

CONDUCT OF THE STUDY

The study was conducted at the Ricerca Biosciences, LLC AgChem Product Development Department Laboratories according to the Ricerca Biosciences, LLC protocol, Document Number 031067-0, "Independent Laboratory Validation (ILV) of an Environmental Chemistry Method for Detection of Bensulide in Water" (Appendix E).

Personnel involved with the study were:

L. Ferguson	Scientist
C. Reed	Associate Scientist I

INTRODUCTION

This protocol describes the Independent Laboratory Validation (ILV) of an environmental chemistry method that had previously been validated for the determination of bensulide in water according to PTRL West Study No. 2256W.

OBJECTIVE/PURPOSE

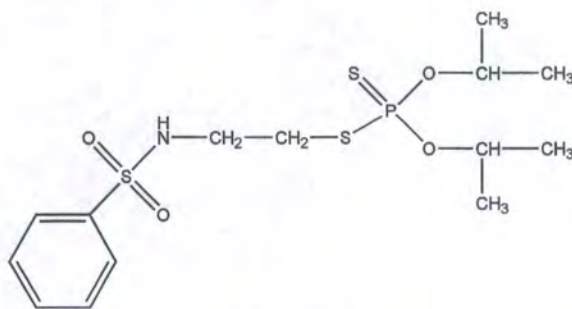
In a recent Data Evaluation Review by the U. S. EPA [4], it was stated that an independent laboratory validation (ILV) was needed for a validated environmental chemistry method for bensulide in water (PTRL West Study No 2256W). That is, while the PTRL West ILV was acceptable to the Agency, the method validation that the ILV was based on was not. Thus, EPA is considering the PTRL West ILV to be the validated method and is requiring an ILV of that method. The purpose of this study is to conduct that ILV on a representative natural water sample (i.e., river water to match the PTRL West Study).

TEST SUBSTANCE

The sponsor supplied the test substance, bensulide. The test substance was characterized by HPLC analysis for purity and LC-MS analysis for identity (the data are included in Appendix A). A Certificate of Analysis (CoA) was issued for the test substance (Appendix A).

The test substance, bensulide, was stored at ambient temperature. Information concerning the test substance including purity is provided as follows:

- **Bensulide**



Bensulide

Common Name: Bensulide

Chemical Name: O,O-diisopropyl S-2-phenylsulfonlaminoethyl
phosphorodithioate
CAS No.: 741-58-2
Molecular Formula: C₁₄H₂₄NO₄PS₃
Molecular Weight: 397.5 g/mole
Lot Number: TQ-BTS-1105-050
Purity: 97.6%
Expiration Date: 5/2015
Storage: Ambient

TEST SYSTEM

The test system, Wyoming river water, was supplied by AGVISE Research, Inc., Northwood, ND. The Wyoming river water was collected from Fremont County, Wyoming (Coordinates: N 43° 15.4713 W 108° 33.9418) on April 15, 2013. The characterization report of the river water is included in Appendix B. The river water was stored refrigerated before use.

SOLVENTS AND REAGENTS

Formic acid, Fisher Optima LC/MS Grade
Acetonitrile (ACN), Fisher Optima Grade
Water, Fisher HPLC or DIUF Grade
Methanol, Fisher HPLC Grade

ACN-water (70:30 v:v): acetonitrile (700 mL) and HPLC water (300 mL) were mixed.

0.1% Formic acid in water: formic acid (1 mL) and HPLC water (1 L) were mixed.

ACN-water (50:50 v:v): acetonitrile (500 mL) and HPLC water (500 mL) were mixed.

STOCK, FORTIFICATION, AND CALIBRATION SOLUTIONS

STOCK SOLUTIONS

Stock solutions of bensulide were prepared at 1.06 and 0.97 mg/mL in acetonitrile. The stock solutions were stored refrigerated when not in use.

FORTIFICATION SOLUTIONS

The fortification solutions were prepared by serial dilution of the 1.06 mg/mL stock solution with ACN-water (70:30 v:v) in "Class A" volumetric flasks, as detailed below:

Fortification solutions			
Standard solution used (µg/mL)	Volume taken (mL)	Final volume (mL)	Nominal concentration (µg/mL)
1060	0.943	10	100
100	1	10	10
10	1	10	1

The fortification solutions were stored refrigerated when not in use.

INTERMEDIATE/CALIBRATION SOLUTIONS

The intermediate solutions were prepared by serial dilution of both stock solutions with ACN-water (70:30 v:v) in "Class A" volumetric flasks, as detailed below:

Solution A			
Standard solution used ($\mu\text{g}/\text{mL}$)	Volume taken (mL)	Final volume (mL)	Nominal concentration ($\mu\text{g}/\text{mL}$)
1060	1	10	106
106	1	10	10.6
10.6	1	10	1.06

Solution B			
Standard solution used ($\mu\text{g}/\text{mL}$)	Volume taken (mL)	Final volume (mL)	Nominal concentration ($\mu\text{g}/\text{mL}$)
970	1	10	97
97	1	10	9.7
9.7	1	10	0.97

The intermediate solutions were stored refrigerated when not in use.

The calibration solutions were prepared by dilution of the intermediate solutions noted in the table with ACN-water (70:30 v:v) in "Class A" volumetric flasks, as detailed below:

Standard solution used	Volume taken (mL)	Final volume (mL)	Nominal concentration (ng/mL)
Solution A 1.06 $\mu\text{g}/\text{mL}$	1	10	106
Solution B 0.97 $\mu\text{g}/\text{mL}$	0.75	10	72.75
Solution A 1.06 $\mu\text{g}/\text{mL}$	0.5	10	53
Solution B 0.97 $\mu\text{g}/\text{mL}$	0.25	10	24.25
106 ng/mL	1	10	10.6
24.25 ng/mL	2	10	4.85

The calibration solutions were stored refrigerated when not in use.

ANALYTICAL METHODOLOGY

FORTIFICATION OF RIVER WATER

Ten river water samples (200 mL each) were fortified with bensulide as described in the table below. Two water samples (200 mL each) serving as controls and one sample with no river water serving as a reagent blank were fortified with ACN-water (70:30 v:v), as described below.

	Fortification Solution	Volume
Reagent Blank	ACN-water (70:30 v:v)	200 μ L
Water Control A	ACN-water (70:30 v:v)	200 μ L
Water Control B	ACN-water (70:30 v:v)	200 μ L
Water 0.001 ppm A	1 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.001 ppm B	1 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.001 ppm C	1 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.001 ppm D	1 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.001 ppm E	1 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.01 ppm A	10 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.01 ppm B	10 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.01 ppm C	10 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.01 ppm D	10 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L
Water 0.01 ppm E	10 μ g/mL Bensulide in ACN-water (70:30 v:v)	200 μ L

SPE CARTRIDGE CLEAN-UP

1. BAKERBOND SPE cartridges (C18, 6 mL, 500 mg) were placed on SPE vacuum manifolds (Supelco Visiprep) and conditioned with two 5 mL aliquots of HPLC methanol followed by two 5 mL aliquots of DI water at a flow rate of 2-3 drops per second. The cartridge was not allowed to go dry.
2. The samples were applied to the cartridges using reservoir adapters.
3. The flasks were rinsed twice with 10 mLs of DI water and the rinse was applied to the cartridges. The cartridges were sucked to dryness for ~5 min. after the entire sample had been applied. The eluents were discarded.
4. 15 mL polypropylene centrifuge tubes were placed under the SPE cartridge to collect eluent.
5. The flasks were rinsed three times with 3 mL of HPLC methanol. The rinses were applied to the cartridges. The cartridge was sucked with vacuum to dryness.
6. 2-3 mL of acetonitrile was added to each methanol eluent.
7. The centrifuge tubes were placed in a Turbo N-evaporate and the solution was taken down to dryness.
8. The residue was reconstituted with 10 mL of ACN-water (70:30 v:v) and vortex to mix.

9. The final extract was microfilterfuged (Osmonics microfuge 0.22 micron, 1.5 mL) and aliquots were transferred to auto sampler vials for analysis on LC-MS/MS. The **0.01 ppm** samples were diluted 10X with ACN-water (70:30 v:v) before LC-MS/MS analysis.

LC-MS/MS ANALYSIS

Separation of the analyte from the matrix was achieved by high performance liquid chromatography (HPLC). Quantitative LC-MS/MS analysis of bensulide in the samples utilized a highly specific and sensitive MRM (Multiple Reaction Monitoring) method. Bensulide precursor ion (m/z 398.03) was monitored in Q1 and the fragment ions were monitored in Q3 (m/z 158.1, primary quantification, m/z 218.0, confirmatory quantification, and m/z 141.1, confirmatory quantification). The analyte was identified by the coincidence of the retention time with that of the calibration standards, and quantified by integration of the peak area relative to the calibration curves.

The following are the LC-MS/MS system (controlled by the operating software, Analyst™ version 1.4.2, a validated system) and the parameters used.

HPLC: Shimadzu Pump LC-10ATvp
Shimadzu System Controller
CTC Analytics LC PAL autosampler

Column: Phenomenex Gemini C6-Phenyl 3 μm 110 Å (50 mm x 2.0 mm ID)

Injection Volume: 5 μL

Solvent System:

Solvent A = 0.1% Formic acid in water

Solvent B = Acetonitrile

Wash solvent 1 = ACN-water (50:50 v:v)

Wash solvent 2 = ACN-water (50:50 v:v)

Solvent Program:

Time (minutes)	Flow Rate (mL/min)	%A	%B
0.0	0.3	50	50
0.5	0.3	50	50
5.0	0.3	5	95
9.0	0.3	5	95
9.5	0.3	50	50
13.0	0.3	50	50

Bensulide Retention Time: 3.26 minute

Mass Spectrometer: SCIEX API 4000QTrap

This mass spectrometer was calibrated monthly.

Mass Spectrometer settings:

Scan Type:	MRM
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Ion Source Gas 1 (GS1):	40 psi
Ion Source Gas 2 (GS2):	40 psi
Curtain Gas (CUR):	10 psi
Collision Gas (CAD):	5 psi
IonSpray Voltage (IS):	5500 V
Temperature (TEM):	450 °C
Declustering Potential (DP):	36 V
Entrance Potential (EP):	10 V

MRM:

Analyte ID	Q1 Mass (amu)	Q3 Mass (amu)	Collision Energy (CE)	Collision Gas Exit Potential (CXP)	Dwell Time (msec)
Bensulide	398.03	158.1	33 V	10 V	150
BensulideC1	398.03	218.0	23 V	16 V	150
BensulideC2	398.03	141.1	47 V	10 V	150

METHODS OF CALCULATION

Recoveries

The recoveries of bensulide from fortified samples were calculated relative to the linearity curve generated with each set:

Linear regression formula from calibration curve $y = mx + b$

$$\text{ng/mL bensulide} = \frac{y - b}{m}$$

Where y = Sample peak area

b = Calibration intercept

m = Calibration slope

$$\text{Sample Concentration (ng/mL)} = \frac{\text{Sample peak area} - \text{intercept}}{\text{Slope}}$$

ppm bensulide =

$$\frac{\text{Sample Conc. (ng/mL)} \times \text{Final Sample Vol. (mL)} \times 0.001 \mu\text{g/ng} \times \text{Dilution Factor}}{\text{Sample weight (g)}}$$

where $\mu\text{g/g}$ is equivalent to mg/kg and ppm.

Percent Recovery =

$$\frac{\text{Conc. of Fortified Sample (ppm)} - \text{Conc. of Control (ppm)}}{\text{Fortification Level (ppm)}} \times 100$$

An example calculation for the recovery of bensulide (0.01 ppm fortification) from water (sample **Water 0.01 ppm C dil 10X**) is shown below:

The bensulide calibration curve equation was $y = 8560x + (4790)$ ($r = 0.9994$)

$$\text{ng/mL bensulide} = \frac{166600 - (4790)}{8560} = 18.9 \text{ ng/mL}$$

The ppm bensulide in water was calculated as shown:

$$\text{ppm bensulide} = \frac{18.9 \text{ ng/mL} \times 10 \text{ mL} \times 0.001 \mu\text{g/ng} \times 10}{200 \text{ g}} = 0.00945 \text{ ppm}$$

The percent recovery of bensulide was calculated as follows:

$$\text{Percent recovery} = \frac{0.00945 \text{ ppm} - 0 \text{ ppm}}{0.01 \text{ ppm}} \times 100 = 94.5\%$$