

BEHQ-0397-1367 4

# The Goodyear Tire & Rubber Company

Akron, Ohio 44316-0001



BEHQ-96-1367 4

PDCM 8896000152

March 3, 1997

(B)

RECEIVED  
OPERATING  
97 MAR -7 8  
11:05

### Certified Mail

OPPT Document Processing Center (TS-790)  
Attn: Section 8(e) Coordinator  
Office Of Pollution Prevention and Toxics (OPPT)  
U.S. Environmental Protection Agency  
401 M Street, S.W.  
Washington, DC 20460



89970000102

**Contains No CBI**

Dear Ladies/Gentlemen:

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

Reference: June 19, 1996, Michael W. Smith Letter to U.S. Environmental Protection Agency

This submitted does not contain Confidential Business Information.

As promised in the above referenced and attached letter, The Goodyear Tire & Rubber Company is providing the EPA with copies of the following final reports:

1. WINGSTAY 100 - Acute Toxicity to Daphnids (Daphnia magna) Under Flow-Through Conditions
2. WINGSTAY 100 - Prolonged (14-Day) Acute Toxicity to Common Carp (Cyprinus carpio) Under Flow-Through Conditions.

The identity of the test material is as follows:

Chemical Abstract Name: 1,4- Benzenediamine, N, N' - mixed Ph and TolyI derivs.

Chemical Abstract Number: 68953-84-4\*

The results of the final studies were the same as the preliminary results that were already submitted to EPA.

RECEIVED  
OPERATING  
17 MAR 1997  
7:05

CSRAD/OPPT  
6/5/97  
nb

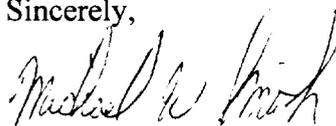
March 3, 1997

Page 2

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  
s7m3c3

Attachments (3)

**WINGSTAY® 100 - ACUTE TOXICITY TO  
DAPHNIDS (*Daphnia magna*) UNDER  
FLOW-THROUGH CONDITIONS**

**Guideline Reference Number: 202**

**Submitted to:**

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

RECEIVED  
GTM-1 PM 3:06

**SLI Report #96-1-6328**

**SLI Study #13537.0995.6123.115**

**Study Director: Arthur E. Putt**

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

**26 June 1996**

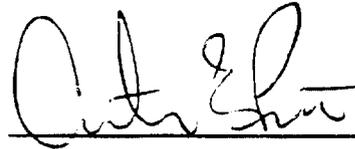
**FINAL REPORT**

---

**GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT**

The data and report prepared for "**Wingstay<sup>®</sup> 100 - Acute Toxicity To Daphnids (*Daphnia magna*) Under Flow-Through Conditions**" were produced and compiled in accordance with all pertinent OECD Good Laboratory Practice regulations with the following exceptions: routine water and food contaminant screening analyses for pesticides, PCBs and toxic metals were conducted using standard U.S. EPA procedures by Lancaster Laboratories, Lancaster, PA. These data were not collected in accordance with Good Laboratory Practice procedures (i.e., no distinct protocol, Study Director, etc.). 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.



6/26/96

---

Arthur E. Putt  
Study Director

Date

---

**TABLE OF CONTENTS**

|  | <b>Page</b> |
|--|-------------|
| <b>GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT</b> ..... | 2           |
| <b>LIST OF TABLES</b> .....                                | 5           |
| <b>LIST OF FIGURES</b> .....                               | 6           |
| <b>SUMMARY</b> .....                                       | 7           |
| <b>1.0 INTRODUCTION</b> .....                              | 9           |
| <b>2.0 MATERIALS AND METHODS</b> .....                     | 9           |
| 2.1 Protocol .....   | 9           |
| 2.2 Test Substance .....                                   | 9           |
| 2.3 Test Organisms .....                                   | 10          |
| 2.4 Test Dilution Water .....                              | 10          |
| 2.5 Test Conditions .....                                  | 11          |
| 2.6 Test Concentrations .....                              | 11          |
| 2.7 Stock Solution Preparation .....                       | 12          |
| 2.8 Test System .....                                      | 12          |
| 2.9 Test Initiation .....                                  | 13          |
| 2.10 Biological Monitoring .....                           | 13          |
| 2.11 Water Quality Measurements .....                      | 13          |
| 2.12 Analytical Measurements .....                         | 14          |
| 2.13 Determination of EC50 and NOEC Values .....           | 15          |
| <b>3.0 RESULTS</b> .....                                   | 15          |
| 3.1 Preliminary Test .....                                 | 15          |
| 3.2 Definitive Test .....                                  | 15          |
| 3.2.1 Evaluation of Test Conditions .....                  | 15          |
| 3.2.2 Analytical Results .....                             | 16          |
| 3.2.3 Biological Results .....                             | 17          |
| <b>PROTOCOL DEVIATION</b> .....                            | 18          |
| <b>QUALITY ASSURANCE UNIT STATEMENT</b> .....              | 19          |
| <b>REFERENCES</b> .....                                    | 20          |
| <b>TABLES</b> .....  | 21          |
| <b>FIGURES</b> .....                                       | 24          |

---

|  |    |
|--|----|
| <b>SIGNATURES AND APPROVAL</b> .....                             | 26 |
| <b>4.0 APPENDIX I - STUDY PROTOCOL</b> .....                     | 27 |
| <b>5.0 APPENDIX II - PRODUCT SPECIFICATION INFORMATION</b> ..... | 38 |
| <b>6.0 APPENDIX III - ANALYTICAL METHODOLOGY</b> .....           | 40 |

## LIST OF TABLES

|  | Page |
|--|------|
| <b>Table 1.</b> Concentrations of Wingstay® 100 measured in replicate (A,B) exposure solutions during the 48-hour flow-through exposure of daphnids ( <i>Daphnia magna</i> ) .....   | 22   |
| <b>Table 2.</b> Mean measured concentrations tested, corresponding cumulative percent of immobilized daphnids ( <i>Daphnia magna</i> ) and observations made during the 48-hour flow-through exposure to Wingstay® 100 ..... | 23   |
| <b>Table 1A.</b> Analytical results for the recovery of Wingstay® 100 from freshwater .....  | 47   |

## LIST OF FIGURES

|   | Page |
|---|------|
| Figure 1. Relationship of mean measured concentrations (analyses at 0 and 48 hours) and the nominal concentrations during the 48-hour flow-through exposure of daphnids ( <i>Daphnia magna</i> ) to Wingstay <sup>®</sup> 100. .... | 25   |
| Figure 1A. HPLC chromatogram of Wingstay <sup>®</sup> 100 in a 0.200 mg/L standard solution .....   | 48   |
| Figure 2A. HPLC chromatogram of Wingstay <sup>®</sup> 100 in a 10.0 µg/L fortified sample .....   | 49   |
| Figure 3A. HPLC chromatogram of a control water sample .....  | 50   |
| Figure 4A. Plot of signal response versus concentration for Wingstay <sup>®</sup> 100 standards linear regression analysis .....  | 51   |

---

**SUMMARY****Wingstay<sup>®</sup> 100 - Acute Toxicity to Daphnids  
(*Daphnia magna*) Under Flow-Through Conditions**

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

**PROTOCOL TITLE:** "Wingstay<sup>®</sup> 100 - Acute Toxicity to Water Fleas (*Daphnia magna*) Under Flow-Through Conditions, Following OECD Guideline #202", Springborn Protocol #: 020195/OECD/115/Goodyear and Protocol Amendment #1 dated 6 December 1995.

**REPORT NUMBER:** 96-1-6328

**STUDY NUMBER:** 13537.0995.6123.115

**TEST SUBSTANCE:** Wingstay<sup>®</sup> 100, Lot No. 137170393, CAS Registry No. 68953-84-4, a gray, flaky substance, was received from Goodyear Research on 7 September 1994. Wingstay<sup>®</sup> 100 was tested on a whole product basis.

**TEST DATES:** 6 to 8 December 1995

**SPECIES:** *Daphnia magna*  
≤24 hours old  
Source: Springborn culture facility

**TEST CONDITIONS:** 48-hour duration, 19 to 21 °C, a photoperiod of 16 hours light:8 hours dark at a light intensity of 60 to 80 footcandles

**DILUTION WATER:** Fortified well water  
pH: 7.9  
Specific conductivity: 500 to 600 µmhos/cm  
Total hardness as CaCO<sub>3</sub>: 170 to 180 mg/L  
Total alkalinity as CaCO<sub>3</sub>: 110 mg/L

**NOMINAL TEST CONCENTRATIONS:** 1.3, 2.2, 3.6, 6.0 and 10 mg/L

**MEAN MEASURED CONCENTRATIONS:** 0.20, 0.36, 0.68, 1.1 and 1.8 mg/L

**RESULTS:**

Based on the results of this study, the 48-hour EC50 for Wingstay® 100 and *D. magna* was empirically estimated to be greater than 1.8 mg/L, the highest mean measured concentration tested. Based on the absence of immobilization and adverse sublethal effects, the No-Observed-Effect Concentration (NOEC) established for this study was 0.36 mg/L.

## 1.0 INTRODUCTION

The purpose of this study was to estimate the acute toxicity (EC50) of Wingstay® 100 to daphnids (*Daphnia magna*) under flow-through conditions. The EC50 is defined as the concentration of test substance in dilution water which causes immobilization of 50% in the exposed test population after a fixed period of time. This value is often used as a relative indicator of potential acute hazards resulting from the release of the test substance into aquatic environments. The study was initiated on 25 October 1995, 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 48-hour definitive toxicity test was conducted from 6 to 8 December 1995 at Springborn Laboratories, Inc. (SLI), *Health and Environmental Sciences*, located in Wareham, Massachusetts. All 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 acute toxicity study followed those described in the Springborn protocol entitled "Wingstay® 100 - Acute Toxicity to Water Fleas (*Daphnia magna*) Under Flow-Through Conditions, Following OECD Guideline #202", Springborn Laboratories Protocol #: 020195/OECD/115/Goodyear and Protocol Amendment #1 dated 6 December 1995 (Appendix I). The methods described in this protocol generally meet the testing requirements of the OECD Guideline for Testing of Chemicals #202, *Daphnia* sp., Acute Immobilization Test and Reproduction Test (OECD, 1984).

### 2.2 Test Substance

The test substance, Wingstay® 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 programs, 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

of the test substance and are reported as milligrams per liter of solution (mg/L). Product specification information is provided in Appendix II. The following information describes the test substance received:

|                      |   |
|----------------------|---|
| Chemical Name:       | diaryl- <i>p</i> -phenylenediamine reaction product |
| Physical Appearance: | gray flake  |
| Lot No.:             | 137170393 NP1017                                    |
| CAS Registry No.:    | 68953-84-4  |
| Molecular Weight:    | 274 g/mol (average)                                 |
| Water Solubility:    | < 5 ppm   |

### 2.3 Test Organisms

*Daphnia magna* (≤24 hours old) was selected as the test species since it is an OECD recommended species and a commonly used freshwater invertebrate in acute toxicity tests. The *Daphnia magna* used in this toxicity test were obtained from laboratory cultures maintained at Springborn. The culture water was prepared by fortifying well water based on the formula for hard water (U.S. EPA, 1975) and filtering it through an Amberlite XAD-7 resin column and a carbon filter. The daphnid culture area received a regulated photoperiod of 16 hours of light and 8 hours of darkness. Light intensity in the culture area ranged from 50 to 70 footcandles (Invertebrate Culture Log, Vol. 11). A waterbath in the culture area was used to maintain the culture solution temperature at  $20 \pm 2$  °C. Daphnids were fed 2.0 mL of a unicellular green algae, *Ankistrodesmus falcatus* ( $4 \times 10^7$  cells/mL) once daily. Daphnids were not fed during the 48-hour exposure. Representative samples of the food source were analyzed periodically for the presence of pesticides, PCBs and toxic metals. None of these compounds have been detected at concentrations considered toxic in any of the samples analyzed. Based on the analysis for pesticides, the food source was considered to be of acceptable quality since the total concentration of pesticides measured was less than 0.3 mg/kg (ASTM, 1985).

### 2.4 Test Dilution Water

The dilution water used during this study was from the same source as the culture water previously described and was characterized as "hard" water. During holding and prior to use, the dilution water was continuously aerated. Representative samples of the dilution water source

were analyzed periodically for the presence of pesticides, PCBs and toxic metals. None of these compounds have been 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 was 1.2 mg/L for the month of December 1995 (TOC and TSS Master Log). Several species of daphnids are cultured and maintained in water from the same source as the dilution water utilized in this study and have successfully survived and reproduced over several generations. The excellent performance of the daphnid cultures, in combination with the previously mentioned analyses, confirmed the acceptability of this dilution water for use during the conduct of bioassays.

## **2.5 Test Conditions**

The toxicity test was conducted using an exposure system consisting of an intermittent-flow proportional diluter (Mount and Brungs, 1967) and a set of 14 exposure vessels. The test system was designed to provide five concentrations of the test substance, a dilution water control and a solvent (acetone, CAS #67-64-1) control. Exposure vessels were maintained in an area illuminated with Duro-Test® Vita-Lite® fluorescent lights. The photoperiod was the same as that of the culture area. Sudden transitions from light to dark and vice versa were avoided. The test was conducted in a temperature controlled room and waterbath which were designed to maintain test solution temperatures at  $20 \pm 1$  °C. Two replicate vessels were established for each treatment level and the controls.

## **2.6 Test Concentrations**

Selection of nominal Wingstay® 100 concentrations for the 48-hour definitive flow-through toxicity test with daphnids was based on toxicity information developed at Springborn during preliminary testing. Based on the preliminary test results (see Section 3.1), nominal Wingstay® 100 concentrations of 1.3, 2.2, 3.6, 6.0 and 10 mg/L were selected for the definitive exposure.

## 2.7 Stock Solution Preparation

A 100 mg/mL primary stock solution was prepared on test days -3 and 1 by dissolving 5.000 g of Wingstay® 100 in 50 mL of acetone. The resulting stock solution was dark brown with no visible sign of undissolved test substance (e.g., precipitate). A 10 mg/L secondary stock solution was prepared on test days -1, 0 and 1, by diluting 12 mL of the 100 mg/mL primary stock solution in 120 L of dilution water. The 10 mg/L secondary stock solution was mixed for one hour and then allowed to settle for one hour before pumping over to the delivery tank. After mixing and settling, the 10 mg/L solution was observed to be light brown with a small amount of precipitate on the surface of the solution. The water accommodated fraction of the 10 mg/L secondary stock solution was then transferred to the diluter systems' holding tank for delivery to the test system's pre-dilution mixing chamber.

In addition, a 0.78 mL/mL solvent stock solution was prepared on test day -1 of the definitive exposure by diluting 39 mL of acetone with distilled water to a total volume of 50 mL.

## 2.8 Test System

Prior to test initiation, a metering pump pre-dilution chamber system was calibrated to deliver a total of 460 mL/cycle of the 10 mg/L Wingstay® 100 secondary stock solution into the diluter system's chemical mixing chamber. The solution contained in the mixing chamber constituted the highest nominal treatment level (i.e., 10 mg/L). The solution contained in the mixing chamber was subsequently diluted (60% dilution factor) to provide the remaining nominal test concentrations: 6.0, 3.6, 2.2 and 1.3 mg/L.

During each cycle of the diluter system, approximately 50 mL of exposure solution was delivered to each replicate test vessel. The system cycled approximately 217 times each day. The function of the diluter system (e.g., diluter flow rates, stock solution consumption) was monitored daily and a visual check was performed twice each day. The exposure system was in proper operation 48 hours prior to test initiation to allow equilibration of the test material in the diluter apparatus and exposure vessels. Two glass capillary tubes with an approximate length of five centimeters (cm) and a diameter of 1-millimeter (mm) (inside diameter) were inserted

through silicone stoppers in the mixing/splitting chambers of the diluter and into the test solution delivery tubes. This tubing served to restrict the flow of the test solutions, minimizing potentially stressful turbulence in the exposure vessels and provided equal distribution of the solutions to the replicate vessels. Each glass test vessel (1.8-L battery jar) maintained a constant solution volume of 1.6 liters and a solution depth of approximately 13 cm. Each replicate vessel received approximately 6.0 solution volume replacements per day. Exposure vessels were labeled to identify the nominal test material concentration and designated replicate.

### **2.9 Test Initiation**

The preliminary and definitive tests were initiated when daphnids ( $\leq 24$  hours old) were impartially selected and introduced, two at a time, to each replicate exposure vessel until each vessel contained 10 daphnids. A total of 20 organisms were exposed to each treatment level and the control(s) solutions.

### **2.10 Biological Monitoring**

The number of immobilized daphnids observed in each replicate test vessel was recorded at 24 and 48 hours during the exposure period. Daphnids were determined immobile if, after 15 seconds of gentle agitation of the test vessel, no movement except for minor appendages was observed (i.e., absence of movement within the solution's water column). Biological observations (e.g., abnormal behavior or appearance of the test organisms) and observations of the physical characteristics of the test solutions (e.g., precipitate, film on the surface of the test solution) were also made and recorded at test initiation and at 24 and 48 hours of exposure.

### **2.11 Water Quality Measurements**

Dissolved oxygen concentration, temperature and pH were measured once daily in all test vessels throughout the exposure period. Total hardness, total alkalinity and specific conductance were measured at test initiation in one replicate vessel of each treatment level and the control solutions. Total hardness concentrations presented in this report were measured by the EDTA titrimetric method; total alkalinity concentrations were determined by potentiometric titration to an endpoint of pH 4.5 (APHA *et al.*, 1989) and specific conductance was measured with a Yellow

Springs Instrument Company (YSI) Model #33 salinity-conductivity-temperature meter and probe; the pH was measured with a Hanna pH meter; the dissolved oxygen concentration was measured with a YSI Model #57 dissolved oxygen meter and probe; and the daily solution temperature was measured with a Fisher alcohol thermometer. Continuous temperature monitoring was performed in one replicate (B) of the solvent control solution using a Fisher Scientific thermometer.

## 2.12 Analytical Measurements

Both replicates of the control, low, middle and high treatment levels were sampled and analyzed for Wingstay<sup>®</sup> 100 concentration twice prior to the start of the definitive exposure. Results of the pretest analyses were used to judge whether sufficient quantities of Wingstay<sup>®</sup> 100 were being delivered and maintained in the exposure aquaria to initiate the definitive test. During the in-life phase of the definitive study, water samples were removed from each replicate test vessel for each treatment level and the controls at 0 hour (test initiation) and at 48 hours (test termination) for analysis of Wingstay<sup>®</sup> 100 concentration. Each exposure solution sample was collected from the approximate midpoint of the test vessel by volumetric pipet.

Three quality control (QC) samples were prepared with Wingstay<sup>®</sup> 100 in dilution water at nominal concentrations which approximated the test concentration range. The QC samples were prepared at each sampling interval and remained with the appropriate set of exposure solution samples throughout the analytical process. The results of the QC analyses were used to judge the precision and the quality control maintained during the analysis of exposure solution samples. All samples were analyzed for Wingstay<sup>®</sup> 100 using a high performance liquid chromatographic (HPLC) procedure according to the method presented in Appendix III. A method validation recovery study, conducted at Springborn prior to the initiation of the definitive test, established an average recovery of Wingstay<sup>®</sup> 100 of  $97.9 \pm 6.68\%$  from freshwater. Conditions and procedures used throughout the analysis of exposure solutions and QC samples during this study were the same as those described in Appendix III.

### **2.13 Determination of EC50 and NOEC Values**

The measured concentrations tested (based on 0- and 48-hour analyses) and the corresponding daphnia immobilization data were used to estimate the 24- and 48-hour median effect concentrations (EC50) and 95% confidence limits. The EC50 is defined as the concentration of the test substance in dilution water which caused immobilization of 50% of the test organism population at the stated time interval. During this study, no concentration caused immobilization  $\geq 50\%$ , therefore, the EC50 value was empirically estimated to be greater than the highest concentration tested and no statistical analyses were performed. The No-Observed-Effect Concentration (NOEC) during the 48-hour exposure period was also determined. The NOEC is defined as the highest concentration tested at and below which there were no toxicant related immobilization or physical and behavioral abnormalities (e.g., lethargy) with respect to the control organisms.

## **3.0 RESULTS**

### **3.1 Preliminary Test**

During preliminary investigations, the diluter system was calibrated to deliver five nominal concentrations of Wingstay<sup>®</sup> 100 at 13, 22, 36, 60 and 100 mg/L. Daphnids ( $\leq 24$ -hours old) were introduced to each treatment level and control vessel. After 48 hours of exposure, immobilization among daphnids exposed to the highest concentration (i.e., 100 mg/L) was 15%. Immobilization of  $\leq 10\%$  was observed among daphnids exposed to the remaining treatment levels. Sublethal effects (e.g., lethargy) were observed among all mobile daphnids exposed to the 22, 36, 60 and 100 mg/L treatment levels. Based on these data and the insoluble nature of the test substance, the following nominal Wingstay<sup>®</sup> 100 concentrations were selected for the definitive exposure: 1.3, 2.2, 3.6, 6.0 and 10 mg/L.

### **3.2 Definitive Test**

**3.2.1 Evaluation of Test Conditions** - Results for the water quality parameters measured in all treatment level and control solutions are as follows: pH range - 8.0 to 8.1, dissolved oxygen concentration range - 9.0 to 9.7 mg/L, temperature - 21 °C, total hardness

- 170 mg/L as CaCO<sub>3</sub>, total alkalinity - 110 mg/L as CaCO<sub>3</sub>, and specific conductance - 500 µmhos/cm. These results established that the water quality parameters measured were unaffected by the concentrations of Wingstay<sup>®</sup> 100 tested and remained within acceptable ranges for the survival of *Daphnia magna*. Continuous temperature monitoring performed in replicate B of the solvent control solution established that the test solution temperature ranged from 19 to 20 °C during the exposure period.

**3.2.2 Analytical Results** - The diluter system which prepared and delivered the test solutions to the exposure aquaria functioned properly during the pretest period and throughout the 48-hour definitive study. Analysis of the exposure solutions during the pretest period established that the expected concentration gradient of Wingstay<sup>®</sup> 100 was maintained and ranged from 4.1 to 18% of the nominal concentration. Throughout the exposure period, the test solutions of the two highest treatment levels, 6.0 and 10 mg/L, were observed to be light brown. Undissolved test substance (e.g., precipitate) was observed in the three highest treatment levels (i.e., 3.6, 6.0 and 10 mg/L) after 24 hours of exposure, and in all of the treatment levels after 48 hours of exposure.

The results of the analysis of the exposure solutions for Wingstay<sup>®</sup> 100 during the in-life portion of the definitive exposure are presented in Table 1. Measured concentrations ranged from 19 to 29% of the nominal fortified levels at test initiation (0 hour). Following 48 hours of exposure, measured concentrations ranged from 9.8 to 13% of the nominal fortified levels, which represented an approximate 50% decrease in measured concentrations. Mean measured concentrations (0- and 48-hour analyses) ranged from 15 to 19% of the nominal fortified levels and were defined as 0.20, 0.36, 0.68, 1.1 and 1.8 mg/L. Analysis of the Quality Control samples resulted in measured concentrations which were consistent with the predetermined recovery range (Appendix III) and ranged from 91.7 to 104% of the nominal fortified levels (0.484 to 2.42 mg/L). These results established that the appropriate quality control was maintained during the analysis of the exposure solutions. The relationship between the nominal treatment levels and the mean measured exposure concentrations is illustrated in Figure 1.

**3.2.3 Biological Results** - The mean measured concentrations tested, the corresponding cumulative percent of immobilized daphnids and observations recorded during the 48-hour definitive test are presented in Table 2. Twenty daphnids were exposed to each treatment level and control tested. Following 48 hours of exposure, 25% immobilization was observed among the daphnids exposed to the highest treatment level tested, 1.8 mg/L. During the same period, immobilization of 5% was observed among daphnids exposed to both the 0.68 and 1.1 mg/L treatment levels. No immobilization was observed among daphnids exposed to the two lowest treatment levels, 0.20 and 0.36 mg/L. No significant adverse effects (e.g., lethargy) were observed among mobile daphnids in all treatment levels. Observations of all mobile daphnids in the 1.1 and 1.8 mg/L treatment levels and several mobile daphnids in the remaining treatment levels (0.20, 0.36 and 0.68 mg/L) were observed to be swimming and carrying particulate matter. In addition, several ( $\leq 5$ ) mobile daphnids in the four lowest treatment levels (0.20 to 1.1 mg/L) were observed to be on the surface of the exposure solution.

Based on the results of this study, the 48-hour EC50 value for Wingstay<sup>®</sup> 100 and *D. magna* was empirically estimated to be >1.8 mg/L, the highest mean measured concentration tested. Based on the absence of immobilization and adverse sublethal effects, the No-Observed-Effect Concentration (NOEC) established for Wingstay<sup>®</sup> 100 and daphnids was 0.36 mg/L.

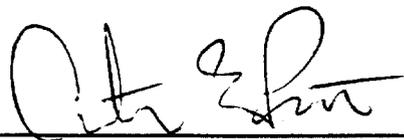
---

**PROTOCOL DEVIATION**

The study protocol states that total dissolved oxygen will not be allowed to drop below 60% or exceed 105% of saturation for the duration of the test. During this study, at 24 and 48 hours of exposure, dissolved oxygen measurements for the controls, 1.3, 2.2 and 3.6 mg/L concentrations ranged from 105 to 109% of saturation.

It is our opinion that this deviation did not adversely impact the results or interpretation of this study.

SPRINGBORN LABORATORIES, INC.



Arthur E. Putt 6/26/96

Arthur E. Putt  
Study Director

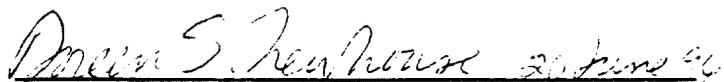
Date

### QUALITY ASSURANCE UNIT STATEMENT

The study conduct, raw data and report for "Wingstay<sup>®</sup> 100 - Acute Toxicity To Daphnids (*Daphnia magna*) Under Flow-Through Conditions" were inspected by the Quality Assurance Unit (QAU) 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>Inspection Type</u>     | <u>Reported to Study Director</u> | <u>Reported to Management</u> |
|------------------------|----------------------------|-----------------------------------|-------------------------------|
| 12/6/95                | phase inspection           | 12/7/95                           | 12/15/95                      |
| 12/29/95               | data audit                 | 12/29/95                          | 12/29/95                      |
| 1/22/96                | data audit                 | 1/22/96                           | 1/26/96                       |
| 2/16/96                | draft report audit         | 2/16/96                           | 2/23/96                       |
| 6/12, 13/96            | revised draft report audit | 6/12, 13/96                       | 6/14/96                       |
| 6/26/96                | final report audit         | 6/26/96                           | 6/26/96                       |

SPRINGBORN LABORATORIES, INC.



Doreen S. Newhouse  
Manager, Quality Assurance Unit

Date

---

**REFERENCES**

- APHA, AWWA, WPCF. 1989. *Standard Methods for the Examination of Water and Wastewater*. 17th Edition, Washington, DC.
- ASTM. 1985. Standard practice for conducting bioconcentration tests with fishes and saltwater bivalve molluscs. Standard E1022-84. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA. 19103
- ASTM. 1989. Conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. Standard E729-88. American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.
- Mount, D.I. and W. A. Brungs. 1967. A simplified dosing apparatus for fish toxicology studies. *Water Res.* 1:20:29.
- OECD. 1981. Good Laboratory Practices acknowledged in the EEC Council Directive 88/320/EEC of 9 June 1988.
- OECD. 1984. Guideline for Testing of Chemicals. *Daphnia* sp., Acute Immobilization Test and Reproduction Test. Guideline #202. Adopted 4 April 1984.
- Sprague, J.B. 1969. Measurement of pollutant toxicity to fish. 1. Bioassay methods for acute toxicity. *Water Res.* 3: 793-821.
- U.S. EPA. 1975. Methods for acute toxicity tests with fish, macroinvertebrates and amphibians. EPA-660/3-75-009. Ecological Research Series. U.S. Environmental Protection Agency. 61 pp.

**TABLES**

**Table 1. Concentrations of Wingstay® 100 measured in replicate (A,B) exposure solutions during the 48-hour flow-through exposure of daphnids (*Daphnia magna*).**

| Nominal Concentration (mg/L) | Measured Concentration (mg/L) |          |                  |          | Mean (SD) <sup>a</sup> | Percent of Nominal (%) <sup>b</sup> |
|------------------------------|-------------------------------|----------|------------------|----------|------------------------|-------------------------------------|
|                              | 0-Hour                        |          | 48-Hour          |          |                        |                                     |
|                              | <u>A</u>                      | <u>B</u> | <u>A</u>         | <u>B</u> |                        |                                     |
| Control                      | < 0.071                       | < 0.071  | < 0.11           | < 0.11   | NA <sup>c</sup>        | NA                                  |
| Solvent Control              | < 0.071                       | < 0.071  | < 0.11           | < 0.11   | NA                     | NA                                  |
| 1.3                          | 0.24                          | 0.26     | 0.14             | 0.15     | 0.20 (0.061)           | 15                                  |
| 2.2                          | 0.49                          | 0.52     | 0.21             | 0.23     | 0.36 (0.17)            | 17                                  |
| 3.6                          | 0.89                          | 0.91     | 0.46             | 0.45     | 0.68 (0.26)            | 19                                  |
| 6.0                          | 1.4                           | 1.8      | 0.72             | 0.69     | 1.1 (0.53)             | 19                                  |
| 10                           | 2.5                           | 2.4      | 1.1              | 1.0      | 1.8 (0.82)             | 18                                  |
| QC <sup>d</sup> #1           | 0.494<br>(0.484) <sup>e</sup> |          | 0.444<br>(0.484) |          |                        |                                     |
| QC #2                        | 1.45<br>(1.45)                |          | 1.43<br>(1.45)   |          |                        |                                     |
| QC #3                        | 2.51<br>(2.42)                |          | 2.42<br>(2.42)   |          |                        |                                     |

<sup>a</sup> Mean measured concentrations were calculated using the actual analytical (unrounded) results obtained at the 0- and 48-hour intervals. Rounded (two significant figures) values presented in this table were not used in calculations. The standard deviation (SD) is presented in parentheses.

<sup>b</sup> Mean percent of nominal = 18%

<sup>c</sup> NA = Not applicable.

<sup>d</sup> QC = Quality Control sample

<sup>e</sup> Nominal fortified concentration for each QC sample is presented in parentheses.

**Table 2. Mean measured concentrations tested, corresponding cumulative percent of immobilized daphnids (*Daphnia magna*) and observations made during the 48-hour flow-through exposure to Wingstay® 100.**

| Mean Measured Concentration (mg/L) | Cumulative Percent Immobilized Organisms <sup>a</sup> |          |                    |           |           |                   |
|------------------------------------|---|----------|--------------------|-----------|-----------|-------------------|
|                                    | 24-Hour   |          |                    | 48-Hour   |           |                   |
|                                    | A   | B        | Mean               | A         | B         | Mean              |
| Control                            | 0<br>(0)  | 0<br>(0) | 0                  | 0<br>(0)  | 0<br>(0)  | 0                 |
| Solvent Control                    | 0<br>(0)  | 0<br>(0) | 0                  | 0<br>(0)  | 0<br>(0)  | 0                 |
| 0.20                               | 0<br>(0)  | 0<br>(0) | 0                  | 0<br>(0)  | 0<br>(0)  | 0 <sup>cdh</sup>  |
| 0.36                               | 0<br>(0)  | 0<br>(0) | 0 <sup>b</sup>     | 0<br>(0)  | 0<br>(0)  | 0 <sup>cde</sup>  |
| 0.68                               | 0<br>(0)  | 0<br>(0) | 0 <sup>cd</sup>    | 0<br>(0)  | 10<br>(1) | 5 <sup>cdh</sup>  |
| 1.1                                | 0<br>(0)  | 0<br>(0) | 0 <sup>cdef</sup>  | 10<br>(1) | 0<br>(0)  | 5 <sup>defi</sup> |
| 1.8                                | 0<br>(0)  | 0<br>(0) | 0 <sup>cdefg</sup> | 30<br>(3) | 20<br>(2) | 25 <sup>di</sup>  |

<sup>a</sup> Twenty daphnids were exposed to each treatment level and control (10 per replicate). The actual number of immobilized daphnids is presented in parentheses.

<sup>b</sup> Two of the mobile daphnids were observed on the surface of the test solution.

<sup>c</sup> Several of the mobile daphnids were observed to be swimming and carrying particulate matter.

<sup>d</sup> Undissolved test substance (e.g., precipitate) was observed in the test vessel.

<sup>e</sup> Several of the mobile daphnids were observed on the surface of the test solution.

<sup>f</sup> Exposure solution were observed to have a slight brown tint.

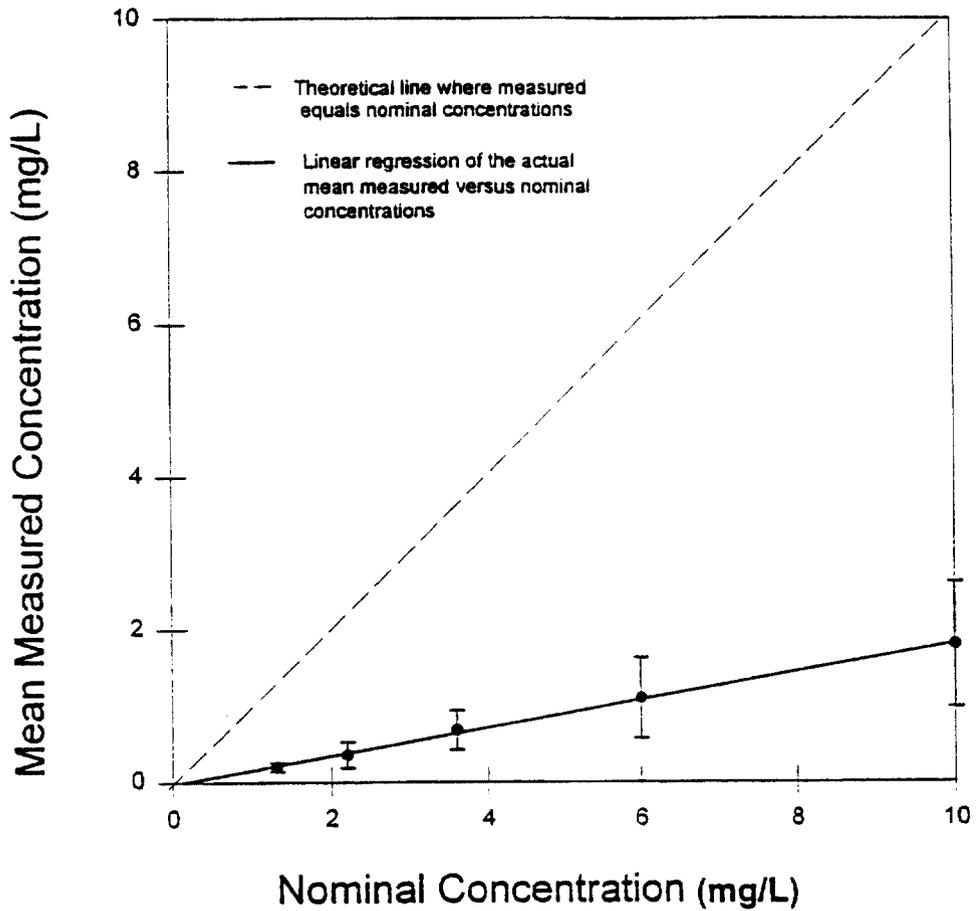
<sup>g</sup> Two of the mobile daphnids were observed to be lethargic.

<sup>h</sup> One of the mobile daphnids was observed on the surface of the test solution.

<sup>i</sup> All of the mobile daphnids were observed to be swimming and carrying particulate matter.

**FIGURES**

Figure 1. Relationship of mean measured concentrations (analyses at 0 and 48 hours) and the nominal concentrations during the 48-hour flow-through exposure of daphnids (*Daphnia magna*) to Wingstay® 100.

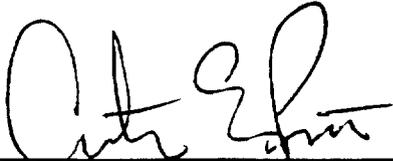


**SIGNATURES AND APPROVAL**

**SUBMITTED BY:**

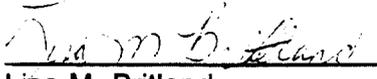
Springborn Laboratories Inc.  
Health and Environmental Sciences  
790 Main Street  
Wareham, Massachusetts 02571-1075

**PREPARED BY:**

  
\_\_\_\_\_  
Arthur E. Putt  
Study Director  
6/26/96  
Date

  
\_\_\_\_\_  
Mark A. Cafarella  
Principal Investigator  
6-26-96  
Date

  
\_\_\_\_\_  
Nigel D. Dix  
Analytical Chemist  
6/26/96  
Date

  
\_\_\_\_\_  
Lisa M. Britland  
Manager, Technical Reporting  
26 June 96  
Date

**APPROVED BY:**

  
\_\_\_\_\_  
Donald C. Surprenant  
Manager, Laboratory Services  
6/26/96  
Date

  
\_\_\_\_\_  
Doreen S. Newhouse  
Manager, Quality Assurance Unit  
26 June 96  
Date

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

#### 4.0 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:** Test Substance - Acute Toxicity to Water Fleas, *Daphnia magna* Under Flow-Through Conditions, Following OECD Guideline #202.

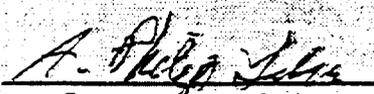
**TO BE COMPLETED BY THE STUDY SPONSOR:**Study Sponsor: The Goodyear Tire and Rubber CompanyAddress: 142 Goodyear Boulevard, Akron, Ohio 44305Phone: (216) 796-1046

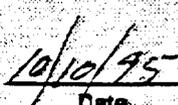
Sponsor Protocol/Project No.: \_\_\_\_\_

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

Analytical Standard: \_\_\_\_\_ Purity: \_\_\_\_\_ CAS# or LOT#: \_\_\_\_\_

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

  
 Sponsor Approval

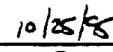
  
 Date
**TO BE COMPLETED BY SPRINGBORN LABORATORIES, INC. PRIOR TO TEST INITIATION:**Testing Facility: Springborn Laboratories, Inc., 790 Main Street, Wareham, MA 02571Study Director: Arthur E. Pitt Study Number: 13537 OFS-6173-115

Test Concentrations: \*

Carrier: \* CAS# or LOT#: \*

Proposed Experimental Schedule: (Start) \* (Completion) \* (Draft Report) \*

  
 Study Director

  
 Date

\* To be provided by protocol amendment.

Springborn Laboratories Protocol #: 020195/OECD/115/Goodyear

Page 1 of 9  


Springborn Laboratories, Inc.

TEST SUBSTANCE - ACUTE TOXICITY TO WATER FLEAS, *Daphnia magna*,  
UNDER FLOW-THROUGH CONDITIONS, FOLLOWING OECD GUIDELINE #202.

## 1.0 OBJECTIVE

The purpose of this test will be to determine the acute effects of a test substance on the water flea, *Daphnia magna*, under flow-through conditions. Test results will be reported as 24- and 48-hour EC50 values, i.e., the median concentration that immobilizes 50% of the number of daphnids exposed, with 95% confidence limits and the No-Observed-Effect-Concentration. The methods described in this protocol generally meet the requirements specified in OECD Guideline For Testing Of Chemicals #202, *Daphnia* sp., Acute Immobilization Test (OECD, 1984). Where applicable, Springborn Laboratories, Inc. Standard Operating Procedures (SOP) will be followed during the conduct of the study.

## 2.0 MATERIALS AND METHODS

### 2.1 CHEMICAL SYSTEM

- 2.1.1. **Test Substance.** Upon arrival at Springborn Laboratories, Inc., the external packaging of the test substance will be inspected for damage. The packaging will be removed and the primary storage container will also be inspected for leakage or damage. The sample identity and percent activity will be recorded and, unless different arrangements are made with the study sponsor, the test substance will be stored in the dark at approximately 20°C until used. The Study Sponsor will be responsible for the characterization of the test substance.
- 2.1.2. **Test Substance Concentration Selection.** Test substance concentrations will be based on the results of a preliminary flow-through range-finding test. 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 substances, one or both levels may not be observed. No attempt will be made to determine the degree of adsorption of the test substance by the test system, as this falls outside the scope and intent of this study. Five test concentrations and a negative control will be used. Each test substance concentration will be at least 45% of the next higher concentration of the test substance. A negative control consists of dilution water without the test substance.
- 2.1.3. **Solvent Control.** An organic solvent may be used as a carrier to solubilize the test substance. In such a case, a solvent control will be included in the test, and consists of dilution water plus the highest concentration of solvent that occurs in any of the test solutions. The solvent concentration will be kept as low as possible, and will not be allowed to exceed 0.1 mL/L.
- 2.1.4. **Stock Solution Preparation.** The test substance will be weighed on an analytical balance for which a calibration log will be maintained. A Chemical Usage Log will also be maintained in which the amount, the date, the intended use and the user's initials will be recorded each time the test substance is used. The stock solution will be prepared according to the following formula:

$$\text{Stock Concentration (mg A.I./L)} = \frac{\text{H.C.} \times \text{M.C.}}{\text{T.D.} \times \% \text{ A.I.}}$$

where: H.C. = high concentration (mg A.I./L)  
M.C. = mixing chamber volume (L/cycle)  
T.D. = toxicant delivery rate (mL/cycle)  
A.I. = % active ingredient

## 2.2. TEST ORGANISMS:

- 2.2.1. **Species.** The water flea, *Daphnia magna*, will be the species used in this test. Test organisms will be  $\leq$  24 hours old at the initiation of the test. Daphnids will be obtained by removing all immature daphnids from the culture vessel, thus isolating sexually mature daphnids 24 hours prior to initiating the test. Young produced by these organisms will be subsequently pipetted into the test beakers.
- 2.2.2. **Justification of Species.** Characteristics which make this test organism suitable for this acute toxicity test are their ease of handling and their sensitivity to a variety of chemical substances, and the extensive data base for this common freshwater invertebrate species.
- 2.2.3. **Origin.** *D. magna* cultures will be maintained at Springborn Laboratories, Inc. Daphnids will be cultured in 1.6-L glass vessels containing 1 L of water. Water used to culture the daphnids will be prepared in the same manner and have the same characteristics as described for dilution water. Culture water will be maintained at  $20 \pm 2^\circ\text{C}$ . Each culture vessel will be cleaned once weekly.
- 2.2.4. **Feeding.** While being maintained in culture prior to the test, organisms will be fed daily a combination of a trout food suspension and a unicellular green algae, *Ankistrodesmus falcatus*. The food solution will be prepared to contain 5 mg/mL trout food suspension and approximately  $4 \times 10^7$  cells/mL of algae. An aliquot of 0.5 mL of trout food suspension and 2 mL of algae will be manually introduced to each culture vessel once daily. Daphnids will not be fed during the 48-hour exposure period. Samples of each food source will be periodically analyzed for the presence of pesticides, PCBs and selected toxic metals.
- 2.2.5. **Handling.** Wide-bore pipets will be used to transfer the daphnids, taking care to minimize possible stress due to handling. Daphnids that are damaged or dropped during transfer will not be used.
- 2.2.6. **Loading.** Biomass will not exceed 0.1 grams for each liter per day of test solution flow.

### 2.3. PHYSICAL SYSTEM:

- 2.3.1. **Test Containers.** The test chambers to be used in the flow-through acute toxicity test will be approximately 1.6 or 2.0 liter clear battery jars. The test chambers will be chemically clean before the test is started. The test chambers will be washed with hot water and a detergent, rinsed with acetone, and then rinsed extensively with dilution water. Each jar will have either a 3 x 8 cm notch or two 2 cm holes drilled in the side of the jar and both the notch and holes will be covered with Nitex<sup>R</sup> 40-mesh screen for drainage. The test solution volume will thus be maintained at approximately 1.4 or 1.8 liters. The test chambers will be labeled to identify the treatment/control and the replicate designation.
- 2.3.2. **Replication and Control of Bias.** Two replicates will be included with each test concentration and control. Each replicate jar will contain ten individuals, a total of 20 daphnids per concentration or control. Daphnids will be added impartially to an intermediate test beaker by adding no more than two daphnids to each beaker until all beakers contain two daphnids. This procedure will be repeated until each beaker contains ten daphnids. The test will be initiated when each beaker of daphnids is added to each respective test vessel.
- 2.3.3. **Dilution Water.** Dilution water will consist of hard fortified well water with a total hardness of 160 to 180 mg/L as CaCO<sub>3</sub>. The well water (total hardness about 30 mg/L as CaCO<sub>3</sub>) will be fortified according to the formulation for hard water presented in "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians" (U.S. EPA, 1975). Dilution water will be filtered through an amberlite XAD-7 resin column and an activated carbon bed prior to delivery to the diluter. The column is 30 cm long and 1.6 cm wide. This filtration will effectively remove any potential organic contaminants from the water. Total hardness, total alkalinity, pH and specific conductance of the diluent water will be monitored on each batch prior to use to assure that these parameters are within the normal acceptable ranges. Total hardness and alkalinity will be determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1985). Ranges for these parameters will generally be: total hardness, 160 to 180 mg/L as CaCO<sub>3</sub>; alkalinity, 110 to 130 mg/L as CaCO<sub>3</sub>; specific conductance, 400 to 600  $\mu$ mhos/cm; and pH, 7.9 to 8.3.
- Quality of the dilution water used to conduct daphnid acute tests will be judged by the ability of the daphnid cultures to survive and reproduce in the water free of stress. The dilution water will be prepared in 1,900-L batches. New batches of diluent water will be prepared when either the previous batch is exhausted, when a water quality parameter (total hardness, alkalinity, etc.) differs from the normal ranges, or after two weeks of holding. The diluent water will be aerated with an air pump and air stones to bring the pH and dissolved gases into equilibrium with the atmosphere. Fiberglass containers will be used to hold the diluent water, and water will be pumped from this holding tank to the diluter. Periodic analysis of representative samples of the dilution water source will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to daphnids.
- 2.3.4. **Diluter.** A 200-mL proportional diluter (e.g., Mount and Brungs, 1967) will be employed to deliver five toxicant concentrations, a control, and a solvent control, to two replicate jars. Each treatment level will be at least 45% of the next higher concentration of the test substance. The exposure system will be constructed entirely of glass, silicone, and nylon.

Based on the solubility of the test substance, the stock solution stability and the range of test concentrations, one of the following toxicant delivery systems may be used: the gas-tight syringe injector metering device (most frequently used); the tube siphon delivery system; and the metering pump/predilution chamber system. The mixing chamber of the diluter will be positioned within an ultrasonic water bath. The contents of the mixing chamber will be gently stirred with a teflon stir bar driven by a submersible pump which will aid in the solubilization of the test material. The solution contained in the mixing chamber will constitute the highest nominal test concentration and will be diluted to produce the remaining nominal test concentrations.

A flow-splitting chamber will be used between the diluter cells and the two replicate test vessels to promote mixing of the toxicant solution and diluent water and to equally split the test solution between the test vessels. Two separate 1 mm (I.D.) glass capillary tubes will exit each splitter cell and enter individual delivery tubes which transfer the test solution to each replicate vessel. The capillary tubes baffle the flow of the test solution and minimize turbulence in the aquaria. Delivery rates of the test substance to each of the test vessels will be equal to 6 to 10 volume replacements per day. This flow rate will be adequate to maintain good water quality and does not stress the organisms due to excessive turbulence.

The calibration of the diluter system will be checked prior to test initiation and at test termination. If there is any indication during the test that the diluter calibration has changed (e.g., diluter malfunction or unexplained differences in dissolved oxygen concentration or temperature in the test vessels), calibration of the necessary diluter components will be checked. During the test, the diluter will be visually inspected at least twice daily. A complete check of diluter functioning will be made once daily. The test substance will be introduced into the diluter and the test vessels for a minimum of 24 hours before the test begins in order to allow the test solution concentration to reach equilibrium. A test will not be started until the diluter and test substance delivery device have been observed to be properly functioning for at least 24 hours prior to the test.

#### 2.4 TEST CONDITIONS

- 2.4.1. **Temperature.** Water temperature of the test solutions will be maintained at  $20 \pm 1^\circ\text{C}$  by conducting the test in a temperature-controlled room or waterbath maintained at the appropriate test temperature.
- 2.4.2. **Lighting.** All tests will be conducted in a light-controlled laboratory. The test will be illuminated to a light intensity of 30 to 100 footcandles using fluorescent bulbs. A 16-hour light, 8-hour dark photoperiod will be maintained with an automatic timer.
- 2.4.3. **Dissolved Oxygen.** Total dissolved oxygen will not be allowed to drop below 60% or exceed 105% of saturation for the duration of the test. Aeration (with oil free air) will be initiated as a last resort, and after Sponsor notification, to raise and maintain the dissolved oxygen concentration at or above 60% of saturation.
- 2.4.4. **Test Initiation.** The test substance will be introduced into the test vessels for a minimum of 24 hours before the test begins in order to allow the test solution to reach an equilibrium

concentration of test substance in the test system. The test begins when all the daphnids have been impartially placed in the test vessels and terminates after 48 hours of exposure.

## 2.5 SAMPLING AND OBSERVATIONS

- 2.5.1. **Sampling.** Water samples of an appropriate volume may be taken from the control, high, middle and low test concentrations at least twice during the pre-exposure period to document water concentrations and the proper functioning of the diluter. During the in-life phase, samples from both replicate vessels of each concentration and control(s) will be taken at the initiation of the test and at test termination for determination of test substance concentration.

Water samples will be taken from a point approximately midway between the surface, bottom and sides of each test vessel and either extracted immediately after sampling or appropriately preserved and stored until analysis can be performed. If analyses will not be performed immediately after sampling, the stability of the test substance in dilution water will be determined during the test system equilibration phase.

Three quality control samples will be prepared at each sampling interval and remain with the set of samples through extraction and analysis. These samples will be prepared in diluent water at three test substance concentrations similar to the treatment level range. Results of the QC analyses indicate the relative accuracy of the analytical methodologies for each sampling period. The analytical method used to measure test material concentration in the exposure solutions will be validated at Springborn Laboratories at the expected nominal concentration range prior to test initiation.

- 2.5.2. **Water Quality Measurements.** At test initiation and daily thereafter, temperature, dissolved oxygen (DO) concentration and pH will be measured and recorded in each test vessel. Total hardness, alkalinity, specific conductance will be determined at test initiation in one replicate of each concentration and control(s). Temperature will be monitored continuously in one test solution by using a minimum-maximum thermometer. Readings of temperature extremes will be recorded daily.
- 2.5.3. **Biological Observations.** The number of immobilized daphnids in each test vessel will be recorded after 24 and 48 hours of test initiation. Immobilization is defined as those daphnids which are not able to swim within 15 seconds after gentle agitation of the test solution. The test will be terminated following 48 hours of exposure. In addition, whenever test organisms are observed, characteristics of the test solutions will also be observed and recorded, e.g., precipitated materials, cloudiness, etc.
- 2.5.4. **Acceptability Criteria.** During the definitive test, immobilization in the control must not exceed 10% at test termination or the test will be considered unacceptable.

## 2.6 LIMIT TEST

The guidelines specify that a limit test may be conducted, using the procedures described in this protocol, to demonstrate that the EC50 will be greater than 100 mg active ingredient/L. The limit test will be conducted using 20 daphnids, with the same number in the controls. If any immobilization occurs, a full study should be conducted.

## 3.0 STATISTICAL ANALYSES

Test results derived from the acute test will be used to statistically estimate a median effective concentration (EC50) and its 95% confidence interval after 24 and 48 hours of exposure. The EC50 is the estimated measured concentration of the test substance in dilution water which produces 50% immobilization in the test populations of daphnids at the stated times of exposure. EC50 values will be computed using mean measured concentrations.

A computer program will be used to estimate EC50 values using three statistical methods: probit analysis, moving average method, and binomial probability. The method selected and reported will be determined by the data base (i.e., presence or absence of 100% response, number of partial responses, etc.). An EC50 value cannot be calculated if the data derived is insufficient according to any of the three statistical methods. The probit method provides values of the slope, including 95% confidence intervals, as well as appropriate statistical tests to evaluate goodness-of-fit. In addition, the highest test concentration at and below which there were no toxicant related mortalities or physical and behavioral abnormalities (e.g., lethargy, erratic swimming), with respect to the control organisms, will be determined and reported as the No-Observed-Effect Concentration (NOEC).

## 4.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but are not be 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.

## 5.0 REPORTING

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

- \* Springborn Laboratories, Inc., report and project numbers, Sponsor study numbers, protocol title and date as well as protocol amendments.

- \* Laboratory and site, the dates of testing and personnel involved in the study, i.e., Quality Assurance Unit, Program Coordinator (if applicable), Study Director, Principal Investigator.
- \* All information pertaining to the test substance which appears on the sample bottle, e.g., its empirical formula, molecular structure, source, percent active ingredient, physical properties, Sponsor's test article I.D., and sample number if available.
- \* Characterization and origin of the dilution water.
- \* Scientific name of the test organisms, source, and culturing information.
- \* Test container volume, dilution water volume, number of replicates used per concentration, and number of daphnids used per treatment.
- \* Description of diluter system, exposure system and stock solution preparation.
- \* Test temperatures, dissolved oxygen concentration, and pH; and photoperiod and light intensity used, as well as specific conductance, total alkalinity and total hardness measured.
- \* Observations of insolubility of the test substance, including the test levels and when observed.
- \* Definition of criteria used to determine the sublethal effects, and general observations on non-quantifiable effects.
- \* Percentage of daphnids that were immobilized in the controls and in each treatment at each observation period, in tabular form.
- \* Description of, or reference to chemical and statistical procedures applied.
- \* Analytical results of test concentration measurements and QC samples.
- \* If applicable, means and standard deviations of measured concentrations of the test compound, as well as nominal test concentrations.
- \* The 24- and 48-hour EC50's with 95 percent confidence limits, and the No-Observed-Effect Concentration (NOEC).
- \* Deviations from the protocol not addressed in protocol amendments will be listed, together with a discussion of the impact on the study.
- \* Good Laboratory Practice (GLP) compliance statement signed by the Study Director.
- \* Date(s) of Quality Assurance audit(s), and certification of report approval.
- \* Location of raw data and report.

#### 6.0 PROTOCOL CHANGES

*Springborn Laboratories Protocol #: 020195/OECD/115/Goodyear*

Page 8 of 9

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor. Protocol amendments and deviations must include the reasons for the change and the impact of the change on results of the study, if any. If necessary, amendments initially may be in the form of verbal authorization, followed by Springborn's written documentation of the amendment. In such a case, the effective date of the amendment will be the date of verbal authorization.

#### 7.0 SPECIAL PROVISIONS

**GOOD LABORATORY PRACTICES (GLP):** All test procedures, documentation, records, and reports comply with the principles of the Good Laboratory Practices (OECD, 1981) as acknowledged in the EEC Council Directive 88/320/EEC of 9 June 1988.

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

**ARCHIVAL:** All raw data and the final report will be archived by the Study Sponsor unless different arrangements are made.

#### 8.0 REFERENCES

- APHA, AWWA, WPCF. 1985. *Standard Methods for the Examination of Water and Wastewater*. 16th Edition, Washington, DC. 2168 pp.
- U.S. EPA. 1975. *Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians*. Ecological Research Series (EPA-660/3-75-009). 61 pp.
- Mount, D.I. and W.A. Brungs. 1967. A simplified dosing apparatus for fish toxicity studies. *Water Research* 1: 21-29.
- OECD, 1981. *Good Laboratory Practice in the Testing of Chemicals*. Paris, France.
- OECD, 1984. *Guideline for Testing of Chemicals. Daphnia sp. Acute Immobilization Toxicity Test. Guideline #202*. Adopted 4 April 1984.

**Springborn Laboratories, Inc.**

Environmental Sciences Division

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

PROTOCOL AMENDMENT

AMENDMENT #: 1

DATE: 6 December 1995

PROTOCOL TITLE: "Wingstay® 100 - Acute Toxicity to Water Fleas, *Daphnia magna* Under Flow-Through Conditions, Following OECD Guideline #202."

SPECIES: *Daphnia magna*

STUDY SPONSOR: The Goodyear Tire & Rubber Company

TEST MATERIAL: Wingstay® 100

SLI STUDY NO: 13537.0995.6123.115

## AMENDMENT(S):

1. The following information is provided as requested on the cover page of the Study Protocol.

Test concentrations: 10, 6.0, 3.6, 2.2 and 1.3 mg/L, plus controls. (nominal) P (1/7/96)

Carrier used: Acetone.

CAS#: 67-64-1

Proposed experimental schedule:

Start: 12-6-95

Completion: 12-8-95

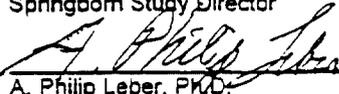
Draft Report: 1-17-96

2. The Study Protocol states that the culture organisms will be fed daily a combination of 0.5 mL trout food suspension, 0.5 mL Selco® suspension and 2 mL of *Ankistrodesmus falcatus*. During this study, the culture organisms will be fed 2.0 mL of *Ankistrodesmus falcatus* ( $4 \times 10^7$  cell/mL) daily. The modification of food type and quantity was done to meet the nutritional requirements of the test organisms.

Approval Signatures:

  
Arthur E. Putt  
Springborn Study Director

11/4/95  
Date

  
A. Philip Leber, Ph.D.  
Sponsor Study Monitor

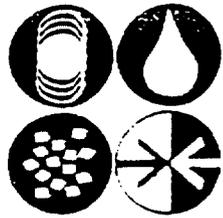
1/7/96  
Date

Springborn Laboratories Protocol #: 020195/OECD/115/Goodyear

Page 1 of 1  
 Springborn  
LABORATORIES

LETTERS AND REPORTS: Springborn Laboratories, Inc. letters and reports are issued for the exclusive use of the clients to whom they are addressed. No questions from reports or use of the Springborn Laboratories, Inc. name is permitted except as expressly authorized in writing. Letters and reports apply only to the specific material, products or processes tested, examined or surveyed and are not necessarily indicative of the quality of apparently identical or similar materials, products or processes. The liability of Springborn Laboratories, Inc. with respect to services rendered shall be limited to the amount of the consideration paid for such services and not include any consequential damages.

**5.0 APPENDIX II - PRODUCT SPECIFICATION INFORMATION**



# PRODUCT SPECIFICATION

THE GOODYEAR TIRE & RUBBER COMPANY • AKRON, OHIO 44316-0001

## WINGSTAY 100

WINGSTAY 100 is a mixture of diaryl-p-phenylenediamines.

| <u>PROPERTY</u>                     | <u>LIMITS</u>     | <u>TEST METHOD</u> |
|-------------------------------------|-------------------|--------------------|
| Appearance                          | Blue-brown flakes | Visual             |
| Iron Content, ppm                   | 750 max           | E-913              |
| Initial Melting Point, deg C        | 87.0 to 97.0      | E-514              |
| Fineness, through 3/8" Screen, %    | 100 min           | E-34               |
| Total Diaryl-p-phenylenediamines, % | 80 min            | E-885              |

---

Chemical Division test methods are available upon request.

---

S-208-5    Ref: 29382    05-27-93

**GOODYEAR**  
CHEMICALS

400C.10

---

Springborn Laboratories, Inc.

## 6.0 APPENDIX III - ANALYTICAL METHODOLOGY

## SUMMARY

Methodology was developed (12 October 1994) to quantify the amount of Wingstay® 100 present in freshwater. Aqueous samples containing the test substance were processed by liquid/liquid extraction with hexane and analyzed on a high performance liquid chromatographic (HPLC) system using ultraviolet (UV) detection.

The test substance, Wingstay® 100, is primarily a mixture of 3 components. These distinct materials elute at different rates under the HPLC conditions used for this analysis. The results presented in this report are based on the total concentration of all 3 of these compounds. The retention times listed are those of the first of these compounds to elute from the column.

This method was validated by fortification of freshwater samples with Wingstay® 100 at nominal concentrations ranging from 5.00 to 50.0 µg/L. Recoveries averaged  $97.9 \pm 6.68\%$  with a limit of quantitation of 3.00 µg/L. Defined limits for acceptance of Quality Control sample performance in subsequent studies were three standard deviations from the mean recoveries obtained in method validation for Wingstay® 100. This three standard deviation range was 77.9 to 118%.

## EXPERIMENTAL

### Equipment

1. Instrument: Hewlett-Packard Chromatographic solvent pump Model 1050 equipped with a Hewlett-Packard Model 1050 Autosampler, Kratos Model 757 UV/VIS variable wavelength detector and Hewlett Packard Model 3396B integrator.
2. Balance: Mettler AE 200, four place analytical balance
3. Laboratory glassware: syringes, volumetric pipets, volumetric flasks, Pasteur pipets, GC vials, separatory funnels, powder funnels, roundbottom flasks and amber serum bottles.

**Reagents**

1. Acetonitrile: Burdick & Jackson, HPLC grade
2. Hexane GC<sup>2</sup>: Burdick & Jackson, HPLC grade
3. Reagent grade water: prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM type IIA requirements).

**Test Substance**

Wingstay® 100, Lot # 137170393, CAS Registry # 68953-84-4, was received from Goodyear Research, Akron, Ohio on 7 September 1994, reported by the Sponsor to have a purity of 100% and used in the preparation of the method validation/recovery samples and analytical standards.

**Instrumental Conditions**

The high performance liquid chromatographic (HPLC) analysis was conducted utilizing the following instrumental conditions:

|                   |  |
|-------------------|--|
| Column:           | Metachem, Inertsil ODS-2 C18 (5 µm), 250 mm (length) x 4.6 mm (I.D.) |
| Mobile Phase:     | 80% acetonitrile; 20% reagent grade water                            |
| Flow Rate:        | 1.0 mL/min   |
| Wavelength:       | 310 nm   |
| Injection Volume: | 100 µL   |
| Instrument        |  |
| Sensitivity:      | 0.020 AUFS   |
| Attenuation:      | 2 <sup>0</sup>   |
| Threshold:        | 1  |
| Peak Width:       | 0.10 seconds   |
| Retention Time:   | Wingstay® 100 ~ 5.4 to 5.6 min.                                      |

## PROCEDURES

### Preparation of Stock Solution

To prepare a Wingstay® 100 primary stock solution with a nominal concentration of 0.250 mg/mL, 0.0250 gram (g) test substance was weighed into a 100-mL volumetric flask and dissolved with 100 mL of hexane GC<sup>2</sup>. This primary stock solution (0.250 mg/mL) was stored in a refrigerator (4 °C) in amber serum bottles fitted with Teflon®-lined lids. This stock was used, with the appropriate dilution, for preparation of analytical standards as well as fortification of recovery samples.

### Sample Fortification

Method validation/recovery samples were prepared in freshwater. The aqueous samples were fortified with dilutions of the primary stock. The fortification levels produced were 5.00, 10.0 and 50.0 µg/L (three replicates at each concentration). An additional three freshwater samples were left unfortified to be used as control samples.

### Extraction

All samples were extracted once by liquid-liquid extraction with hexane GC<sup>2</sup>. Exactly 4.00 mL hexane GC<sup>2</sup> was added to 50.0 mL of reagent grade water that had been fortified to the appropriate concentration in 60 mL separatory funnels. The sample was shaken by hand for 3 minutes and the phases allowed to separate. An aliquot of the hexane extract was analyzed by high performance liquid chromatography (HPLC) using ultraviolet (U.V.) detection.

### Sampling Techniques

Laboratory studies are generally conducted in glass volumetric flasks, centrifuge tubes or aquaria. Sampling procedures typically include siphoning (using silicone tubing) from the midpoint of the test container into graduated cylinders for volumes greater than 100 mL, and pipetting (using volumetric pipets) from the midpoint of the test container for sample volumes less than or equal to 100 mL. Deviations from these practices, if any, are identified in the study report.

## ANALYSIS

### Preparation of Standard Curve

Calibration standards were prepared from the primary Wingstay® 100 stock. The concentrations of the standards were 0.0500, 0.100, 0.150, 0.200 and 0.500 mg/L. Two sets of standards were analyzed with each sample set, one prior to analysis of the samples and one immediately following the samples. Samples were also run at several points during the analysis. Injection of samples and standards onto the chromatographic system was performed by programmed injection.

A standard curve was constructed by plotting the peak area of each Wingstay® 100 standard against the concentration (mg/L) of the standard injected. The coefficient of determination, slope, y-intercept and limit of quantitation were calculated. The concentration of Wingstay® 100 in each sample was determined using the linear regression analysis and the peak area of the sample.

## CALCULATIONS

The following equations were utilized in the calculation of measured concentrations and analytical results:

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

$$DC \times DF = A$$

where:

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

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

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

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

where:

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

## RESULTS AND DISCUSSION

The mean recovery of Wingstay<sup>®</sup> 100 in freshwater was  $97.9 \pm 6.68\%$ , for samples with nominal concentrations ranging from 5.00 to 50.0  $\mu\text{g/L}$ . The limit of quantitation was 3.00  $\mu\text{g/L}$ . The LOQ can vary from one analysis interval to another, since it is dependent upon the linear regression of the standards and the peak response (area) of the low standards. These parameters, while relatively constant, do vary somewhat among runs and produce small variations in the LOQ. Recovery results from this method validation were used to evaluate Quality Control samples prepared during subsequent studies involving Wingstay<sup>®</sup> 100. Quality Control sample recovery expectations were three standard deviations from the mean recoveries obtained in method validation. The acceptable range for Wingstay<sup>®</sup> 100 was 77.9 to 118%.

Analytical results for the recovery of Wingstay<sup>®</sup> 100 from freshwater are presented in Table 1A. A representative chromatogram showing the analysis of Wingstay<sup>®</sup> 100 in a standard solution is shown in Figure 1A. A representative chromatogram of a Wingstay<sup>®</sup> 100 fortified freshwater sample is shown in Figure 2A. An analysis of a control water sample is presented

---

in Figure 3A. A typical linear regression analysis for Wingstay<sup>®</sup> 100 standards is presented in Figure 4A.

**Table 1A. Analytical results for the recovery of Wingstay<sup>®</sup> 100 from freshwater.**

| Fortified Concentration (µg/L) | Recovered Concentration (µg/L) | Percent Recovery <sup>a</sup> (%) |
|--------------------------------|--------------------------------|-----------------------------------|
| Control                        | < 3.00                         | NA                                |
| Control                        | < 3.00                         | NA                                |
| Control                        | < 3.00                         | NA                                |
| 5.00                           | 5.22                           | 105                               |
| 5.00                           | 5.35                           | 107                               |
| 5.00                           | 5.41                           | 108                               |
| 10.0                           | 9.57                           | 95.7                              |
| 10.0                           | 9.24                           | 92.4                              |
| 10.0                           | 9.45                           | 94.5                              |
| 50.0                           | 46.5                           | 93.1                              |
| 50.0                           | 45.4                           | 90.8                              |
| 50.0                           | 47.7                           | 95.4                              |

NA = Not Applicable

Mean recovery:  $97.9 \pm 6.68\%$ , (N = 9).

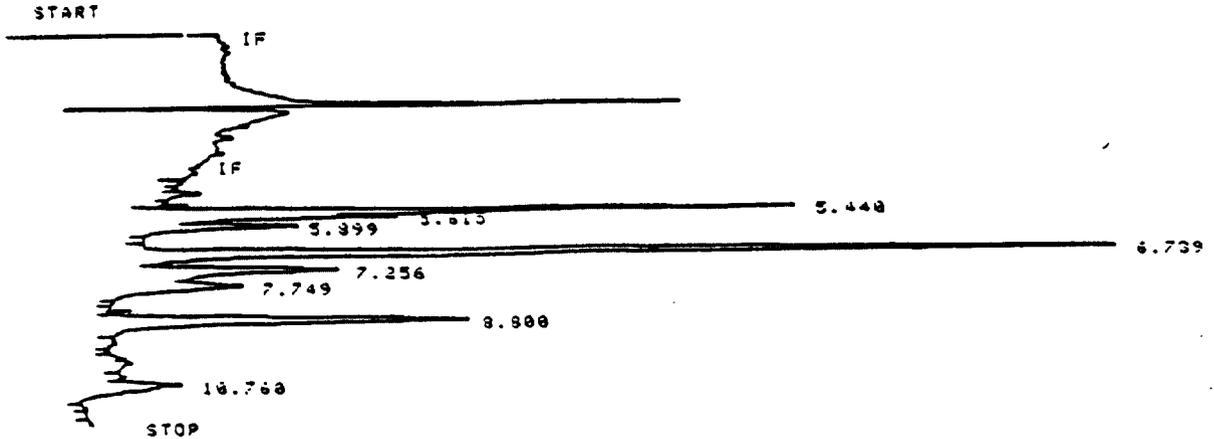
Limit of quantitation has been determined to be 3.00 µg/L.

Concentrations expressed as less than values are below the limit of quantitation (LOQ). The LOQ for each sample is dependent upon the sample volume, dilution factor and standard concentration range.

<sup>a</sup> Values presented are based on unrounded analytical results rather than the rounded values presented in this table.

Figure 1A. HPLC chromatogram of Wingstay® 100 in a 0.200 mg/L standard solution.

RUN # 5 OCT 12, 1994 12:58:46



Closing signal file M:SIGNAL .BNC

RUN # 5 OCT 12, 1994 12:58:46

SIGNAL FILE: M:SIGNAL.BNC

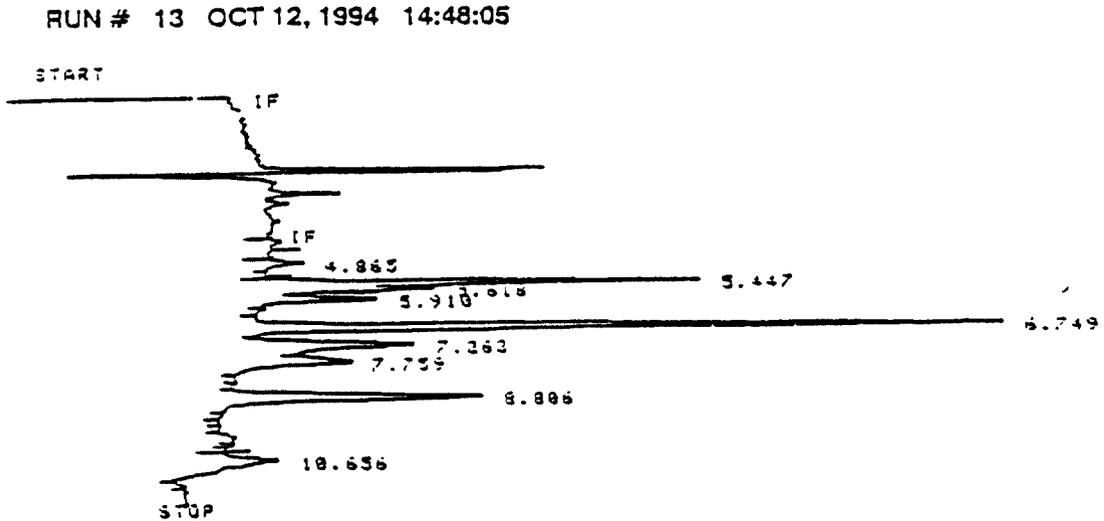
ESTD-HEIGHT

| RT     | TYPE | AREA  | WIDTH | HEIGHT | CAL# | AMOUNT | NAME |
|--------|------|-------|-------|--------|------|--------|------|
| 5.440  | BV   | 38264 | .123  | 5205   | 1R   | .191   | MG/L |
| 5.615  | VV   | 16911 | .144  | 1959   |      | .000   |      |
| 5.899  | VV   | 11567 | .160  | 1206   |      | .000   |      |
| 6.739  | PV   | 83760 | .158  | 8820   | 2    | .184   | MG/L |
| 7.256  | VV   | 25717 | .252  | 1699   |      | .000   |      |
| 7.749  | VV   | 16773 | .277  | 1010   |      | .000   |      |
| 8.800  | BV   | 35839 | .213  | 2807   | 3    | .176   | MG/L |
| 10.760 | VP   | 14632 | .309  | 790    |      | .000   |      |

TOTAL HEIGHT= 23496

MUL FACTOR= 1.0000E+00

Figure 2A. HPLC chromatogram of Wingstay® 100 in a 10.0 µg/L fortified sample.



Closing signal file M:SIGNAL .BNC

RUN # 13 OCT 12, 1994 14:48:05

SIGNAL FILE: M:SIGNAL.BNC

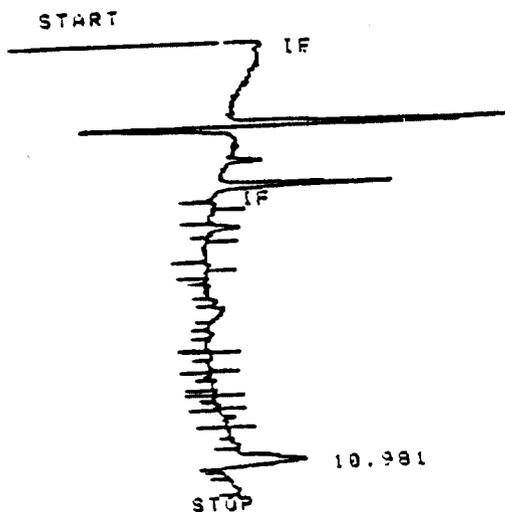
ESTD-HEIGHT

| RT     | TYPE | AREA  | WIDTH | HEIGHT | CAL# | AMOUNT | NAME |
|--------|------|-------|-------|--------|------|--------|------|
| 4.865  | PP   | 1690  | .103  | 274    |      | .000   |      |
| 5.447  | BV   | 24026 | .119  | 3374   | 1R   | .124   | MG/L |
| 5.618  | VV   | 10885 | .140  | 1299   |      | .000   |      |
| 5.910  | VV   | 8478  | .158  | 894    |      | .000   |      |
| 6.749  | PV   | 51832 | .149  | 5784   | 2    | .121   | MG/L |
| 7.263  | VV   | 20636 | .263  | 1306   |      | .000   |      |
| 7.759  | VV   | 13672 | .257  | 886    |      | .000   |      |
| 8.806  | VP   | 24690 | .211  | 1952   | 3    | .123   | MG/L |
| 10.656 | BP   | 11600 | .344  | 562    |      | .000   |      |

TOTAL HEIGHT= 16331  
 MUL FACTOR= 1.0000E+00

**Figure 3A. HPLC chromatogram of a control<sup>1</sup> water sample.**

RUN # 8 OCT 12, 1994 13:39:51



Closing signal file M:SIGNAL .BNC

RUN # 8 OCT 12, 1994 13:39:51

SIGNAL FILE: M:SIGNAL.BNC

NO CALIB PEAKS FOUND

AREA%

| RT     | AREA | TYPE | WIDTH | AREA%     |
|--------|------|------|-------|-----------|
| 10.981 | 9356 | VP   | .252  | 100.00000 |

TOTAL AREA= 9356

MUL FACTOR= 1.0000E+00

---

<sup>1</sup> No peak equivalent to retention time for Wingstay® 100. Wingstay® 100 is ordinarily detected at a retention time of approximately 5.4 to 5.6 minutes.

Figure 4A. Plot of signal response versus concentration for Wingstay<sup>®</sup> 100 standards linear regression analysis.

