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The Goodyear Tire & Rubber Company

Akron, Ohio 44316-0001

PCN: 8595-200074

March 3, 1997

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Dear Ladies/Gentlemen:

Subject: **Supplemental Information Regarding a TSCA Section 8(e) Notice**

Reference: EPA Document Control Number: 8EHQ-94-13284

This submitted does not contain Confidential Business Information.

As promised in the above referenced document, The Goodyear Tire & Rubber Company is providing EPA with the following final report:

WINGSTAY 100 - Bioaccumulation Study with Carp (Cyprinus carpio), Phase 1

The unexpected mortalities observed in the first exposure at the 50 ppb target concentration (Initial submittal - 8EHQ-94-13284) were not seen in the subsequent exposure at a similar target concentration of the test material. The deaths observed in the first exposure were isolated to one aquarium. No mortality or adverse behavior were noted in any of the other aquaria associated with the study. In an effort to identify whether or not the deaths were related to the test material, a second exposure was conducted. In this second exposure, at 79 ppb no mortalities or adverse effects were observed.

Additionally, the bioaccumulation study demonstrated that the test material accumulates to a high degree in fish (consistent with the high fat solubility for this chemical product), but is readily eliminated following cessation of exposure.

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March 3, 1997

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My address and telephone number are as follows:

The Goodyear Tire & Rubber Company  
Department 100D  
1144 East Market Street  
Akron, Ohio 44316-0001  
Telephone: (330) 796-2362

Sincerely,

A handwritten signature in cursive script, appearing to read "Michael W. Smith".

Michael W. Smith  
Section Manager, Chemical Information  
Systems & Regulatory Affairs

MWS/jh  
s7m3a3

Enclosure (1)

## EXECUTIVE SUMMARY

## ABSTRACT

This study investigated the potential for Wingstay® 100 to be concentrated in the tissue of freshwater fish. Japanese Carp were exposed to two different concentrations of Wingstay® 100. During the time the fish were in water containing Wingstay® 100, the test substance was measured in the tissues of the exposed fish. Concentrations of Wingstay® 100 in tissue were approximately 3,000 - 5,000 times greater than the concentration of Wingstay® 100 in the water. Following this part of the experiment, the fish were placed in clean water containing no Wingstay® 100. After 14 days in clean water, the fish had essentially eliminated all Wingstay® 100 from their tissues. This experiment demonstrates the fact that Wingstay® 100 accumulates to a high degree in fish (consistent with the high fat solubility for this chemical product), but is readily eliminated following cessation of exposure.

## EXECUTIVE SUMMARY

This study investigated the potential for Wingstay® 100 to bioaccumulate in freshwater fish. The measure of bioaccumulation was performed using the Japanese carp (*Cyprinus carpio*), following OECD Guideline 305E ("Bioaccumulation: Flow-Through Fish Test", OECD, 1981b) and MITI test guidelines ("Methods for Testing the Bioaccumulation of Chemical Substances in Fish", MITI, 1993). Exposure to two aqueous concentrations of Wingstay® 100 was provided via a continuous flow diluter system. Following exposure, the rate of depuration of the accumulated residues was measured by transferring the exposed populations to clean flowing water (i.e., without test substance). Selection of the target exposure concentrations for the initial investigation (Springborn Report #95-5-5882) was based on the results of an acute toxicity test (96-hour LC50 of > 1.0 mg/L for Wingstay® 100 and a representative freshwater fish). Concentrations selected for bioaccumulation estimation (i.e., 50 and 5.0 µg/L) were 5 and 0.5% of the established LC50 value.

Exposure of carp to 50 µg/L (nominal) Wingstay® 100 resulted in unexpected mortality (i.e., 18%) by day 7. Due to the unexpected mortality, exposure of the population of carp to the 50 µg/L treatment level was terminated. No mortalities or adverse effects were observed during the exposure of carp to 5 µg/L of Wingstay® 100 or the control solution, which continued through 36 days, followed by 14 days of depuration. Analysis of the exposure solution established an average measured concentration of Wingstay® 100 of 3.24 µg/L or 65% of the target

concentration. Measurement for residues in the fish tissue determined that steady state concentrations in the edible (11,800  $\mu\text{g}/\text{kg}$ ), non-edible (20,500  $\mu\text{g}/\text{kg}$ ) and whole body (15,700  $\mu\text{g}/\text{kg}$ ) tissues were achieved by exposure day 15. Based on these data, the measured (tissue / water) bioconcentration factor (BCF) for each tissue type was; 3640 (edible), 6310 (nonedible) and 4850 (whole body). These measured BCF's were consistent with the predicted values based on the uptake ( $k_u$ ) and depuration ( $k_d$ ) constants, i.e., 3790 (edible), 6450 (nonedible) and 4970 (whole body). Elimination of Wingstay<sup>®</sup> 100 from all tissue types was continuous during the 14-day depuration period. The half-life (i.e., the time required to eliminate 50% of the accumulated residues determined at the termination of the exposure period) occurred in the whole body tissue prior to day 7. Elimination of 95% of the residues in the whole body tissue was achieved by day 14. Based on the lipid content (5.90%), the BCF for whole body tissue was calculated to be 82,200, a factor which represents theoretical accumulation in fatty tissue.

Due to the unexpected mortality during the initial exposure to 50  $\mu\text{g}/\text{L}$  Wingstay<sup>®</sup> 100, this portion of the study was repeated (Phase II Exposure, Springborn Report # 95-4-5826) to determine if the observed mortality was test material related and to establish the BCF for Wingstay<sup>®</sup> 100 at a second exposure concentration. No mortalities were observed during the supplemental exposure which extended through 28 days, followed by a 14-day depuration period. The absence of mortality during the supplemental test indicated that the previously observed mortality was associated with a condition unrelated to toxicity of the test material. Analysis of the exposure solution established an average measured concentration of Wingstay<sup>®</sup> 100 of 79.0  $\mu\text{g}/\text{L}$  or 158% of the target concentration. Analysis of the fish tissue established that steady state was achieved by day 16 of the exposure period. Steady state concentrations determined during the supplemental test were as follows: (198,000  $\text{ug}/\text{Kg}$ ) edible, (291,000  $\text{ug}/\text{Kg}$ ) nonedible, (246,000  $\text{ug}/\text{Kg}$ ) whole body.

Based on these data, the measured bioconcentration factor (BCF) for each tissue type was 2510 (edible), 3680 (non-edible), and 3110 (whole body). These measured BCF's were consistent with the predicted values based on the uptake ( $k_u$ ) and depuration ( $k_d$ ) constants, i.e., 1890 (edible), 2910 (non-edible), and 2400 (whole body). Elimination of Wingstay<sup>®</sup> 100 from all tissue types was continuous during the 14-day depuration period. The half-life occurred in the whole body tissue prior to day 7. Elimination of 94% of the residues in the whole body tissue was achieved by day 14. Based on the lipid content, the BCF for whole body tissue was calculated to be 52,700 which represents theoretical accumulation in fatty tissue.

These two studies together provide data pertaining to the bioconcentration of Wingstay® 100 in fish. Exposures at 3.24 µg/L and 79.0 µg/L yielded BCF's ranging from 2,510 to 6,310 depending on exposure concentration and tissue type. This range of variability is well within that expected for bioaccumulation studies and demonstrates that the bioconcentration of Wingstay® 100 is independent of exposure concentration. The unexpected mortality observed in the first exposure at the 50 ug/L target concentration were not seen in the subsequent exposure at a similar target concentration of Wingstay® 100. The deaths observed in the first exposure were isolated to one aquarium. No mortality or adverse behavior were noted in any of the other aquaria associated with the study. In an effort to identify whether or not the deaths were related to Wingstay® 100 effects, a second exposure was conducted. In this second exposure, at 79.0 µg/L no mortalities or adverse effects were observed. Therefore, based on the orange-red killifish exposure and the two bioconcentration studies there does not appear to be any adverse biological effects caused by exposure of fish to Wingstay® 100. The following tables and figure further illustrate the comparison between the predicted and measured BCFs and uptake and depuration rates observed during the two bioaccumulation exposures.

**Table 1. Summary of the Steady State Concentrations Determined for Wingstay® 100 in Carp During Two Separate Exposures to Mean Measured Water Concentrations of 3.24 ug/L and 79 ug/L.**

Study Phase	Report #	Mean Measured Water Concentration	Steady State Concentrations			
			Steady State	Edible	Nonedible	Whole Body
Phase I Bioaccumulation	95-5-5882	3.24 µg/L	day 15	11,800	20,500	15,700
Phase II Bioaccumulation	95-4-5826	79 µg/L	day 16	198,000	291,000	246,000

**Table 2. Summary of the Residue Concentrations for Wingstay® 100 in Carp After 14 Days of Depuration Following Two Separate Exposures to Mean Measured Water Concentrations of 3.24 ug/L and 79 ug/L.**

Study Phase	50% Depuration	Percent Depuration (14 day)	Residue Concentrations after 14 Days Depuration			
			Edible	Nonedible	Whole Body	BCF Lipid Content (whole body)
Phase I Bioaccumulation	before day 7	95%	3640	6310	4850	82,200
Phase II Bioaccumulation	before day 14	94%	2510	3680	3110	52,700

**Table 3. Summary of the Predicted and Measured Bioconcentration Factors (BCF) for Wingstay® 100 in Carp Determined During Two Separate Exposures to Mean Measured Water Concentrations of 3.24 ug/L and 79 ug/L.**

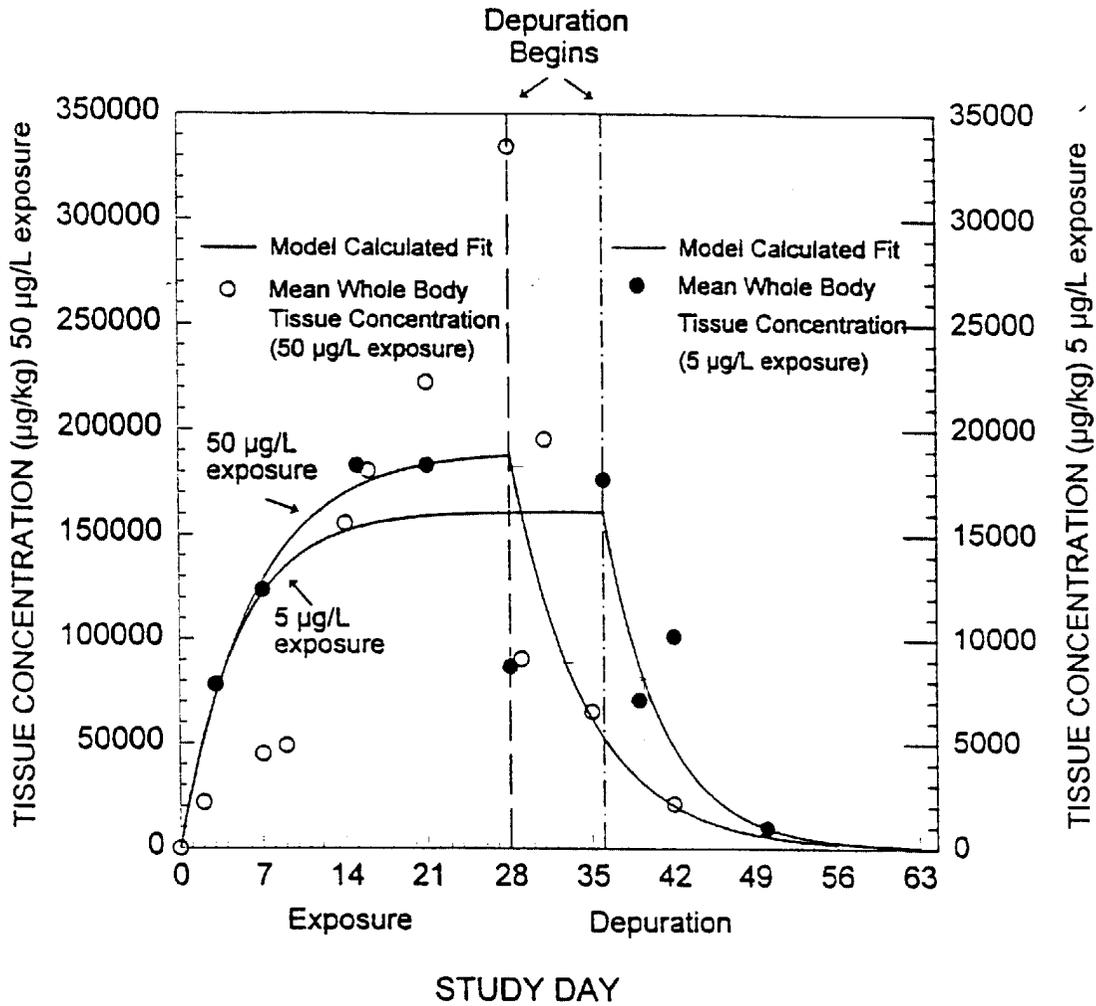
## Phase I

Mean Measured Water Concentration During Exposure	Tissue type	$K_o$	$K_d$	Predicted BCF	Measured BCF
3.24 µg/L	Edible	602	0.159	3790	3640
	Nonedible	1370	0.212	6450	6310
	Whole Body	1000	0.201	4970	4850

## Phase II

Mean Measured Water Concentration During Exposure	Tissue type	$K_o$	$K_d$	Predicted BCF	Measured BCF
79 µg/L	Edible	318	0.169	1890	2510
	Nonedible	439	0.151	2910	3680
	Whole Body	376	0.156	2400	3110

Figure 1. Residue Levels in Carp During and After Wingstay® 100 Exposures Under Water Flow-through Conditions in Laboratory Aquaria.



*Handwritten notes:*  
 14, 47, 20, 50, 41  
 5, 3, 4, 5

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**WINGSTAY® 100 - BIOACCUMULATION  
STUDY WITH CARP (*Cyprinus carpio*), PHASE I**

**MITI Test Guidelines and  
OECD Guideline Reference Number 305E**

**Submitted to:**

**The Goodyear Tire & Rubber Company  
142 Goodyear Boulevard  
Akron, Ohio 44305**

**SLI Study 13537.0994.6100.143**

**SLI Report #95-5-5882**

**Study Director: John Mao, Ph.D.**

**Springborn Laboratories, Inc.  
*Health and Environmental Sciences*  
790 Main Street  
Wareham, Massachusetts 02571-1075**

**11 September 1996**

**FINAL REPORT**

**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

The data and report prepared for "**Wingstay<sup>®</sup> 100 - Bioaccumulation Study with Carp (*Cyprinus carpio*), Phase I**" were produced and compiled in accordance with all pertinent OECD Good Laboratory Practice Regulations with the following exceptions. 1) Routine water and food contaminant screening analyses for pesticides, PCBs and metals were conducted by either Lancaster Laboratories, Lancaster, Pennsylvania or Woodson-Tenent Laboratories, Inc., Memphis, Tennessee, using standard U.S. EPA procedures. These data were not collected in accordance with Good Laboratory Practice procedures (i.e., no distinct protocol, Study Director, etc). 2) The study protocol was signed by the Study Sponsor on 31 August 1994. Because of an unexpected delay in the transfer of the original protocol, the study director did not sign the protocol until 7 October 1994, after some preliminary screening work had taken place. 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.

John Mao, Ph.D.  
Study Director

9/11/96

Date

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**SUMMARY****Wingstay® 100 - Bioaccumulation Study with Carp (*Cyprinus carpio*), Phase I**

**SPONSOR:** The Goodyear Tire & Rubber Company

**PROTOCOL TITLE:** Wingstay® 100: Measurement of Bioaccumulation in Japanese Carp (*Cyprinus carpio*) Following OECD and MITI Guidelines, Springborn Laboratories Protocol No.: 081194/OECD-MITI-BCF and Protocol Amendment Nos. 1 and 2, dated 7 October 1994 and 8 June 1995, respectively.

**REPORT NO. :** 95-5-5882

**STUDY NO.:** 13537.0994.6100.143

**TEST SUBSTANCE:** Wingstay® 100, Lot No. 137170393 NP1017, CAS Registry No. 68953-84-4, a gray, flaky substance with a purity of 100% reported by the Study Sponsor, was received from Goodyear Research on 7 September 1994

**TEST DATES:** Exposure : 25 October to 30 November 1994  
Depuration: 1 December 1994 to 16 December 1994  
(last analytical sampling was on 14 December 1994)

**TEST SPECIES:** Japanese Carp (*Cyprinus carpio*) . SLI Lot #  
Mean wet weight = 12 g (range 7.0 to 19 g)  
Mean total length = 97 mm (range 76 to 115 mm)  
Source = Owens and Williams Fish Farm, a commercial supplier in Hawkinsville, Georgia

**TEST CONDITIONS:** 36 days exposure, 14 days depuration, 23 to 25 °C, illumination of 16 hours light:8 hours dark at 50 to 70 footcandles

**DILUTION WATER:** Well water  
pH: 6.9 to 7.2  
Specific conductivity: 110 to 130 µmhos/cm  
Total hardness as CaCO<sub>3</sub>: 30 to 36 mg/L  
Total alkalinity as CaCO<sub>3</sub>: 21 to 27 mg/L

**TARGET TEST CONCENTRATION:** 5.0 µg/L Wingstay® 100

**MEAN MEASURED  
CONCENTRATION:** 3.24 µg/L

**ANALYTICAL DETECTION  
LIMIT:** approximately 2 to 3 µg/L (Section 5.1)

**RESULTS AND  
DISCUSSION:**

Carp (*Cyprinus carpio*) were exposed to a target concentration of 5 µg/L of Wingstay® 100 for 36 days to determine bioaccumulation (bioconcentration) factors (BCF) in edible (fillet), nonedible (viscera/carcass) and whole body tissues. A 14-day depuration period was also conducted, after steady-state conditions had been reached, to determine the elimination rate of Wingstay® 100 from fish tissues at this time.

The actual Wingstay® 100 concentration in water over the 36-day exposure period was determined by high performance liquid chromatography (HPLC) to be  $3.24 \pm 1.57$  µg/L. Steady state conditions were reached on Day 15. The mean tissue concentrations at this time were determined by HPLC to be 11,800, 20,500 and 15,700 µg/kg for edible, nonedible and whole body tissues, respectively. BCF values based on mean measured steady state tissue concentrations and mean measured exposure concentration (3.24 µg/L) were calculated to be 3640, 6310 and 4850 in edible, nonedible and whole body tissues, respectively. By Day 14 of depuration, 94.5% of the Wingstay®100 had been eliminated from fish tissues.

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**Predicted and measured bioaccumulation  
(bioconcentration) factors for each tissue type**

Tissue type	$K_u$	$K_d$	Predicted BCF	Measured BCF
Edible	602	0.159	3790	3640
Nonedible	1370	0.212	6450	6310
Whole Body	1000	0.201	4970	4850

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## 1.0 INTRODUCTION

The purpose of this study was to measure the bioaccumulation (accumulation from water) of the test substance using a representative freshwater fish species, carp (*Cyprinus carpio*). A static acute toxicity test with orange-red killifish (*Orizias latipes*) was performed prior to initiation to allow selection of test concentrations for the bioaccumulation study. Based on the acute test, concentrations of 5.0 and 50 µg/L Wingstay® 100 were selected for the bioaccumulation study. However, the 50 µg/L exposure was discontinued after 9 days due to unexpected fish mortality. Thus, only bioconcentration factors from the 5.0 µg/L bioaccumulation exposure are presented in this report. To replicate the high concentration exposure and to investigate the cause of fish mortality at this concentration, a separate 28-day study was conducted and reported in Springborn Report #95-4-5826 entitled "Wingstay® 100 - Bioaccumulation Study with Carp (*Cyprinus carpio*), Phase II". For comparison purposes, results from that study are also included in this report. These reports are summarized below.

Study Phase	Report #	Test Type	Species	Target Concentration	Test Dates
Pilot	95-5-5882	static acute	orange-red killifish	1.0 mg/L	28 September to 2 October 1994
Bioaccumulation Phase I	95-5-5882	flow-through acute	carp	5.0 and 50 µg/L <sup>a</sup>	25 October to 14 December 1994
Bioaccumulation Phase II	95-4-5826	flow-through acute	carp	50 µg/L	11 January to 24 February 1995

<sup>a</sup> Unexpected fish mortality was observed at the 50 µg/L treatment level.

The study was initiated on 7 October 1994, 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 50-day definitive test was conducted from 25 October 1994 to 14 December 1994 at

Springborn Laboratories, Inc. (SLI), *Health and Environmental Sciences*, located in Wareham, Massachusetts. All original raw data and the final report produced during this study are stored in Springborn's archives at the above location.

## 2.0 MATERIALS AND METHODS

### 2.1 Protocol

Procedures used in this study followed those described in the Springborn protocol entitled "Wingstay<sup>®</sup> 100: Measurement of Bioaccumulation in Japanese Carp (*Cyprinus carpio*) Following OECD and MITI Guidelines", Springborn Protocol No. 081194/OECD-MITI-BCF and Protocol Amendments No. 1 and 2, dated 7 October 1994 and 8 June 1995, respectively (Appendix I). The methods described in this protocol generally meet the testing requirements of the OECD Guideline for Testing of Chemicals #305E (OECD, 1981) and MITI test guidelines, Method for Testing the Bioaccumulation of Chemical Substances in Fish (MITI, 1993).

### 2.2 Test Substance and Standard Reagents

**2.2.1 Test Substance.** The test substance, Wingstay<sup>®</sup> 100, was received from Goodyear Research, Akron, Ohio on 7 September 1994. Upon receipt at Springborn, the test substance was stored at room temperature (approximately 20 °C) in a dark, ventilated cabinet. At the termination of the testing program, a sample of the test substance will be maintained at Springborn. The remaining test substance will be sent to the Study Sponsor. Test concentrations were not adjusted for purity and are reported as micrograms per liter of solution (µg/L). The structure of the test chemical's components is presented in Figure 1. Product specification information is provided in Appendix II. The following information describes the test substance received:

Chemical Name:	mixed diaryl- <i>p</i> -phenylenediamines
Physical Appearance:	gray flake
Lot No.:	137170393 NP1017
CAS Registry No.:	68953-84-4
Purity:	100% (Certificate of Analysis, Appendix II)
Molecular Weight:	274 g/mol (average)
Water Solubility:	< 5 ppm
Vapor Pressure:	not available

**2.2.2 Standard Reagents.** All aqueous solutions were prepared using water (meeting ASTM Type IIA requirements) obtained with a Sybron/Barnstead NANOpure® II system. The filter-sterilized water typically shows greater than 16.7 Mohm-cm resistivity and less than 1 mg/L total organic carbon, which is the detection limit. All solvents were of HPLC grade from commercial sources. All other chemicals were at least reagent grade from commercial sources.

### 2.3 Test Dilution Water

The dilution water used throughout this study was from the same source as the water which flowed into the holding tank and was characterized as having total hardness and alkalinity ranges (as CaCO<sub>3</sub>) of 30 to 36 mg/L and 21 to 27 mg/L, respectively, a pH range of 6.9 to 7.2, and a specific conductance range of 110 to 130 µmhos/cm (Gravity Feed Tank Water Quality Analysis Notebook, Volume 9). Representative samples of the dilution water source were analyzed periodically for the presence of pesticides, PCBs and toxic metals. None of these compounds were detected at concentrations that are considered toxic in any of the water samples analyzed, in agreement with ASTM (1989) standard practice. In addition, representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. The TOC concentration of the dilution water source ranged from 0.39 to 0.56 mg/L during the period of September to December 1994 (Springborn TOC Master Log). The minimum instrumental operating range is 0.1 mg/L. 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 during this study and have successfully survived and reproduced over several generations. The excellent performance of the cultured daphnids, in combination with the previously mentioned analyses, confirmed the acceptability of this dilution water for use in the bioaccumulation study.

### 2.4 Test Food

For the acute toxicity test, the orange-red killifish were fed a commercial dry, pelleted food daily, *ad libitum*, supplemented with live brine shrimp (*Artemia* sp.) and flake food.

For the definitive bioaccumulation study, the carp were fed a commercial dry, pelleted food twice daily, *ad libitum*, except during the 24 hours prior to test initiation. Fish were not fed during these periods to eliminate the possibility of large amounts of gut contents being present, which could bias measurement of tissue residue concentrations. All fish were fed at the rate of approximately 1% of their total biomass per feeding. Representative samples of the food sources were analyzed for the presence of pesticides, PCBs and toxic metals. Food sources were considered to be of acceptable quality since the total concentration of pesticides was below the detection limit, *i.e.*, less than 0.3 mg/kg (ASTM, 1985), for the pesticides that were assayed.

## 2.5 Test Conditions

For each phase of the study, the acute toxicity and the bioaccumulation test, the aquaria were placed in a temperature-controlled water bath designed to maintain the temperature at  $25 \pm 1$  °C. The circulating water was automatically heated by two Pro-Tec quartz 1000 watt Omega Thermoregulators or cooled with a Frigid Unit 1/3 horsepower chiller, as necessary.

Illumination was provided by Duro-Test® Vita-Lite® fluorescent bulbs located over the test aquaria. Light intensity was measured with a General Electric Model 214 light meter. Sixteen hours of light were provided each day, avoiding sudden transitions from light to dark and vice versa.

## 2.6 Water Quality and Test Monitoring

Throughout the study temperature and dissolved oxygen concentration were measured daily in each test aquarium. Temperature was also continuously monitored in the control aquarium. Measurements of pH were performed daily during the acute toxicity test and three times a week during the bioaccumulation study. Hardness (as CaCO<sub>3</sub>) was measured in the treatment and control aquaria at initiation of the acute toxicity test and the bioaccumulation study.

The pH was measured with a Jenco Model 601A pH meter, the dissolved oxygen concentration was measured with a Yellow Springs Instrument Model #57 dissolved oxygen meter and probe, and the temperature was measured with a Fisher Brand alcohol thermometer. In

addition, temperature was continuously monitored using a Fisher Submersible Min/Max thermometer. Total hardness was measured according to APHA *et al.* (1985).

### **3.0 ACUTE TOXICITY TEST WITH ORANGE-RED KILLIFISH (*Orizias latipes*)**

#### **3.1 Test Organisms**

Orange-red killifish (*Orizias latipes*) was selected as the acute toxicity test species, since it is an OECD Guideline for Testing of Chemicals (1981)-recommended species and a commonly used freshwater fish in acute toxicity tests. The orange-red killifish used during this study (SLI Lot # 94A102) were obtained from Aquatic Research Organisms, a commercial supplier in Hampton, New Hampshire. Prior to testing, these fish were held in a 18.9-L fiberglass tank under a photoperiod of 16 hours of light and 8 hours of darkness. The test fish were maintained under standard test conditions for a minimum of 14 days prior to test initiation. The temperature in the holding tank was 24 °C during this 14-day period. Mortality observed among the killifish test fish population during the 4 days prior to testing (Springborn Daily Record of Fish Holding Conditions) was less than 1%. A representative sample of the killifish from the test population had a mean wet weight of 0.34 g (range 0.29 to 0.43 g) and a mean total length of 32 mm (range 29 to 35 mm) (Springborn Fish Weight and Lengths Log).

#### **3.2 Stock Solution Preparation**

The target exposure concentration selected for the acute toxicity test was 1.0 mg/L. To achieve a Wingstay<sup>®</sup> 100 concentration of 1.0 mg/L in 15 L of water, 1.5 mL of a 10.0 mg/mL stock solution was used. The 10 mg/mL Wingstay<sup>®</sup> 100 solution was prepared by dissolving and diluting 100.8 mg of Wingstay<sup>®</sup> 100 to 10 mL with acetone (CAS #67-64-1).

#### **3.3 Test System**

The acute toxicity test was conducted using an exposure system consisting of a temperature-controlled water bath and a Wingstay<sup>®</sup> 100 exposure aquarium, a solvent control (acetone) aquarium and a blank control aquarium. Each clear glass aquarium contained a total solution volume of 15 L. The solution depth in each aquarium was maintained at 18.4 cm. The

surface area of the solutions' air-water interface was 814 cm<sup>2</sup>. The test aquaria were labeled to identify the target test concentrations.

### 3.4 Test Procedures

The 96-hour acute exposure of orange-red killifish to Wingstay<sup>®</sup> 100 was conducted from 28 September to 2 October 1994. The study was initiated by impartially selecting and distributing ten killifish to each of three replicate aquaria (e.g., 30 fish per treatment level and control). One treatment level (1.0 mg/L, nominal), a dilution water control and a solvent control were included. Dissolved oxygen concentration, pH, temperature and conductivity were monitored once daily in each exposure aquarium. Observations were made on the appearance and behavior of the fish and the physical appearance of the test solutions at 0, 4, 8, 24, 48, 72 and 96 hours of exposure.

## 4.0 BIOACCUMULATION STUDY WITH CARP (*Cyprinus carpio*)

### 4.1 Test Organisms

Carp (*Cyprinus carpio*) was selected as the test species for the definitive bioaccumulation study, based on the OECD Guideline for Testing of Chemicals (1981). Characteristics which make carp suitable for bioaccumulation tests are ease of handling, ready availability, convenient size, and the extensive data on this common fish species. The carp used during this study (SLI Lot # 94A110a) were obtained from Owens and Williams Fish Farm, a commercial supplier in Hawkinsville, Georgia. Prior to testing, these fish were held in a 500-L fiberglass tank under a photoperiod of 16 hours of light and 8 hours of darkness. The well water which flowed into this holding tank was characterized as having total hardness and alkalinity ranges (as calcium carbonate, CaCO<sub>3</sub>) of 33 to 35 mg/L and 23 to 26 mg/L, respectively, and a specific conductance range of 110 to 140 (µmhos/cm). Other parameters determined in the holding tank were a pH range of 7.0 to 7.4, a dissolved oxygen concentration range of 59 to 114% of saturation and a flow rate range of 20.4 to 43.2 tank volume replacements/day. The test fish were maintained under these conditions for a minimum of 14 days prior to test initiation. The temperature in the holding tank ranged from 23 to 24 °C during this 14-day period.

No mortality was observed among the carp test fish population during the 48 hours prior to

testing (Springborn Daily Record of Fish Holding Conditions). A representative sample (N = 30) of the carp from the test population had a mean wet weight of 12 g (range 7.0 to 19 g) and a mean total length of 97 mm (range 76 to 115 mm) (Springborn Fish Weight and Lengths Log). The length range was within acceptable length in accordance with OECD guidelines. The fish used in this study were smaller than limits recommended in the OECD guidelines (OECD 305C recommends using 20- to 40-gram fish). For this study, smaller fish were used in order to minimize biomass loading. Furthermore, the analytical methods used were sensitive enough to obtain reproducible results with smaller tissue samples.

#### 4.2 Test Concentrations

The target exposure concentrations selected for the bioaccumulation study were 5.0 and 50 µg/L, based on the acute toxicity test with orange-red killifish (*Orizias latipes*).

#### 4.3 Stock Solutions Preparation

For the 5.0 µg/mL exposure, a 463 µg/mL primary stock solution was prepared by dissolving and diluting 92.6 mg Wingstay® 100 to 200 mL with acetone. A diluter stock was prepared by combining 80 mL of the 463 µg/mL solution (37.04 mg) with 80 mL of reagent water and diluting to a final volume of 200 mL with acetone. No visible precipitate was observed. This yielded a diluter stock with a nominal concentration of 185 µg/mL.

For the 50.0 µg/mL exposure, a 4630 µg/mL primary stock solution was prepared by dissolving and diluting 925.9 mg Wingstay® 100 to 200 mL with acetone. A diluter stock was prepared by combining 80 mL of the 4630 µg/mL solution (370.4 mg) with 80 mL reagent water and diluting to a final volume of 200 mL with acetone. This yielded a diluter stock with a nominal concentration of 1850 µg/mL.

Subsequent primary and diluter stock solutions of Wingstay® 100 were prepared in a manner similar to that described above. Primary stock solutions were prepared every 7 to 10 days, as needed; diluter stocks, every 3 to 5 days, as needed. Concentrations of the diluter stocks were confirmed by high performance liquid chromatography with ultra-violet detection (HPLC-UV).

The solvent control stock solution was prepared by combining 120 mL of acetone with 80 mL reagent water in a 200-mL volumetric flask. This stock was prepared every 3 to 4 days (total of 12 preparations), as needed in the definitive test.

A 0.250 mg/mL primary stock solution was prepared by dissolving and diluting 25.0 mg Wingstay® 100 to 100 mL with hexane. This stock solution was used, with dilution, in hexane to prepare HPLC calibration standards for water analyses. Five calibration standards (0.05 to 0.500 mg/L) were prepared in hexane for Days 0 through 8 in the bioaccumulation study. HPLC analysis performed on Days 15 through 36 required calibration standards prepared in hexane at a lower range (40.8 to 204 µg/L) to accommodate water sample analysis.

A 1.00 mg/mL primary stock solution was prepared by dissolving and diluting 100.1 mg Wingstay® 100 to 100 mL with acetonitrile. This stock solution was used, with dilution, to prepare HPLC calibration standards for tissue analyses. Five calibration standards (0.500 to 10.0 mg/L) were prepared in 80:20 acetonitrile:reagent water for tissue analysis in the bioaccumulation study.

#### 4.4 Test System

The bioaccumulation study was conducted using an exposure system consisting of a continuous chemical delivery system, an adaptation of a diluter described by Benoit *et al.* (1982), a temperature-controlled water bath, two Wingstay® 100 exposure aquaria, a solvent control (acetone) aquarium and a blank control aquarium. Each clear glass aquarium was constructed at Springborn using glass and silicone adhesive and measured 75 x 39 x 30 cm (length x width x height). The water level in the test aquaria was maintained at a depth of 25 cm for a total solution volume of 73 L. The test aquaria were labeled to identify the target concentration of the test substance.

The diluter system was calibrated prior to test initiation and at termination of the exposure phase (Test Day 36) by measuring delivery volumes of each stock solution (toxicant and solvent). The function of the diluter system (e.g., flow rates, stock solution consumption) was monitored daily and a visual check was performed each day. In addition, analysis of the exposure solutions for the

concentration of Wingstay<sup>®</sup> 100 was also used to verify proper operation of the diluter system. The diluter systems continuously delivering Wingstay<sup>®</sup> 100 at concentrations of 5.0 and 50 µg/L and solvent control were in operation from mid-to-late October 1994 prior to the addition of the test organisms. The aquaria were sampled and analyzed for Wingstay<sup>®</sup> 100 on three separate days to confirm the appropriate concentration of test substance in the exposure water.

The toxicant delivery system for the 5.0 µg/L Wingstay<sup>®</sup> 100 exposure consisted of a Sage<sup>®</sup> syringe pump equipped with two 50-mL gas-tight syringes, each calibrated to deliver 0.027 mL/min of the Wingstay<sup>®</sup> 100 or solvent control stock solution to a flow of 1000 mL/min of test dilution water. This delivery rate was equivalent to approximately 20 aquarium volumes per day or a 90% aquarium volume replacement each approximately 2½-hour period (Sprague, 1969). On Pretest #3, the toxicant delivery was increased to 0.038 mL/min to achieve the target concentration of 5.0 µg/L. The concentration of solvent in the acetone solvent control aquarium was equal to that in the exposure aquaria. Since the stock solution used to prepare the exposure solutions consisted of 60% acetone (by volume) in reagent water, the delivery system initially provided a maximum concentration of solvent (acetone) to exposure solutions of 0.016 to 0.023 mL/L.

#### 4.5 Test Procedures

On 25 October 1994, the exposure was initiated by impartially selecting and distributing 38 carp to each of four aquaria. The total fish biomass was 456 grams (0.31 gram per liter of the 24-hour flow-through volume of the aquaria) at test initiation and decreased throughout the study as fish were removed for tissue residue analysis. Water quality parameters were measured daily for two weeks prior to initiation and through study termination. Daily observations were made on the appearance and behavior of the fish and the physical appearance of the test solution. Exposure water solutions were sampled on Days 0, 3, 7, 8, 15, 21, 24, 28, 31 and 35 of exposure for analysis by HPLC-UV. Three fish per aquarium were sampled on Days 0, 3, 7, 15, 21, 28 and 36 of exposure and Days 3, 7, and 14 of depuration for analysis by HPLC-UV.

#### 4.6 Analytical Measurements

To monitor the concentration of Wingstay® 100 residues in the exposure water, 50-mL water samples were collected on 15 separate days during the month prior to test initiation. During the in-life phase, water samples were removed on Days 0, 3, 7, 8, 15, 17, 21, 24, 28, 31 and 35 of exposure. Each 50-mL sample was collected from the approximate midpoint of the aquarium and extracted with 4.0 mL of hexane. An aliquot, 1 to 2 mL, of the diluted extract was transferred into an amber glass vial and analyzed by HPLC-UV. This procedure is detailed in Appendix III.

Since Wingstay® 100 contains three major diaryl-*p*-phenylenediamine components, multiple peaks were observed in the HPLC chromatograms. A representative HPLC chromatogram of Wingstay® 100 is shown in Figure 2, depicting the separation of the three diaryl-*p*-phenylenediamines. The sum of the three major components was determined in both exposure water and fish tissue and used to calculate the BCFs.

Quality Control (QC) water samples were prepared and analyzed with the water samples at each interval to determine the accuracy and quality control maintained during the analytical process. The QC water samples were prepared by fortification of a known amount of Wingstay® 100 into 50 mL of test dilution water which was then analyzed in the same manner as the exposure solutions. A method validation/recovery study performed prior to test initiation resulted in a mean recovery of  $97.9 \pm 6.68\%$  (N=9) from water at Wingstay® 100 concentrations of 5 and 50 µg/L. QC samples having a recovery range of 77.9 to 118% (representing three standard deviations from the mean) were considered acceptable. Conditions and procedures used throughout the analysis of exposure solutions and QC samples during the bioaccumulation study were similar to those described in Appendix III.

To quantify the concentration of Wingstay® 100 residues in the carp tissue during the exposure period, three fish were collected from each aquarium and analyzed on Days 0, 3, 7, 15, 21, 28 and 36. The fish were dissected into 2 portions: edible (muscle) and nonedible (viscera and carcass). Each tissue sample was extracted with acetonitrile as follows: a volume of acetonitrile approximately 5 times the wet weight of the tissue portion was combined with the tissue portion,

homogenized three times for two minutes using a biohomogenizer, vortexed for one minute and centrifuged at 1500 revolutions per minute for approximately 30 minutes. Aliquots of the supernatant were transferred into glass vials prior to HPLC analysis. This procedure and analytical conditions are detailed in Appendix IV (with the exceptions that tissues were extracted only once, and Wingstay® 100 was monitored at 280 or 310 nm).

QC tissue samples were prepared and analyzed with the tissue samples at each interval. The QC tissue samples were prepared by fortification of a known amount of Wingstay® 100 onto blank control fish tissue which was then extracted and analyzed in the same manner as the exposure tissue samples. A method validation/recovery study performed prior to test initiation resulted in a mean recovery of  $96.0 \pm 5\%$  (N=9) from tissue. Samples with recovery in the range of 81.0 to 111% (representing three standard deviations from the mean) were considered acceptable. Conditions and procedures used throughout the analysis of tissue and QC samples during the bioaccumulation study were similar to those described in Appendix IV (with the exceptions that tissues were extracted only once, and Wingstay® 100 was monitored at 280 or 310 nm).

#### **4.7 Determination of Lipid Content**

To reduce variability in test results, particularly for those substances with high lipophilicity, bioconcentration should be expressed in relation to lipid content as well as whole body weight. The lipid content in carp was determined in duplicate according to the following procedure. Two fish were weighed and separately homogenized in a centrifuge tube with 20 mL of chloroform and 40 mL of methanol for a period of two minutes. A second 20-mL volume of chloroform was added and the sample was homogenized for an additional two minutes. The homogenized samples and chloroform were then filtered (Whatman GF/F glass microfibre filters) through a Buchner funnel and the filtrates transferred to a 500-mL separatory funnel. The residues remaining on the Buchner funnel were transferred to the centrifuge tube and vortexed for 3 minutes with 80 mL of 1:1 chloroform and methanol mixture. The vortexed mixture was again filtered through a Buchner funnel. The filtrate was combined with the first filtrates in the same 500-mL separatory funnel and

exactly 60 mL of NANOpure<sup>®</sup> water added. The samples were shaken by hand for three minutes, transferred to centrifuge tubes and centrifuged at 1500 rpm for 2 hours.

The top layer from each sample was decanted into 250-mL glass (clear) bottles before the second aliquot was added to the centrifuge tubes. The samples were refrigerated overnight and then centrifuged at 1500 rpm for 6 hours. Approximately 50 mL of the lower chloroform layer was removed using a glass syringe. To avoid contamination, the syringe was inserted through the upper methanol/water layer while expelling air. An additional volume of chloroform (20 mL) was added to each tube and the samples were refrigerated overnight. The samples were removed from the refrigerator and centrifuged at 1500 rpm for 4 hours. A portion (25 mL) of the methanol water (top) layer was removed and reagent grade water and chloroform (25 mL of each) were added to each tube. The samples were centrifuged at 1500 rpm for 1.5 hours and stored refrigerated overnight. The samples were centrifuged the next day for 3 hours at 1500 rpm. The methanol/water layer was removed to expose the chloroform (lower) layer. The chloroform layer was removed and combined with the two aliquots previously removed and dried through anhydrous sodium sulfate into preweighed 250-mL roundbottom flasks. The chloroform was then rotary evaporated and the roundbottom flask, containing the remaining lipid residues, was placed in a vacuum desiccator and allowed to dry overnight. The roundbottom flask was then reweighed to determine the weight of the dry lipid sample. The weight of the dried lipid sample was divided into the original tissue weight to determine the percent lipid content.

#### 4.8 Calculation of Bioaccumulation (Bioconcentration) Factors

Bioaccumulation (bioconcentration) factors for edible, nonedible and whole body fish tissue for Wingstay<sup>®</sup> 100 were determined by dividing the mean measured equilibrium (steady state) tissue concentration for each tissue type by the corresponding mean measured water concentration for the entire exposure period. For comparison, an additional method of calculating - bioaccumulation (bioconcentration) factors where the ratio of the uptake constant ( $K_u$ ) to the depuration constant ( $K_d$ ) equals bioaccumulation (bioconcentration) factor (BCF) was performed. The uptake and depuration constants,  $K_u$  and  $K_d$ , were established based on the measured tissue and water concentrations. They were calculated using an iterative computer program (i.e., Eureka,

Version 1.0, Borland Software). This program selected the values for  $K_u$  and  $K_d$ , which best fit the experimental data.

For the uptake phase:

$$C_t = \frac{K_u}{K_d} \times C_w \times [1 - e^{(-K_d t)}] \quad (1)$$

For the depuration phase:

$$C_t = \frac{K_u}{K_d} \times C_w \times [e^{(-K_d t)}] \quad (2)$$

Where:

- $K_d$  = depuration constant ( $\text{day}^{-1}$ )
- $K_u$  = uptake constant ( $\text{day}^{-1}$ )
- $t$  = time in days
- $C_t$  = tissue concentration at time  $t$  ( $\mu\text{g}/\text{kg}$ )
- $C_w$  = water concentration at time  $t$  ( $\mu\text{g}/\text{L}$ )
- $\text{BCF} = K_u/K_d$

BCFs based on lipid content were calculated as:

$$\text{lipid content BCF} = \frac{\text{BCF}}{\text{lipid content}} \quad (3)$$

## 5.0 RESULTS

### 5.1 Analytical Method Development

Analytical methods were developed for analysis of Wingstay<sup>®</sup> 100 in water and fish tissue using reversed-phase HPLC. The three main components of Wingstay<sup>®</sup> 100, (R-59, R-1679 and R-898) were well separated under the chromatographic conditions. The sum of the three components was determined and used to quantify the amount of Wingstay<sup>®</sup> 100 in both water and tissue. The method limit of quantitation (LOQ) for water sample analysis was determined to be approximately 2 to 3 µg/L (the sum of three components) based on the method validation (Appendix III). During the study, the concentrations of Wingstay<sup>®</sup> 100 were measured as low as 1.9 µg/L. The apparent difference in LOQs was attributable to the variance in the calibration curve (linear regression). The definition of LOQ is described in Appendix V.

During method development, Wingstay<sup>®</sup> 100 was found to be rather unstable in water at low concentrations. The extent of degradation appeared to be intermittent but highly concentration dependent. Although considerable efforts were devoted to the investigation, the mechanism of the apparent degradation was not clear. It was suspected that oxidation was involved in the degradation of Wingstay<sup>®</sup> 100 in water at low concentrations (approximately 3 to 5 µg/L). The ability to recover Wingstay<sup>®</sup> 100 from water through solvent extraction was evaluated. Hexane extracts showed the least amount of degradation.

### 5.2 Acute Toxicity Test with Killifish

At a nominal test concentration of 1.0 mg/L, no lethal or sub-lethal effects were observed. The LC<sub>50</sub> was estimated to be greater than 1.0 mg/L. Based on this information and the detection limit of the analytical methods, Wingstay<sup>®</sup> 100 exposure concentrations of 5.0 and 50.0 µg/L (representing 0.1 and 1% of the expected toxicity level, respectively) were selected for the bioaccumulation study.

During the 96-hour static acute toxicity test, the measured water quality parameters varied minimally and remained within acceptable ranges for the maintenance of killifish in the control, solvent control and 1.0 mg/L Wingstay<sup>®</sup> 100 exposure aquaria. Dissolved oxygen concentration ranged from 7.4 to 9.1 mg/L. Temperature, monitored continuously, was measured to be 25 °C. The pH ranged from 7.2 to 7.6. Conductivity ranged from 170 to 180 µmhos/cm. Characterization

of the dilution water at test initiation established a total hardness of 30 mg/L as CaCO<sub>3</sub> and an alkalinity of 24.

### 5.3 Evaluation of the Bioaccumulation (Bioconcentration) Factor

Solution samples were removed from the test aquaria on each of three days before the exposure was initiated. Analyses of these samples resulted in measured concentrations which were acceptable. Based on these results, the exposure was initiated.

Throughout the study, no undissolved test substance was observed in the dilution system or the test aquarium. During the exposure no mortalities occurred among the test organisms exposed to 5 µg/L Wingstay® 100. In general, the fish appeared healthy and exhibited normal behavior throughout the study.

By Day 7, seven deaths had occurred among the test organisms exposed to 50 µg/L (target) Wingstay® 100. Due to the unexpected mortality rate, the 50 µg/L Wingstay® 100 exposure was discontinued. The fish were maintained in the aquarium through the end of the study. Six additional mortalities occurred within seven days of discontinuing the Wingstay® 100 exposure. This result was considered to be an anomaly since no mortality or adverse effects were observed in a subsequent 28-day study (SLI Report #95-4-5826, Phase II) at concentrations exceeding 50 µg/L (target).

During the 50-day bioaccumulation study (36 days exposure, 14 days depuration at 5.0 µg/L Wingstay® 100), the measured water quality parameters varied minimally and remained within acceptable ranges for the maintenance of carp. Dissolved oxygen concentration averaged approximately 7.5 mg/L (range = 5.4 to 8.8 mg/L) and was equivalent to 88% of saturation at 24 °C. Dissolved oxygen concentrations dropped to approximately 55% saturation in the solvent and blank control aquaria on day 1 of the study. Aeration was initiated and dissolved oxygen concentrations were maintained above 60% saturation for the remainder of the study. Continuous monitoring of the temperature (monitored in the solvent control aquarium located in the same water bath) throughout the test resulted in a temperature range of 22 to 27 °C. The temperature was also measured daily in each aquarium. The pH range was 6.9 to 7.6. Characterization of the dilution water at test initiation established a total hardness range of 31 to 36 mg/L as CaCO<sub>3</sub>. Water quality

parameters (mean dissolved oxygen concentration, mean daily temperature and pH range) for each aquarium are detailed in Table 1.

The concentrations of the Wingstay® 100 diluter stocks were determined prior to use and are presented in Table 2. The concentrations of the 185 mg/L diluter stock ranged from 99.6% to 103% of nominal. The concentrations of the 1850 mg/L diluter stock ranged from 98.2% to 102% of nominal.

The concentrations of Wingstay® 100 measured in the exposure water during the pretest and 36-day exposure are presented in Table 3. A representative chromatogram of an exposure water sample is shown in Figure 3. The mean ( $\pm$  S.D.) measured concentration of Wingstay® 100 over the Day 0 to Day 36 exposure period was 3.24 ( $\pm$  1.57)  $\mu$ g/L which represented 65% of the target Wingstay® 100 concentration. As indicated by the data, the Wingstay® 100 concentrations in exposure water were both somewhat less than targeted and somewhat variable over the 36-day exposure period. The variable mean measured concentration of Wingstay® 100 for the 36-day exposure period was not unexpected because water concentrations were close to the 3  $\mu$ g/L LOQ of the analytical system. Results of the solvent control water analyzed during the 36-day exposure were below the detection limit of the analytical system. A representative chromatogram of a solvent control water sample is shown in Figure 5.

Analysis of the aqueous QC samples for Wingstay® 100 at each aquaria water sampling interval resulted in a mean percent of nominal ( $\pm$  S.D.) of 93.2 ( $\pm$  15.9)% (n = 42). These results are presented in Table 4 and Figure 6 and were consistent with the acceptable recovery range of 77.9 to 118% determined during the method validation (Appendix III). Recovery of Wingstay® 100 outside the acceptable range was not unexpected because QC water sample concentrations of 5  $\mu$ g/L were close to the LOQ of the analytical system.

The concentrations of residues measured in the edible, nonedible and whole body tissue of the carp sampled during the exposure and depuration periods are presented in Tables 5 (exposure) and 6 (depuration). Representative chromatograms of edible and nonedible tissue samples are presented in Figures 7 and 8, respectively. Steady state concentrations for edible, nonedible and whole body tissues were determined by subjecting measured (or calculated as in

whole body) concentrations for three consecutive sampling intervals to analyses of variance until no statistically significant difference was found between the three concentrations. Steady state concentrations were reached by day 15 as determined by statistical analysis of variance ( $p = 0.05$ ) and Tukey's Test (Neter et al., 1985; Zar, 1984). The mean steady state concentrations (Day 15 through Day 36) in edible tissue, nonedible tissue and whole fish were determined to be  $11,800 \pm 4,580 \mu\text{g}/\text{kg}$ ,  $20,500 \pm 8,570 \mu\text{g}/\text{kg}$  and  $15,700 \pm 5,980 \mu\text{g}/\text{kg}$ , respectively. Results of the solvent control fish tissue analyzed during the 36-day exposure and 14-depuration were below the detection limit of the analytical system. A representative chromatogram of a solvent control tissue extract is shown in Figure 9.

Tissue QC samples for Wingstay<sup>®</sup> 100 at each exposure sampling interval resulted in a mean percent recovery (S.D.) of  $94.7 (\pm 7.29)\%$  for nonedible tissue and  $92.0 (\pm 6.44)\%$  for edible tissue (Table 7) and, with two exceptions, were within the acceptable recovery range of 81.0 to 111% determined during the method validation (Appendix IV). Tissue QC sample results for Wingstay<sup>®</sup> 100 during depuration are presented in Table 8 and Figure 10. The results of QC samples throughout the study established that satisfactory precision and quality control were maintained during the analytical process. A representative chromatogram of a tissue QC extract is shown in Figure 11.

Bioaccumulation (bioconcentration) factors for each tissue type were calculated using the mean measured water exposure concentration,  $3.24 \mu\text{g}/\text{L}$  Wingstay<sup>®</sup> 100, and the mean measured steady state tissue concentration. **Predicted** bioaccumulation (bioconcentration) factors for edible, nonedible and whole body tissue were also calculated. Equations 1 and 2 (Section 4.8) were used to determine the uptake ( $K_u$ ) and the depuration constant ( $K_d$ ) for each tissue type.  $K_u/K_d$  established the predicted BCF. **Predicted** and **measured** bioaccumulation (bioconcentration) factors for each tissue type are presented in the table below. The model predictions for edible, nonedible and whole body tissue, as presented in Figures 12, 13 and 14, respectively, are in relatively close agreement with the actual bioconcentration factors determined during the study.

**Predicted and measured bioaccumulation  
(bioconcentration) factors for each tissue type**

Tissue type	$K_u$	$K_d$	Predicted BCF	Measured BCF
Edible	602	0.159	3790	3640
Nonedible	1370	0.212	6450	6310
Whole Body	1000	0.201	4970	4850

#### 5.4 Depuration Phase

Analyses of the edible and nonedible tissue portions of carp transferred to a depuration aquarium (Wingstay® 100-free) in the diluter system after 36 days of exposure indicated continuous elimination of Wingstay® 100 from all tissue types over the 14-day depuration period (Table 6; Figures 12 to 14).

The half-life, or time when 50% of the accumulated residues were eliminated, was reached before Day 7 post-exposure in the whole body tissue. By Day 14, elimination in the whole body tissue was 94.5%.

#### 5.5 Lipid Content

The lipid content in carp was determined as described in the materials and methods section. The percent lipid content from each of two carp from Springborn Lot #94A110 was calculated by dividing the weight of the final lipid residue by the initial weight of the fish. The results of the percent lipid determination were used to calculate BCFs in both the Wingstay®100 bioaccumulation Phase I study (SLI Report #95-5-5882) and the Wingstay® 100 bioaccumulation Phase II study (SLI Report #5826). BCFs based on lipid content are calculated by dividing the measured BCF by the lipid content. Results of duplicate lipid content determinations were 5.63% and 6.16%, average 5.90%. Based on relation to lipid content, the BCF (whole body) was calculated to be 82,200, a factor which represents accumulation in fatty tissue.

## 6.0 CONCLUSION

The bioconcentration of Wingstay® 100 in carp (*Cyprinus carpio*) was investigated for 36 days in a flow-through system at a target exposure concentration of 5 µg/L. Concentrations of Wingstay® 100 in both exposure water and fish tissue were measured by HPLC-UV at 310 nm (exposure water) and 280 or 310 nm (fish tissue). During the 36-day exposure, the concentration of Wingstay® 100 in water averaged 3.24 µg/L. The steady state was reached after 15 days of exposure. The steady state tissue concentration averaged 11,800 µg/kg for edible tissue, 20,500 µg/kg for nonedible tissue, and 15,700 µg/kg for whole body tissue. Based on these values, the bioconcentration factors (BCF) were calculated to be 3,640 for edible tissue, 6,310 for nonedible tissue, and 4,850 for whole body tissue. These BCF values were consistent in the model calculations using equations specified in Section 4.8.

The lipid content of the carp population used in this study was estimated to be 5.9%. The whole body BCF, calculated based on the estimated lipid content, was 82,200. This lipid content BCF can be used as a reference BCF value for comparison to fish with different lipid contents.

The elimination of Wingstay® 100 by carp during depuration was rapid. The depuration half-life was less than 7 days. By day 14 of depuration, 94.5% of Wingstay® 100 had been eliminated from the whole body tissue.

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**PROTOCOL DEVIATIONS**

1. Due to unexpected fish mortality, the test at high concentration (50 µg/L) was terminated after 9 days of exposure. Instead, a 28-day study was conducted at higher concentrations of Wingstay® 100 under a separate study protocol (SLI Protocol #010495/GOODYEAR).
2. The lipid content of a representative sample of the fish population was measured after test termination, instead of within two weeks of the start of the exposure period as described in the study protocol.
3. To better understand the chemical behavior of Wingstay® 100 in exposure water and fish tissue, the analytical sampling schedule was adjusted in the definitive study. Exposure water was sampled on Days 0, 3, 7, 8, 15, 17, 21, 24, 28, 31 and 35 of exposure. Fish were sampled on Days 0, 3, 7, 15, 21, 28 and 36 of exposure.
4. Section 4.5.3: Test Conditions; Temperature of the study protocol states that the water temperature of the test solutions will be maintained at  $25 \pm 2^\circ\text{C}$ . On Day 8 of depuration the temperature in the solvent control and 5 µg/L aquaria was measured to be  $22.0^\circ\text{C}$ . The water temperature was gradually increased throughout the day to within  $25 \pm 2^\circ\text{C}$ .
5. In Section 4.5.3, Test Conditions, Dissolved Oxygen, the protocol states that the total dissolved oxygen is not allowed to drop below 60% saturation for the duration of the test and aeration will be used maintain this saturation level. On Day 1 of the study, the dissolved oxygen concentrations in the solvent and blank control aquaria

dropped to 4.7 and 4.5 mg/L, respectively. At 25°C, these concentrations yield 57% and 54% saturation (solvent and blank controls, respectively). Aeration was initiated and the dissolved oxygen concentrations were maintained above 60% for the remainder of the study.

These deviations from the protocol are not expected to impact the results of the study.

SPRINGBORN LABORATORIES, INC.

  
\_\_\_\_\_  
John Mao, Ph.D.                      9/11/96  
Study Director                      Date

**QUALITY ASSURANCE UNIT STATEMENT**

The study conduct, raw data and report for "**Wingstay® 100 - Bioaccumulation Exposure In Carp (*Cyprinus carpio*)**" were inspected by the Quality Assurance Unit at Springborn Laboratories, Inc., *Health and Environmental Sciences*, to determine adherence with the study protocol and laboratory standard operating procedures. Dates of study inspections, dates reported to the Study Director and to Management are listed below.

<u>Inspection Date</u>	<u>Reported to Study Director</u>	<u>Reported to Management</u>
9/30/94	9/30/94	9/30/94
10/25/94	10/28/94	11/4/94
10/26/94	10/26/94	11/4/94
11/9,14,16/94	11/16/94	11/18/94
11/29/94	11/29/94	12/2/94
12/20/94	12/20/94	12/30/94
12/30/94	12/30/94	12/30/94
1/4,5/95	1/5/95	1/13/95
1/13/95	1/13/95	1/13/95
2/6,7/95	2/7/95	2/10/95
6/6/95	6/6/95	6/16/95
6/7/95	6/7/95	6/16/95
6/9-12/95	6/12/95	6/16/95
6/12,13/95	6/13/95	6/16/95
6/13-19/95	6/19/95	6/30/95
6/20,21/95	6/21/95	6/30/95
3/27,28/96	3/28/96	4/5/96
4/1,2/96	4/2/96	4/5/96
4/11,12/96	4/12/96	4/19/96
7/24/96	7/24/96	7/26/96
8/1/96	8/1/96	8/1/96
9/11/96	9/11/96	9/11/96

SPRINGBORN LABORATORIES, INC.

*Doreen S Newhouse 11 Sep 96*

Doreen S, Newhouse  
Manager,  
Quality Assurance Unit

Date

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**Table 3. Wingstay® 100 concentrations measured in exposure water during 36 days of exposure of carp (*Cyprinus carpio*) to 5.0 µg/L (target) Wingstay® 100.**

Day	Water Concentration (µg/L)			Mean	Standard Deviation	Population (n)
	Replicate A	Replicate B	Replicate C			
Pretest #1	BDL <sup>a</sup>	BDL	BDL	NA <sup>b</sup>	NA <sup>b</sup>	3
Pretest #2	4.04	3.83	3.82	3.90	0.12	3
Pretest #3	3.24	3.70	3.60	3.51	0.24	3
0	5.42	5.05	6.44	5.63	0.72	3
3	3.93	4.85	4.05	4.28	0.50	3
7 <sup>c</sup>	2.11	1.80	2.51	2.05	0.23	3
8	2.53	2.72	2.52	2.59	0.12	3
15	2.33	2.63	2.75	2.57	0.22	3
17	2.96	2.58	2.51	2.68	0.25	3
21	8.89	3.13	2.30	4.77	3.59	3
24	2.69	2.02	2.87	2.53	0.45	3
28	1.97	2.07	BDL	2.02	NA	2
31	BDL	2.21	BDL	2.21	NA	1
35	3.08	3.44	BDL	3.26	NA	2

<sup>a</sup> Value was below limit of detection.

<sup>b</sup> NA = Not applicable. Value cannot be calculated.

<sup>c</sup> Two HPLC analyses were conducted on day 7. The diluter system went static overnight and values from the first sampling were all below the limit of detection. Thus, values from the first sampling are not included in this table.

**Table 4. Analytical results for Quality Control samples analyzed concurrently with water samples during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.**

Test Day	Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Percent of Nominal
Pretest #1	5.00	3.85	77.0 <sup>a</sup>
	25.0	29.6	118
	50.0	49.8	99.6
Pretest #2	5.00	4.49	89.8
	25.0	24.3	97.2
	50.0	47.2	94.4
Pretest #3	5.00	6.49	130 <sup>a</sup>
	25.0	23.6	94.4
	50.0	47.1	94.1
0	5.00	4.87	97.4
	25.0	25.7	103
	50.0	46.1	92.3
3	5.00	4.64	92.8
	25.0	24.6	98.4
	50.0	49.1	98.3
7	5.00	4.62	92.5
	25.0	21.5	86.2
	50.0	45.3	90.6
8	5.00	4.44	88.7
	25.0	24.3	97.0
	50.0	48.3	96.6
15	5.00	3.83	76.5 <sup>a,b</sup>
	5.00	3.77	75.5 <sup>a,b</sup>
	5.00	3.72	74.3 <sup>a,b</sup>

<sup>a</sup> Percent recovery for this QC sample is outside the standard acceptable range established by this laboratory (i.e.,  $\pm 3$  standard deviations from the mean percent recovery determined during the method validation/recovery study, 77.9 to 118%, Appendix V). These values, however, were used in the calculation of the mean recovery to demonstrate the overall method accuracy and precision.

<sup>b</sup> Low recovery is not unexpected for QC samples intentionally prepared at 5 µg/L in the water phase of the test system. This concentration is close to the LOQ of the analytical system.

**Table 4. Continued. Analytical results for Quality Control samples analyzed concurrently with water samples during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.**

Test Day	Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Percent of Nominal
17	5.06	6.42	127 <sup>a</sup>
	5.06	5.45	108
	5.06	6.03	119 <sup>a</sup>
21	5.06	4.40	86.9
	5.06	3.88	76.6 <sup>a</sup>
	5.06	4.44	87.7
24	5.06	5.32	105
	5.06	4.62	91.4
	5.06	6.19	122 <sup>a</sup>
28	5.06	4.57	90.4
	5.06	5.45	108
	5.06	3.63	71.8 <sup>a</sup>
31	5.06	2.90	57.2 <sup>a,b</sup>
	5.06	3.13	61.8 <sup>a,b</sup>
	5.06	3.66	72.3 <sup>a,b</sup>
35	5.06	4.19	82.7
	5.06	4.69	92.7
	5.06	5.00	98.7
Mean			93.2
Standard Deviation			15.9
% Coefficient of variance			17.1
N			42

<sup>a</sup> Percent recovery for this QC sample is outside the standard acceptable range established by this laboratory (i.e.,  $\pm 3$  standard deviations from the mean percent recovery determined during the method validation/recovery study, 77.9 to 118%, Appendix V). These values, however, were used in the calculation of the mean recovery to demonstrate the overall method accuracy and precision.

<sup>b</sup> Low recovery is not unexpected for QC samples intentionally prepared at 5 µg/L in the water phase of the test system. This concentration is close to the LOQ of the analytical system.

**Table 5. Wingstay® 100 concentrations measured in fish tissue portions and exposure water during 36 days of exposure of carp (*Cyprinus carpio*) to 5.0 µg/L (target) Wingstay® 100.**

Test Day	Water Concentration (µg/L)	Tissue Concentration (µg/kg)		
		Edible	Nonedible	Whole Body <sup>a</sup>
0	5.42	< 211 <sup>b</sup>	< 234	NA <sup>c</sup>
	5.05	< 222	< 242	NA
	6.44	< 238	< 237	NA
<b>Mean</b>	<b>5.63</b>	<b>NA</b>	<b>NA</b>	<b>NA</b>
<b>S.D.</b>	<b>0.72</b>	<b>NA</b>	<b>NA</b>	<b>NA</b>
3	3.93	7,190	17,800	11,120
	4.85	4,450	5,860	5,270
	4.05	5,710	8,710	7,080
<b>Mean</b>	<b>4.28</b>	<b>5,790</b>	<b>10,800</b>	<b>7,840</b>
<b>S.D.</b>	<b>0.50</b>	<b>1,370</b>	<b>6,210</b>	<b>3,030</b>
7	2.11 <sup>b</sup>	7,380	13,500	10,600
	1.80 <sup>b</sup>	9,260	19,900	14,700
	2.25 <sup>b</sup>	8,380	15,200	11,800
<b>Mean</b>	<b>2.05<sup>b</sup></b>	<b>8,340</b>	<b>16,200</b>	<b>12,400</b>
<b>S.D.</b>	<b>0.23</b>	<b>937</b>	<b>3,320</b>	<b>2,090</b>
15	2.33 <sup>b</sup>	10,600	26,600	17,900
	2.63 <sup>b</sup>	14,700	21,600	18,100
	2.75 <sup>b</sup>	13,900	24,000	18,900
<b>Mean</b>	<b>2.57<sup>b</sup></b>	<b>13,100</b>	<b>24,100</b>	<b>18,300</b>
<b>S.D.</b>	<b>0.22<sup>b</sup></b>	<b>2,160</b>	<b>2,540</b>	<b>531</b>

<sup>a</sup> Based on calculations using sample weights and tissue concentrations of Wingstay® 100 measured in the edible and nonedible tissue portions.

<sup>b</sup> All tissue concentration values were below the limit of quantitation for test day 0.

<sup>c</sup> NA = Not applicable. Value cannot be calculated.

<sup>d</sup> Water values presented are Day 35 values.

**Table 5. Continued. Wingstay® 100 concentrations measured in fish tissue portions and exposure water during 36 days of exposure of carp (*Cyprinus carpio*) to 5.0 µg/L (target) Wingstay® 100.**

Test Day	Water Concentration (µg/L)	Tissue Concentration (µg/kg)		
		Edible	Nonedible	Whole Body <sup>a</sup>
21	8.89	11,400	26,100	17,500
	3.13	18,900	37,900	26,200
	2.30	8,410	15,200	11,300
<b>Mean</b>	<b>4.77</b>	<b>12,900</b>	<b>26,400</b>	<b>18,300</b>
<b>S.D.</b>	<b>3.59</b>	<b>5,410</b>	<b>11,400</b>	<b>7,490</b>
28	1.97	6,840	12,700	9,540
	2.07	3,290	4,370	3,970
	BDL <sup>b</sup>	10,400	15,100	12,600
<b>Mean</b>	<b>2.02</b>	<b>6,850</b>	<b>10,700</b>	<b>8,710</b>
<b>S.D.</b>	<b>NA</b>	<b>3,570</b>	<b>5,640</b>	<b>4,390</b>
36	3.08 <sup>d</sup>	19,100	26,100	23,000
	3.44	11,100	18,400	14,700
	BDL <sup>b</sup>	13,100	17,600	15,200
<b>Mean</b>	<b>3.26</b>	<b>14,400</b>	<b>20,700</b>	<b>17,700</b>
<b>S.D.</b>	<b>NA</b>	<b>4,170</b>	<b>4,690</b>	<b>4,670</b>

<sup>a</sup> Based on calculations using sample weights and tissue concentrations of Wingstay® 100 measured in the edible and nonedible tissue portions.

<sup>b</sup> Below limit of quantitation.

<sup>c</sup> NA = Not applicable. Value cannot be calculated.

<sup>d</sup> Water values presented are Day 35 values.

**Table 6. Wingstay® 100 concentrations measured in fish tissue portions during 14 days of depuration.**

Test Day	Tissue Concentration (µg/kg)			Percent Elimination <sup>b</sup>
	Edible	Nonedible	Whole Body <sup>a</sup>	
3	10,900	19,200	14,800	
	2,890	4,660	3,770	
	2,040	3,370	2,760	
<b>Mean</b>	<b>5,270</b>	<b>9,090</b>	<b>7,100</b>	<b>59.8</b>
<b>S.D.</b>	<b>4,880</b>	<b>8,810</b>	<b>6,670</b>	
7	7,640	12,600	10,100	
	6,780	8,920	7,850	
	9,910	15,500	12,600	
<b>Mean</b>	<b>8,110</b>	<b>12,300</b>	<b>10,200</b>	<b>42.3</b>
<b>S.D.</b>	<b>1,620</b>	<b>3,280</b>	<b>2,360</b>	
14	403	490	447	
	1,120	1,040	1,080	
	1,070	1,710	1,370	
<b>Mean</b>	<b>862</b>	<b>1,080</b>	<b>967</b>	<b>94.5</b>
<b>S.D.</b>	<b>399</b>	<b>610</b>	<b>474</b>	

<sup>a</sup> Based on calculations using sample weights and tissue concentrations of Wingstay® 100 measured in the edible and nonedible tissue portions.

<sup>b</sup> Based on mean measured whole body concentration on Day 36 of exposure (17,700 µg/kg).

**Table 7. Analytical results for Quality Control samples analyzed concurrently with fish tissue samples during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.**

Test Day	Edible			Nonedible		
	Nominal Concentration (mg/kg)	Measured Concentration (mg/kg)	Percent Theoretical	Theoretical Concentration (mg/kg)	Measured Concentration (mg/kg)	Percent Theoretical
Exposure						
0	8.81	9.66	110	10.6	10.0	94.4
	4.53	4.51	99.4	4.52	4.31	95.3
	0.878	0.816	93.0	0.835	0.870	104
3	49.6	42.4	85.6	50.4	45.5	90.2
	21.8	19.1	87.5	27.3	25.3	92.6
	5.36	4.66	86.8	5.44	5.88	108
7	98.0	91.4	93.3	102	105	103
	45.9	42.6	92.8	49.9	49.8	99.9
	9.53	9.20	96.5	11.1	11.1	99.7
15	18.9	15.3	81.1	20.0	16.9	84.6
	10.1	8.08	80.0 <sup>a</sup>	9.83	7.99	81.3
	2.07	1.98	95.7	1.97	2.12	108
21	44.5	41.6	93.4	48.0	47.2	98.2
	17.7	17.0	96.5	18.4	17.3	93.8
	6.72	6.40	95.3	6.08	5.78	95.0
28	6.77	6.33	93.5	6.00	5.42	90.3
	18.1	16.4	90.7	17.8	15.5	87.3
	47.4	43.9	92.7	45.5	43.6	95.7
36	6.43	5.70	88.6	6.36	5.47	86.0
	24.4	22.3	91.2	23.9	21.6	90.5
	44.3	39.1	88.3	50.3	45.8	91.0
Mean			92.0			94.7
Standard Deviation			6.44			7.29
%Coefficient of variance			7.00			7.70
N			21			21

<sup>a</sup> Percent recovery for this QC sample is outside the standard acceptable range established by this laboratory (i.e.,  $\pm 3$  standard deviations from the mean percent recovery determined during the method validation/recovery study, 81.0 to 111%, Appendix VI).

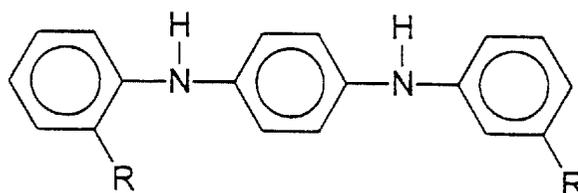
**Table 8. Analytical results for Quality Control samples analyzed concurrently with fish tissue samples during the 14-day depuration period of carp (*Cyprinus carpio*) to Wingstay® 100.**

Test Day	Edible			Nonedible		
	Theoretical Concentration (mg/kg)	Measured Concentration (mg/kg)	Percent Theoretical	Theoretical Concentration (mg/kg)	Measured Concentration (mg/kg)	Percent Theoretical
<b>Depuration</b>						
3	1.67	1.52	90.8	1.80	1.53	85.1
	8.97	8.02	89.4	8.60	7.78	90.4
	13.6	12.0	88.4	14.0	13.4	95.6
7	0.672	0.892	133 <sup>a</sup>	0.609	0.614	101
	2.59	2.38	91.8	2.77	2.67	96.5
	4.97	4.65	93.6	4.89	4.66	95.2
14	0.610	0.663	109	0.632	0.591	93.5
	2.48	2.13	85.9	2.55	2.26	88.7
	5.00	4.46	89.3	4.83	4.43	91.7
Mean		96.8			93.1	
Standard Deviation		15.1			4.7	
%Coefficient of Variance		15.6			5.0	
N		9			9	

<sup>a</sup> Percent recovery for this QC sample is outside the standard acceptable range established by this laboratory (i.e.,  $\pm 3$  standard deviations from the mean percent recovery determined during the method validation/recovery study, 81.0 to 111%, Appendix VI).

FIGURES

Figure 1. Diagram of the chemical structure of Wingstay<sup>®</sup> 100 components.



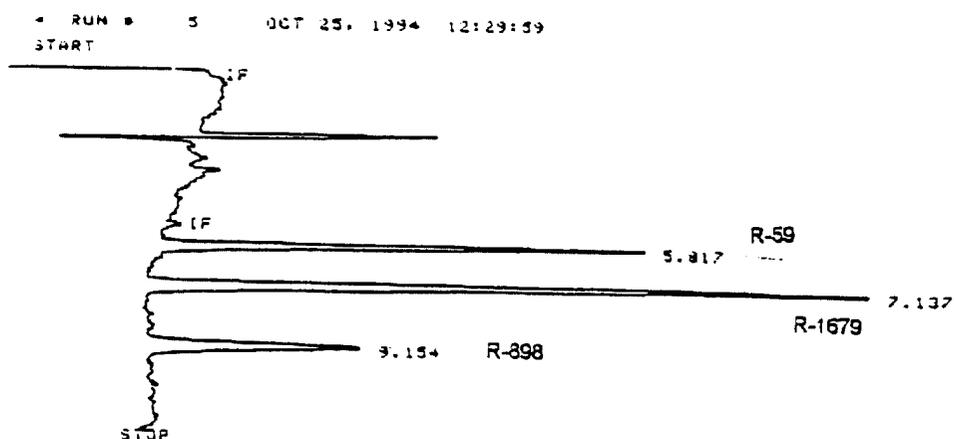
R = H or CH<sub>3</sub>

R-59      R<sub>1</sub> and R<sub>2</sub> = H

R-1679    R<sub>1</sub> = H, R<sub>2</sub> = CH<sub>3</sub>

R-898     R<sub>1</sub> and R<sub>2</sub> = CH<sub>3</sub>

Figure 2. Representative HPLC chromatogram of a Wingstay<sup>®</sup> 100 water calibration standard (0.125 mg/L) depicting the separation of the three diaryl-*p*-phenylenediamines.



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RUN# 3 OCT 25, 1994 12:29:39

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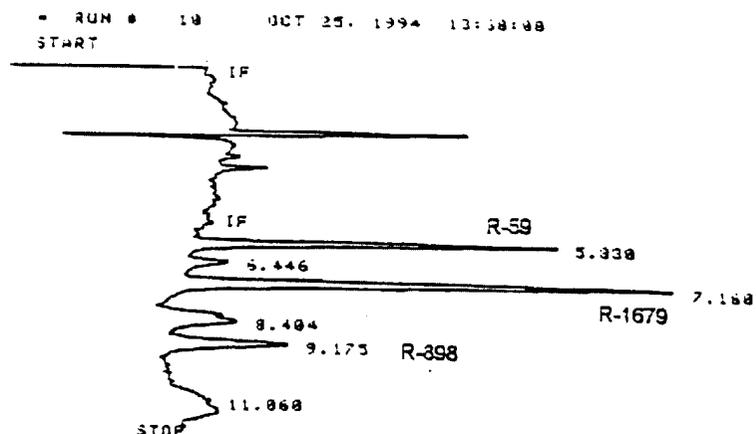
ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	AMOUNT	NAME
5.817	PV	44670	.169	4418	1R	.119	MG/L
7.137	PP	74663	.192	6495	2	.111	MG/L
9.154	VV	30644	.272	1879	3	.099	MG/L

TOTAL HEIGHT= 12791

AVL FACTOR=1.0000E+00

Figure 3. Representative HPLC chromatogram of an aquarium water sample analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.



Closing signal file M:SIGNAL .BNC

RUN# 10 OCT 25, 1994 13:38:00

SIGNAL FILE: M:SIGNAL.BNC

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	AMOUNT	NAME
5.338	PS	38738	.156	3293	1R	.089	MC/L
5.446	SV	5337	.237	375		.000	
7.168	VB	52137	.195	4456	2	.075	MC/L
8.404	BP	3294	.191	303		.000	
9.175	PP	19879	.298	1895	3	.053	MC/L
11.068	VV	6873	.346	331		.000	

TOTAL HEIGHT= 9843

MUL FACTOR=1.0000E-00

Figure 4. Representative HPLC chromatogram of a solvent control aquarium sample analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay<sup>®</sup> 100.

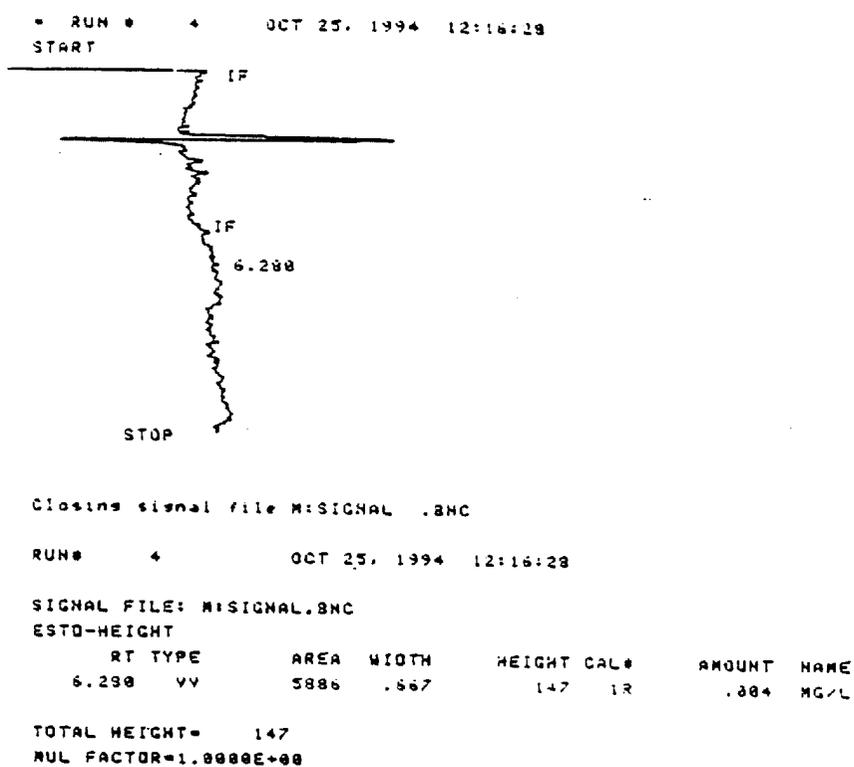
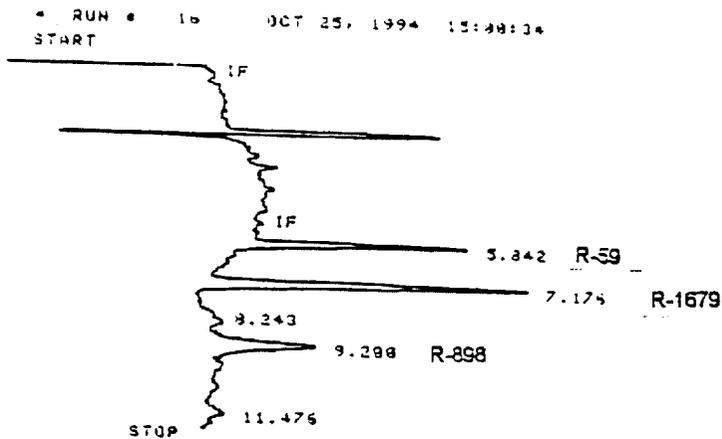


Figure 5. Representative HPLC chromatogram of a quality control sample analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.



Closing signal file M:SIGNAL .BNC

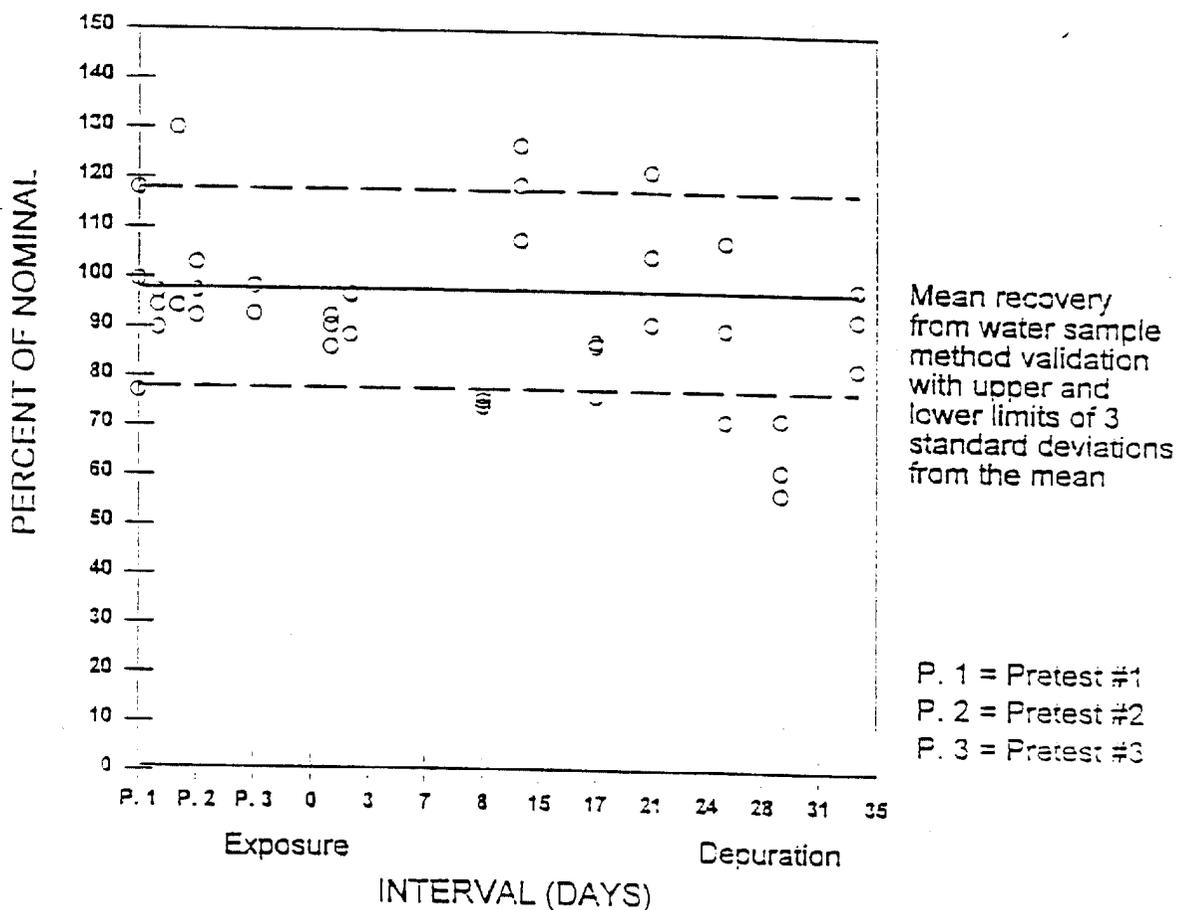
RUN# 16    OCT 25, 1994 15:00:34

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 ESTD-HEIGHT

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7.176	PP	33171	.191	2395	2	.050	MG/L
8.243	PV	3464	.340	173		.000	
9.288	VV	23988	.399	1825	1	.049	MG/L
11.476	VV	3178	.300	175		.000	

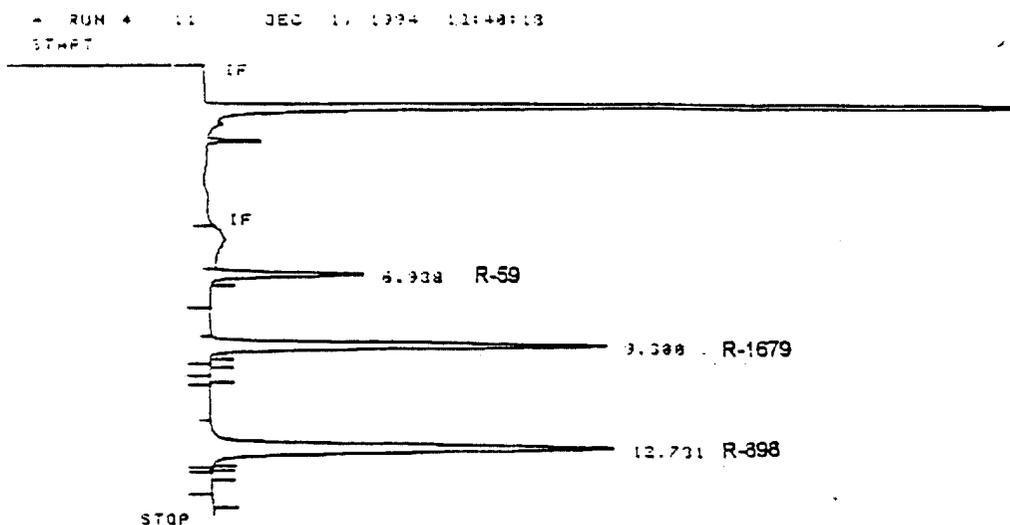
TOTAL HEIGHT= 6318  
 MUL FACTOR=1.0000E+00

Figure 6. Analytical results for Quality Control samples analyzed concurrently with water samples during three pretests and 36 days of exposure of carp (*Cyprinus carpio*) exposed to Wingstay® 100.



Note: It was not unexpected that some of the QC samples fell outside the acceptable recovery range. QC samples were prepared to match concentrations in the water phase of the test system close to the 3 µg/L LOQ of the analytical system.

Figure 7. Representative HPLC chromatogram of an edible tissue extract analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay<sup>®</sup> 100.



Closing signal file N:SIGNAL.BNC

RUN# 11 DEC 1, 1994 13:48:13

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SIGNAL FILE: N:SIGNAL.BNC

NO CALIB PEAKS FOUND

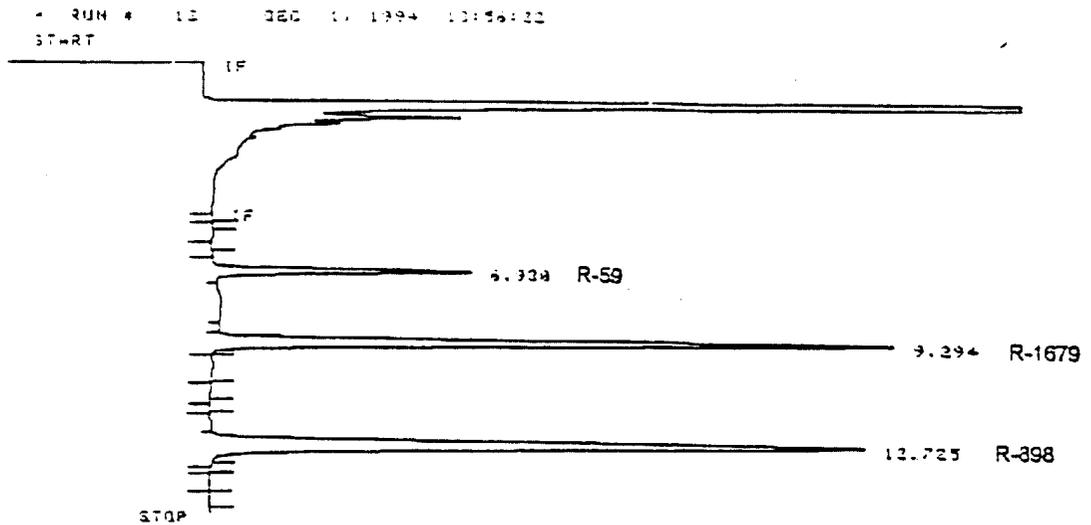
AREA:

RT	AREA	TYPE	WIDTH	AREA%
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9.308	187455	VB	.221	37.34714
12.731	258446	PS	.300	51.49085

TOTAL AREA= 501926

MUL FACTOR=1.0000E+00

Figure 8. Representative HPLC chromatogram of a nonedible tissue extract analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.



C:\aslon\signal\file\M:SIGNAL.BNC

RUN# 12 DEC 1, 1994 13:56:32

METHOD NAME: M=GENERIC.MET

SIGNAL FILE: M:SIGNAL.BNC

NO CALIB PEAKS FOUND

AREAS

RT	AREA	TYPE	WIDTH	AREA%
6.938	96479	VB	.177	11.69232
9.294	323169	VB	.222	39.16720
12.725	405439	VB	.291	49.13949

TOTAL AREA= 825078

MUL FACTOR=1.0000E+00

Figure 9. Representative HPLC chromatogram of a solvent control tissue extract analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.

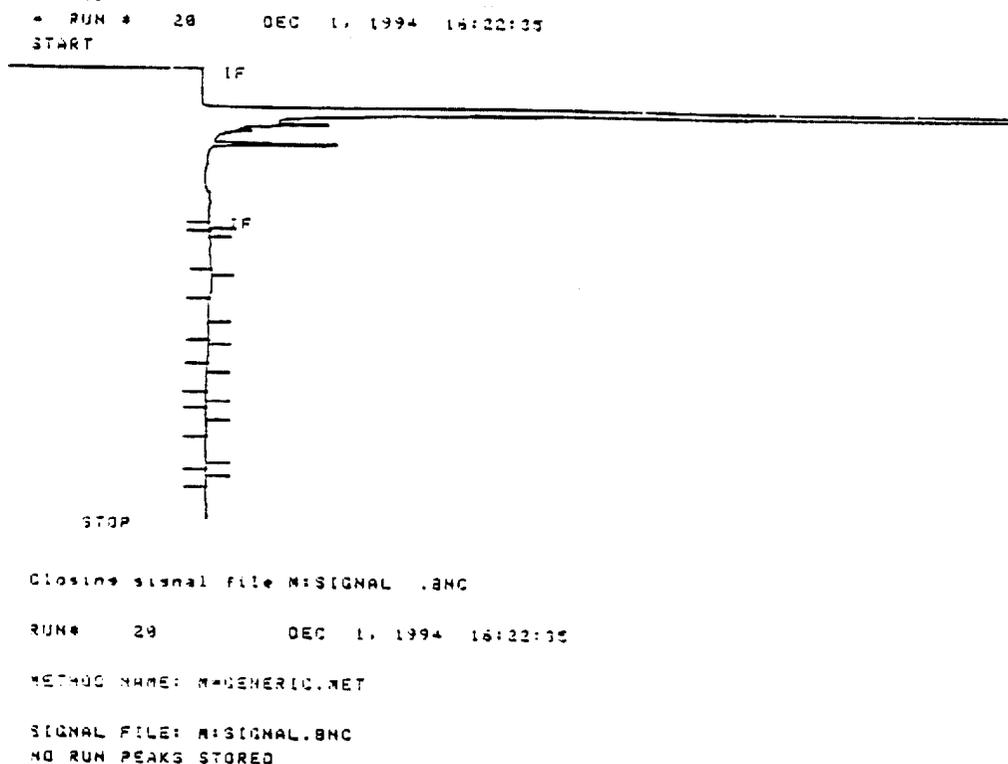


Figure 10. Analytical results for Quality Control samples analyzed concurrently with tissue samples during 36 days of exposure and 1 to 14 days depuration of carp (*Cyprinus carpio*) exposed to Wingstay® 100.

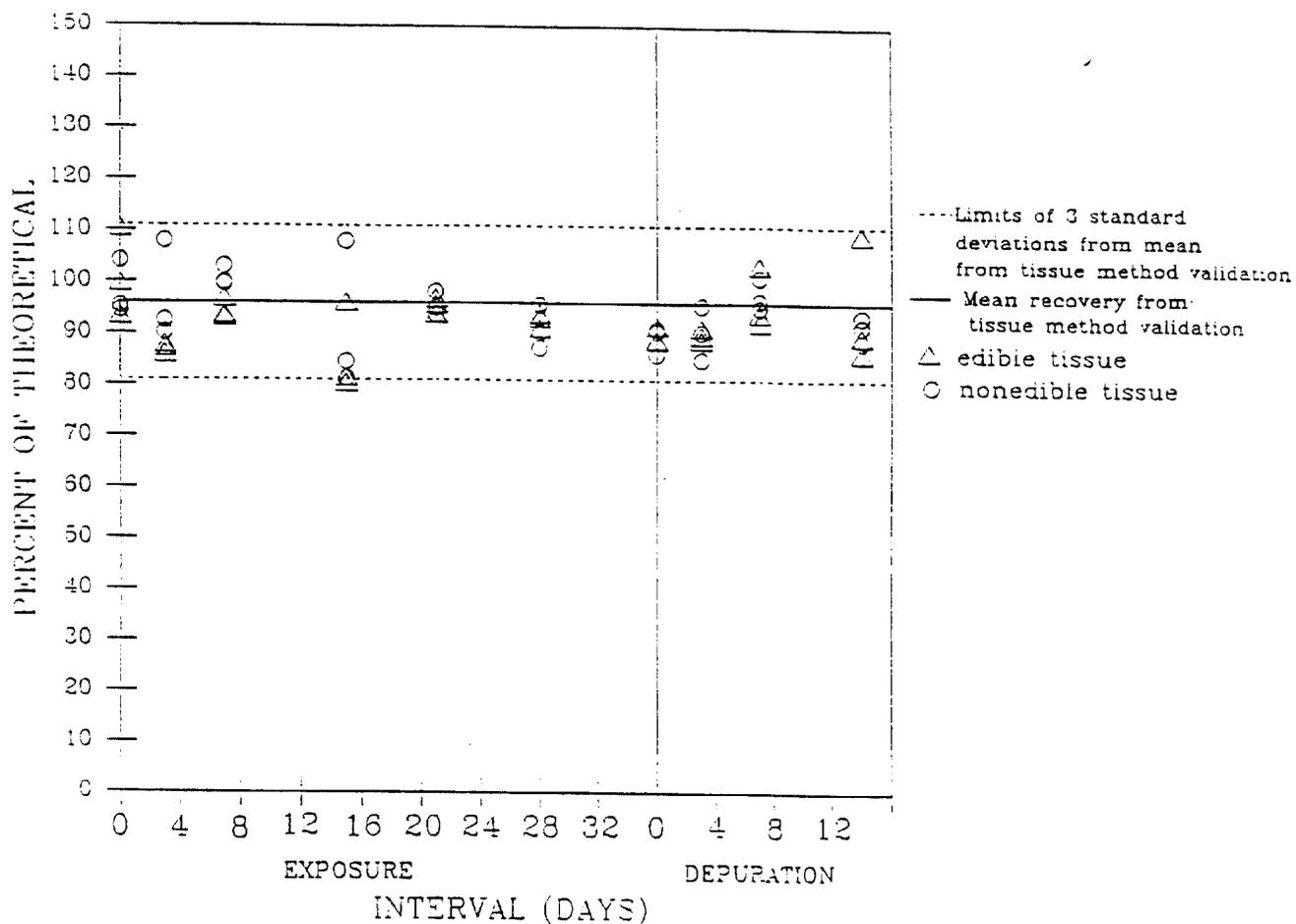
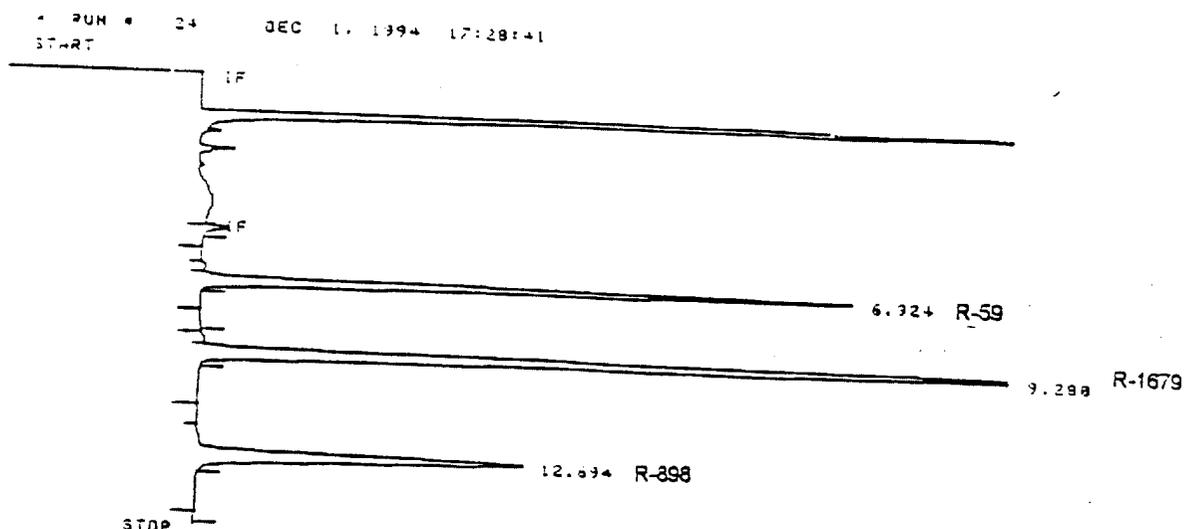


Figure 11. Representative HPLC chromatogram of a Quality Control tissue extract analyzed during the 36-day exposure of carp (*Cyprinus carpio*) to Wingstay® 100.

\* indicates peak of interest



Closing signal file M:SIGNAL.BNC

RUN# 24 DEC 1, 1994 17:23:41

METHOD NAME: M-GENERIC.MET

SIGNAL FILE: M:SIGNAL.BNC

NO CALIB PEAKS FOUND

AREA#

RT	AREA	TYPE	WIDTH	WPEAK
6.324	357573	V8	.185	29.02129
9.299	461753	V9	.223	50.23393
12.634	139873	P8	.299	21.74477

TOTAL AREA= 919295

MUL FACTOR=1.0000E-08

Figure 12. Measured tissue concentrations and model calculations of Wingstay® 100 in edible tissue of carp (*Cyprinus carpio*).

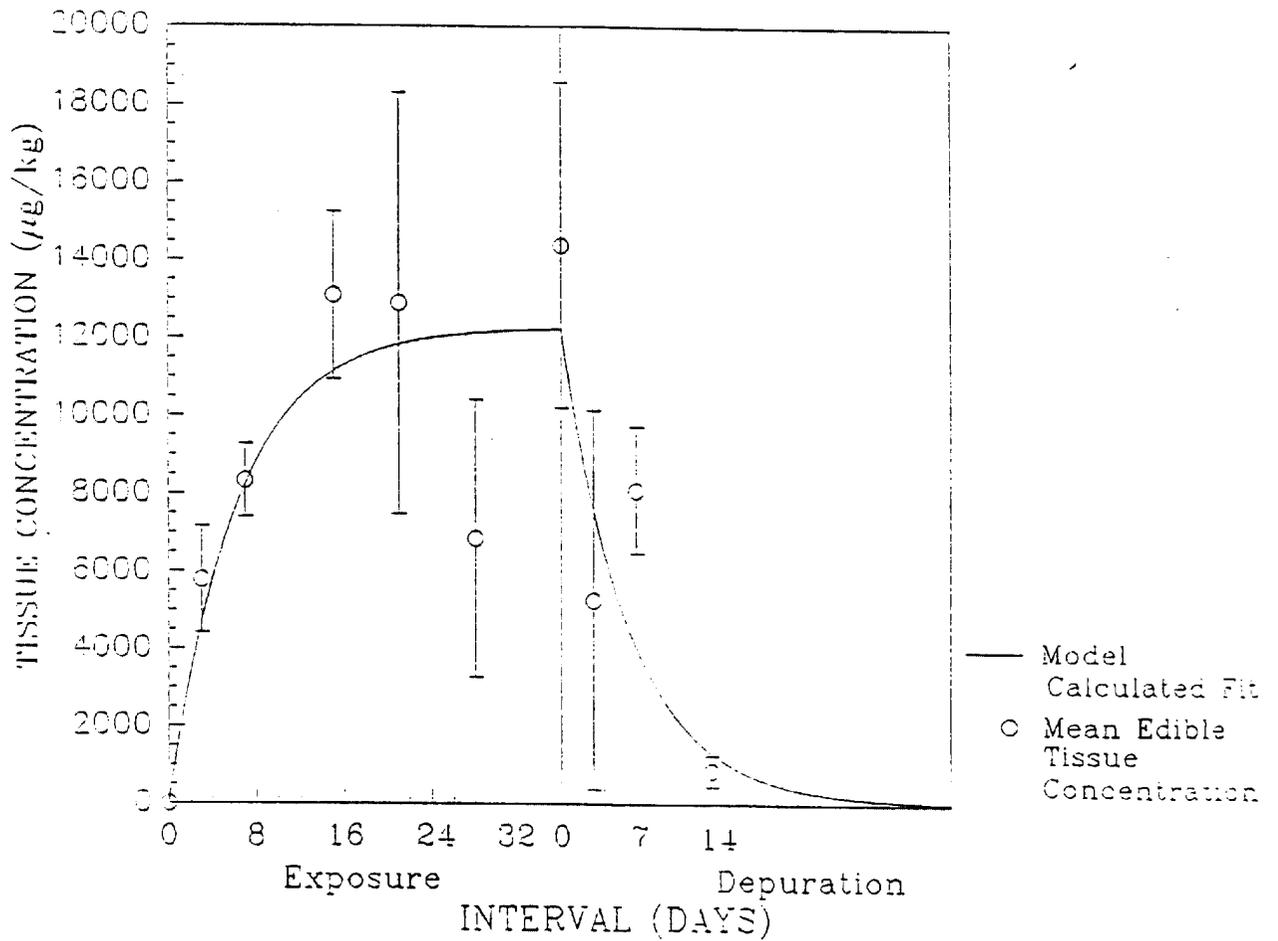


Figure 13. Measured tissue concentrations and model calculations of Wingstay® 100 in nonedible tissue of carp (*Cyprinus carpio*).

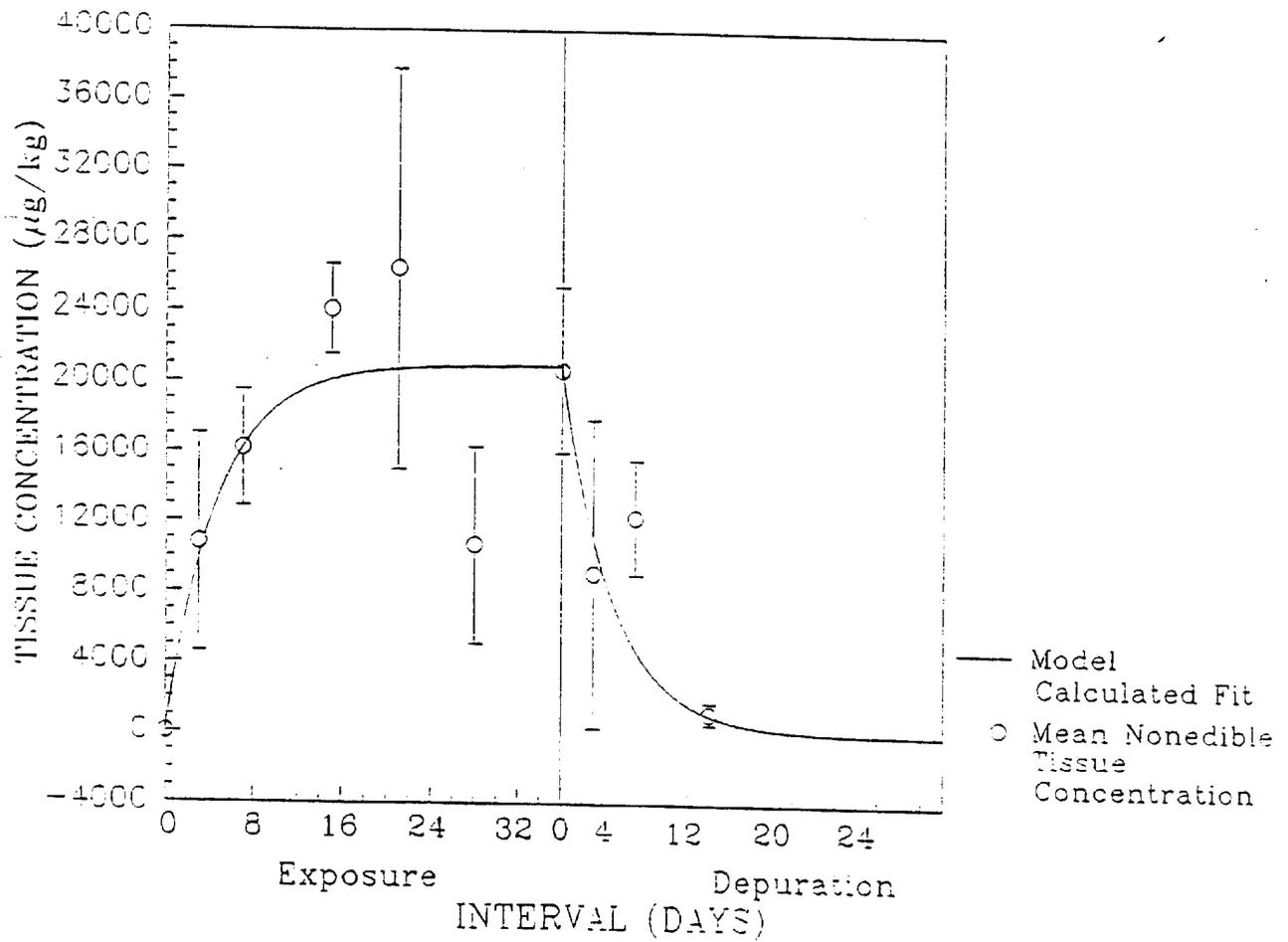
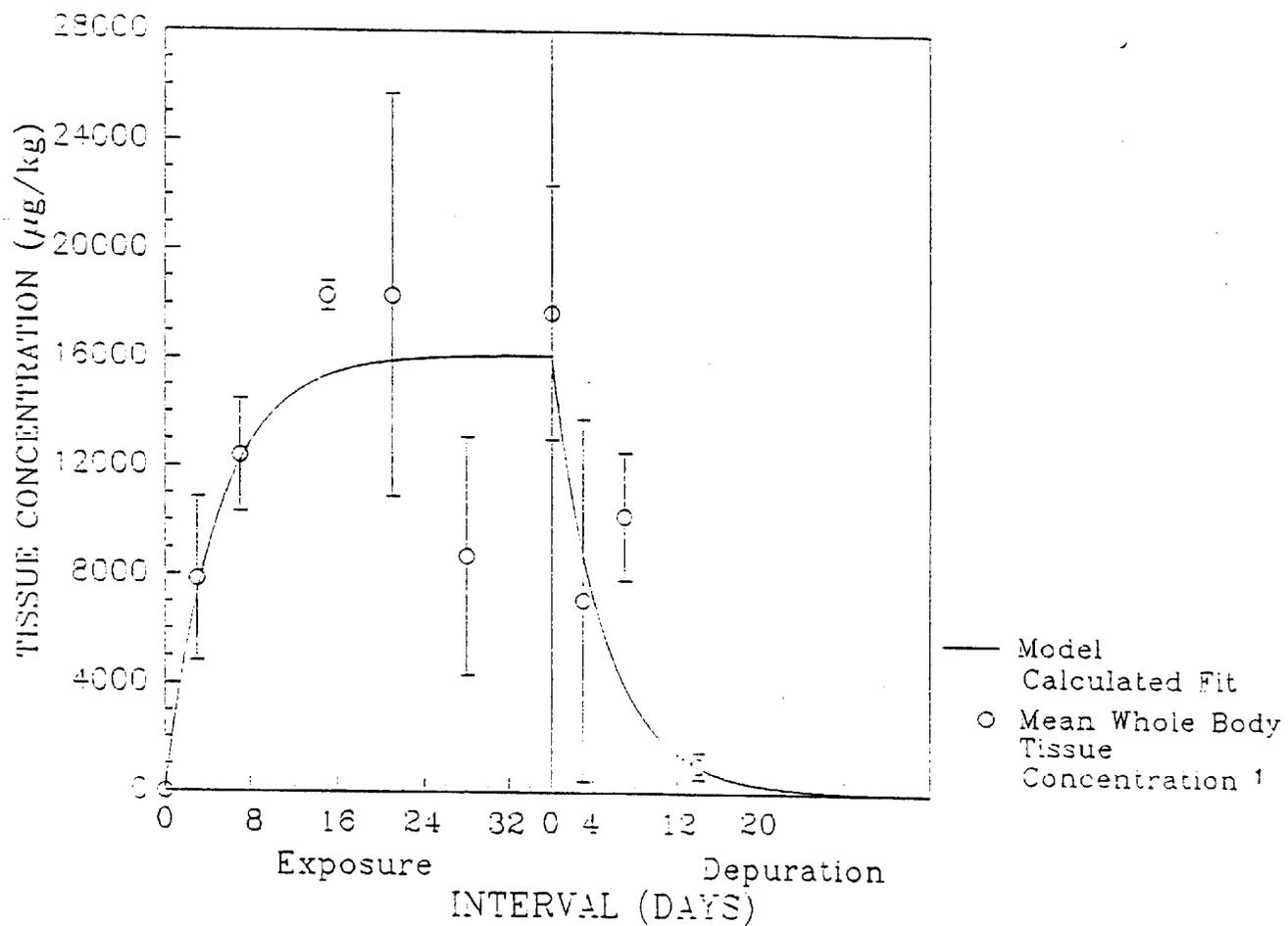


Figure 14. Measured tissue concentrations and model calculations of Wingstay<sup>®</sup> 100 in whole body tissue of carp (*Cyprinus carpio*).



<sup>1</sup> Mean whole body tissue concentration was calculated based on sample weights and tissue concentrations of Wingstay<sup>®</sup> 100 measured in the edible and nonedible tissue portions.

## 7.0 APPENDIX I - STUDY PROTOCOL

**Springborn Laboratories, Inc.**

Environmental Sciences Division

790 Main Street • Wareham, Massachusetts 02571-1075 • (508) 295-2550 • Telex 4436041 • Facsimile (508) 295-8107

**TEST PROTOCOL**

**PROTOCOL TITLE:** Wingstay® 100: Measurement of Bioaccumulation in Japanese Carp  
(Cyprinus carpio) Following OECD and MITI Guidelines

**TO BE COMPLETED BY THE STUDY SPONSOR:**

Study Sponsor: The Goodyear Tire & Rubber Company

Address: 142 Goodyear Blvd.

Allston, OH 44305 Phone: 216-796-1046

Sponsor Protocol/Project No.: —

Test Substance: Wingstay 100

Purity: 100% CAS# or LOT#: 68953-84-4

Reference Substance: \_\_\_\_\_ CAS# or LOT#: \_\_\_\_\_

Additional Comments and/or Modifications: \_\_\_\_\_

A. Philip \_\_\_\_\_  
Sponsor Approval Date 5/31/94

**TO BE COMPLETED BY SPRINGBORN PRIOR TO TEST INITIATION:**

Testing Facility: Springborn Laboratories, Inc.

Study Director: John Mao Ph.D. Study Number: 13537-0994-Lim-143

Test Concentrations: \*

Solvent Used: \* CAS# or LOT#: \*

Proposed Experimental Start: \* Termination: \*

John Mao \_\_\_\_\_  
Study Director Date 10/7/94

\* To be provided by amendment

Wingstay® 100: Measurement of Bioaccumulation in  
Japanese Carp (*Cyprinus carpio*) Following OECD and MITI Guidelines

## 1.0 PURPOSE AND OBJECTIVES

### 1.1 Purpose

The purpose of this test is to measure the bioconcentration (accumulation from water) of the test material using a representative freshwater fish species, the Japanese carp (*Cyprinus carpio*), following OECD Guideline 305E ("Bioaccumulation: Flow-Through Fish Test") (OECD, 1981b) and MITI test guidelines ("Method for Testing the Bioaccumulation of Chemical Substances in Fish") (MITI, 1993). The bioaccumulation of the test material is determined at two test concentrations. The test concentrations are selected based on the results of an acute TL<sub>m</sub> study with orange-red killifish (*Orizias latipes*) as described in Section 3 of this protocol. Following the uptake phase, carp are kept in clean flowing water for 14 days to study the rate of depuration of the chemical from their tissues.

### 1.2 Objective

Information on the uptake of a chemical from water and its retention in fish tissues can be useful in assessing the propensity of the chemical to enter and persist in aquatic food chains. Bioaccumulation studies are conducted in order to obtain an estimate of the maximum amount of the test material which can be expected to accumulate in fish tissues during continuous exposure. Test results are reported as the concentration factor, defined as the ratio of test material concentration in tissue (edible tissue, nonedible tissue, and whole body) to the test material concentration in the water. Rates of uptake and depuration of the test material are also reported. This study will follow OECD GLP guidelines (OECD, 1981a).

### 1.3 Justification of Test System

Characteristics which make carp suitable for bioaccumulation tests are their ease of handling, their ready availability and convenient size, and the extensive existing data for this common fish species.

## 2.0 TEST MATERIAL

### 2.1 Identification and Characterization

The name, CAS or Lot number, and purity of the test material and reference materials are provided by the Study Sponsor on the cover page of the protocol. The Sponsor also provides any available information on water solubility, vapor pressure, storage stability, methods of analysis, MSDS and safe handling procedures, and a verified expiration or reanalysis date, if applicable.

### 2.2 Receipt and Handling

Upon arrival at Springborn Laboratories, all test materials, reference substances, and standards are received by the Test Material Center. Records are maintained by GLP standards, and a Chain-of-Custody is established. The condition of the external packaging of the test material is recorded and any damage is noted. The packaging is removed, the primary storage container is also inspected for leakage or damage, and the condition is recorded. Any damage is reported to the Sponsor and/or manufacturer. Each test material sample is given a unique sample identification number and is stored under the conditions specified by the Sponsor or manufacturer.

## 3.0 ACUTE TOXICITY TEST WITH ORANGE-RED KILLIFISH (*ORIZIAS LATIPES*)

### 3.1 Purpose

Before the bioaccumulation study begins, the acute toxicity of the test material to the orange-red killifish, *Orizias latipes*, is measured to allow selection of test concentrations for the bioaccumulation study. Results of the acute toxicity test are reported as the 24-, 48- and 96-hour TLm values (the concentrations of test material that kill 50% of the test population within the stated exposure period).

### 3.2 Test Organism

Mature orange-red killifish are used, weighing 0.15 g to 0.5 g each. Diseased fish or those with abnormal appearance are not used. All fish used in a single test are obtained from the same source and are of approximately the same age. The length of the largest fish used in a test does not exceed that of the smallest fish by more than 1.5. The sex of the fish is not determined.

**3.2.1 Source and Acclimation.** The fish are obtained from a reliable commercial supplier. They are gradually acclimated to the test conditions over a ten-day period before testing, and are held for a minimum of one week at the required test temperature before testing. During the 4 days before the test period the number of dead or ill fish must not exceed ten percent or the entire batch is not used.

**3.2.2 Feeding.** The fish are fed at least once daily until 48 hours before the test. They are not fed during the final 48 hours before the test nor during the test. Fish are fed frozen brine shrimp (*Artemia* sp.) and flaked fish food.

**3.2.3 Handling.** Fish are transferred with fine-mesh dip nets. Care is taken to minimize handling stress. Fish that are damaged or dropped during transfer are not used in the test.

### 3.3 Exposure System

**3.3.1 Test Chambers.** The test chambers used in the acute toxicity test are 19-L clear glass aquaria which are chemically clean. Each aquarium contains approximately 15 liters of test medium. This size is adequate to meet the maximum allowable biomass loading rate of 1 g/L. One aquarium is used for each test concentration. Each test chamber is labeled with the study number and test concentration.

**3.3.2 Dilution Water.** Dilution water consists of unadulterated water obtained from a 100-meter bedrock well and pumped to an approximately 6,000-gallon concrete reservoir. It is

supplemented in varying proportions with untreated Town of Wareham well water, aerated to bring the pH and dissolved gases into equilibrium with the atmosphere, and pumped through aged PVC piping to the test system. The water is characterized as soft water with a typical total hardness of 20 to 40 mg/L as CaCO<sub>3</sub>, and alkalinity of 20 to 35 mg/L as CaCO<sub>3</sub>. The pH range is 6.9 to 7.6, and the specific conductivity ranges between 20 and 150 µmhos/cm. Total hardness, total alkalinity, pH and specific conductivity of the dilution water are monitored weekly at a central location in the laboratory to assure that these parameters are within the normal acceptable ranges.

**3.3.3 Test Concentrations.** Toxicant concentrations for the acute toxicity test are selected based on a preliminary static range-finding test. The preliminary test consists of several widely spaced concentrations, usually of 3-L volume, each containing five test fish. 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. A geometric series of five concentrations and one control are used for each definitive test, with ten fish (in a single aquarium) exposed at each concentration. If a carrier solvent is used, a solvent control is included also. Each dose level is at least 60% of the next higher concentration of the test material.

### 3.4 Test Procedures

**3.4.1 Test Initiation.** Test solutions are prepared in the test chambers by mixing appropriate volumes of test material and dilution water and brought to test temperature ( $25 \pm 2^\circ\text{C}$ ). Ten fish are impartially distributed to each test chamber within 30 minutes of solution preparation.

**3.4.2 Test Conditions.** The temperature of the test solutions is maintained at  $25 \pm 2^\circ\text{C}$  by a temperature-controlled water bath. Fluorescent bulbs provide approximately 25 footcandles illumination in the test area. A 16-hour light:8-hour dark photoperiod is maintained

with an automatic timer. If the dissolved oxygen concentration in any test chamber falls below 4 mg/L during the test, all test chambers are aerated with oil-free air for the duration of the test.

**3.4.3 Observations.** At the start of the test, and 4, 3, 24, 48 and 96 hours thereafter, observations of stress, abnormal behavior and mortality are made on the fish in each test chamber, and dead fish are removed. Physical characteristics of the test solutions (such as presence of precipitates, surface films, or cloudiness) are also observed and recorded. The test is terminated after 96 hours of exposure.

**3.4.4 Water Quality Analysis.** Conductivity, pH, temperature and dissolved oxygen are measured at the start of the test and at 24-hour intervals thereafter in all test chambers where there are surviving fish. If 100% mortality occurs in a chamber, water quality is measured on that day but not at subsequent observation intervals. The temperature of the water bath is monitored continuously with a minimum-maximum thermometer and/or a recording thermometer.

### 3.5 Statistics

Test results are used to calculate the median lethal concentration (TL<sub>50</sub>, the concentration of test material that kills 50% of the test population) after 24, 48 and 96 hours of exposure. A computer program estimates TL<sub>50</sub> values using one of three statistical methods: probit analysis, moving average angle method, and binomial probability. The method selected is determined by the data (e.g., presence or absence of 100% response, number of partial responses, etc.). If the test data are statistically inadequate for all three of these statistical methods, alternative approaches (such as graphical interpolation) may be used. 95% confidence limits are reported when the data permit their calculation.

#### 4.0 BIOACCUMULATION STUDY WITH JAPANESE CARP (*CYPRINUS CARPIO*)

##### 4.1 Test Organism

Yearling Japanese carp, *Cyprinus carpio*, are used to conduct the bioaccumulation test. 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, and have an average weight between ten and twenty grams. The sex of the fish is not determined. The lipid content of a representative sample of the fish population is measured within two weeks of the start of the exposure period.

**4.1.1 Origin and Acclimation.** The fish are obtained from a reliable commercial fish hatchery and are gradually acclimated to the test conditions (<3 °C/day) under flowing water conditions. They are held for at least an additional 14 days in the dilution water prior to testing. They are held for a minimum of five days at the required test temperature, during which time total mortality or evidence of weakening of the fish must not exceed five percent, or the population is not used. During acclimation, diseased fish are removed.

**4.1.2 Feeding.** The fish are fed a commercial fish food twice daily prior to and during the bioaccumulation test. The food is analyzed routinely for metals, pesticides, and PCBs to ensure its suitability for use. During the bioaccumulation study the fish are fed twice daily at a rate of approximately one percent of total biomass per day. Feed is provided slowly to assure that all feed is eaten. They are not fed 24 hours prior to initiation of the test, nor 24 hours prior to each sampling, to eliminate the possibility of large amounts of gut contents which could bias the tissue residue concentrations measured.

**4.2.3 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.

**4.2.4 Biomass Loading.** Fish biomass to solution ratio ("loading") does not exceed 0.5 gram per liter passing through the test system per 24 hours. Thirty-eight fish are included in

each aquarium to allow sampling of three fish per interval for each of the ten planned sampling intervals, with additional fish to allow for a maximum of twenty percent mortality throughout the experimental period.

#### 4.3 Exposure System

4.3.1 **Test Containers.** The test chambers used in the flow-through bioaccumulation test are clear glass aquaria constructed with silicone adhesive, and measure approximately 75 x 39 x 30 cm (length x width x height). Water depth is maintained by a constant level overflow drain approximately 25 cm from the bottom of each test aquarium. The total test solution volume in each aquarium is approximately 73 L. One test container is used for each test concentration and for the controls.

4.3.2 **Cleaning.** The test aquaria are chemically cleaned using standard laboratory procedures to remove any residual contamination before the test is started. Test aquaria are cleaned daily during the test by removing material adhering to the walls of the aquaria and siphoning out accumulated debris.

4.3.3 **Dilution Water.** Dilution water consists of soft, unadulterated well water obtained from a 100-meter bedrock well, and pumped to an approximately 6,000-gal concrete reservoir. It is supplemented with varying amounts of untreated town of Wareham well water, aerated to bring the pH and dissolved gases into equilibrium with the atmosphere, and gravity fed through aged PVC piping to the test system.

Total hardness, total alkalinity, pH and specific conductance of the diluent water are routinely monitored. Total hardness and alkalinity are determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1989). In general, ranges for soft water are: total hardness, 25 - 36 mg/L CaCO<sub>3</sub>; alkalinity, 22 - 30 mg/L CaCO<sub>3</sub>; specific conductivity, 100 - 170  $\mu$ mhos/cm; and pH, 6.9 - 7.6.

**4.3.4 Diluter.** A continuous flow chemical delivery system, an adaptation of a diluter described by Benoit *et al.* (1982), is used to deliver approximately 1,000 mL/min of treated dilution water (exposure aquaria), untreated dilution water (control aquarium) or solvent treated dilution water (solvent control aquarium) to the test aquaria. This flow rate is adequate to meet the maximum allowable loading requirements (see above under "Biomass Loading"), and provides a turnover rate of approximately twenty aquarium volumes per 24 hours. A Sage syringe pump metering device, equipped with a glass, gas tight syringe, is used to continuously deliver the appropriate amount of a chemical stock solution or solvent to the 1,000 mL/minute dilution water flow to produce the test or solvent control solutions.

**4.3.5 Solvent Control.** When a solvent is used in the chemical stock preparation, a solvent control aquarium is maintained instead of a control aquarium and the delivery system adds solvent to the control water at the same rate (and concentration) as that of the treatment aquaria.

**4.3.6 System Calibration.** The calibration of the diluter system is checked prior to test initiation. If there is any indication during the test that the diluter calibration has changed (e.g. changes in measured concentration or differences in dissolved oxygen concentration), calibration of the necessary diluter components is rechecked. A test is not started until the diluter and toxicant delivery device have been observed to be properly functioning for at least 48 hours prior to the test.

#### 4.4 Test Solutions

**4.4.1 Selection of Toxicant Concentrations.** The exposure concentrations for the bioaccumulation test are selected based on the acute toxicity of the chemical to the orange-red killifish (determined under Section 3 of this protocol) and physical/chemical characterizations of the test substance. The concentrations selected are intended to exert no toxicity during continuous exposure, yet to result in residue of the test material in the fish tissues which can be measured. The targeted nominal concentrations are 1/1,000th and 1/10,000th of the 96-hour

TLm, but may be higher if necessary depending on the analytical detection limit of the test material.

**4.4.2 Stock Preparation.** A stock solution of the test substance is prepared by transferring a measured weight or volume into a volumetric flask containing the appropriate solvent (usually acetone or distilled water). Initially, sufficient stock solution is prepared to replenish the syringe injector system volume for the entire duration of the study. The final volume and concentration of the stock solution depends on the selected exposure concentration the calibrated delivery volume of the chemical delivery system. The test material concentration in the stock solution is measured.

#### 4.5 Test Procedure

**4.5.1 Equilibration Period.** The test system is allowed to operate for at least two days prior to the introduction of test fish so that the chemical delivery system and diluter operations can be checked and the desired test concentrations confirmed. Triplicate aliquots of treated water are analyzed to confirm the test concentrations on two separate days preceding test initiation.

**4.5.2 Test Initiation and Duration.** The test begins when thirty-eight fish are impartially distributed to each test chamber. The exposure of carp to the test material is continued for 56 days or until a steady-state tissue residue concentration has been established. This is determined by subjecting the measured tissue residue concentrations for three consecutive sampling intervals to analysis of variance until no statistically significant difference is found between the three sets of residue concentrations. The data are also examined graphically, and if an increasing trend is suspected the data may be analyzed by regression analysis to confirm that the slope of concentration vs. time is not significantly different from zero. The fish remaining in the exposure aquarium at the end of the exposure period are transferred to an aquarium containing flowing dilution water (identical to the control aquarium) for 14 days of depuration.

4.5.3 Test Conditions. The following conditions are maintained during the bioaccumulation study:

Dissolved Oxygen. Total dissolved oxygen is not allowed to drop below 60 percent saturation for the duration of the test. Aeration is used, with non-volatile test materials, to raise and maintain the dissolved oxygen concentration at or above 60% of saturation.

Temperature. Water temperature of the test solutions is maintained at  $25 \pm 2$  °C by resting the aquaria in a water bath maintained at the appropriate test temperature.

Lighting. A combination of fluorescent bulbs is used to illuminate the aquaria with a wide spectrum of light, simulating the spectrum of natural sunlight. An 8-hours dark and 16-hours light photoperiod is maintained during the test.

4.5.4 Sampling. During the course of the exposure, three fish are collected from each aquarium on days 0, 3, 7, 14, 28, 42 and 56, if necessary, to demonstrate maximum accumulation. At the end of the exposure period, the remaining fish are transferred to an aquarium containing flowing dilution water (identical to the control aquarium) for 14 days of depuration. During the depuration period, three fish are collected from each aquarium on days 3, 7 and 14. For each sample, fish selected impartially from the surviving fish in the aquarium are removed from the aquarium, wiped lightly with gauze, and divided into two portions, i.e., fillets (edible portion) and viscera/carcass (nonedible portion). Each portion is weighed and frozen. The tissue is homogenized before analysis. Analytical techniques will be developed and validated before the samples are analyzed.

Water samples are collected from each aquarium two times per week (days 0, 3, 7, 10, 14, 17, 21, 24, 28, etc.) and analyzed for the test material. The water samples are taken from a point approximately midway between the surface, bottom and sides of each aquarium. Analytical techniques will be developed and validated before the samples are analyzed.

**4.5.5 Quality Control Samples.** For every sampling interval in which water samples are collected and analyzed for test material residues, three samples consisting of aliquots of water spiked with a known amount of test material are also analyzed. During every interval in which fish are sampled and analyzed, three tissue samples are spiked with a known amount of test material and analyzed. Quality Control (QC) samples are stored (in the case of fish), processed and analyzed in parallel with the water and fish samples from the test system.

**4.5.6 Water Quality Analysis.** At test initiation, water quality variables (temperature, pH, hardness and dissolved oxygen concentrations) are recorded in each test aquarium. Subsequent to this, aquarium temperatures and dissolved oxygen concentration are monitored daily, and pH is measured at least three times per week.

#### 4.6 Computations

Measured concentration factors for edible and nonedible fish tissue are determined by dividing the mean measured equilibrium test material concentration by the mean measured water concentration for the entire exposure period (yielding the "mean equilibrium" or "steady state" concentration factor). Concentration factors for whole fish are calculated in the same way, using average tissue concentrations calculated from the concentrations and weights of the edible and nonedible portions.

The uptake constant ( $K_u$ ) and depuration constant ( $K_d$ ) are determined by obtaining the best fit of the mean measured water and tissue concentrations during the uptake and depuration phases with the following simultaneous equations:

For uptake: 
$$\frac{C_t}{C_w} = \left( \frac{K_u}{K_d} \right) \times [1 - e^{-(K_d - K_u)t}]$$

For depuration:

$$\frac{C_t}{C_w} = \frac{K_u}{K_u - K_d} (1 - e^{-(K_u - K_d)t})$$

where  $C_t$  = tissue concentration at time  $t$  (units  $\mu\text{g}/\text{kg}$ ),  $C_w$  = mean water concentration during uptake phase (units  $\mu\text{g}/\text{L}$ ),  $K_u$  = uptake constant (units  $\text{days}^{-1}$ ), and  $K_d$  = depuration constant (units  $\text{days}^{-1}$ ).

#### 5.0 RECORDS TO BE MAINTAINED

Records to be maintained include, but are not limited to, correspondence and other documents relating to the interpretation and evaluation of data, as well as all raw data and documentation generated as a result of the study.

#### 6.0 REPORTING

The raw data and final draft of the report are reviewed by the Quality Assurance Unit and Study Director. One copy of the draft report is submitted to the Sponsor for review and comments. Upon acceptance by the Sponsor, three copies of the final report are issued. All reports include, but are not limited to, the following information:

- The report and project numbers from Springborn Laboratories, Inc. and Sponsor study number, if any.
- Laboratory site, dates of testing and personnel involved in the study, i.e., Quality Assurance Unit, Program Coordinator (if applicable), Study Director, Principal Investigator.