SUMMARY

Methodology was developed to quantify the amount of total pyrethrins present in freshwater (hard reconstituted and soft water), seawater (filtered and unfiltered), acetone and fish (fathead minnow) tissue. Initially, samples of each of the four matrices (hard reconstituted water, soft water, filtered seawater and unfiltered seawater) were fortified with Pyrethrin 1 (Code NK 9212) and extracted to compare and evaluate the relative extraction efficiencies. The analysis of these samples resulted in recoveries which indicated that the extraction efficiency was statistically similar for all four matrices. In addition, the extraction efficiency of Pyrenone Crop Spray (Sample Ref. 13650) was determined using the same procedures developed for the extraction of Pyrethrum Extract (FEK-99). Statistical comparison of analytical recoveries obtained for samples fortified with Pyrethrum Extract (FEK-99) and samples fortified with Pyrenone Crop Spray (Sample Ref. 13650) indicated similar extraction efficiencies. Based on these data, method validation procedures were completed for a representative freshwater matrix (hard reconstituted water) and a representative seawater matrix (filtered seawater) using Pyrethrum Extract (FEK-99). All samples were analyzed by gas chromatography using electron capture detection (GC-ECD). Due to chromatographic interference with one of the Pyrethrin II congeners during the analysis of actual test samples, the quantitation of total pyrethrins was based on the combined area of the Pyrethrin I congeners (Pyrethrin 1, Cinerin 1, and Jasmolin 1). Due to the high adsorptive properties of pyrethrins, all procedures unless otherwise specified, were performed using silanized glassware.

Freshwater and seawater samples were fortified with Pyrethrum Extract (FEK-99) at nominal concentrations ranging from 0.250 to 70.0 µg total pyrethrins/L and 0.0600 to 70.0 µg total pyrethrins/L, respectively. Aqueous samples containing the test substance were extracted two times with hexane and rotary evaporated to dryness. Fortified samples with concentration factors greater than 0.004 were adjusted to the appropriate final volume with hexane. Those samples having a concentration factor of 0.004 or less were adjusted to a known volume with acetonitrile, to which an equal volume of reagent grade water was added. The sample matrix was removed using C-18 Solid Phase Extraction (SPE) clean-up procedures. Aqueous samples

were extracted and concentrated to final concentrations ranging between 50.0 to 750 µg/L.

Fish (fathead minnow) tissue (approximately 0.5 grams) was homogenized with dry ice and fortified with Pyrethrum Extract (FEK-99) at nominal concentrations of approximately 200 mg/kg. The tissue containing the test substance was thoroughly mixed with a solution of 50/50 acetonitrile/ASTM type II water. Following centrifugation, an aliquot of the supernatant was extracted with hexane. The phases were allowed to separate and an aliquot of the hexane was transferred to a GC vial for analysis.

1.0 INTRODUCTION

The purpose of this study was to validate the analytical methodology needed to support aquatic toxicological testing in matrices of freshwater, seawater and acetone and fish (fathead minnow) tissue. This study was initiated on 26 March 1993, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental phase of the method validation was conducted from 1 April through 22 November 1993 at Springborn Laboratories, Inc. (SLI), Environmental Sciences Division, Wareham, Massachusetts. All original raw data and the final report produced for this study are stored at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol and Amendment

Procedures used in this method validation followed those described in the SLI protocol entitled "Development and Validation of an Analytical Methodology for the Determination of Total Pyrethrins in Water, Acetone and Fish Tissue", Springborn Laboratories Protocol #:032293/FIFRA/250 and SLI Protocol Amendments #1 and #2 dated 7 April and 15 September 1993, respectively (Appendix I). The method development and validation consisted of establishing instrumental conditions, determining an extraction scheme and performing the recovery studies. The validation process followed the guidelines presented in the Springborn Laboratories, Inc. Standard Operating Procedures (SOP) # 577-1.

2.2 Test Substance

2.2.1 Pyrethrum Extract (FEK-99). A sample of the test material, Pyrethrum Extract (FEK-99), was received from Roussel UCLAF Corporation, Lincoln Park, New Jersey, on 12 March 1993. The following information describes the test substance received:

CAS Registry Number:	8003-34-7
Physical appearance:	brown liquid
Lot #:	R92-254
Sample Ref.:	13677; FEK-99

Percent Active Ingredient:	57.488% total pyrethrins (see Appendix II for Certificate of Analysis and Letter of Transmittal)
Water Solubility:	approximately 800 ppb (personal communication with Sponsor)
Expiration Date:	August, 1996

Upon receipt at Springborn, the sample of test material was covered with aluminum foil to protect the sample from degradation from ultraviolet light and stored under freezing conditions. The sample of Pyrethrum Extract (FEK-99) received was used to prepare recovery samples and analytical standards. Two hours prior to use, the sample of Pyrethrum Extract (FEK-99) was allowed to warm the room temperature, then vigorously shaken for one minute. All usage of the test material was documented in the Test Material Usage Log (Vol. 31).

2.2.2. Pyrenone Crop Spray. A sample of the test material, Pyrenone Crop Spray (Sample Ref. 13650), was received from Roussel UCLAF Corporation, Middleport, New York, on 24 February 1993. The following information describes the test material received:

Physical appearance:	brown liquid
Lot/Batch #:	M60008JX
Sample Ref.:	13650; FER-10
Percent Active Ingredient:	6.02% total pyrethrins, 60.25% piperonyl butoxide; (see Appendix III for Certificate of Analysis and Letter of Transmittal)
Water Solubility:	emulsifiable
Expiration Date:	30 June 1995

Upon receipt at Springborn, the sample was stored in a dark, ventilated cabinet at room temperature (approximately 20 °C) and was used to prepare recovery samples and analytical standards. The stability of the test substance was based on the above expiration date. All usage of the test material was documented in the Test Material Usage Log (Vol. 31).

2.2.3 Pyrethrin 1 (Code NK 9212). A stock solution (35 mg/mL) of the test substance, Pyrethrin, in hexane/ethyl acetate (19/1), was received from Biological Test Center, Irvine, California on 17 February 1993. The Pyrethrum 1 (Code NK 9212) was synthesized by

Amersham Life Sciences as a component of a commercial product, the stability is expected to be similar to Pyrethrum Extract. The following information describes the test substance received:

Physical appearance:	colorless liquid
Sample Ref.:	NK 9212
Percent Active Ingredient:	97.0% (see Appendix IV for Certificate of Analysis)

Upon receipt at Springborn, the sample of test material was stored in a freezer at approximately 0 °C. The sample of Pyrethrin 1 (Code NK 9212) received was used to prepare preliminary samples and analytical standards. All usage of the test material was documented in the Test Material Usage Log (Vol. 31).

2.3 Test System

The validation process included determination of instrumental conditions, an extraction scheme and method precision. and accuracy.

2.3.1 Equipment

1. Instrument:	Hewlett Packard Model 5890 gas chromatograph equipped with an Ni63 electron capture detector, a Hewlett Packard Model 7673A autosampler and a Hewlett Packard Model 3396 series II integrator or a Hewlett Packard Model 5890 series II gas chromatograph equipped with an Ni63 electron capture detector, a Hewlett Packard Model 7673 autosampler and a Hewlett Packard Chemstation Model 3365A-01
2. Balance:	Mettler AE 200, four place analytical balance
3. Rotary evaporator:	Brinkman Buchi
4. Laboratory glassware:	syringes, volumetric pipets, Pasteur pipets, volumetric flasks, graduated cylinders, separatory funnels, round bottom flasks, glass funnels, glass wool, powder funnel, test tubes, GC vials and amber serum bottle
5. Solid Phase Extraction column:	C-18, Burdick & Jackson, glass, 1000 mg
6. Solid Phase Extraction	

Apparatus: Burdick & Jackson equipped with a vacuum pump

7. Tissue Homogenizer: Jake & Kankel Model A-10 S2
8. Ultra Centrifuge: Baxter Biofuge 17, Model 2742

2.3.2 Reagents

1. Acetone GC[2]: Burdick & Jackson, Baxter, GC grade
2. Acetonitrile: Burdick & Jackson, Chromatography grade
3. Hexane GC[2]: Burdick & Jackson, Baxter, GC grade
4. Methanol: Burdick & Jackson, Chromatography grade
5. Reagent grade water: prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM type IIA requirements)
6. Sodium sulfate: anhydrous, Mallinckrodt
7. Dichlorodimethyl Silane: Sigma
8. Toluene: Baxter, Chromatography grade

2.4. Preparation of Stock Solutions

To prepare the primary stock solution (1.00 mg/mL) of Pyrethrum Extract (FEK-99), approximately 0.1740 grams (0.1000 g as total pyrethrins) was weighed into a 100-milliliter (mL) silanized volumetric flask, dissolved and diluted to volume with acetone.

To prepare a primary stock solution (1.00 mg/mL) of Pyrenone Crop Spray (Sample Ref. 13650), approximately 1.6666 g (0.1003 g as total pyrethrins) was weighed into a 100-milliliter (mL) silanized volumetric flask, dissolved and diluted to volume with acetone.

To prepare a primary stock solution (34.0 mg/mL) of Pyrethrin 1 (Code NK 9212), approximately 0.0300 gram (0.00170 g as active ingredient, pyrethrin 1) was weighed into a 50-milliliter (mL) silanized volumetric flask, dissolved and diluted to volume with acetone.

These primary stock solutions were stored in a freezer (< 0 °C) in silanized amber serum bottles fitted with teflon-lined lids. Serial dilutions of these stocks into hexane and acetone were prepared in Class A glassware for use as analytical standards as well as for fortification of recovery samples.

2.5 Sample Fortification

2.5.1 Preliminary. Two 100-mL samples of each of the four matrices to be evaluated (hard reconstituted water, soft reconstituted water, unfiltered seawater and filtered seawater) were fortified with the Pyrethrin 1 (Code NK 9212) primary stock solution at a nominal concentration of 25.5 µg/L.

2.5.2 Aqueous Method Validation. Method validation/recovery samples (100 - 1000 mL) were prepared in freshwater (fortified to increase hardness to 160 - 180 mg/L as $CaCO_3$) and seawater (filtered through a 5.0 µ fiber filter). Freshwater and seawater samples were fortified with a dilution of the Pyrethrum Extract (FEK-99) primary stock, producing fortification levels of 0.250, 25.0 and 70.0 µg/L (six replicates at each concentration), and 0.0600, 0.250, 25.0 and 70.0 µg/L (six replicates at each concentration), respectively. An additional six freshwater and six seawater samples (1000 mL) were left unfortified to be utilized as control samples.

The freshwater fortification levels of Pyrenone Crop Spray (Sample Ref. 13650) produced were 0.500, 25.0 and 70.0 µg/L (three replicates at each concentration). An additional three freshwater samples (1000 mL) were left unfortified to be utilized as control samples.

2.5.3 Acetone Method Validation. Recovery stock samples prepared in acetone, were prepared by diluting the Pyrethrum Extract (FEK-99) primary stock producing a quantification level of 0.250 mg/L. An additional six solvent samples were left unfortified to be used as control samples.

2.5.4 Tissue Method Validation. Tissue (fathead minnow) samples (approximately 0.5 grams) were fortified at nominal concentrations of approximately 200 mg/kg (nine replicates).

2.6 Extraction

2.6.1 Aqueous. All preliminary method validation aqueous samples were extracted two times with hexane using silanized extraction glassware. After each addition of hexane, the sample was shaken for approximately two minutes by hand and the phases allowed to separate. The organic phase (hexane) was drained through anhydrous sodium sulfate (prerinsed with hexane) to remove residual water. The extract was then collected into an appropriately sized silanized round bottom flask. For those samples having a concentration factor less than 0.004, the extract was collected into an appropriately sized non-silanized round bottom flask and the volume of the extract was reduced to dryness using rotary evaporation. The remaining residue was diluted with an appropriate volume of hexane and an aliquot was removed for analysis by gas chromatography utilizing electron capture detection (GC-ECD).

For samples having a concentration factor of 0.004 or less (freshwater: 0.250 µg/L and the controls, seawater: 0.250, 0.0600 µg/L and the controls), the residues were dissolved in acetonitrile and an equal volume of reagent grade water. Matrix interferences were removed from the sample using solvent prepared C-18 Solid Phase Extraction (SPE) clean-up procedures. The C-18 solid phase columns were conditioned with 2 column volumes of acetonitrile followed by 2 column volumes of reagent grade water. Following addition of the samples to the C-18 columns, the columns were washed with 200 mL reagent grade water and dried for 10 minutes under vacuum. The test material was then eluted off of the column with 10 mL of acetone. The extracts were then reduced to dryness under a gentle stream of nitrogen, and then brought to final volume using hexane. An aliquot was removed from each sample and placed in a silanized GC vial for analysis by gas chromatography utilizing electron capture detection (GC-ECD).

2.6.2 Tissue. Homogenized tissue samples were thoroughly vortexed for two minutes following the addition of 10.0 mL of 50:50 acetonitrile:reagent grade water and then centrifuged for 5 minutes at 3000 RPM. An aliquot of the supernatant (5.0 mL) was transferred to a second

silanized test tube and extracted with 10.0 mL of hexane and vortexed thoroughly. The phases were allowed to separate and an aliquot of the hexane was removed for analysis by gas chromatography utilizing electron capture detection (GC-ECD).

3.0 ANALYSIS

3.1 Preparation of Standard Curve

3.1.1 Preliminary Analysis. Calibration standards were prepared using the primary Pyrethrin 1 (Code NK 9212) stock solution (34.0 mg/L). The concentrations of the standards prepared were 51.0, 102, 255, 510 and 748 µg/L. Two sets of standards, and solvent (hexane) blanks were analyzed with each sample set, one prior to analysis of the samples and one immediately following the samples. Injection of samples and standards onto the chromatographic system was performed by programmed injection.

A standard curve was constructed by plotting the peak height of Pyrethrin 1 (Code NK 9212) against the concentration (µg/L) of the standard injected. The correlation coefficient, slope, y-intercept and the limit of quantitation were calculated. The concentration of Pyrethrin 1 (Code NK 9212) in each sample was determined using the linear regression and the peak height of the sample.

3.1.2 Method Validation. Calibration standards were prepared from dilutions of the primary Pyrethrum Extract (FEK-99) and Pyrenone Crop Spray (Sample Ref. 13650) stocks (1.00 mg/mL). The concentrations of the standards were 50.0, 100, 250, 500 and 750 µg total pyrethrins/L. Two sets of standards, and solvent (hexane) blanks were analyzed with each sample set, one prior to analysis of the samples and one immediately following the samples. Injection of samples and standards onto the chromatographic system was performed by programmed injection.

A standard curve was constructed by plotting the total peak height of the three Pyrethrin 1 congeners (Pyrethrin 1, Cinerin 1 and Jasmolin 1) against the concentration (µg/L) of the standard injected. The correlation coefficient, slope, y-intercept and the limit of quantitation were

calculated. The concentration (total Pyrethrins) of Pyrethrum Extract (FEK-99) or Pyrenone Crop Spray (Sample Ref. 13650) in each sample was determined using the linear regression and the total peak height of the three congeners Pyrethrin 1, Cinerin 1 and Jasmolin 1 in the sample.

Baseline construction and integration was performed automatically by the computer. Following analysis the chromatographic data was reviewed by the chemist; peak symmetry, peak resolution, retention time and signal to noise ratio were evaluated. When the computerized integration of the peaks of interest was not deemed to be an accurate representation, a new baseline was constructed and chromatogram reintegrated. Following acceptance of the chromatographic data, the chromatograms were batch processed and a total percent height report, totaling the three Pyrethrin 1 congener peaks was generated.

3.2 Instrumental Conditions

The gas chromatographic (GC) analysis was conducted utilizing the following instrumental conditions:

Column:	J & W, DB-1 (100% methyl silicone), 15 m (length) x 0.25 mm I.D., 0.1 µm film thickness
Gas flows:	Carrier gas - Helium, 1.5 to 2.5 mL/min.
	Make-up gas - Ar/CH_4, 62.0 mL/min.
Temperatures:	Injector - 220 °C
	Column - 100 to 270/300 °C, ramp 10 degrees per minute
	Detector - 300 °C
Injection Volume:	5 µL, 2 µL
Attenuation:	2^1, 2^0
Threshold:	1, 2, 4
Peak Width:	0.05
Retention Time:	Pyrethrin 1 ≈ 10.3 to 10.6 min.
	Cinerin 1 ≈ 11.0 to 11.3 min.
	Jasmolin 1 ≈ 11.2 to 11.6 min.

4.0 CALCULATIONS

The following equations were used to calculate measured concentrations and analytical results:

$$\frac{(\text{signal} - b)}{m} = DC$$

$$DC \times DF = A$$

where:

- signal = peak signal (height or total height) from chromatogram
- b = y-intercept from regression analysis
- m = slope from regression analysis
- DC = detected concentration (µg/L) in the diluted or extracted sample on GC
- DF = dilution factor (final volume of the diluted or extracted sample divided by the original aqueous volume, if appropriate)
- A = analytical result (µg/L), concentration in the original aqueous sample

The limit of quantitation (LOQ) was calculated using the following equation:

$$\frac{((0.5 \times A_{LS}) - b)}{m} = LOQ_{INST}$$

$$LOQ_{INST} \times DF_{CNTL} = LOQ$$

where:

- A_{LS} = The mean signal response of the low concentration standard (two injections)
- b = y-intercept from regression analysis
- m = slope from regression analysis
- LOQ_{INST} = The minimum detected level on the instrument (extract)
- DF_{CNTL} = The dilution factor of the control samples (smallest dilution factor used)
- LOQ = The minimum detectable concentration reported for samples in the regression analysis (limit of quantitation)