American Petroleum Institute

Health and Environmental Sciences Department

STEP Strategies for Today's Environmental Partnership

Tert-Amyl Methyl Ether (TAME)—Acute Toxicity to Rainbow Trout *(Oncorhynchus mykiss)* Under Flow-through Conditions

TSCA Guideline §797.1400

FEBRUARY 1995

TOXICOLOGY REPORT NUMBER 408
CAIS ABSTRACT NO. 42-1521

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QUALITY ASSURANCE/GLP COMPLIANCE STATEMENT

Study Title: Tert-Amyl Methyl Ether (TAME) - Acute Toxicity to Rainbow Trout *(Oncorhynchus mykiss)* Under Flow-Through Conditions

Testing Facility: Springborn Laboratories, Inc.

SLI Study Number: 12827.0692.6104.108

This study was reviewed by API Quality Assurance personnel under the direction of API Management on the dates indicated below for compliance with EPA (TSCA) Good Laboratory Practice (GLP) regulations. These studies were conducted in accordance with EPA GLP regulations, with the exceptions* listed below, as well as those listed on the Springborn GLP Compliance Statement.

Copies of reports by API Quality Assurance personnel are available upon written request to the Director of the Health and Environmental Sciences Department of the American Petroleum Institute or his designee.

Date(s) of Inspection/Review	Type of Inspection	Date of Report to Management
8/28/92	Protocol Evaluation	8/28/92
5/5/93	Draft Report Audit	5/5/93
4/27-28/94	Draft Report Audit	4/29/94
10/15/94	Revised Final Draft Report Acceptance	10/15/94
12/20/94	Final Report Acceptance	12/20/94

Christine Sexsmith
Quality Assurance Coordinator

12/20/94
Date

*Test article characterization, other than that received by the manufacturer (98.8% pure TAME) was not performed, storage stability for this test article is not known, and the method of fabrication is maintained with the test article supplier.

TERT-AMYL METHYL ETHER (TAME) - ACUTE TOXICITY TO RAINBOW TROUT (*Oncorhynchus mykiss*) UNDER FLOW-THROUGH CONDITIONS

TSCA GUIDELINE § 797.1400

Submitted to:

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1220 L Street, Northwest
Washington, D.C. 20005

SLI Report # 93-3-4682

Study # 12827.0692.6104.108

Study Director: Mark W. Machado

Springborn Laboratories, Inc.
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19 December 1994

FINAL REPORT

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

The data and report presented for "**Tert-Amyl Methyl Ether (TAME) - Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Flow-Through Conditions**" were produced and compiled in accordance with all pertinent EPA Good Laboratory Practice Regulations (40 CFR, Part 792) with the following exceptions: routine water and food contaminant screening analyses for pesticides, PCBs and metals are conducted using standard U.S. EPA procedures by Lancaster Laboratories, Lancaster, PA. These data were not collected in accordance with Good Laboratory Practice procedures (i.e., no distinct protocol, Study Director, etc.). Storage stability, characterization and verification of the test substance identity and maintenance of these records on the test substance are the responsibility of the Study Sponsor. At the termination of the testing program, all remaining test material will be sent to the Study Sponsor. Archival of a sample of the test material is the responsibility of the Study Sponsor.

SPRINGBORN LABORATORIES, INC.

Mark W. Machado 2/19/94

Mark W. Machado Date
Study Director

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SUMMARY

The purpose of this study was to estimate the acute toxicity (LC50) of Tert-Amyl Methyl Ether (TAME) to rainbow trout (*Oncorhynchus mykiss*) under flow-through conditions. The LC50 is defined as the concentration of the test material in dilution water which causes mortality of 50% in the exposed test population after a fixed period of time. Twenty organisms (ten per replicate) were exposed in duplicate test aquaria to each of five concentrations of TAME and a dilution water control for 96-hours. During the test, nominal concentrations of 950, 570, 340, 210 and 120 mg A.I./L were maintained by introducing approximately 6.5 aquarium volumes per day of newly prepared test solution via a modified constant-flow serial diluter apparatus (Benoit, 1982). Each replicate solution was sampled and analyzed for TAME concentration at 0-hour (test initiation) and 96-hours (test termination) of exposure. Based on the results of these analyses, the mean measured exposure concentrations were defined as 640, 560, 310, 150 and 78 mg A.I./L. Biological observations and observations of the physical characteristics of the exposure solutions were made and recorded at test initiation and every 24 hours thereafter until the test was terminated. Throughout the exposure period, treatment level solutions were observed to be clear and colorless and contained no visible sign of undissolved test material (e.g., precipitate).

Following 72-hours of exposure, 100% mortality was observed among fish exposed to the highest mean measured concentration tested (640 mg A.I./L). At test termination (96-hours), mortality of 30% was observed among fish exposed to the 560 mg A.I./L treatment level. In addition, sublethal effects (e.g., loss of equilibrium, darkened pigmentation) were observed among all of the surviving fish exposed to this treatment level. No mortality or sublethal effects were observed among fish exposed to the remaining concentrations tested (310, 150 and 78 mg A.I./L). The LC50 values and the 95% confidence intervals determined throughout the exposure period are summarized in the following table. The No-Observed-Effect Concentration (NOEC) established during this study was 310 mg A.I./L.

TEST RESULTS

LC50 (mg A.I./L)[ab]				No-Observed-Effect Concentration Through 96 Hours (mg A.I./L)[a]
24-Hour[c]	48-Hour[c]	72-Hour[d]	96-Hour[de]	
600 (580 - 620)	600 (570 - 620)	580 (560 - 640)	580 (310 - 640)	310

a Based on mean measured concentrations of TAME (as active ingredient).
b Corresponding 95% confidence interval is presented in parentheses.
c LC50 value and 95% confidence interval calculated by probit analysis.
d LC50 value estimated by nonlinear interpolation; 95% confidence interval calculated by binomial probability.
e Since the 96-hour LC50 was not less than 50% of the 48-hour value, the study was not extended beyond 96-hours to determine the incipient LC50.

1.0 INTRODUCTION

The purpose of this study was to determine the acute toxicity of Tert-Amyl Methyl Ether (TAME) to rainbow trout (*Oncorhynchus mykiss*) under flow-through conditions. The LC50 is defined as the concentration of the test material in dilution water which causes mortality of 50% in the exposed test population after a fixed period of time. This value is often used as a relative indicator of the potential acute hazards resulting from release of the test material into aquatic environments. The study was initiated on 14 October 1992, 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 96-hour definitive flow-through toxicity test was conducted from 27 February - 3 March 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 SLI.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this acute toxicity test followed those described in the SLI protocol entitled "Protocol for Conducting a Flow-Through Acute Toxicity Test with Rainbow Trout following TSCA 797.1400", SLI Protocol #:091192/TSCA 797.1400 and Protocol Amendments #1 and #2 dated 10 and 24 March 1993, respectively (Appendix I). The methods described in this protocol generally follow the standard procedures outlined in the EPA/OTS guideline (§ 797.1400) for acute toxicity testing with fish (U.S. EPA, 1985, amended 1987).

2.2 Test Material

Two samples of Tert-Amyl Methyl Ether (TAME) (CAS # 994-05-8), a clear liquid, were received from Experimental Pathology Labs, Inc., Herndon, Virginia. The first sample, Lot # 02814BZ, was received at SLI on 17 August 1992 and was used to prepare analytical standards during the method validation/recovery study and to prepare Quality Control samples during the definitive exposure. The sample was identified by Aldrich Chemical to contain 98.8% active ingredient A.I. (Certificate of Analysis, Appendix II). The second sample, Lot # 07905KZ, was

received at SLI on 2 November 1992 and was used to prepare exposure solutions during the preliminary and definitive exposures. The sample was identified by Aldrich Chemical to contain 98.7% active ingredient A.I. (Certificate of Analysis, Appendix II). Upon receipt at SLI, the samples of test material were stored in a dark, ventilated cabinet at room temperature (approximately 20 °C). Test concentrations are expressed as milligrams of test material (as active ingredient) per liter of test solution and are reported as mg A.I./L.

At the request of the Study Sponsor, mass spectral analysis was conducted on the initial batch of TAME received at program initiation, and the additional batches received throughout the course of the program. The purpose of the mass spectral analysis evaluation was to determine test material integrity throughout the duration of the program. Initial evaluation of test material (i.e., lot # 02814BZ) was conducted on 3 December 1992. Following completion of the flow-through acute toxicity test with mysids (SLI Report # 94-5-5269), spectral analysis was conducted on 20 July 1994 on each of the remaining two lots (lot # 02814BZ and lot # 07905KZ). The spectral analysis conducted on 20 July 1994 on the two remaining lots in comparison to the initial spectral analyses of lot # 02814BZ established that negligible change in test material composition had occurred during storage at Springborn Laboratories, Inc. (i.e., approximately 24 months).

2.3 Test Organisms

Rainbow trout (*Oncorhynchus mykiss*) were selected as the test species since it is a recommended species (U.S. EPA, 1985) and a commonly used cold water fish in flow-through freshwater fish toxicity tests. The rainbow trout (SLI lot #93B3) used during the study were obtained from Spring Creek Trout Hatchery, a commercial supplier located in Lewistown, Montana. Prior to testing, these fish were held in a 500-L fiberglass tank under a photoperiod of 16 hours light and 8 hours darkness. The well water which flowed into this holding tank was characterized as having total hardness and total alkalinity ranges as calcium carbonate ($CaCO_3$) of 25 - 27 mg/L and 20 - 22 mg/L, respectively, and a specific conductance of 110 μmhos/cm (Gravity Feed Tank Water Quality Log). Other parameters monitored in the holding tank were pH with a range of 6.8 - 6.9, dissolved oxygen concentration with a range of 80 - 87% of saturation

and the flow rate with 8.2 - 11 tank volume replacements/day (Weekly Record of Fish Holding Characteristics). Fish used during the definitive exposure were maintained under similar conditions for a minimum of 14 days prior to testing. The temperature in the holding tank ranged from 11 - 12 °C during this 14-day period. All fish were fed a dry commercial pelleted food, *ad libitum*, daily except during the 48 hours prior to, and during the definitive test. Representative samples of the food source were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix III). Food sources were considered to be of acceptable quality since the total concentration of pesticides was less than 0.3 mg/kg. Mortality of 0.20% was observed in the test fish population during the two days prior to testing (Daily Record of Fish Holding Conditions). The rainbow trout used during this study were all of the same year class and a representative sample (N = 30) had a mean (range) wet weight and total length of 0.47 (0.28 - 0.75) grams and 37 (30 - 48) millimeters, respectively (Fish Lengths and Weights Log).

2.4 Test Dilution Water

The dilution water used during this study was from the same source as the water which flowed into the fish holding tank and was characterized as having total hardness and total alkalinity (as $CaCO_3$) ranges of 20 - 22 and 25 - 27 mg/L, respectively, a pH range of 6.9 - 7.0 and a specific conductance of 110 μmhos/cm (Gravity Feed Tank Water Quality Log). Representative samples of the dilution water source were analyzed for the presence of pesticides, PCBs and toxic metals (Appendix IV). None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM standard practice. In addition, representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. The results of these analyses demonstrated that the TOC concentration of the dilution water ranged from 0.82 - 1.3 mg/L for the months of September 1992 - February 1993 (TOC and TSS Master Log, Vol 1). Several species of daphnids (a representative freshwater organism generally recognized to be sensitive to chemical challenges) are maintained in water from the same source as the dilution water utilized in this study and have successfully survived and reproduced over several generations. This, in combination with the previously mentioned analyses, confirms the acceptability of this dilution water for bioassays.

2.5 Test Conditions

The toxicity test was conducted using an exposure system consisting of a constant-flow serial diluter (Benoit, 1982), with a 60% dilution factor, a temperature-controlled water bath and a set of 12 exposure aquaria. The test system was designed to provide five concentrations of the test material and a dilution water control. Calibration of the diluter system was confirmed prior to test initiation and again subsequent to test termination. All treatment levels and the controls were maintained in duplicate. Each glass test aquarium measured 39 x 20 x 25 centimeters (cm) with a 14.5 cm high standpipe which maintained a constant test solution volume of 11 L. The flow of exposure solutions to each test aquarium was approximately 50 mL/min, which provided approximately 6.5 volume replacements per aquarium every 24 hours. Test aquaria were labeled to identify the nominal test concentration and designated replicate. Test vessels were not covered during the exposure period. Test vessels were impartially positioned in a water bath containing circulating water cooled by a Frigid Unit® chiller designed to maintain the test solution temperatures at 12 ± 1° C. Test solutions were not aerated. A photoperiod of 16 hours light and 8 hours dark provided light with an intensity of 30 - 80 footcandles at the test solution surface throughout the study period. Sudden transitions from light to dark and vice versa were avoided. Lighting was provided by Duro-Test® Vitalite fluorescent bulbs.

2.6 Test Concentrations

Selection of nominal TAME concentrations for the 96-hour flow-through definitive toxicity test with rainbow trout was based on toxicity information developed at SLI through preliminary testing.

2.7 Test Solution Preparation and Delivery

During the exposure period, the test material was delivered directly to each replicate test aquarium for each treatment level. The concentration of test material that was delivered to each aquarium was determined to be 759.99 mg A.I./mL based on the test material's density (0.770 g/mL) and percent active ingredient (98.7% A.I.). A series of Sage syringe pumps in conjunction with Glenco® gas-tight syringes were calibrated to deliver the appropriate amount of test material (759.99 mg A.I./mL) directly into the delivery tube for each replicate aquarium. Each

individual delivery tube also received 0.050 L/min of dilution water. Delivery of the test material and dilution water at this point in the system aided in the mixing and solubilization of the test material. Proportional dilution (60%) across the range of nominal concentrations established (i.e., 950, 570, 340, 210 and 120 mg A.I./L) was accomplished by adjusting the size of syringes used (i.e., 20 - 50 mL) and the flow of each pump (0.0625, 0.0375, 0.0225, 0.0135 and 0.0081 mL/min).

The diluter system was calibrated prior to test initiation and at test termination by measuring delivery volumes of toxicant and dilution water. The function of the diluter system (e.g., flow rates, stock consumption) was monitored daily and a visual check was performed twice daily. In addition, analysis of the exposure solutions for TAME concentration was also used to verify proper operation of the diluter system. The exposure system was in proper operation for several days prior to test initiation to allow equilibration of the test material in the diluter apparatus and exposure vessels.

3.0 TEST PROCEDURES

3.1 Test Initiation

The test was initiated when rainbow trout were impartially selected and distributed two at a time to each replicate aquarium until each replicate contained 10 fish (20 fish per treatment level and the control). At any given time during the exposure period, the maximum organism loading concentration was 0.065 g of biomass per liter of flowing test solution per day.

3.2 Test Monitoring

The diluter was visually inspected at least twice daily during the definitive exposure period. Biological observations of the exposed rainbow trout and observations of the physical characteristics of the test solutions (e.g., precipitate, film on the solution's surface) were made at test initiation and at each subsequent 24-hour interval until test termination (96-hours). Effects for this study were based on death, defined as the lack of movement by the exposed organisms (i.e., absence of gill movement and reaction to gentle prodding). Mortalities were recorded and removed from each aquarium every 24 hours during the exposure period.

3.3 Water Quality Measurements

Dissolved oxygen concentration, temperature and pH were measured once daily in each replicate of each treatment level and the controls throughout the exposure period. The pH was measured using a Jenco Model 601A pH meter and combination electrode; the dissolved oxygen concentration was measured with a YSI Model #57 dissolved oxygen meter and probe and the daily solution temperature was measured with a Brooklyn alcohol thermometer. Light intensity was measured with a General Electric Model 214 light meter. Test solution temperature was continuously monitored in one replicate (B) of the dilution water control solution using a Brooklyn Min/Max thermometer.

3.4 Analytical Measurements

Both replicate solutions of the high, middle and low treatment levels and the control were sampled and analyzed for TAME concentration prior to the start of the definitive exposure. Results of these pretest analyses were used to judge whether sufficient quantities of test material were being delivered and maintained in the exposure aquaria to initiate the definitive test. During the in-life phase of the definitive study, water samples were removed from both replicate test solutions of each treatment level and the controls at 0- and 96-hours of exposure for analysis of TAME concentration. Each exposure solution sample was collected from the approximate midpoint of the aquarium with a volumetric pipet. In addition, three Quality Control (QC) samples were prepared at each sampling interval and remained with the exposure solution samples throughout the analytical process. These QC samples were prepared in dilution water at TAME concentrations similar to the exposure concentration range. Results of the analyses of the QC samples were used to judge the precision and quality control maintained during the analysis of exposure solution samples. All samples were analyzed for TAME using a gas chromatographic (GC) procedure according to the methodology described in Appendix V. A method validation study, conducted at SLI prior to the initiation of the definitive test, established an average recovery of TAME of 102 ± 10% from hard reconstituted water.

4.0 STATISTICS

The mean measured concentrations (0- and 96-hour analysis) and the corresponding mortality data derived from the definitive test were used to estimate the median lethal concentration (LC50) and 95% confidence interval at each 24-hour interval of the exposure period. The LC50 is defined as the concentration of the test material in dilution water lethal to 50% of the test animal population at the stated exposure interval. If at least one test concentration caused mortality of greater than or equal to 50% of the test population, then a computer program (Stephan, 1982, personal communication) was used to calculate the LC50 values and 95% confidence interval. Additionally, the data was evaluated to estimate an incipient LC50. The incipient LC50 is defined as the concentration that is lethal to 50% of a test population when exposure to the test substance is continued until the mean increase in mortality does not exceed 10% in any concentration over a 24-hour period. Since the 96-hour LC50 was not less than 50% of the estimated 48-hour LC50, the duration of the exposure was not continued to determine the incipient LC50 value (U.S. EPA, 1987).

Three statistical methods were available in the computer program: moving average angle analysis, probit analysis, and nonlinear interpolation with 95% confidence interval calculated by binomial probability. Moving average angle and probit analyses yield statistically sound results only if at least two concentrations produce a mortality of between 0 and 100% of the test organism population. The selection of reported LC50 values and 95% confidence interval was based upon an examination of the data base and the results of the computer analysis. Selection criteria included the establishment of a concentration-effect relationship (mortality), the number of concentrations causing partial responses, and the span of responses bracketing the LC50 value. If two or more statistical methods produced acceptable results, then the method which yielded the smallest 95% confidence interval was selected. The No-Observed-Effect Concentration (NOEC) during the 96-hour exposure period was also determined. The NOEC is defined as the highest concentration tested at and below which there were no toxicant-related mortalities or physical and behavioral abnormalities (e.g., lethargy, loss of equilibrium, darkened pigmentation), with respect to the control organisms.

5.0 RESULTS

5.1 Preliminary Testing

Prior to initiating the definitive study, several preliminary range-finding studies were conducted at SLI. During these preliminary studies, the test material was delivered to the test aquaria using conventional delivery methods in a Benoit-style diluter (i.e., mixing chamber, chemical cells). Test vessels were not covered during any of the exposure. In three separate tests, rainbow trout were exposed to nominal concentrations of TAME ranging from 570 - 15, 1100 - 89 and 1700 - 130 mg A.I./L. After 120 hours of exposure, no toxicant related mortality or sublethal effects were observed among fish exposed to 570 - 15 mg A.I./L treatment levels. Following 72 hours of exposure at treatment levels of 1100 - 89 mg A.I./L, 10% was observed among fish exposed to the highest treatment level. Surviving fish at this treatment exposure concentration (1100 mg A.I./L) were described as exhibiting darkened pigmentation and complete loss of equilibrium. During the third exposure, mortality of 80% was observed among fish exposed to the 1700 mg A.I./L treatment level following 24-hours of exposure. No mortality or sublethal effects were noted among organisms exposed to the remaining test concentrations.

Following the completion of the initial preliminary investigations, the diluter system and toxicant delivery method were modified in an attempt to compensate for the volatile nature of the test material and to maximize the measured to nominal test material concentration in solution. This modification consisted of eliminating the mixing chamber and chemical cells of the Benoit-style diluter and injecting the material directly into the delivery tubing for each replicate aquarium. Using this delivery method, rainbow trout were exposed to a single concentration of 950 mg A.I./L. Following 24-hours exposure, 100% mortality was observed among fish in the treatment level tested. Based on the results of these preliminary tests, it was determined that the definitive study would be conducted utilizing the non-conventional delivery of the test material to the exposure aquaria. Nominal concentrations chosen for the definitive study were 950, 570, 340, 210 and 120 mg A.I./L.

5.2 Definitive Test

The water quality parameters (pH, dissolved oxygen concentration and temperature) measured during the definitive study are presented in Table 1. All water quality parameters measured were unaffected by the concentrations of TAME tested and remained within acceptable ranges for the survival of rainbow trout. Daily monitoring of the test solutions established a temperature range of 11 - 12 °C throughout the exposure period. Continuous temperature monitoring of one replicate (B) of the control solution established that the test solution temperature ranged from 11 - 13 °C throughout the exposure period.

The diluter system which prepared and delivered the test solutions to the exposure aquaria functioned properly throughout the 96-hour study. Analyses of the exposure solutions during the pretest period established that the concentrations of TAME in the exposure solutions were generally consistent between replicate samples and that the delivery apparatus maintained the expected concentration gradient (approximately 60% dilution factor). Analyses of the pretest samples resulted in measured concentrations which averaged 65% of nominal. Throughout the exposure period, no visible signs of undissolved test material (e.g., precipitate) was observed in either the diluter system or in the exposure solutions.

The results of the analysis of the exposure solutions for TAME concentration during the in-life portion of the definitive exposure are presented in Table 2. Throughout the exposure period, analytical measurements between replicate solutions were generally consistent and established the expected concentration gradient (60%). Mean measured concentrations averaged 79% of the nominal concentrations and defined the treatment levels as 640, 560, 310, 150 and 78 mg A.I./L. Coefficients of variation averaged 12% for all mean measured concentrations. Analysis of the Quality Control (QC) samples during the definitive study resulted in measured concentrations which were consistent with the predetermined recovery range established during the method validation/recovery study (Appendix V) and averaged 110% of the nominal fortified concentration range (950 - 120 mg/L). Based on the results of these analyses, it was established that the appropriate quality control was maintained during the analyses of the exposure solutions.

The relationship between the nominal treatment levels and the mean measured concentrations established by the diluter apparatus during this study is illustrated in Figure 1.

The mean measured concentrations tested, the corresponding percent mortalities and the observations made during the definitive study are presented in Table 3. Following 72-hours of exposure, 100% mortality was observed among fish exposed to the highest mean measured concentration tested (640 mg A.I./L). At test termination (96-hours), mortality of 30% was observed among fish exposed to the 560 mg A.I./L treatment level. In addition, sublethal effects (e.g., loss of equilibrium, darkened pigmentation) were observed among all of the surviving fish exposed to this treatment level. No mortality or sublethal effects were observed among fish exposed to the remaining concentrations tested (310, 150 and 78 mg A.I./L). Based on these data, it was established that the effects observed during this study were clearly concentration-dependent. Figure 2 presents the 96-hour concentration-response (mortality) curve established for this study. The slope of this curve was calculated to be 5.7417. Table 4 summarizes the 24-, 48-, 72- and 96-hour LC50's and the 95% corresponding confidence interval. The 96-hour LC50 value for rainbow trout exposed to TAME was estimated by nonlinear interpolation to be 580 mg A.I./L (95% confidence intervals calculated by binomial probability of 310 - 640 mg A.I./L). The No-Observed-Effect Concentration (NOEC) for rainbow trout exposed to TAME was determined to be 310 mg A.I./L. An incipient LC50 was not estimated since the 96-hour LC50 value was not less than 50% of the 48-hour LC50 value. Copies of raw data used to establish the maintained exposure conditions (e.g., water quality, test material concentration analyses) and the concentration-effect response used to determine the reported LC50 and NOEC values for this study are presented in Appendix VI.

PROTOCOL DEVIATION

1. The study protocol states that dissolved oxygen concentration exceeds 90% of saturation at the initiation of the test. For this study, dissolved oxygen concentration ranged from 89 - 91% at test initiation.

2. The study protocol states that the dilution water used during this study had a total hardness range of 25 to 40 mg/L (as $CaCO_3$). During this study, the total hardness of the dilution water ranged from 20 to 22 mg/L ($CaCO_3$).

It is our opinion that these deviations did not effect the results or interpretation of this study.

SPRINGBORN LABORATORIES, INC.

Mark W. Machado
Study Director

12/19/94
Date

QUALITY ASSURANCE UNIT STATEMENT

The raw data and report **"Tert-Amyl Methyl Ether (TAME) - Acute Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) Under Flow-Through Conditions"** were inspected by the Springborn Laboratories, Inc., Environmental Sciences Division, Quality Assurance Unit (QAU) to assure compliance with the study protocol, laboratory standard operating procedures and the pertinent EPA Good Laboratory Practice Regulations. Dates of study inspections, dates reported to Study Director and to Management are listed below.

It is the opinion of the QAU that this report accurately reflects the raw data generated during this study.

Inspection Date	Reported to Study Director	Reported to Management
3/1/93	3/1/93	3/12/93
3/11/93	3/12/93	3/12/93
3/12/93	3/12/93	3/12/93
3/15/93	3/15/93	3/26/93
9/19/94	9/19/94	9/23/94
9/26-28/94	9/28/94	10/7/94
12/19/94	12/19/94	12/19/94

SPRINGBORN LABORATORIES, INC.

Patricia D. Royal 12/14/94
Patricia D. Royal Date
Manager, Regulatory Affairs
and Quality Assurance Unit

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U.S. EPA. 1989. Toxic Substance Control Act Test Guidelines (40 CFR, Part 792). Federal Register 54 (158): 34052-34074, August 17, 1989.

TABLES

Table 1. The water quality parameters measured during the 96-hour flow-through toxicity test exposing rainbow trout (*Oncorhynchus mykiss*) to TAME.

Nominal Concentration (mg A.I./L)	0-Hour A	0-Hour B	24-Hour A	24-Hour B	48-Hour A	48-Hour B	72-Hour A	72-Hour B	96-Hour A	96-Hour B
					pH					
950	7.1	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
570	7.1	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
340	7.1	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
210	7.1	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
120	7.1	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
Control	7.1	7.1	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
					Dissolved Oxygen Concentration, mg/L (% Saturation)					
950	9.8 (91)	9.6 (89)	9.6 (87)	9.4 (85)	9.0 (83)	9.3 (86)	9.8 (89)	9.6 (87)	9.0 (83)	9.1 (84)
570	9.6 (89)	9.7 (90)	9.6 (87)	9.6 (87)	9.2 (85)	9.0 (83)	9.7 (88)	9.4 (85)	9.1 (82)	8.9 (82)
340	9.8 (91)	9.8 (91)	9.6 (87)	9.4 (85)	9.3 (86)	9.4 (87)	9.6 (87)	9.6 (87)	9.1 (82)	9.1 (82)
210	9.8 (91)	9.7 (90)	9.5 (86)	9.4 (85)	9.4 (87)	9.4 (87)	9.8 (89)	9.7 (88)	9.6 (87)	9.0 (81)
120	9.8 (91)	9.8 (91)	9.4 (85)	9.6 (87)	9.0 (83)	9.2 (83)	9.6 (87)	9.7 (88)	8.9 (81)	9.0 (81)
Control	9.6 (89)	9.7 (90)	9.5 (86)	9.6 (87)	9.1 (84)	9.2 (83)	9.5 (86)	9.6 (87)	8.9 (82)	8.9 (81)
					Temperature (°C)[a]					
	12		11		11 - 12		11		11 - 12	

[a] Value presented represents the daily range of temperature measured (Brooklyn alcohol thermometer) in all test concentrations and the controls at the stated observation interval. Continuous monitoring of replicate B of the dilution water control established a test solution temperature range of 11 - 13 °C throughout the exposure period.

Table 2. **Concentrations of TAME measured in replicate (A,B) test solutions during the 96-hour flow-through exposure of rainbow trout (*Oncorhynchus mykiss*).**

Nominal Concentration (mg A.I./L)	0-Hour Measured Concentration (mg A.I./L)		96-Hour Measured Concentration (mg A.I./L)		Mean Measured Concentration[a] (mg A.I./L)
	A	B	A	B	
950	740	700	560	580	640 (87)
570	510	110[b]	560	610	560[c] (51)
340	280	340	320	280	310 (29)
210	150	150	160	140	150 (6.7)
120	56	89	76	93	78 (17)
Control	< 5.3	< 5.3	< 5.2	< 5.2	
QC #1[d]	1215 (950)[e]		898 (950)		
QC #2	357 (350)		454 (350)		
QC #3	128 (120)		118 (120)		

[a] Mean measured concentrations are presented with the standard deviations in parentheses and were calculated using the unrounded analytical results and not the rounded (two significant figures) values presented in this table.

[b] The lower than expected concentration for this sample is due to an error during the analytical process and is not considered representative of exposure conditions. This value was not included in the calculation of the mean measured concentration.

[c] N = 3

[d] QC = Quality Control sample.

[e] Value in parentheses represents the nominal fortified concentration for the corresponding QC sample.

Table 3. **Mean measured concentrations tested, corresponding mortalities and observations made during the 96-hour flow-through exposure of rainbow trout (*Oncorhynchus mykiss*) to TAME.**

Mean Measured Concentration (mg A.I./L)	Cumulative Mortality (%)											
	24-Hour			48-Hour			72-Hour			96-Hour		
	A	B	Mean	A	B	Mean	A	B	Mean	A	B	Mean
640	100	70	85[a]	100	70	85[d]	100	100	100	100	100	100
560	30	0	15[bc]	40	0	20[ce]	40	0	20[d]	40	20	30[d]
310	0	0	0	0	0	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0	0	0	0	0	0
78	0	0	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0	0	0

[a] All of the surviving fish exhibited complete loss of equilibrium,
[b] Several of the surviving fish exhibited complete loss of equilibrium.
[c] Several of the surviving fish were observed to be lethargic.
[d] All of the surviving fish exhibited darkened pigmentation and complete loss of equilibrium.
[e] Several of the surviving fish exhibited darkened pigmentation and complete loss of equilibrium.

Table 4. **The LC50 values (95% confidence interval) and No-Observed-Effect Concentration for rainbow trout (*Oncorhynchus mykiss*) exposed to TAME under flow-through conditions.**

LC50 (mg A.I./L)[ab]				No-Observed-Effect Concentration Through 96 Hours (mg A.I./L)[a]
24-Hour[c]	48-Hour[c]	72-Hour[d]	96-Hour[de]	
600 (580 - 620)	600 (570 - 620)	580 (560 - 640)	580 (310 - 640)	310

[a] Based on mean measured concentrations of TAME (as active ingredient).
[b] Corresponding 95% confidence interval is presented in parentheses.
[c] LC50 value (95% confidence interval) calculated by probit analysis.
[d] LC50 value estimated by nonlinear interpolation; 95% confidence interval calculated by binomial probability.
[e] Since the 96-hour LC50 value was not less than 50% of the 48-hour value, the study was not extended beyond 96-hours to determine the incipient LC50.

FIGURES

Figure 1. **Graphical illustration of the relationship between mean measured concentrations (analyses at 0- and 96-hours) and the nominal treatment levels established during the 96-hour flow-through exposure of rainbow trout (*Oncorhynchus mykiss*) to TAME.**

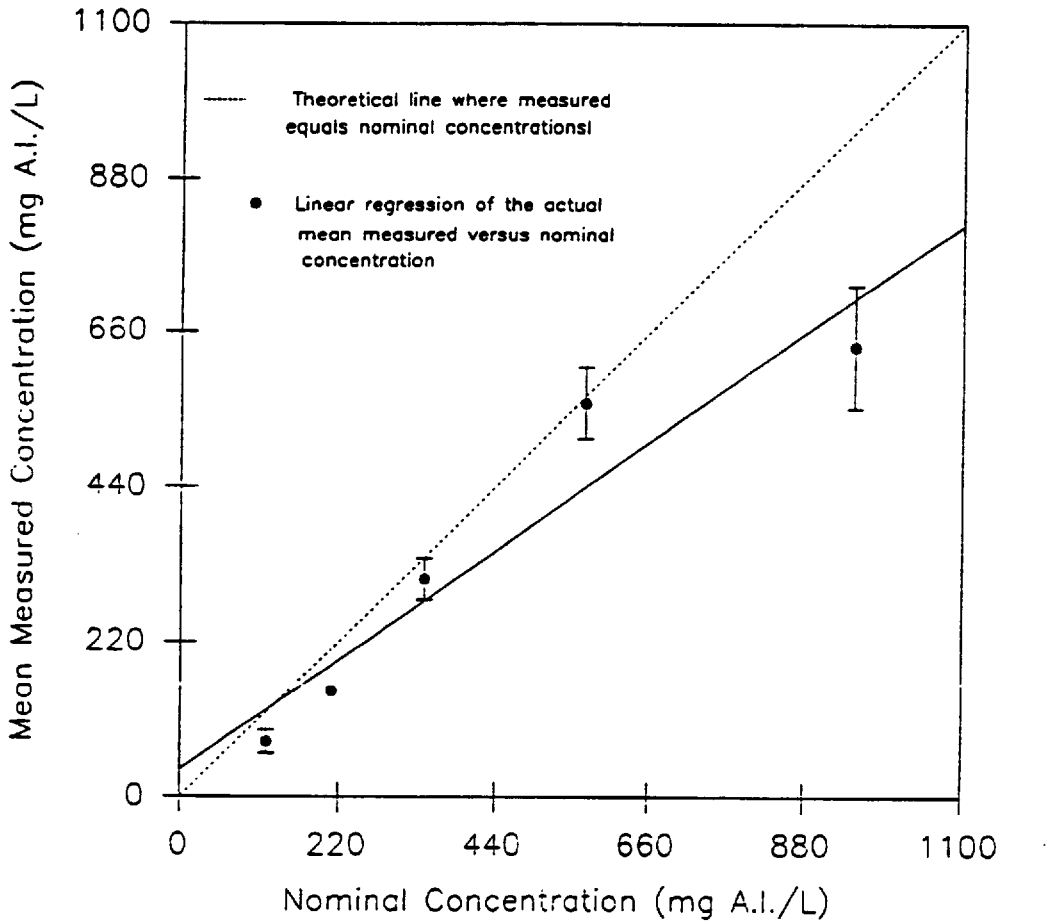
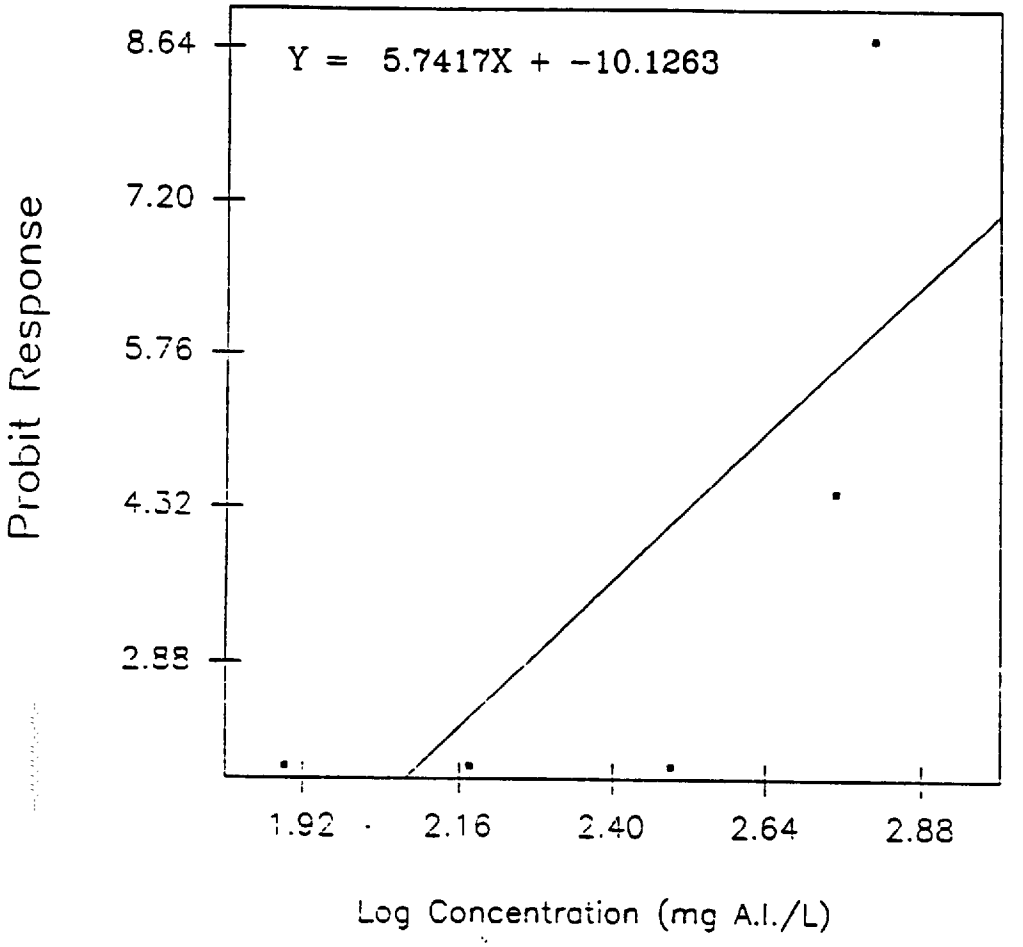


Figure 2. **The 96-hour concentration-response (mortality) curve for rainbow trout (*Oncorhynchus mykiss*) exposed to TAME.**



SIGNATURES AND APPROVAL

SUBMITTED BY:

Springborn Laboratories Inc.
Environmental Sciences Division
790 Main Street
Wareham, Massachusetts 02571-1075

PREPARED BY:

Mark W. Machado

Mark W. Machado 12/19/94

Study Director Date

Lisa M. Thibault

Lisa M Thibault 19 Dec 94

Coordinator, Data Management and Reporting Unit Date

Mark da Silva

Mark da Silva 12-19-94

Analytical Chemist Date

APPROVED BY:

Patricia D. Royal

Patricia D Royal 12/19/94

Manager, Regulatory Affairs and Quality Assurance Unit Date

Donald C. Surprenant

Robert B. Foster for DCS 12/19/94

Program Manager, Environmental Toxicology Date

This final report has been signed in accordance with SLI SOP No. 4.3.07(1).

6.0 APPENDIX I - STUDY PROTOCOL

R

Springborn Laboratories, Inc.
Environmental Sciences Division
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TEST PROTOCOL

PROTOCOL TITLE: Protocol for Conducting a Flow-Through Acute Toxicity Test with Rainbow Trout following TSCA 797.1400.

TO BE COMPLETED BY THE STUDY SPONSOR:

Study Sponsor: American Petroleum Institute

Address: 1220 L Street, Northwest

Washington, D.C. 20005 Phone: (202) 682-8300

Sponsor Protocol/Project No.:

Test Substance: Tert-Amyl Methyl Ether, (TAME)

Purity: 94% CAS# or LOT#: 02814BZ

Additional Comments and/or Modifications:

Robert A. Clarke 10/6/92
Sponsor Approval Date

TO BE COMPLETED BY SLI PRIOR TO TEST INITIATION:

Testing Facility: Springborn Laboratories, Inc. Project #: 12827-0692-6104-108

Study Director: MARK W. MACHADO

Test Concentrations: 950, 570, 340, 210, 120 mg A.I./L plus Control

Solvent Used: NA CAS# or LOT#: NA

Proposed Schedule: (Start) 2/27/93 (Completion) 3/3/93

Additional Comments and/or Modifications:

Mark W. Machado 10/14/92
Study Director Date

PROTOCOL FOR CONDUCTING A FLOW-THROUGH ACUTE TOXICITY TEST WITH RAINBOW TROUT FOLLOWING TSCA 797.1400

OBJECTIVE

The purpose of this test is to determine the acute lethal effects of a test material on a representative cold water fish species under flow-through conditions. The methods described in this protocol generally follow the standard procedures described in the EPA/OTS guidelines for testing the effects of chemicals on fish (U.S. EPA, 1985, amended 1987). Test results are reported as LC50 values together with 95% confidence limits, as No Observed Effect Level (NOEL), and as the incipient LC50, i.e., that value which indicates an LC50 when exposure to the test substance is continued until the mean increase in mortality does not exceed ten percent in any concentration over a 24-hour period.

MATERIALS AND METHODS

TEST ORGANISMS:

1. Species. Juvenile rainbow trout *Salmo gairdneri*, are used to conduct the dynamic acute toxicity test with cold water fish. The fish are of approximately the same size and age, i.e., the length of the largest fish does not exceed the length of the smallest fish by more than two-fold.

2. Origin and Acclimation. The fish are obtained from a reliable commercial supplier and are gradually acclimated to the test conditions. They are held for at least an additional 14 days in the dilution water prior to testing, a minimum of 7 days of which at the required test temperature. During the final 48 hours of fish holding, total mortality must not exceed three percent, or the batch will not be used.

3. Feeding. The fish are fed at least once daily prior to the test, but are not fed during the final 48 hours before the test, nor during the first 96-hours of the in-life test.

4. Handling. Fine-mesh dip nets are used to transfer the fish, taking care to minimize possible stress due to handling. Fish that are damaged or dropped during transfer are not used.

5. Loading. Fish biomass to solution ratio ("loading") does not exceed 0.5 grams per liter per 24 hours.

PHYSICAL SYSTEM:

1. Test Containers. The test chambers used in the flow-through acute bioassay are 19-L clear glass aquaria which are chemically clean. Each aquarium maintains a consistent solution volume (11 or 15 liters of test medium). This size is adequate to meet the maximum allowable loading requirements (see above).

2. Cleaning. The test aquaria are chemically cleaned before the test is started following standard laboratory procedures.

3. Dilution Water. Water from a 100 meter bedrock well is pumped to a concrete reservoir where it is supplemented on demand with untreated, unchlorinated, Town of Wareham well water and aerated before flowing to the exposure system through aged PVC pipe. The pH, total hardness, alkalinity, and specific conductance of this water are measured and recorded weekly in Springborn Laboratories' GFT Laboratory Notebook. The water is characterized as being "soft" with a pH range of 6.9 - 7.2, a total hardness of 25 - 40 mg/L and a specific conductance of 80 - 150 µmhos/cm. During any one month, weekly analysis of the dilution water should show that the water quality characteristics of hardness, alkalinity and specific conductance do not vary by more than 10% from the respective monthly average and the monthly pH range should be less than 0.4 pH units. At least twice a year, analyses of representative samples of dilution water are conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to the fish. None of these compounds have been detected at concentrations which may be harmful to the fish. None of these compounds have been detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with US EPA and ASTM standard practices. In addition, TOC, COD, particulate matter, unionized ammonia and organic chlorine analyses are conducted at least twice each year in the dilution water.

4. Replication. Two replicates are included with each test concentration and control. Test aquaria are positioned inside the water bath by stratified random design, and labeled by replicate and concentration (or control). Fish are added impartially to the test aquaria by adding no more than two fish to each replicate until all aquaria contain two fish. This procedure is repeated until each aquarium contains ten fish (20 fish per concentration or control).

CHEMICAL SYSTEM:

1. Test Material. Upon arrival at Springborn Life Sciences, the external packaging of the test material is inspected for damage. The packaging is removed and the primary storage container is also inspected for leakage or damage. The sample identity and percent active ingredient are recorded and, unless different arrangements are made with the study Sponsor, the material is stored in the dark at approximately 20°C until used.

2. Toxicant Concentration Selection. Toxicant concentrations for the acute toxicity test are selected based on information provided by the Sponsor or obtained from a preliminary static or flow-through range-finding test. The preliminary test consists of three widely spaced concentrations, usually of 3-L volume, each containing at least three fish. A geometric series of five concentrations and one control are used for each definitive test, each concentration consisting of twenty test fish (see Section "Replication", above). Each dose level is 60% of the next higher concentration of the test material. The range of concentrations selected for the definitive test is intended to include both 100% effect and no-effect levels, but due to the nature of some test materials, one or both levels may not be observed.

3. Diluter. A proportional or serial diluter (e.g., Mount and Brungs, 1967) is employed to deliver five toxicant concentrations, a control, and a solvent control, if necessary, to duplicate aquaria. If no solvent control is required, a sixth toxicant concentration may be added. Based on the solubility of the test material, the stock solution stability and the range of test concentrations, one of the following toxicant delivery systems is used: the gas-tight syringe injector metering device; the Mariotte bottle/"dipping bird" system, or the metering pump/predilution chamber system.

   A flow-splitting chamber is used between the diluter cells and the aquaria to promote mixing of the toxicant solution and diluent water. In each chamber, two separate standpipes are employed to equally split the test solution between the A and B duplicate test aquaria.

   The calibration of the diluter system is checked prior to test initiation. During the test, the diluter is visually inspected twice daily. If there is any indication during the test that the diluter calibration has changed (e.g.; diluter malfunction or unexplained differences in dissolved oxygen concentration or temperature in the aquaria), calibration of the necessary diluter components is checked. A test is not started until the diluter and toxicant delivery device have been observed to be properly functioning for at least 24 hours prior to the test. During a test, the flow rates shall not vary by more than 10% from one replicate test chamber to another.

4. Stock Preparation. The stock solution is prepared according to the following formula:

$$\text{Stock concentration} = \frac{\text{H.C. x M.C.}}{\text{B.D. x \% A.I.}}$$

where:

H.C. = high concentration (mg/L)

M.C. = mixing chamber volume (L)

B.D. = bird or syringe delivery (mL)

A.I. = % active ingredient

The test material is weighed on an analytical balance for which a calibration log is maintained. A Chemical Usage Log is also maintained in which the amount, the date, the intended use and the user's initials are recorded each time test material is used.

5. Solvent Control. If a solvent is used, a solvent control is established which contains a concentration of solvent equal to the amount present in the test concentrations, but not to exceed 0.1 mL/L. The solvent concentration is kept as low as possible. If >0.1 mL/L solvent is needed to solubilize a required quantity of the test material, the Sponsor will be notified, and the solvent concentration used will be identified on the cover page of the protocol. Reagent grade or highest quality triethylene glycol (TEG), acetone, ethanol, or dimethyl formamide (DMF), in this order of preference, is used.

SAMPLING AND OBSERVATIONS:

1. Sampling. Unless specified differently by the Sponsor, water samples of an appropriate volume are taken from one replicate of the high, middle and low test concentrations at least once during the pre-exposure period to document water concentrations and the proper functioning of the diluter. Samples from both replicate aquarium of each concentration and control(s) are taken at the initiation, mid-term (48 hours) and termination of the test (96 hours) for determination of toxicant concentrations. The test solution of at least one appropriate test aquarium is measured whenever a malfunction is detected in any part of the test delivery system. Prior to analysis, and, if possible, within 30 minutes of sampling, samples are passed through a 0.45 µm filter to remove any material which may be associated with particulate matter. The filters and filter holders are pre-rinsed with distilled water and finally with test solution prior to use. Three quality control samples are prepared at each sampling interval and remain with the set of samples through extraction, storage and analysis. These samples are prepared in diluent water at test material concentrations similar to the treatment level range. Results of these analyses indicate the relative accuracy of the analytical methodologies for each sampling period. Water samples are taken from a point approximately midway between the surface, bottom and sides of each aquarium and either extracted immediately after sampling or appropriately preserved and stored until analysis can be performed.

2. Measurement of Water Quality Variables. At test initiation and every 24 hours thereafter, water quality variables (temperature, pH, and dissolved oxygen concentrations) are recorded in each test aquarium. The following water quality conditions are maintained during the test:

   Dissolved Oxygen. Total dissolved oxygen exceeds 90% of saturation at the initiation of the test, and is maintained at ≥8.2 mg/L for the duration of the test. Aeration (with oil free air) would be initiated as a last resort to raise and maintain the dissolved oxygen concentration at acceptable levels.

   Temperature. Water temperature of the test solutions is maintained at 12 ± 2 °C by maintaining the aquaria in a water bath at the appropriate test temperature. Temperature is monitored continuously in one aquarium by using a minimum-maximum thermometer which is read and recorded daily.

Lighting. A combination of fluorescent bulbs is used to illuminate the aquaria which provides a wide spectrum of light, simulating the spectrum of natural sunlight. Light intensity at the water surface is within the range of 20-100 foot candles. An 8-hours dark and 16-hours light photoperiod is maintained during the test.

3. Biological Data. Observations of stress, abnormal behavioral activity and mortality are made daily. Dead fish are removed from test solutions at these intervals. In addition, characteristics of the test solutions are also observed and recorded, e.g., precipitated materials, cloudiness, etc.

4. Acceptability Criteria. The test is unacceptable if more than 10 percent of the control fish die in 96 hours.

STATISTICS

Mortality data derived from the acute test is used to statistically estimate a median lethal concentration (LC50) and its 95% confidence interval after each 24-hour interval of exposure. The LC50 is the estimated nominal or measured concentration of the test material in dilution water which produces 50% mortality in the test fish population at the stated times of exposure. The incipient LC50 is determined also, i.e., that value which indicates an LC50 when exposure to the test substance is continued until the mean increase in mortality does not exceed ten percent in any concentration over a 24-hour period. LC50 values are computed using measured concentrations, if available.

The computer program utilized estimates LC50 values using one of three statistical methods: probit analysis, moving average method, or binomial probability. The method selected is determined by the data base (i.e., presence or absence of 100% response, number of partial responses, etc.). An LC50 value cannot be calculated if the mortality data derived is insufficient according to any of the three statistical methods. The method provides values of the slope, including 95% confidence intervals, for the probit analysis, as well as appropriate statistical tests to evaluate goodness-of-fit.

In addition, the highest test concentration that shows no statistically significant difference from the control (No Observed Effect Concentration, NOEC) is determined and reported.

REPORTING

The raw data and final drafts of the report are reviewed by the Quality Assurance Unit and Study Director. All values of chemical and water quality measurements are reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. A single copy of the draft report will initially be submitted to the study Sponsor for review. Upon acceptance by the Sponsor, three copies of the final report will be submitted. All reports include, but are not limited to, the following information.

- Springborn Laboratories, Inc., report and project numbers and if applicable, Sponsor protocol and project numbers and the dates of when the definitive test was conducted..
- Laboratory and site, the dates of testing and personnel involved in the study, i.e., Quality Assurance Unit, Program Coordinator, Study Director, Principal Investigator.
- All information pertaining to the test material which appears on the sample bottle, e.g., its source and percent activity, if available.
- Characterization and origin of the dilution water.
- Scientific name of the test organism, source, percent mortality of the fish population 48 hours prior to testing, and acclimation temperature, pH, and DO range.
- Description of stock preparation.
- Information regarding test temperatures, dissolved oxygen concentration, pH and photoperiod.
- Observations of insolubility of the test material, including the test levels and when observed.
- Number of fish that showed lethality in the controls and in each treatment at each observation period, as well as percent mortality at test termination, in tabular form.
- Description or reference (or inclusion as an appendix) to chemical and statistical procedures applied.
- The LC50 value for each day it can be calculated, with 95 percent confidence limits, the incipient LC50, and the No Observed Effect Level (NOEL).
- Analytical results of test concentration measurements and QC samples.
- Deviations from the protocol not addressed in protocol amendments will be listed, together with a discussion of the impact on the study and signed by the Study Director.
- Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- Dates of Quality Assurance reviews, signed by the QA Unit.

SPECIAL PROVISIONS

GOOD LABORATORY PRACTICES (GLP): All test procedures, documentation, records, and reports will comply with the U. S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the Toxic Substances Control Act (*FEDERAL REGISTER*, Part III, August 17, 1989)

TEST MATERIAL DISPOSAL: After 60 days of the issuance of the final test report, the test material will be returned to the Sponsor's project officer, at Sponsor expense, unless different arrangements are made.

REFERENCES

APHA, AWWA, WPCF. 1985. *Standard Methods for the Examination of Water and Wastewater.* 16th Edition, Washington, DC. 2168 pp.

Mount, D.I. and W.A. Brungs. 1967. A simplified dosing apparatus for fish toxicological studies. Water Research 1: 21-39.

U.S. Environmental Protection Agency. 1985. *Toxic Substances Control Act Test Guidelines.* Federal Register 50(188):39252-39516, September 27, 1985. Amended in Fed. Reg. 52:19062 (1987).

Springborn Laboratories, Inc.
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PROTOCOL AMENDMENT

AMENDMENT #: 1

DATE: 10 March, 1993

PROTOCOL TITLE: "Protocol for Conducting a Flow-Through Acute Toxicity Test with Rainbow Trout following TSCA 797.1400."

SPECIES: *Oncorhynchus mykiss*

STUDY SPONSOR: American Petroleum Institute

TEST MATERIAL: Tert-Amyl Methyl Ether, ("TAME")

SLI STUDY NO: 12827.0692.6104.108

AMENDMENT(S):

1. The protocol states that a proportional or a serial diluter is employed to deliver five toxicant concentrations, a control and solvent control, if necessary, to duplicate aquaria and that a flow-splitting chamber is used between the diluter cells and aquaria to promote mixing of the toxicant solution and diluent water.

   During the conduct of this study, however, it became necessary to modify the serial diluter and toxicant delivery method in order to compensate for the volatile nature of the test material and to maximize the concentration of test material in solution. This modification consisted of circumventing the mixing chamber and chemical cells of the diluter and changing the point of entry of the test material stock solution such that the toxicant pumps delivered the solution directly into the individual delivery tubes exiting each of the splitter cell compartments. This modification effectively eliminated the necessity for the mixing chamber and subsequent dilution accomplished in the chemical cells and splitters. The 60% dilution series necessary to provide the five treatment levels to conduct the study was accomplished by adjusting the delivery of each toxicant pump.

2. The protocol states that samples from both replicate aquaria of each concentration and control(s) are taken at the initiation, mid-term (48 hours) and termination of the test (96 hours) for determination of toxicant concentrations.

   During this study the mid-term sampling interval was eliminated. A 48-hour sampling interval was never intended for this study, but was inadvertently included in the study protocol.

3. The protocol incorrectly states that juvenile rainbow trout, *Salmo gairdneri*, are used in the test. The scientific name for rainbow trout has been changed to *Oncorhynchus mykiss*.

Approval Signatures: Mark W. Machado 3/22/93

Mark W. Machado
SLI Study Director

Date

Richard A. Rhoden 3/25/93

Richard A. Rhoden, Ph.D.
Sponsor Study Monitor

Date

Springborn Laboratories, Inc.
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PROTOCOL AMENDMENT

AMENDMENT #: 2

DATE: 24 March, 1993

PROTOCOL TITLE: "Protocol for Conducting a Flow-Through Acute Toxicity Test with Rainbow Trout following TSCA 797.1400."

SPECIES: *Oncorhynchus mykiss*

STUDY SPONSOR: American Petroleum Institute

TEST MATERIAL: Tert-Amyl Methyl Ether, ("TAME")

SLI STUDY NO: 12827.0692.6104.108

AMENDMENT(S):

1. The protocol incorrectly states that the test chambers used in the flow-through acute bioassay are 19-L clear glass aquaria. Test chambers used in this study were 19.5 L all glass aquaria.

2. The protocol states that test results are reported as LC 50 values together with 95% confidence limits, along with a No Observed Effect Level (NOEL) and the incipient LC50, i.e., that value which indicates on LC50 when exposure to the test substance is continued until the mean increase in mortality does not exceed ten percent in any concentration over a 24-hour period. Based on the criteria established in the Environmental Effects Testing Guidelines under TSCA (40 CFR, Part 797, § 797-1400) an incipient LC50 was not calculated because the 96-hour LC50 value for this study was not less than 50% of the 48-hour LC50 value and the test was not continued beyond the 96-hour interval.

3. The protocol states that samples will be passed through a 0.45 μm filter to remove any material which may be associated with particulate matter. Due to the test material characteristics, use of a filtration apparatus was anticipated to cause significant test material loss and deterioration of membrane filters. For these reasons, filtration was not utilized.

Approval Signatures: Mark W. Machado 3/24/93

Mark W. Machado
SU Study Director

Date

Richard A. Rhoden 3/25/93

Richard A. Rhoden, Ph.D.
Sponsor Study Monitor

Date

7.0 APPENDIX II - CERTIFICATE OF ANALYSIS

aldrich chemical co.
chemists helping chemists in research & industry

SPRINGBURN LABORTORIES
508 295 8107
PAULA LECONTE

PO NBR:

PRODUCT INFORMATION

PRODUCT NUMBER: 28309-6 LOT NUMBER: 02814BZ

PRODUCT NAME: TERT-AMYL METHYL ETHER, 94%

FORMULA: C6H14O FORMULA WEIGHT: 102.18

APPEARANCE	COLORLESS LIQUID
REFRACTIVE INDEX AT 20 DEG C	1.3885
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 268A OF EDITION I, VOLUME 3 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
GAS LIQUID CHROMATOGRAPHY	98.8 %

Aldrich Chemical Company
DAVID SWESSEL
NOVEMBER 11, 1992

aldrich chemical co.
P.O. Box 355, Milwaukee, Wisconsin 53201 USA • (414) 273-3850

aldrich chemical co. (926)

chemists helping chemists in research & industry

AMER PETRO INST
202 682 8270
DR DICK RHODEN

PO NBR: 82000300BA13

12827.0692.602-05

PRODUCT INFORMATION

PRODUCT NUMBER: 28309-6 LOT NUMBER: 07905KZ

PRODUCT NAME: TERT-AMYL METHYL ETHER, 94%

FORMULA: C6H14O FORMULA WEIGHT: 102.18

APPEARANCE	COLORLESS LIQUID
REFRACTIVE INDEX AT 20 DEG C	1.3876
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 268A OF EDITION I, VOLUME 3 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
GAS LIQUID CHROMATOGRAPHY	98.7 %

Aldrich Chemical Company
DAVID SWESSEL
NOVEMBER 6, 1992

aldrich chemical co.
P.O. Box 355, Milwaukee, Wisconsin 53201 USA • (414) 273-3850

8.0 APPENDIX III - CULTURE FOOD ANALYSIS

Zeigler Brothers, Inc. Salmon Starter Feed Sample*		
Date Submitted:11/13/92 Date Reported: 12/1/92		
Analysis	**Final Result**	**Limit of Quantitation**
Pesticide Screen I;II;III	Result as Received	
Alpha BHC	< 0.01 mg/kg	0.01
Beta BHC	< 0.01 mg/kg	0.01
Gamma BHC - Lindane	< 0.01 mg/kg	0.01
Delta BHC	< 0.01 mg/kg	0.01
Heptachlor	< 0.01 mg/kg	0.01
Aldrin	< 0.01 mg/kg	0.01
Heptachlor Epoxide	< 0.01 mg/kg	0.01
DDE	< 0.01 mg/kg	0.01
DDD	< 0.01 mg/kg	0.01
DDT	< 0.01 mg/kg	0.01
HCB	< 0.01 mg/kg	0.01
Mirex	< 0.01 mg/kg	0.01
Methoxychlor	< 0.05 mg/kg	0.05
Dieldrin	< 0.01 mg/kg	0.01
Endrin	< 0.01 mg/kg	0.01
Telodrin	< 0.01 mg/kg	0.01
Chlordane	< 0.05 mg/kg	0.05
Toxaphene	< 0.1 mg/kg	0.1
PCBs	< 0.2 mg/kg	0.2
Ronnel	< 0.01 mg/kg	0.01
Ethion	< 0.02 mg/kg	0.02
Trithion	< 0.05 mg/kg	0.05
Diazinon	< 0.1 mg/kg	0.1
Methyl Parathion	< 0.02 mg/kg	0.02
Ethyl Parathion	< 0.02 mg/kg	0.02
Malathion	< 0.05 mg/kg	0.05
Endosulfan I	< 0.01 mg/kg	0.01
Endosulfan II	< 0.01 mg/kg	0.01
Endosulfan Sulfate	< 0.03 mg/kg	0.03
Chlorpyrifos	< 0.01 mg/kg	0.01
* Analyzed by Lancaster Laboratories, Inc.		

Zeigler Brothers Inc. Salmon Starter Feed Sample*		
Date Submitted:11/13/92 Date Reported:12/1/92		
Analysis	Final Result	Limit of Quantitation
Pesticide Screen I;I;II	attached	
Arsenic	2.1 ppm	0.1
Cadmium	0.4 ppm	0.1
Copper	2.1 mg/100g	0.2
Lead	0.4 ppm	0.2
Mercury	0.10 ppm	0.02
Zinc	29.4 mg/100g	0.2
Selenium (fluorometric)	1.6 ppm	0.1
* Analyzed by Lancaster Laboratories, Inc.		

9.0 APPENDIX IV - DILUTION WATER ANALYSIS

GFT Grab Water Sample*		
Date Sampled: 1/28/93 Date Reported: 2/12/93		
Pesticide Screen I;II;III	Result As Received	Limit of Quantitation
Alpha BHC	< 0.01 µg/l	0.01
Beta BHC	< 0.01 µg/l	0.01
Gamma BHC - Lindane	< 0.01 µg/l	0.01
Delta BHC	< 0.01 µg/l	0.01
Heptachlor	< 0.01 µg/l	0.01
Aldrin	< 0.01 µg/l	0.01
Heptachlor Epoxide	< 0.01 µg/l	0.01
DDE	< 0.01 µg/l	0.01
DDD	< 0.01 µg/l	0.01
DDT	< 0.01 µg/l	0.01
HCB	< 0.01 µg/l	0.01
Mirex	< 0.01 µg/l	0.01
Methoxychlor	< 0.05 µg/l	0.05
Dieldrin	< 0.01 µg/l	0.01
Endrin	< 0.01 µg/l	0.01
Telodrin	< 0.01 µg/l	0.01
Chlordane	< 0.3 µg/l	0.3
Toxaphene	< 4. µg/l	4.
PCBs	< 1. µg/l	1.
Ronnel	< 0.01 µg/l	0.01
Ethion	< 0.02 µg/l	0.02
Trithion	< 0.05 µg/l	0.05
Diazinon	< 0.1 µg/l	0.1
Methyl Parathion	< 0.02 µg/l	0.02
Ethyl Parathion	< 0.02 µg/l	0.02
Malathion	< 0.05 µg/l	0.05
Endosulfan I	< 0.01 µg/l	0.01
Endosulfan II	< 0.01 µg/l	0.01
Endosulfan Sulfate	< 0.03 µg/l	0.03
* Analyzed by Lancaster Laboratories, Inc.		

GFT Grab Water Sample*		
Date Sampled:1/28/93 Date Reported: 2/12/93		
Analysis	**Result As Received**	**Limit of Quantitation**
Pesticide Screen I,II,III	attached	
Mercury	< 0.0002 mg/l	0.0002
Arsenic	< 0.2 mg/l	0.2
Selenium	< 0.2 mg/l	0.2
Boron	< 0.04 mg/l	0.04
Thallium	< 0.3 mg/l	0.3
Aluminum	< 0.2 mg/l	0.2
Antimony	< 0.2 mg/l	0.2
Barium	< 0.1 mg/l	0.1
Beryllium	< 0.01 mg/l	0.01
Cadmium	< 0.01 mg/l	0.01
Calcium	7.8 mg/l	0.2
Chromium	< 0.05 mg/l	0.05
Cobalt	< 0.05 mg/l	0.05
Copper	< 0.02 mg/l	0.02
Iron	< 0.1 mg/l	0.1
Lead	< 0.1 mg/l	0.1
Magnesium	2.3 mg/l	0.1
Manganese	0.02 mg/l	0.01
Molybdenum	< 0.1 mg/l	0.1
Nickel	< 0.05 mg/l	0.05
Potassium	1.0 mg/l	0.5
Silver	< 0.02 mg/l	0.02
Sodium	14.9 mg/l	0.4
Titanium	< 0.01 mg/l	0.01
Vanadium	< 0.01 mg/l	0.01
Zinc	< 0.04 mg/l	0.04
* Analyzed by Lancaster Laboratories, Inc.		

10.0 APPENDIX V - ANALYTICAL METHODOLOGY

SUMMARY

An analytical methodology is presented for the measurement of TAME (Tert-Amyl Methyl Ether) in AAP media, filtered seawater and freshwater (reconstituted to increase hardness). All water samples were analyzed either by direct sampling into a purge and trap liquid sample concentrator or vial sampling system. The water phase was stripped of TAME with a high flow of helium gas and trapped on an active support material. The TAME was then thermally desorbed from the support and transferred through a heated line onto a gas chromatograph for separation and quantitation. TAME was detected utilizing a flame ionization detector. Quantitation was performed using various fitting techniques both on and off the instrument.

Mean recovery from AAP media was 89.7 ± 2.3%, 104 ± 11% for filtered seawater and 102 ± 5% for freshwater, however, the analyte purging efficiency from a hard reconstituted water matrix presents a greater degree of instrumental variability. Therefore the standard deviation acceptance criteria has been increased to 10% to more accurately represent the recovery data. Repeatability of TAME analysis showed a 5.4% relative standard deviation (%RSD) at 0.026 mg/L from water.

EQUIPMENT AND REAGENTS

Equipment

1. Balance: Mettler AE 200 182, four-place analytical
2. Volumetric flask: grade A, assorted sizes
3. Wheaton vials with teflon-lined crimp top lids, assorted sizes
4. Syringes: Hamilton, assorted sizes, gas tight and valved
5. Absorbent Trap: 25 cm x 0.125 O.D. stainless steel column packed with 1 cm 3% OV -1, 15 cm tenax and 8 cm silica gel.
6. Purge and Trap Liquid Sample Concentrator: Tekmar model LSC-2000
7. Vial Sampling System: Tekmar Model ALS2050
8. Gas chromatograph: Hewlett-Packard 5890A equipped with a capillary injection port and 105 m x 0.53 mm I.D. 3 μm film RT_x 502.2 column and Flame ionization detector.

Reagents

1 Methanol: reagent grade solvent

2. TAME: Lot # 02814BZ, was received from Experimental Pathology Labs, Inc., on 17 August 1992 and was identified by Aldrich Chemical Company to be 98.8% pure.

3. Water: All solutions were prepared using water from a Sybron/Barnstead NANOpure II® (meets ASTM Type IIA specifications) filtered and sterilized water purification system. The filtered sterilized water typically shows greater than 16.7 Mohm-cm resistivity and less than 1 mg/L total organic carbon.

4. AAP Media

5. Filtered seawater

6. Hard Reconstituted water

PROCEDURE

Preparation of Stock Solution

Primary standards were prepared by placing approximately nine and a half milliliters (mL) of methanol into a 10 mL volumetric flask. The flask was allowed to stand unstoppered to allow any methanol along the neck to evaporate and was weighed to the nearest 0.1 milligram (mg). TAME was immediately added to the flask using a microliter syringe, making sure the primary material fell directly into the alcohol. The vessel was reweighed, diluted to the mark, stoppered, and finally mixed by inverting the flask several times.

The solution was transferred to a 10 mL crimp top bottle with a Teflon lined lid and stored in a refrigerator until used. This stock was used with further dilution for sample fortification and standard(s) preparation. All stock solutions and dilutions were stored in Wheaton vials with Teflon lined crimp tops in a refrigerator.

Preparation of Standards for Purge & Trap

Secondary standards (104, 26.0 and 5.20 mg/L in methanol) were drawn into a microliter syringe and spiked directly into water in a 5 mL gas tight Luer lock syringe. These aqueous

standards were added directly to the purge vessel and analyzed immediately. Calibration and check standards were prepared just prior to analysis. Standards were prepared in a 5 mL gas-tight syringe using TAME working standards. Examples of formulation working standard formulation are outlined below:

Stock Concentration (mg/L)	Volume Taken (μL)	Nominal Concentration (mg/L)
5.20	25.0	0.026
26.0	25.0	0.130
26.0	50.0	0.260
26.0	100	0.520
26.0	250	1.30
104	250	5.20
104	500	10.4

Sample Fortification

Method validation/recovery samples were prepared using AAP media, filtered seawater and freshwater (reconstituted to increase hardness). Samples were fortified with dilutions of the TAME stock in volumetric flasks and loaded onto a automatic liquid sample autosampler (LSC 2050). The fortified levels produced were 0.052, 4.16 and 10.4 mg/L TAME in AAP media, 0.026, 4.16 and 10.4 mg/L in filtered seawater and 49.7, 248 and 695 mg/L in freshwater (reconstituted to increase hardness). Three replicates at each level were prepared for each experiment along with three unfortified matrix blanks.

Liquid Sampler

Samples were loaded into 40 mL vials. Vials were placed in vial sampler. Five milliliters sample was transferred from the vial samples into the purge vessel attached in-line with the activated sorbent support matrix (EPA method 624 trap) and the stripping program initiated with a high flow of helium (60 mL/min) bubbled through the vessel. The sorbent trapped gaseous TAME from the helium carrier gas. This approach was effective because the compound is highly

volatile. After the water phase had been stripped for four or six minutes, the sorbent trap was heated and TAME stripped into the carrier and brought through a heated capillary transfer line (0.53 mm I.D fused silica) onto the top of the gas chromatographic column located in a capillary injection port of the gas chromatograph.

TAME was separated chromatographically using a temperature program after splitless injection from the purge and trap liquid sample concentrator.

Liquid Sample Concentrator: Tekmar LSC-2000.

Programmed Purge & Trap Conditions

Standby Temperature: 40 ° C

	Time (minutes)	Temperature (° C)
Purge:	4 or 6	< 40
Desorption Preheat:	NA	175
Desorption:	4.0	180
Bake:	8.0	225

Heating Zones

	Temperature °C
Valve:	200
Mount:	40
Transfer Line:	200

Gas Chromatography

Gas chromatographic analysis was conducted utilizing a directly coupled liquid sample concentrator (purge and trap) into the capillary injection port. The samples were introduced by

programmed injection from the purge and trap. The refocusing of sample entered the column occurred at the head of the column as a function of the film thickness of the RT_x 502.2 column.

Gas Chromatograph: Hewlett Packard 5890A gas chromatograph equipped with a split/splitless capillary injection port operated in the splitless mode.

Column:	105 m x 0.53 mm ID x 3 μm film
	Temperature (°C): Injector: 200
	column temperature programmed: 40 - 250
	Rate: 10 °C per minute from 40 to 70 °C
	25 °C per minute from 70 - 250 °C
Gas (mL/minute):	Helium
	Carrier Gas: ca. 9
Makeup gas(mL/minute):	Helium (28)
	Run Time: 16 minutes
	Retention Time: ca. 12.4 minutes

Integrator: Hewlett Packard 3396A II programmable integrator

Analysis

TAME was analyzed utilizing purge and trap thick film capillary (0.53 mm I.D.) gas chromatography flame ionization detection (GC/FID). Water samples were loaded onto the purge vessel (5 mL) of the LSC-2000 using a 5 mL gas tight syringe or vial transfer line from the vial sampler. The purge program was initiated and the systems allowed to sequence through the preprogrammed methods (purge and trap, gas chromatograph and integrator).

RESULTS AND DISCUSSION

Analytical results for the recovery of TAME from AAP media, filtered seawater and freshwater (reconstituted to increase hardness) are presented in Table 1A, 2A and 3A, respectively. System performance was tested for system repeatability in water. Results of repeatability studies are presented in Table 4A. Run time for samples was approximately 27 minutes. Samples were introduced through the capillary injection port operated in the splitless mode onto the gas chromatographic column. The split vent was closed for the 4 minutes of desorb on the purge and trap. Figure 1A is a representative chromatogram of TAME analysis by purge and trap GC-FID.

TAME analysis was generally linear (correlation coefficient, r^2, greater than 0.98) from 0.25 mg/L TAME in water through 5.0 mg/L (Figure 2A). Detector response was not linear, rather there is a notable curve apparent in detector response from 0.026 though 10.4 mg/L TAME (Figure 3A). The integrator had software to fit calibration data to polynomial fit. Recovery samples for AAP media and filtered seawater were calculated using a least squares polynomial analysis performed on the height response. Recovery from freshwater (reconstituted to increase hardness) samples were calculated using a least squares linear regression analysis performed on the height response.

The reports generated by the integrator were categorized in a report with concentration (mg/L) calibrated from a 5-mL sample. Check standards were evaluated periodically and providing up-to-date evaluation of system calibration. Calibration was monitored utilized a series of stock standards in methanol. Evaluation was based on the trend of results and the reported value for that standard. Working standards were prepared around the concentration range of interest and stored along with other operating information on the integrator. Calibration could be conducted using linear, polynomial or point to point fitting techniques.

Table 1A. Analytical results for the recovery of TAME from AAP media.

Nominal Concentration (mg/L)	Volume Purged (mL)	Concentration Recovered (mg/L)	Percent Recovered (%)
10.4	5.00	8.92	85.8
10.4	5.00	9.17	88.1
10.4	5.00	9.39	90.3
4.16	5.00	3.79	91.1
4.16	5.00	3.88	93.2
4.16	5.00	3.84	92.3
0.052	5.00	0.0462	88.9
0.052	5.00	0.0462	88.9
0.052	5.00	0.0462	88.9
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA

Mean Recovery: 89.7 ± 2.3%

The minimum detectable concentration was 0.026 mg/L for a 5.00 mL sample which is the lowest standard used in the polynomial fit.

Table 2A. Analytical results for the recovery of TAME from filtered seawater.

Nominal Concentration (mg/L)	Volume Purged (mL)	Concentration Recovered (mg/L)	Percent Recovered (%)
10.4	5.00	10.0	96.3
10.4	5.00	12.1	116
10.4	5.00	12.1	117
10.4	5.00	11.9	114
4.16	5.00	3.79	91.1
4.16	5.00	3.78	90.9
4.16	5.00	3.79	91.2
0.026	5.00	0.027	105
0.026	5.00	0.027	105
0.026	5.00	0.028	109
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA
Control	5.00	< 0.026	NA

Mean Recovery: 104 ± 11%

The minimum detectable concentration was 0.026 mg/L for a 5.00 mL sample which is the lowest calibration standard used in the polynomial fit.

Table 3A. Analytical results for the recovery of TAME from freshwater (reconstituted to increase hardness).

Nominal Concentration (mg/L)	Dilution Factor	Volume Purged (mL)	Concentration Recovered (mg/L)	Percent Recovered (%)
695	200	5.00	694	99.8
695	200	5.00	693	99.6
695	200	5.00	705	101
248	100	5.00	268	108
248	100	5.00	258	104
248	100	5.00	265	107
49.7	20.0	5.00	50.9	102
49.7	20.0	5.00	44.9	90.3
49.7	20.0	5.00	51.7	104
Control	1.00	5.00	< 0.248	NA
Control	1.00	5.00	< 0.248	NA
Control	1.00	5.00	< 0.248	NA

Mean Recovery: 102 ± 5% (10%)[1]

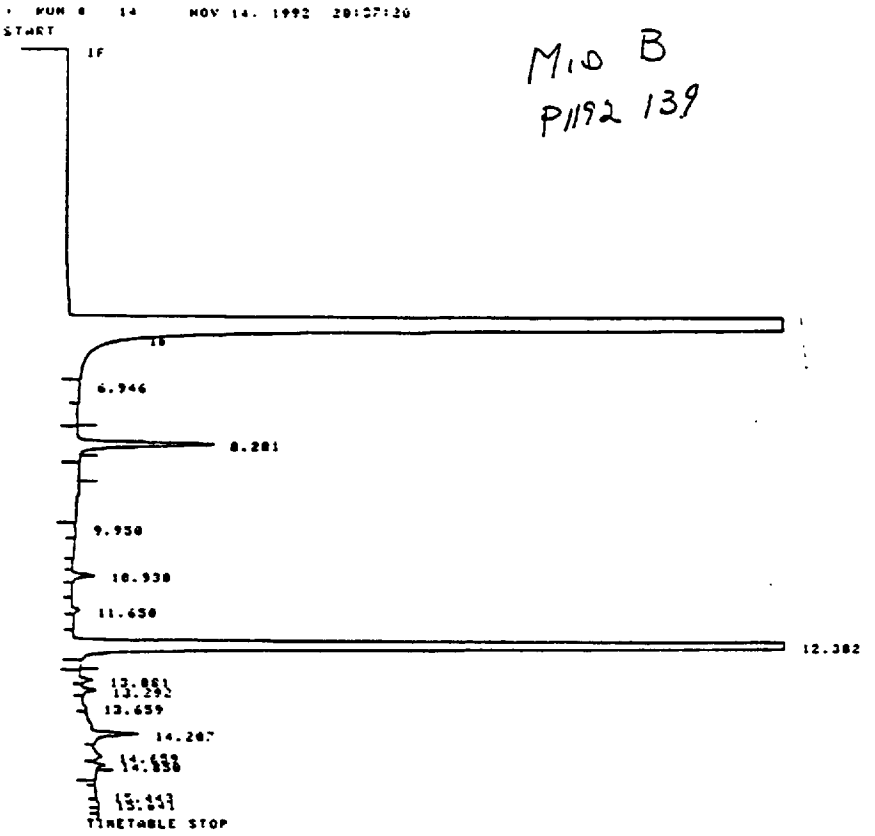
The minimum detectable concentration was 0.248 mg/L for a 5.00 mL sample which is the lowest standard used in the linear regression analysis.

[1] The analyte purging efficiency from a hard reconstituted water matrix presents a greater degree of instrumental variability. Therefore the standard deviation acceptance criteria has been increased to 10% to more accurately represent the recovery data.

Table 4A. Repeatability of TAME analysis from ASTM Type II water at 0.026 mg/L.

Replicate	Area	Height
1	47510	5725
2	54711	6099
3	46909	5631
4	36628	5646
5	36305	5699
6	55640	6292
7	54256	6365
Mean:	47423	5922
Std Dev.:	8243	320
% RSD:	17.4	5.4

Figure 1A. **A representative chromatogram of TAME purge and trap GC/FID analysis.**



RUN# 14 NOV 14, 1992 20:37:20

METHOD NAME: M-TAMEPGTP.MET

IDENTIFIER : :3140A39449

~~CALIBRATION~~ TAME
ER 9/14/92

ESTD-WEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	AMT	NAME
8.281	PB	156375	.107	24263		.000	
10.930	PP	10711	.082	3817		.000	
12.382	PB	3787178	.103	612842	1R	3.596	TAME
13.292	VP	11181	.071	2618		.000	
14.207	VV	69086	.129	8923		.000	
14.659	VV	17942	.164	1825		.000	

TOTAL WEIGHT= 654286
MUL FACTOR=1.0000E+00

Figure 2A. A representative linear regression analysis from standard TAME analysis.

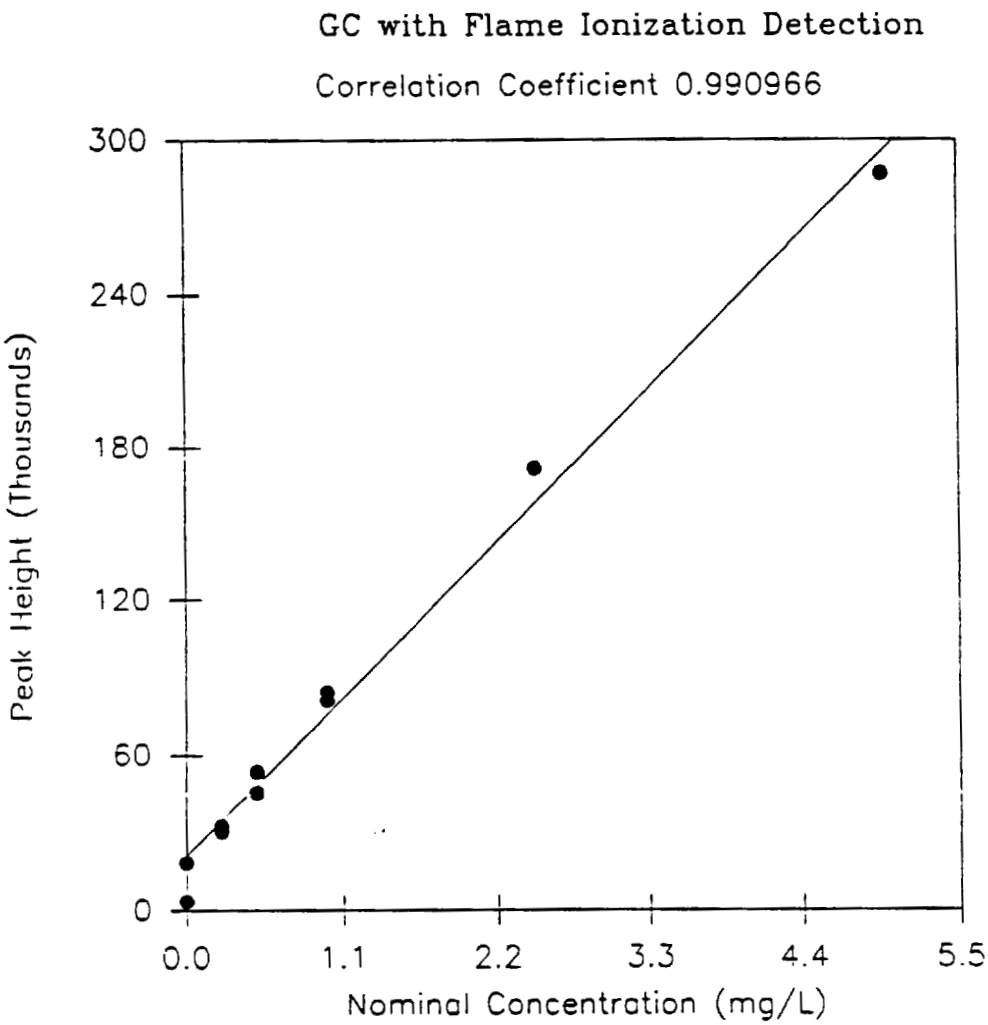


Figure 3A. A representative polynomial regression analysis from standard TAME analysis.

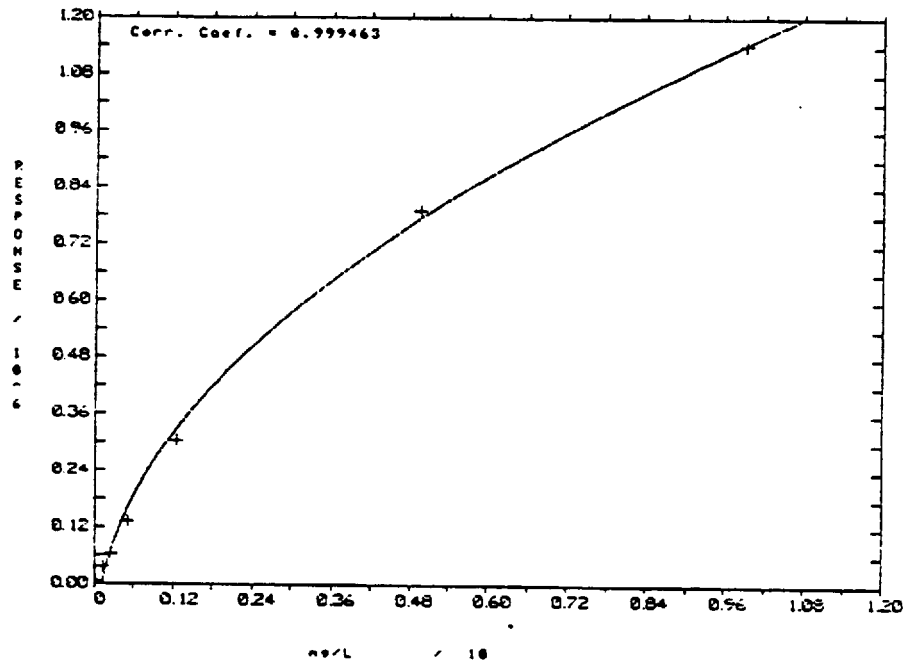
```
WELCOME TO THE HP3396 CALIBRATION CURVE PLOTTING PROGRAM Rev. B.02.00

At any prompt:    '0' [ENTER] Quits
                  '5' [ENTER] Starts Over

Load which method or calib. file [Current active*]:

Plot the calibration curve for which CAL # [All*]:

                    ng/L       vs. Response for Cal # 1
ng/L      = +1.16E-01 +1.37E-06 (RESPONSE) +6.26E-12 (RESPONSE^2)
```




```
Plot additional peaks [Y*/N]: N

* EDIT CALIB #

1 = CALIB PROCEDURE
2 = RETENTION TIME WINDOWS
3 = TABLE ENTRIES
4 = PEAK GROUPS
5 = CALIB OPTIONS
```

11.0 APPENDIX VI - RAW DATA

SPRINGBORN LABORATORIES, INC.

ACUTE TOXICITY TEST - BIOLOGICAL OBSERVATIONS AND WATER QUALITY FORM

Date Test Started: 2/27/93 | Species: Oncorhynchus mykiss | SLI Study #: 12827-0692-6104-108

Time (Fish)/Mysids Added: | Source: T-10 | Study Director: MARK MACHADO

#(Fish)/Mysids Added Per Replicate: 10 | Principle Investigator: NA

SUBJECT: BIOLOGICAL OBSERVATIONS AND WATER QUALITY MEASUREMENTS

Test Hour	0-HR						24-Hour						48 HOUR					
Date	2/27/93						2/28/93						3-1-93					
Time	1230						1215						1245					
Date By	RHD						RHD						AC					
Test Conc. (mL/L)	Rep.	ppm D.O.	pH	°C Tem	Sol.	Observations	ppm D.O.	pH	°C Tem	Sol.	Observations	# Dd/cum	ppm D.O.	pH	°C Tem	Sol.	Observations	# Dd/cum
950	1A	9.8	7.1	12	NA	None	9.6	7.2	11	NA	None	10/10	9.0	7.2	12	NA	NONE	—/10
	1B	9.6	7.1	12		None	9.4	7.2	11		CLE	7/7	9.3	7.2	12		DRK CLE	•/7
570	2A	9.6	7.1	12		None	9.6	7.2	11		CLE	3/3	9.2	7.2	12		DRK CLE	1/4
	2B	9.7	7.1	12		None	9.6	7.2	11		CLETH	0/0	9.0	7.2	12		CLETH	0/0
340	3A	9.8	7.1	12		None	9.6 to 9.4	7.2	11		None	0/0	9.3	7.2	12		NONE	0/0
	3B	9.8	7.1	12		None	9.4	7.2	11		None	0/0	9.4	7.2	12		NONE	0/0
210	4A	9.8	7.1	12		None	9.5	7.2	11		None	0/0	9.4	7.2	12		NONE	0/0
	4B	9.7	7.1	12		None	9.4	7.2	11		None	0/0	9.4	7.2	12		NONE	0/0
120	5A	9.8	7.1	12		None	9.4	7.2	11		None	0/0	9.0	7.2	12		NONE	0/0
	5B	9.8	7.1	12		None	9.6	7.2	11		None	0/0	9.2	7.2	11		NONE	0/0
Control	6A	9.6	7.1	12		None	9.5	7.2	11		None	0/0	9.1	7.2	12		NONE	0/0
	6B	9.7	7.1	12		None	9.6	7.2	11		None	0/0	9.2	7.2	11		NONE	0/0
S. Cont.	7A	NA	NA	NA		NA	—	—	—		NA	NA/NA	—	—	—		NA	NA/NA
	7B	NA	NA	NA	↓	↓	—	—	—	↓	↓	NA/NA	—	—	—	↓	↓	NA/NA

Rhodes 2/27/93

H.C.K. 3-2-93

12827 0692 6104 108

160

SPRINGBORN LABORATORIES, INC.

ACUTE TOXICITY TEST - BIOLOGICAL OBSERVATIONS AND WATER QUALITY FORM

SUBJECT: BIOLOGICAL OBSERVATIONS AND WATER QUALITY MEASUREMENTS (continued)

		72 HOUR						96 HOUR											
Test Hour		72 HOUR						96 HOUR											
Date		3-2-93						3-3-93											
Time		1245						1230											
Data By		MC						MC											
(PPM) AI Test Conc.	Rep.	PPM D.O.	pH	°C Tem	Sal.	Observations	#Dd/Cm	PPM D.O.	pH	°C Tem	Sal.	Observations	#Dd/Cm	D.O	pH	Te	Sal.	Observation	#Dd/Cm
950	1A	9.8	7.2	11	NA	NONE	– / 10	9.0	7.2	12	NA	NONE	– / 10						
	1B	9.6	7.2	11		NONE	3 / 10	9.1	7.2	12		NONE	– / 10						
570	2A	9.7	7.2	11		DRK+CLE	0 / 4	9.1	7.2	11		DRK+CLE	0 / 4						
	2B	9.4	7.2	11		DRK+CLE	0 / 0	8.9	7.2	12		DRK+CLE	2 / 2						
340	3A	9.6	7.2	11		NONE	0 / 0	9.1	7.2	11		NONE	0 / 0						
	3B	9.6	7.2	11		NONE	0 / 0	9.1	7.2	11		NONE	0 / 0						
210	4A	9.8	7.2	11		NONE	0 / 0	9.6	7.2	11		NONE	0 / 0						
	4B	9.7	7.2	11		NONE	0 / 0	9.0	7.2	11		NONE	0 / 0						
120	5A	9.6	7.2	11		NONE	0 / 0	8.9	7.2	11		NONE	0 / 0						
	5B	9.7	7.2	11		NONE	0 / 0	9.0	7.2	11		NONE	0 / 0						
Control	6A	9.5	7.2	11		NONE	0 / 0	8.9	7.2	12		NONE	0 / 0						
	6B	9.6	7.2	11		NONE	0 / 0	8.9	7.2	11		NONE	0 / 0						
S. Cont.	7A	–	–	–		–	– / –	–	–	–	–	–	– / –						
	7B	–	–	–		–	– / –	–	–	–	–	–	– / –						

NA MC 3-4-93

RIF PL 3-3-93

12827 0692 6104 108 162

12827 0692 6104 108

SPRINGBORN LABORATORIES, INC.

ACUTE TOXICITY TEST - BIOLOGICAL OBSERVATIONS AND WATER QUALITY FORM

SUBJECT: BIOLOGICAL OBSERVATIONS AND WATER QUALITY MEASUREMENTS (continued)

OBSERVATION KEY

GENERAL BEHAVIOR	SWIMMING	RESPIRATION
AS At the Surface	ERR Erratic	RA Rapid
MSP Muscle Spasm	GY Gyrating	G Gulping
CLE Complete Loss of Equilibrium	SK Skittering	SOLUTION
PLE Partial Loss of Equilibrium	PIGMENTATION	PRE Precipitate
LETH Lethargic	DRK Dark	FOS Film on Surface
PFAE Pect. Fins Anteriorly Extended	INTEGUMENT	UC Undissolved Chemical
EXO Exophthalamus	MS Mucous Shedding	
EA Extended Abdomen	EMP Excessive Mucous Production	
SU Surfacing	HEM Hemorrhagic	
HYP Hyperactive		

Additional Comments/Observations (Sign and Date each entry):

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Springborn Laboratories, Inc.

SPRINGBORN LABORATORIES, INC. Page 3

RESULTS OF CHROMATOGRAPHIC ANALYSIS
MEAN MEASURED TABLE

Sponsor: AMERICAN PETROLEUM INC.
Test Material: TAME
Project No.: 12827-0692-6104-108
Test Type: 96HR FIA W/ RT
Data Entered By: MD /MD
Date Program Run: 22-Feb-93

Sample ID	Nominal Concentration (MG/L)	INTERVAL (HR)	Analytical Result (MG/L)	MEAN	N	STD.DEV.
2-93-1193CNT	0	ONR	< 5.2799	NA	4	NA
2-93-1194	0	ONR	< 5.2799			
3-93-115	0	96HR	< 5.2074			
3-93-116	0	96HR	< 5.2074			
2-93-1191	120	ONR	5.565E+01	78.2	4	16.7
2-93-1192	120	ONR	8.862E+01			
3-93-117	120	96HR	7.578E+01			
3-93-118	120	96HR	9.267E+01			
2-93-1189	210	ONR	1.483E+02	150	4	6.73
2-93-1190	210	ONR	1.534E+02			
3-93-119	210	96HR	1.566E+02			
3-93-120	210	96HR	1.411E+02			
2-93-1187	340	ONR	2.849E+02	305	4	28.6
2-93-1188	340	ONR	3.356E+02			
3-93-121	340	96HR	3.233E+02			
3-93-122	340	96HR	2.771E+02			
2-93-1185	570	ONR	5.068E+02	559	3	51.3
2-93-1186	570	ONR	1.110E+02 *			
3-93-123	570	96HR	5.618E+02			
3-93-124	570	96HR	6.094E+02			
2-93-1183	950	ONR	7.391E+02	644	4	87.0
2-93-1184	950	ONR	6.968E+02			
3-93-125	950	96HR	5.614E+02			
3-93-126	950	96HR	5.804E+02			
2-93-1195	760000	ONR	2.051E+05 *	962572	1	NA
3-93-130	760000	96HR	9.626E+05			

* SAMPLES ARE LOW DUE TO INJECTOR ERROR. A FULL SAMPLE WAS NOT INJECTED AND WILL NOT BE INCLUDED IN THE STATISTICAL ANALYSIS.

SPRINGBORN LABORATORIES, INC. Page 4

RESULTS OF CHROMATOGRAPHIC ANALYSIS
QA SUMMARY TABLE

Sponsor: AMERICAN PETROLEUM INC.
Test Material: TAME
Project No.: 12827-0692-6104-108
Test Type: 96HR FTA W/ RT
Data Entered By: MD
Date Program Run: 22-Feb-93

Sample ID	Nominal Concentration (MG/L)	INTERVAL (HR)	Analytical Result (MG/L)	Percent of Nominal
2-93-1196	120	0HR	1.275E+02	106
2-93-1197	350	0HR	3.571E+02	102
2-93-1198	950	0HR	1.215E+03	128
3-93-127	120	96HR	1.182E+02	98.5
3-93-128	350	96HR	4.543E+02	130
3-93-129	950	96HR	8.977E+02	94.5

MEAN	110
N=	6
STD.DEV.	15

American Petroleum Institute
1220 L Street, Northwest
Washington, D.C. 20005

Order No. 848-00408