

Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pesticide Name: Pyrethrin

MRID #: 438841-02

Matrix: water

Analysis: GC/ECD

This method is provided to you by the Environmental Protection Agency's (EPA) Environmental Chemistry Laboratory (ECL). This method *is not* an EPA method but one which was submitted to EPA by the pesticide manufacturer to support product registration. EPA recognizes that the methods may be of some utility to state, tribal, and local authorities, but makes no claim of validity by posting these methods. Although the Agency reviews *all* Environmental Chemistry Methods submitted in support of pesticide registration, the ECL evaluates only about 30% of the currently available methods. Most methods perform satisfactorily but some, particularly the older methods, have deficiencies. Moreover, the print quality of the methods varies considerably because the methods originate from different sources. Therefore, the methods offered represent the best available copies.

If you have difficulties in downloading the method, or further questions concerning the methods, you may contact Elizabeth Flynt at 228-688-2410 or via e-mail at flynt.elizabeth@epa.gov.

**DEVELOPMENT AND VALIDATION OF AN
ANALYTICAL METHODOLOGY FOR THE
DETERMINATION OF TOTAL PYRETHRINS
IN WATER, ACETONE AND FISH TISSUE**

438841-02

Submitted to:

**Pyrethrin Joint Venture/
Chemical Specialties Manufacturer's Association
1913 Eye Street, Northwest
Washington, DC 20006**

SLI Report No. 93-9-4922

Study No. 11572.0193.6105.250

Study Director: Joseph P. St. Laurent

**Springborn Laboratories, Inc.
Environmental Sciences Division
790 Main Street
Wareham, Massachusetts 02571**

19 December 1995

FINAL REPORT

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1)(A), (B), or (C).

Company: Pyrethrin Joint Venture/CSMA

Company Agent: Ralph Engel
(Typed Name)

Date: 12/29/95

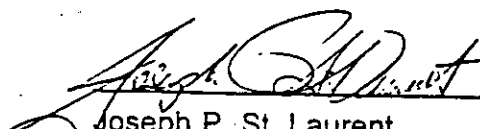
President, CSMA
(Title)

Stephen S. Kellner
vice President CSMA
for Ralph Engel
(Signature)

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The data and report presented for "Development and Validation of an Analytical Methodology for the Determination of Total Pyrethrins in Water, Acetone and Fish Tissue" were produced and compiled by SLI in accordance with all pertinent EPA Good Laboratory Practice Regulations (40 CFR, Part 160) with the following exception: the stability of the Pyrethrum 1 (Code NK 9212) was not determined prior to the experimental start date or concomitantly with the study conduct. Stability, characterization, and verification of the test substance identity and maintenance of records on the test substance are the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES, INC.

 12/15/95
Joseph P. St. Laurent Date
Study Director



 Vice President CSMA
 12/29/95
Ralph Engel, President Date
Chemical Specialties
Manufacturers Association
Sponsor/Submitter

TABLE OF CONTENTS

	PAGE
STATEMENT OF <u>NO</u> DATA CONFIDENTIALITY CLAIMS	2
GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT	3
LIST OF TABLES	6
LIST OF FIGURES	7
SUMMARY	9
1.0 INTRODUCTION	11
2.0 MATERIALS AND METHODS	11
2.1 Protocol and Amendment	11
2.2 Test Substance	11
2.2.1 Pyrethrum Extract (FEK-99)	11
2.2.2 Pyrenone Crop Spray	12
2.2.3 Pyrethrin 1 (Code NK 9212)	12
2.3 Test System	13
2.3.1 Equipment	13
2.3.2 Reagents	14
2.4 Preparation of Stock Solutions	14
2.5 Sample Fortification	15
2.5.1 Preliminary	15
2.5.2 Aqueous Method Validation	15
2.5.3 Acetone Method Validation	15
2.5.4 Tissue Method Validation	16
2.6 Extraction	16
2.6.1 Aqueous	16
2.6.2 Tissue	16
3.0 ANALYSIS	17
3.1 Preparation of Standard Curve	17
3.1.1 Preliminary Analysis	17
3.1.2 Method Validation	17
3.2 Instrumental Conditions	18
4.0 CALCULATIONS	19
5.0 RESULTS AND DISCUSSION	20
PROTOCOL DEVIATIONS	23
QUALITY ASSURANCE UNIT STATEMENT	24
TABLES	25

FIGURES	36
SIGNATURES AND APPROVAL	53
7.0 APPENDIX I - STUDY PROTOCOL AND AMENDMENTS	54
8.0 APPENDIX II - CERTIFICATE OF ANALYSIS - PYRETHRUM EXTRACT	73
9.0 APPENDIX III - CERTIFICATE OF ANALYSIS - PYRENONE CROP SPRAY	77
10.0 APPENDIX IV - CERTIFICATE OF ANALYSIS - PYRETHRIN 1 (Code NK 9212) ..	80
11.0 APPENDIX V - DILUTION WATER CHARACTERIZATION - FRESHWATER AND SEAWATER	84

LIST OF TABLES

Page

Table 1.	Chromatographic analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the freshwater fortified recovery study samples	26
Table 2.	Analytical results for the recovery of Pyrethrum Extract (FEK-99) from freshwater (reconstituted to increase hardness)	27
Table 3.	Chromatographic analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the seawater fortified recovery study samples	28
Table 4.	Analytical results for the recovery of Pyrethrum Extract (FEK-99) from seawater (filtered through a 0.5 μ fiber filter)	29
Table 5.	Chromatographic analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the acetone fortified recovery study samples	30
Table 6.	Analytical results for the recovery of Pyrethrum Extract (FEK-99) from acetone	31
Table 7.	Chromatographic analysis of the analytical standards used to quantitate Pyrenone Crop Spray (Sample Ref. 13650) in the freshwater fortified recovery study samples	32
Table 8.	Analytical results for the recovery of Pyrenone Crop Spray (Sample Ref. 13650) from freshwater (reconstituted to increase hardness)	33
Table 9.	Chromatographic analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the fish tissue method validation	34
Table 10.	Analytical results for the recovery of Pyrethrum Extract (FEK-99) from fish tissue	35

LIST OF FIGURES

Page

Figure 1.	GC chromatogram of Pyrethrum Extract (FEK-99) in a 100 µg/L standard solution	37
Figure 2.	GC chromatogram of Pyrethrum Extract (FEK-99) in a 0.250 µg/L freshwater fortified sample	38
Figure 3.	GC chromatogram of a freshwater control sample	39
Figure 4.	The linear regression analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the freshwater fortified recovery study samples	40
Figure 5.	GC chromatogram of Pyrethrum Extract (FEK-99) in a 0.250 µg/L seawater fortified sample	41
Figure 6.	GC chromatogram of a seawater control sample	42
Figure 7.	The linear regression analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the seawater fortified recovery study samples	43
Figure 8.	GC chromatogram of Pyrethrum Extract (FEK-99) in a 1.0 mg/mL acetone fortified sample	44
Figure 9.	The linear regression analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the acetone fortified recovery study samples	45
Figure 10.	GC chromatogram of Pyrenone Crop Spray (Sample Ref. 13650) in a 100 µg/L standard solution	46
Figure 11.	GC chromatogram of Pyrenone Crop Spray (Sample Ref. 13650) in a 25.0 µg/L freshwater fortified sample	47
Figure 12.	The linear regression analysis of the analytical standards used to quantitate Pyrenone Crop Spray (Sample Ref. 13650) in the freshwater fortified recovery study samples	48
Figure 13.	GC chromatogram of Pyrethrum Extract (FEK-99) in a 10.0 mg/L standard solution	49

Figure 14.	GC chromatogram of Pyrethrum Extract (FEK-99) in a fortified tissue sample	50
Figure 15.	GC chromatogram of a tissue control sample	51
Figure 16.	The linear regression analysis of the analytical standards used to quantitate Pyrethrum Extract (FEK-99) in the fortified tissue recovery study samples	52

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. Freshwater recoveries averaged $86.4 \pm 7.4\%$ with a limit of quantitation of 0.0562 µg/L. Defined limits for acceptance of Quality Control sample performance in subsequent studies were three standard deviations from the mean recoveries obtained in method validation for Pyrethrum Extract (FEK-99). The range was 64.3 to 108%. Seawater recoveries averaged $100 \pm 11\%$ with a limit of quantitation of 0.0128 µg/L. Defined limits for acceptance of Quality Control sample performance in subsequent studies were three standard deviations from the mean recoveries obtained in method validation for Pyrethrum Extract (FEK-99). This range was 66.5 to 134%.

Acetone samples were fortified at a concentration of 1.00 mg/mL. Acetone recoveries averaged $99.8 \pm 7.5\%$, providing an expected range of recovery for analysis of stock solutions utilized during subsequent toxicological studies.

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. Recoveries from fathead minnow tissue averaged $85.2 \pm 7.7\%$ with a limit of quantitation of approximately 5 mg/kg. Defined limits for acceptance of Quality Control sample performance in subsequent studies were three standard deviations for the mean recovery. This range was 62.1 to 108%.

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 ($< 0^{\circ}\text{C}$) 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 $\mu\text{g/L}$.

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 $\mu\text{g/L}$ (six replicates at each concentration), and 0.0600, 0.250, 25.0 and 70.0 $\mu\text{g/L}$ (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 $\mu\text{g/L}$ (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 $\mu\text{g/L}$. 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 ($\mu\text{g/L}$) 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 ($\mu\text{g/L}$) 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 μ m 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 μ L, 2 μ L
Attenuation:	2 ¹ , 2 ⁰
Threshold:	1, 2, 4
Peak Width:	0.05
Retention Time:	Pyrethrin 1 \approx 10.3 to 10.6 min. Cinerin 1 \approx 11.0 to 11.3 min. Jasmolin 1 \approx 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 ($\mu\text{g/L}$) 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 ($\mu\text{g/L}$), 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)

5.0 RESULTS AND DISCUSSION

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. The methodology was developed and validated to support aquatic toxicological testing with both Pyrethrin Extract (FEK-99) and Pyrenone Crop Spray (Sample Ref. 13650) in hard reconstituted water, soft water, filtered seawater and unfiltered seawater. Results were used to evaluate Quality Control samples prepared during the toxicological studies. Due to the highly absorptive nature of pyrethrins, all glassware used during extraction and analysis of the method validation and subsequent samples was silanized unless otherwise indicated.

Initially, two samples of each of the four matrices (hard reconstituted water, soft reconstituted 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 mean (standard deviation) recoveries of $86.8 \pm 2.03\%$. Statistical analysis, using Allen's Criteria, indicated that the extraction efficiencies were identical for all four matrices. In addition, the relative extraction efficiency of Pyrenone Crop Spray (Sample Ref. 13650) was evaluated in freshwater (hard reconstituted water) using the same procedures developed in the extraction of Pyrethrum Extract (FEK-99). Statistical comparison of recoveries of Pyrethrum Extract (Sample Ref. 13650) from hard reconstituted water were evaluated using a two sample, two tailed Welch's T-test. Results of this analysis indicated that there was no statistical difference between the extraction efficiencies of Pyrethrum Extract (FEK-99) and Pyrenone Crop Spray (Sample Ref. 13650). 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). Initial chromatographic results indicated a peak which co-eluted with one of the Pyrethrin II congeners. The peak was not present in the appropriate proportion to the other components of the chromatogram and was determined to be a contaminant. The presence of this

contaminant peak resulted in artificially high analytical results, especially samples with lower concentrations. Due to chromatographic interference and following consultation with the Study Sponsor's Representative, the quantitation of total pyrethrins was based on the combined area of the Pyrethrin I congeners (Pyrethrin 1, Cinerin 1 and Jasmolin 1).

Freshwater recoveries averaged $86.4 \pm 7.4\%$ with a limit of quantitation of $0.0562 \mu\text{g/L}$. Defined limits of acceptance of Quality Control sample performance in subsequent studies were three standard deviations from the mean recoveries obtained in method validation for Pyrethrum Extract (FEK-99) and Pyrenone Crop Spray (Summary Ref. 13650). This range was 64.3 to 108%. Seawater recoveries averaged $100 \pm 11\%$ with a limit of quantitation of $0.0128 \mu\text{g/L}$. Defined limits for acceptance of Quality Control sample performance in subsequent studies with Pyrethrum Extract (FEK-99) and Pyrenone Crop Spray (Summary Ref. 13650) were three standard deviations from the mean recoveries obtained in method validation for Pyrethrum Extract (FEK-99). This range was 66.5 to 134%. Tissue recoveries averaged $85.2 \pm 7.7\%$. Defined limits for acceptance of Quality Control sample performance in subsequent studies would be three standard deviations from the mean recoveries obtained in method validation. This range is 62.1 to 108%.

The Limit of quantitation (LOQ) may vary slightly from one analysis interval to another, since it is dependent upon the linear regression of the standards and the peak response (height) of the low standards. These parameters, while relatively constant, do vary somewhat among analyses and produce small variations in the LOQ.

The chromatographic analysis of the Pyrethrum Extract (FEK-99) analytical standards analyzed during freshwater method validation is presented in Table 1. Analytical results for the recovery of Pyrethrum Extract (FEK-99) from freshwater are presented in Table 2. The chromatographic analysis of the Pyrethrum Extract (FEK-99) analytical standards analyzed during seawater method validation is presented in Table 3. Analytical results for the recovery of Pyrethrum Extract (FEK-99) from seawater are presented in Table 4. The chromatographic analysis of the Pyrethrum Extract (FEK-99) analytical standards analyzed during acetone method

validation is presented in Table 5. Analytical results for the recovery of Pyrethrum Extract (FEK-99) from acetone are presented in Table 6. The chromatographic analysis of the Pyrenone Crop Spray (Sample Ref 13650) analytical standards analyzed during the preliminary study is presented in Table 7. Analytical results for the recovery of Pyrenone Crop Spray (Sample Ref. 13650) from freshwater are presented in Table 8. The chromatographic analysis of the Pyrethrum Extract (FEK-99) analytical standards analyzed during the tissue method validation study is presented in Table 9. Analytical results for the recovery of Pyrethrum Extract (FEK-99) from fish tissue are presented in Table 10. A representative chromatogram showing the analysis of Pyrethrum Extract (FEK-99) in one of the standards is shown in Figure 1. A representative chromatogram showing the recoveries of Pyrethrum Extract (FEK-99) from one of the fortified freshwater samples is shown in Figure 2. The analysis of a freshwater control is presented in Figure 3. A typical linear regression analysis for Pyrethrum Extract (FEK-99) freshwater standards is presented in Figure 4. A representative chromatogram showing the recoveries of Pyrethrum Extract (FEK-99) from one of the fortified seawater samples is shown in Figure 5. The analysis of a seawater control is presented in Figure 6. A typical linear regression analysis for Pyrethrum Extract (FEK-99) seawater standards is presented in Figure 7. A representative chromatogram showing the analysis of Pyrethrum Extract (FEK-99) in one of the fortified acetone samples is shown in Figure 8. A typical linear regression analysis for Pyrethrum Extract (FEK-99) acetone standards is presented in Figure 9. A representative chromatogram showing the analysis of Pyrenone Crop Spray (Sample Ref. 13650) in one of the standard solutions is shown in Figure 10. A representative chromatogram showing the recoveries of Pyrenone Crop Spray (Sample Ref. 13650) from one of the fortified freshwater samples is shown in Figure 11. A typical linear regression analysis for Pyrenone Crop Spray (Sample Ref. 13650) standards is presented in Figure 12. A representative chromatogram showing the analysis of Pyrethrum Extract (FEK-99) in one of the standard solutions is shown in Figure 13. A representative chromatogram showing the recoveries of Pyrethrum Extract (FEK-99) from one of the fortified tissue samples is shown in Figure 14. The analysis of a tissue control is presented in Figure 15. A typical linear regression analysis for Pyrethrum Extract (FEK-99) standards is presented in Figure 16.