Sigma-Aldrich  
Batch number: 3223X  
Purity: 99.9%  
Expiry date: 11 August 2010  
Appearance: White solid

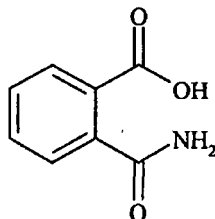
Certificate of Analysis is presented in Annex 1.

**PHTHALAMIC ACID**

Common name: Phthalamic acid

CAS registry number: 88-97-1

Structure:



Molecular formula: C<sub>8</sub>H<sub>6</sub>NO<sub>3</sub>

Molecular weight: 165.2

Storage conditions: Approximately +4°C

Supplier: Sigma-Aldrich

Batch number: 07625LH

Purity: 99.1%

Expiry date: 20 January 2010

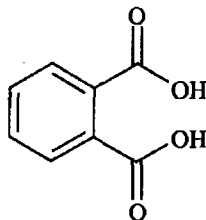
Appearance: White powder

Certificate of Analysis is presented in Annex 1.

## PHTHALIC ACID

Common name: Phthalic acid  
Chemical name (IUPAC): 1,2-Benzenedicarboxylic acid  
CAS registry number: 88-99-3

Structure:



Molecular formula:  $C_8H_6O_4$   
Molecular weight: 166.1  
Storage conditions: Approximately +4°C  
Supplier: Sigma-Aldrich  
Batch number: 1357930  
Purity: 100.1%  
Expiry date: 20 January 2010  
Appearance: White crystalline powder

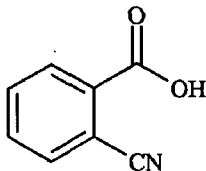
Certificate of Analysis is presented in Annex 1.

## 2-CYANOBENZOIC ACID

Common name: 2-cyanobenzoic acid

CAS registry number: 3839-22-3

Structure:



Molecular formula:  $C_8H_5NO_2$

Molecular weight: 147.1

Storage conditions: Approximately +4°C

Supplier: Sigma-Aldrich

Batch number: 02530EJ

Purity: 94.7%

Expiry date: 20 January 2010

Appearance: White powder

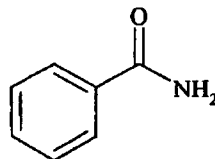
Certificate of Analysis is presented in Annex 1.

## BENZAMIDE

Common name: Benzamide

CAS registry number: 55-21-0

Structure:



Molecular formula: C<sub>7</sub>H<sub>7</sub>NO

Molecular weight: 121.1

Storage conditions: Ambient

Supplier: Sigma-Aldrich

Batch number: S42483

Purity: 99.7%

Expiry date: 20 January 2010

Appearance: White powder

Certificate of Analysis is presented in Annex 1.

**FOLPAN 80 WDG**

Common name: Folpan 80 WDG  
Formulation type: Water dispersible granules  
Active ingredient and purity: Folpet at 80%  
Storage conditions: Ambient  
Supplier: Sponsor  
Batch number: 20131608  
Expiry date: March 2010

Certificate of Analysis is presented in Annex 1.

**2.2 Control matrices**

Control pond water and sediment used for the validation of the analytical method was obtained from the Cambridge Environmental Assessments microcosm facilities.



### 3. Methods

#### 3.1 Validation

Sub-samples of pond water were fortified with known concentrations of folpet, phthalimide, phthalamic acid, phthalic acid, 2-cyanobenzoic acid and benzamide and analysed according to the following regime:

Number of replicates	Fortification level ( $\mu\text{g/L}$ )		
	wrt Folpet	wrt Phthalimide, Benzamide and 2-Cyanobenzoic acid	wrt Phthalamic acid and Phthalic acid
2	Untreated	Untreated	Untreated
5	1	0.5	2.5
5	10	5	5
5	1000	500	500

Sub-samples of pond sediment were fortified with known concentrations of folpet, phthalimide, phthalamic acid, phthalic acid, 2-cyanobenzoic acid and benzamide and analysed according to the following regime:

Number of replicates	Fortification level ( $\text{ng/g}$ )	
	wrt Folpet, 2-cyanobenzoic acid and benzamide	wrt Phthalimide, Phthalamic acid and Phthalic acid
2	Untreated	Untreated
5	5	20
5	50	50
5	500	500

These samples were then subjected to the analytical methodology described.

#### 3.2 Active substance content of the formulated product

Duplicate weighed amounts of Folpan 80 WDG were assayed for folpet content and used to generate a certificate of analysis for the formulated product.

#### 3.3 Analytical methodology

##### 3.3.1 General principles

Due to the rapid hydrolysis of folpet, the extraction of folpet from water and sediment samples was performed as quickly as possible to avoid any potential degradation.

## WATER

Folpet was partitioned out of water samples by shaking with toluene prior to quantification for folpet using gas chromatography with mass spectrometric detection (GC-MS). Phthalimide was partitioned out of water samples by shaking with dichloromethane. The dichloromethane extracts were diluted with 2% diglyme in toluene prior to quantification for phthalimide using gas chromatography with mass spectrometric detection (GC-MS). An aliquot of the aqueous phase was directly quantified for phthalamic acid, phthalic acid and benzamide using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). A further aliquot of the aqueous phase was cleaned up using a NH<sub>2</sub> solid phase extraction cartridge prior to quantification of 2-cyanobenzoic acid by LC-MS/MS.

## SEDIMENT

Folpet and phthalimide were extracted out of sediment samples by shaking with toluene after the addition of water. The toluene extracts were further cleaned up by passing through an ENVI-Carb solid phase extraction cartridge (SPE) prior to quantification for folpet and phthalimide using gas chromatography with mass spectrometric detection (GC-MS). An aliquot of the remaining aqueous phase was filtered prior to being directly quantified for phthalamic acid, phthalic acid, 2-cyanobenzoic acid and benzamide using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

## ACTIVE SUBSTANCE CONTENT

Folpan 80 WDG was dissolved in dichloromethane prior to quantification by high performance liquid chromatography with ultra violet detection (HPLC-UV).

### 3.3.2 Materials

Materials	Grade
Acetone	Analytical reagent
Acetonitrile	HPLC
Ammonia (S.G. 0.88)	Analytical reagent
Ammonium formate	Analytical reagent
Dichloromethane	HPLC
Diglyme	Analytical reagent
ENVI-Carb solid phase extraction cartridges, 500mg/6 mL	
Formic acid	HPLC
Hexane	Glass distilled
Methanol	HPLC
NH <sub>2</sub> solid phase extraction cartridges, 500mg/3 mL	
Orthophosphoric acid (S.G. 1.7)	HPLC
Sodium chloride	Analytical reagent
Sodium sulphate (anhydrous)	Analytical reagent
Toluene	Glass distilled
Water	HPLC

### 3.3.3 Test substances stock and fortifying solutions

Appropriate amounts of the test substances (corrected for purity) were accurately weighed and dissolved in a suitable solvent to give a stock standard solution. Appropriate dilutions of the stock standard solutions were made with acetonitrile to give a series of fortification standard solutions.

For the determination of the content of active substance in the formulated product, an appropriate amount of folpet analytical standard was accurately weighed and dissolved in dichloromethane to give a stock standard solution. Appropriate dilutions of this stock standard solution were made with dichloromethane to produce a series of instrument calibration solutions in the range 500 to 1000  $\mu\text{g/mL}$  for HPLC-UV.

Folpet stock solutions were progressively diluted with 2% diglyme in toluene to produce a series of instrument calibration solutions in the range 0.5 to 50  $\text{ng/mL}$  for the quantification of water and sediment samples by GC-MS.

Phthalimide stock solutions were progressively diluted with 1% diglyme in toluene:dichloromethane (50:50 v/v) to produce a series of instrument calibration solutions in the range 0.5 to 50  $\text{ng/mL}$  for the quantification of water samples by GC-MS.

Phthalimide stock solutions were progressively diluted with 2% diglyme in toluene to produce a series of instrument calibration solutions in the range 0.5 to 50  $\text{ng/mL}$  for the quantification of sediment samples by GC-MS.

The phthalamic acid, phthalic acid, 2-cyanobenzoic acid and benzamide stock solutions were progressively diluted with water to produce a series of instrument calibration solutions in the range 0.25 to 100  $\text{ng/mL}$  for LC-MS/MS.

### 3.3.4 Preparation of reagents

#### Preparation of 1% diglyme in toluene:dichloromethane (50:50):

Diglyme (10 mL) and toluene (500 mL) were added to dichloromethane (500 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

#### Preparation of 2% diglyme in toluene:

Diglyme (10 mL) was added to toluene (500 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

#### Preparation of 1M formic acid solution:

Formic acid (3.8 mL) was added to water (100 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

Preparation of 0.01M formic acid solution:

Formic acid solution (1M, 1 mL) was added to water (100 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

Preparation of 10% ammonia in methanol:

Ammonia solution (50 mL) was added to methanol (450 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

Preparation of 1N orthophosphoric acid solution:

Orthophosphoric acid (22 mL) was added to water (980 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

Preparation of saturated sodium chloride solution:

Sodium chloride was added to water (~900 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly. If all sodium chloride dissolves more sodium chloride was added and shaken again. This was repeated until some sodium chloride remained undissolved.

Preparation of Mobile Phase A – water:methanol (90:10 v:v) with 0.01M ammonium formate and 0.1 % formic acid:

Methanol (100 mL) was added to water (900 mL); ammonium formate (0.6 g) and formic acid (1 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

Preparation of Mobile Phase B – 0.1% formic acid in methanol:

Formic acid (1 mL) was added to methanol (1000 mL) in a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

Preparation of Mobile Phase C – dichloromethane:hexane (70:30 v:v):

Dichloromethane (700 mL) and hexane (300 mL) were added to a suitably sized glass bottle. The bottle was capped and shaken thoroughly.

NOTE – to prepare different volumes of the above reagents, the ratio of the individual components was maintained.

### 3.3.5 Procedures

#### Procedure – Folpet in water samples

##### 1. Extraction

- 1.1. Transfer pond water (25 mL) to a polypropylene tube (50 mL).
- 1.2. Fortify as required.
- 1.3. Add aliquots of saturated sodium chloride solution (5 mL), orthophosphoric acid (1 mL, 1 N) and toluene (5 mL) to the sample, cap and shake vigorously by hand for approximately 1 minute.
- 1.4. Allow the phases to separate and transfer the upper organic phase to a scintillation vial containing anhydrous sodium sulphate (~2 g).
- 1.5. Add a further aliquot of toluene (5 mL) to the remaining aqueous phase and shake vigorously by hand for approximately 1 minute.
- 1.6. Allow the phases to separate and transfer the upper organic phase to the scintillation vial containing the first toluene extract.
- 1.7. Transfer the combined extracts to a graduated polypropylene tube (15 mL).
- 1.8. Add diglyme (200 $\mu$ L) and make to volume (10 mL) by the addition of toluene prior to quantification of folpet by GC-MS. If further dilution necessary, dilute with 2% diglyme in toluene.

**Procedure – Degradates in water samples****2. Extraction**

- 2.1. Transfer pond water (25 mL) to a polypropylene tube (50 mL).
- 2.2. Fortify as required.
- 2.3. Add an aliquot of dichloromethane (5 mL) to the sample, cap and shake vigorously by hand for 1 minute.
- 2.4. Allow the phases to separate and transfer the lower dichloromethane phase to a scintillation vial containing anhydrous sodium sulphate (~2 g).
- 2.5. An aliquot (1 mL) of the dichloromethane phase was diluted to volume (2 mL) with the addition of 2% diglyme in toluene prior to quantification of phthalimide by GC-MS. If further dilution necessary, dilute with 1% diglyme in toluene:dichloromethane (50:50).
- 2.6. Transfer an aliquot (1 mL) of the aqueous phase to a HPLC vial prior to quantification of phthalamic acid, phthalic acid and benzamide by LC-MS/MS. If further dilution necessary, dilute with water.
- 2.7. Condition a  $\text{NH}_2$  SPE cartridge with methanol and 0.01M formic acid (3 mL of each).
- 2.8. Add formic acid (200  $\mu\text{L}$ , 1M) to an aliquot (20 mL) of the aqueous phase and pass through the SPE cartridge discarding the eluate.
- 2.9. Pull the SPE cartridge dry for 5 minutes.
- 2.10. Elute the cartridge with an aliquot of 10% ammonia in methanol (6 mL) and collect the eluate in a scintillation vial.
- 2.11. Transfer to a round bottom flask (100 mL) and wash the vial with methanol (~5 mL) and add the washing to the round bottom flask.
- 2.12. Rotary evaporate at approximately 40°C to a low volume (~0.5 mL).
- 2.13. Transfer to a graduated polypropylene tube (15 mL) and wash the round bottom flask with two aliquots of acetone (~2.5 mL) and add the washing to the polypropylene tube.
- 2.14. Remove the solvent under a steady stream of nitrogen at approximately 40°C until the volume reaches ~0.5 mL.
- 2.15. Dilute to volume (2 mL) with the addition of water and shake to mix.
- 2.16. Transfer to HPLC vial prior to quantitation by LC-MS/MS. If further dilution necessary, dilute with water.

### Procedure – sediment samples

#### 3. Extraction

- 3.1. Weigh an aliquot (2.5 g) of sediment into a polypropylene tube (50 mL).
- 3.2. Fortify as required.
- 3.3. Add aliquots of water (10 mL) and toluene (5 mL) to the sample, cap the tube and shake vigorously by hand for approximately 1 minute.
- 3.4. Centrifuge at 3500 rpm for 2 minutes.
- 3.5. Place anhydrous sodium sulphate (~1 g) on top of the packing of an ENVI-Carb SPE cartridge and condition with one column volume of toluene.
- 3.6. Transfer the upper organic phase to the SPE cartridge and collect the eluant in a polypropylene tube (15 mL).
- 3.7. Add a further aliquot (5 mL) of toluene to the aqueous phase and shake vigorously by hand for approximately 1 minute.
- 3.8. Centrifuge at 3500 rpm for 2 minutes.
- 3.9. Transfer the upper organic phase to the SPE cartridge and collect the eluant in the same polypropylene tube (15 mL) as the first extraction.
- 3.10. Retain the aqueous phase for further processing.
- 3.11. Add diglyme (250 µL) to the organic extract and dilute to volume (12.5 mL) with toluene.
- 3.12. Transfer an aliquot to a HPLC vial prior to quantitation of folpet and phthalimide by GC-MS. If further dilution necessary, dilute with 2% diglyme in toluene.
- 3.13. Decant the aqueous phase into a polypropylene tube and dilute to volume (12.5 mL) with water.
- 3.14. Filter an aliquot (~3 mL) through a 0.2 µm syringe filter.
- 3.15. Transfer an aliquot (~1 mL) to HPLC vial prior to quantitation of phthalamic acid, phthalic acid and 2-cyanobenzoic acid by LC-MS/MS. If further dilution necessary, dilute with water.
- 3.16. Dilute an aliquot (1 mL) the filtered aqueous phase to volume (1 mL) with water and transfer to HPLC vial prior to quantitation of benzamide by LC-MS/MS. If further dilution necessary, dilute with water.

### Procedure – active substance content

#### 4. Extraction

- 4.1. Weigh an aliquot (50 mg) of Folpan 80 WDG into a glass weighing boat.
- 4.2. Quantitatively transfer to a volumetric flask (50 mL) using dichloromethane and make to approximately 40 mL.
- 4.3. Sonicate for 30 minutes to disperse.
- 4.4. Remove from the sonicate bath and allow to cool.
- 4.5. Dilute to volume (50 mL) with the addition of dichloromethane.
- 4.6. Filter through a 0.45 µm PTFE filter, discarding the first 2 mL.
- 4.7. Transfer an aliquot to an HPLC vial prior to quantification folpet by HPLC-UV.

### 3.3.6 Instrumentation

#### GC-MS

Instrument:	Hewlett Packard 5890 Gas Chromatograph with a Trio 1000 mass spectrometric detector (GC-MS)
Ionisation mode:	Negative chemical ionisation (CI -)
Ion monitoring details:	Folpet <i>m/z</i> 146 Phthalimide <i>m/z</i> 146
Column:	Rxi-5ms or equivalent (30 m x 0.25 mm x 0.25µm film thickness)
Temperature programme:	100°C held for 2 minutes then ramped at 15°C/minute to 150°C then ramped at 30°C/minute to 300°C, held for 3 minutes
Injector temperature:	250°C
Transfer line:	260°C
Injection volume:	1 µL (Splitless)
Gas flow rate:	Carrier (Helium) at 1 mL/min
Retention time:	Folpet Approximately 10 minutes Phthalimide Approximately 7 minutes
LOD:	0.5 ng/mL (equivalent to 0.2 µg/L in water for both folpet and phthalimide and 2.5 ng/g in sediment for both folpet and phthalimide)
LOQ:	1 µg/L in water for folpet 0.5 µg/L in water for phthalimide 5 ng/g in sediment for folpet 20 ng/g in sediment for phthalimide



**LC-MS/MS conditions**

**Instrument:** Waters Acquity TQD  
**Ionisation mode:** Benzamide - positive electrospray  
 Phthalamic acid, phthalic acid and 2-cyanobenzoic acid – negative electrospray  
**Ion monitoring details:** Benzamide MRM  $m/z$  122>79  
 Phthalamic acid  $m/z$  164>120  
 Phthalic acid  $m/z$  165>121  
 2-Cyanobenzoic acid  $m/z$  146>102  
**Column:** Phenomenex Luna C<sub>8</sub> (15cm x 2 mm id)  
**Mobile phase A:** Water:methanol:formic acid (90:10:0.1 v:v:v) containing 0.01 M ammonium formate  
**Mobile phase B:** 0.1% formic acid in methanol  
**Gradient:**

Time (minutes)	%A	%B
0	100	0
6	0	100
10	0	100
11	100	0
15	100	0

  
**Cycle time:** 15 minutes  
**Flow rate:** 0.2 mL/minute  
**Injection volume:** 20  $\mu$ L  
**Retention time:** Benzamide Approximately 5 minutes  
 Phthalamic acid Approximately 4 minutes  
 Phthalic acid Approximately 5 minutes  
 2-Cyanobenzoic acid Approximately 5 minutes  
**LOD:** 0.25 ng/mL for Benzamide  
 (equivalent to 0.25  $\mu$ g/L in water, 2.5 ng/g in sediment)  
 0.5 ng/mL for Phthalamic acid and Phthalic acid  
 (equivalent to 1  $\mu$ g/L in water, 2.5 ng/g in sediment)  
 0.5 ng/mL for 2-Cyanobenzoic acid  
 (equivalent to 0.1  $\mu$ g/L in water, 2.5 ng/g in sediment)  
**LOQ:** 0.5  $\mu$ g/L in water and 5 ng/g in sediment for Benzamide and 2-Cyanobenzoic acid  
 2.5  $\mu$ g/L in water and 20 ng/g in sediment for Phthalamic acid and Phthalic acid

**HPLC-UV conditions**

Instrument: Agilent 1100  
Column: Lichrosorb Si-100-5 $\mu$  (25 cm x 4.6 mm id)  
Column Temperature: 30°C  
Mobile phase: Isocratic – dichloromethane:hexane (70:30 v:v)  
Flow rate: 1.6 mL/min  
Detector wavelength: 254 nm  
Injection volume: 10  $\mu$ L  
Retention time: approximately 4.2 minutes

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### 3.4 Calculation of results for validation samples

For folpet, phthalimide, phthalamic acid, phthalic acid and 2-cyanobenzoic acid test samples were quantified using the following equation:

$$\text{Residue found } (\mu\text{g/L or ng/g}) = x \times \frac{1}{M} \times D$$

Where  $x$  (residue concentration in final solution) was calculated using the linear regression

$$y = m x + c \quad \text{where } x \text{ (concentration in ng/mL)} = \frac{y - c}{m}$$

- $c$  = intercept
- $m$  = slope
- $y$  = peak area of sample
- $M$  = matrix concentration (mL/mL or g/mL)
- $D$  = dilution factor

Example calculation of folpet in an untreated pond water sample fortified at 1000  $\mu\text{g/L}$ , sample identification 09/00/0766 F1000 A.

Linear regression  $y = m x + c$

$$y = 41.7268x - 10.3375$$

where

$$y = 899.833$$

$$m = 41.7268$$

$$c = -10.3375$$

Therefore, concentration of folpet ( $x$ )

$$= \frac{899.833 + 10.3375}{41.7268} = 21.81 \text{ ng/mL}$$

Matrix concentration = 2.5 mL/mL

Dilution factor = 100

Folpet detected ( $\mu\text{g/L}$ )

$$= \frac{21.81 \text{ ng/mL} \times 100}{2.5 \text{ mL/mL}} = 872.5 \mu\text{g/L}$$

Recovery of folpet (%)

$$= \frac{872.5 \mu\text{g/L} \times 100\%}{1000 \mu\text{g/L}} = 87\%$$

For benzamide test samples were quantified using the following equation:

$$\text{Residue found } (\mu\text{g/L or ng/g}) = x \times \frac{1}{M} \times D$$

Where x (residue concentration in final solution) was calculated using the quadratic regression

$$x = \frac{-b + \sqrt{(b^2 - 4a(c-y))}}{2a}$$

- y = peak area of sample  
 a, b, c = coefficients from quadratic regression analysis  
 M = matrix concentration (mL/mL or g/mL)  
 D = dilution factor

Example calculation of benzamide in an untreated pond water sample fortified at 500  $\mu\text{g/L}$ , sample identification 09/00/0766 HIGH A.

Quadratic regression  $y = ax^2 + bx + c$

$$y = -2.34251x^2 + 1396.72x + 241.334$$

where

$$\begin{aligned}
 y &= 55304.695 \\
 a &= -2.34251 \\
 b &= 1396.72 \\
 c &= 241.334
 \end{aligned}$$

Therefore, concentration of benzamide (x)

$$\begin{aligned}
 &= \frac{-1396.72 + \sqrt{(1396.72^2 - 4 \times -2.34251(241.334 - 55304.695))}}{2 \times -2.34251} = 42.4 \text{ ng/mL}
 \end{aligned}$$

Matrix concentration = 1 mL/mL

Dilution factor = 10

$$\begin{aligned}
 \text{Benzamide detected } (\mu\text{g/L}) &= \frac{42.4 \text{ ng/mL} \times 10}{1 \text{ mL/mL}} = 424 \mu\text{g/L}
 \end{aligned}$$

$$\begin{aligned}
 \text{Recovery of benzamide } (\%) &= \frac{424 \mu\text{g/L} \times 100\%}{500 \mu\text{g/L}} = 85\%
 \end{aligned}$$