Analytical Method

The analytical method used for the determination of BAJ 2740 in freshwater was developed by Wildlife International, Ltd.

Aqueous samples were volumetrically transferred to separatory funnels followed by 100 mL of dichloromethane. The separatory funnels were shaken for approximately one minute and the phases allowed to separate before the lower organic layer was drained into a roundbottom flask. The extraction was repeated by adding an additional 100 mL of dichloromethane to each separatory funnel, shaking for approximately one minute and draining the organic layer into its respective roundbottom flask. The combined extracts were rotary evaporated to approximately 1 mL, evaporated to dryness under a gentle stream of nitrogen and reconstituted to volume with 50% acetonitrile: 50% water: 0.1% phosphoric acid. Samples were analyzed using high performance liquid chromatography (HPLC) with UV detection (Table 1). A method flow chart is provided in Figure 1.

Stock and Standard Preparation

A primary stock solution of the test substance was prepared by accurately weighing 0.1015 g (weight corrected for purity) of the test substance on an analytical balance. The test substance was quantitatively transferred to a 100-mL volumetric flask and the flask brought to volume with acetone. The resulting stock (1.00 mg a.i./mL) was serially diluted in acetone to produce stock solutions with concentrations of 0.100 and 0.0100 mg a.i./mL. The 0.0100 mg a.i./mL stock solution was used to fortify method verification samples.

An additional primary stock solution of the test substance was prepared by accurately weighing 0.2026 g (weight corrected for purity) of the test substance on an analytical balance. The test substance was quantitatively transferred to a 100-mL volumetric flask and the flask brought to volume with acetone. The resulting stock (2.00 mg a.i./mL) was used to fortify samples for the solubility trial.

A primary stock solution of the analytical standard was prepared by accurately weighing 0.1019 g (weight corrected for purity) of the analytical standard on an analytical balance. The analytical standard was transferred to a 100-mL volumetric flask and the flask brought to volume with acetonitrile. The resulting stock (1.00 mg a.i./mL) was serially diluted in acetonitrile to produce a secondary analytical

standard stock solution at 0.100 mg a.i./mL. These stocks were used to prepare calibration standards for the study.

Calibration standards were prepared in 50% acetonitrile: 50% water: 0.1% phosphoric acid and analyzed concurrently with each sample set. The calibration standards were prepared with the 0.100 and the 1.00 mg a.i./mL analytical stocks using the following dilution scheme.

Stock Concentration mg a.i./mL	Aliquot (mL)	Final Volume (mL)	Standard Concentration (µg a.i./L)
0.100	0.0500	100	50.0
0.100	0.150	100	150
0.100	0.250	100	250
0.100	0.350	100	350
0.100	0.500	100	500
1.00	1.00	100	1000

Calibration Curve and Limit of Quantitation (LOQ)

Calibration standards of BAJ 2740 ranging in concentration from 50.0 to 500 µg a.i./L were analyzed with the method verification sample set. Calibration standards of BAJ 2740 ranging in concentration from 50.0 to 1000 µg a.i./L were analyzed during the solubility trial. Linear regression equations were generated using the peak area responses versus the respective concentrations of the calibration standards. The concentration of BAJ 2740 in the samples was determined by substituting the peak area responses into the applicable linear regression equation. The limit of quantitation, (LOQ) was defined as 1.25 µg a.i./L calculated as the product of the low standard (50.0 µg a.i./L) and the dilution factor of the matrix blank samples (0.0250). A representative calibration curve is presented in Figure 2. Representative chromatograms of low and high calibration standards are presented in Figures 4 and 5, respectively.

Reagent and Matrix Blank Samples

Three reagent and three matrix blank samples were analyzed concurrently with the method verification samples to determine possible interference. No interference was observed at or above the limit of quantitation (LOQ) during the sample analyses (Table 2). Representative chromatograms of reagent and matrix blank samples are presented in Figures 6 and 7, respectively.

Method Verification Samples

Freshwater was fortified in triplicate at 3.00, 10.0, 30.0 and 60.0 µg a.i./L using a 0.0100 µg a.i./µL stock solution containing BAJ 2740 in acetone. Samples were prepared by fortifying freshwater contained in separatory funnels as follows:

Sample I.D. (149C-110-)	Nominal Concentration (µg a.i./L)	Stock Concentration (µg a.i./µL)	Fortification Volume (µL)	Sample Volume (mL)
VMAS 1-3	3.00	0.0100	60.0	200
VMAS 4-6	10.0	0.0100	200	200
VMAS 7-9	30.0	0.0100	300	100
VMAS 10-12	60.0	0.0100	600	100

Samples fortified at 3.00, 10.0, 30.0 and 60.0 µg a.i./L yielded mean recoveries of 92.7, 94.2, 92.1 and 92.1%, respectively (Table 2). Representative chromatograms of low and high-level matrix fortifications are presented in Figures 8 and 9, respectively.

Example Calculations

The analytical result and percent recovery for method verification sample number 149C-110-VMAS-5, nominal concentration of 10.0 µg a.i./L, was calculated using the following equations.

$$\text{BAJ 2740 (µg a.i./L) in sample} = \frac{\text{Peak area - (Y-intercept)}}{\text{Slope}} \text{ X Dilution factor}$$

Peak area = 71960.8
Y-intercept = 391.5795
Slope = 185.53
Initial Volume (V_i): = 200 mL
Final Volume (V_f): = 5.00 mL
Dilution Factor (V_f/V_i): = 0.0250

$$\text{BAJ 2740 (µg a.i./L) in sample} = \frac{71960.8 - 391.5795}{185.53} \text{ X } 0.0250$$

BAJ 2740 (µg a.i./L) in sample = 9.64

$$\text{Percent of nominal concentration} = \frac{\text{BAJ 2740 in sample(µg a.i./L)}}{\text{BAJ 2740 fortified conc. (µg a.i./L)}} \text{ X } 100$$

$$\text{Percent of nominal concentration} = \frac{9.64}{10.0} \text{ X } 100$$

$$= 96.4\%$$

Table 1

Typical HPLC Operational Parameters

INSTRUMENT:	Hewlett-Packard Model 1090 High Performance Liquid Chromatograph (HPLC) equipped with a Waters Model 486, a Jasco Model 975 or a Hewlett Packard Series 1100, UV Detector
ANALYTICAL COLUMN:	YMC – Pack ODS – AM (150 mm x 4.6 mm, 3 µm particle size)
FLOW RATE:	1.00 ml/minute
OVEN TEMPERATURE:	40°C
MOBILE PHASE A:	Acetonitrile: NANOpure® water: Phosphoric acid (10:90:0.1, v/v)
MOBILE PHASE B:	Acetonitrile: NANOpure® water: Phosphoric acid (95:5:0.1, v/v)
GRADIENT PROFILE:	Time (Minutes) / %A / %B 0.01 / 50.0 / 50.0 1.00 / 50.0 / 50.0 9.00 / 0.0 / 100.0 12.0 / 0.0 / 100.0 12.1 / 50.0 / 50.0 17.0 / 50.0 / 50.0
ANALYTICAL WAVELENGTH:	200 nm
INJECTION VOLUME:	150µL
BAJ 2740 PEAK RETENTION TIME:	Approximately 12.8 minutes

Method Outline For The Processing Of BAJ 2740 In Freshwater

Rinse separatory funnels and roundbottom flasks with dichloromethane.

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Transfer the requisite volume of freshwater to the separatory funnels. Fortify recovery samples with the appropriate BAJ 2740 stock solution (s). Freshwater will serve as the matrix blank.

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Using a graduated cylinder or tiltapet, add 100 mL of dichloromethane to each sample. Stopper and shake each sample (with venting) for approximately one minute. Allow the organic and aqueous layers to separate. Drain the organic (lower) layer into a roundbottom flask.

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Add 100 mL of dichloromethane to the separatory funnel. Stopper and shake each sample (with venting) for approximately one minute. Drain the organic layer into the roundbottom flask.

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Rotary evaporate each sample to approximately 1.0-3.0 mL using a waterbath maintained at ~40°C. Do not rotary evaporate to dryness.

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Evaporate the samples to dryness under a gentle stream of nitrogen.

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Volumetrically add the requisite volume of 50% acetonitrile: 50% water: 0.1% phosphoric acid to each roundbottom flask and swirl well in order to dissolve all residues.

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Transfer each extract to an autosampler vial. Submit samples for HPLC/UV analysis.

Figure 1. Analytical method flow chart for the analysis of BAJ 2740 in freshwater.