Battelle
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Chemical Repository Services for the EDSP

EPA Contract No. 68-W-01-023

DRAFT

1.0 TITLE AND APPROVAL

Chemistry Report for WA 2-17
17β-Estradiol in Bird Feed

June 22, 2005

Prepared By: Approved By:

_____   _____   _____   _____
Michael E. Cobb.   Date   Richard M. Ecker   Date
Chemical Repository Study Director   Director, Marine Sciences Laboratory

Battelle Marine Sciences Laboratory

1529 West Sequim Bay Road

Sequim, WA 98382

Submitted to:

Crystal Driver

Battelle Richland Operations

Richland, Washington

2.0 QA REVIEWER AND CONTRIBUTOR PAGE

Chemistry Report for WA 2-17
17β-Estradiol in Bird Feed

Reviewed by: _____

Mary Lynn, EDSP Quality Assurance Representative
Windward Company, Gig Harbor

Date: _____

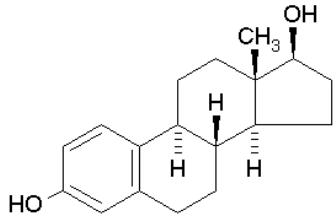**Personnel participating in this study:**

Analyst: Waverly Thorsen

Statistician: Valerie Cullinan

Chemical Repository Study Director: Michael Cobb

3.0 EXECUTIVE SUMMARY

Chemistry Report for WA 2-17
17β-Estradiol in Bird Feed

Table 1. Study Test and Reference Substance Procurement Information

Parameter	Study Test and Reference Substance	Substance Structure
Compound Name	17β-Estradiol	
CAS #	50-28-2	
Central File No.	1932	
Initial Receipt Date	02/26/2003	
Expiration Date	02/26/2008 (set by CR)	
Manufacturer	Sigma	
Lot Number	052K1370	
Purity per supplier	100%	
Battelle Study #	WA 2-17	
Method	SOP, MSL-O-018-00	

Executive Summary

Work Assignment (WA) 2-17 of the Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP) describes a reproductive and developmental study on Japanese quail exposed to 17β-estradiol (estradiol) via spiked feed. The Chemical Repository (CR) has the responsibility for carrying out the purity, stability, and in-life concentration determinations for EDSP studies. The test substance purity of estradiol, as determined by the manufacturer, was 100%. The analytical method developed in-house for the estradiol stability study and in-life samples utilized Gas Chromatography/Mass Spectrometry (GC/MS). To provide analytical compatibility with the GC/MS system, the estradiol required derivatization prior to analysis. To minimize the complexity of the measurement for the less demanding purity analysis, the compound was tested using High Performance Liquid Chromatography (HPLC). The Battelle-Sequim purity result by HPLC was 96.17%.

The stability of the feed dosed with Estradiol (333 ng/g) was assessed 7 or over 28 days under three different storage conditions; refrigerated, kept in the bird feed troughs, and stored at room temperature (RT) in a sealed container. The statistician reported that the concentration of estradiol was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 19 days when refrigerated. Though the statistical analysis suggests a stability of 19 days, the data clearly indicates there is no degradation over the 28 day evaluation period. In speaking to the statistician about the issue, she indicated the low result for day 7 biased the regression downward, and use of a conservative 95% confidence interval resulted in the conclusion of 19 days stability. The day 7 result is just shy of being 3 standard deviations from the mean of all 8 stability determinations. When comparing the individual stability results to the day zero results, day 7 varies by a negative 12.4%, whereas the remaining 6 determinations are all within 1.7% of day zero. Therefore, in spite of the statistical conclusion, the feed is considered stable for a minimum of 28 days. For the trough study, the concentration of estradiol was expected to stay greater than or equal to 80% of the Day 0 concentration for up to an estimated 4 days. For the bucket study, the concentration of estradiol was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 5 days.

Exceptions:

o   Due to the method development phase being conducted concurrently with the stability testing, the reported sample data is from samples that were re-analyzed with a filtration clean-up step. This does not impact the stability data.

o   The Certificate of Analysis indicated that estradiol used in the study had a shelf life of 2 years, ending January 2004. The study continued beyond this date and Sigma (the manufacturer) was contacted to determine if additional stability data were available. In response, (attached as Appendix A) their technical service stated that no additional testing had been carried out but that, "a shelf-life is not an expiration date." They further stated, "Since most Sigma products are intended for research use, it is impractical and inappropriate to assign formal expiration dates." Later in the response, they state, "If the recommendation is to store an item at room temperature, then the item is not temperature-sensitive and will usually have a shelf-life of at least five years." Our standard approach within the Repository is to assign test substances expiration dates 5 years from date of receipt when the MSDS indicates that they are "stable," as is the case with 17β-Estradiol.

4.0 TABLE OF CONTENTS

5.0 INTRODUCTION

The goal of the Battelle-Sequim, Marine Sciences Laboratory (MSL) Chemical Repository (CR) for the Endocrine Disruptor Screening Program is to provide the participating laboratory with requested chemicals of documented quality at required concentrations and in a matrix appropriate for different toxicological tests. The CR supplies the manufacturer's information regarding purity and stability, the material safety data sheet (MSDS) chemical information, and independent analysis of purity and stability in a matrix specified by the Study Plan: *EDSP Purity Analysis and Stability Testing Plan WA 2-17*, developed in collaboration with the requesting Principal Investigator. Under Work Assignment (WA) 2-17, Crystal Driver of Battelle Richland requested purity and stability testing of estradiol (Figure 1).
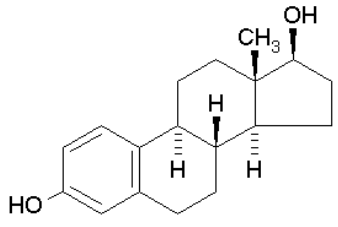
6.0 GENERAL METHODS

Methods of standard operation of the CR are currently addressed in MSL SOPs numbered R-001 through R-017. These procedures address test substance procurement including procurement of controlled substances, when applicable, which have unique permitting, ordering, handling, inventory, and storage requirements; test substance receipt and chain of custody, test substance log-in and labeling, inventory, test substance storage; stock solution preparation, documentation and archiving; test solution preparation, documentation and shipping; test substance disposal, and repository maintenance over time. The quality assurance (QA) requirements for procurement of test substances for use in the CR are addressed in the Quality Assurance Project Plan for CR.

6.1 TEST SUBSTANCE PROCUREMENT
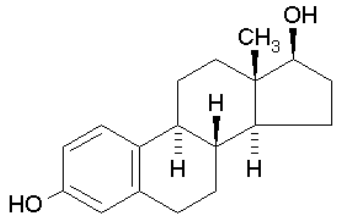
As requested (Figure 1) by Crystal Driver, the principal investigator for the study, 17β-estradiol (estradiol), (CAS No. 50-28-2), was purchased for purity and stability analysis and used as the test substance, blended with bird feed, in a two generation avian endocrine disruption study. The estradiol was purchased from Sigma, Inc. and the first shipment of lot number 052K1370 was received on 11/26/02 with a stated shelf life[1] (on the Certificate of Analysis) of two years from January 2002 (Table 1). The test substance was logged in to the Chemical Management System (CMS) and given a CMS barcode and unique log-in (central file) number (CF-1932-1) as per the QA Project Plan (QAPP) for the CR. The test substance was stored in the Repository in a clean, dry location at room temperature, away from direct sunlight.
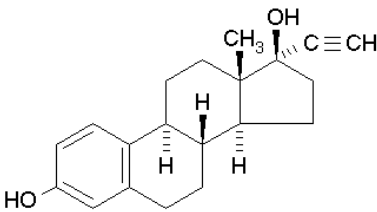
Table 2. Test and Reference Substance Procurement Information

Parameter	Study Test and Reference Substance	Substance Structure
Compound Name	17β-Estradiol	
CAS #	50-28-2	
Central File No.	1932	
Initial Receipt Date	02/26/2003	
Expiration Date	02/26/2008 (set by CR)	
Manufacturer	Sigma	
Lot Number	052K1370	
Purity per supplier	100%	
Battelle Study #	WA 2-17	
Method	SOP, MSL-O-018-00	

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[1] See Attachment 1 for manufacturer's explanation of "shelf life" claims.

Table 2. Test and Reference Substance Procurement Information (cont.)

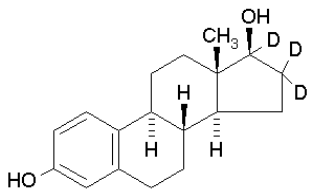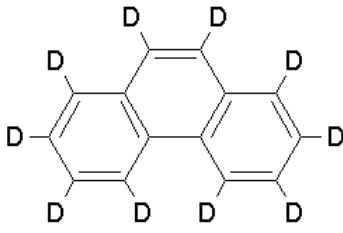| Parameter	Reference Substance	Substance Structure
Compound Name	17β-Estradiol	
CAS #	50-28-2	
Central File No.	2083-1	
Initial Receipt Date	03/22/1999	
Expiration Date	10/05/2005 (set by CR)	
Manufacturer	Sigma	
Lot Number	086H1138	
Purity per supplier	99.7%	
Battelle Study #	WA 2-17	
Method	SOP, MSL-O-018-00	

Parameter	Reference Substance*	Substance Structure
Compound Name	17α-Ethynlestradiol	
CAS #	57-63-6	
Central File No.	2064-1	
Initial Receipt Date	07/16/2003	
Expiration Date	07/16/2005 (set by CR)	
Manufacturer	Sigma-Aldrich	
Lot Number	05013CB	
Purity per supplier	98%	
Battelle Study #	WA 2-17	
Method	SOP, MSL-O-018-00	

*Used as Surrogate internal standard (SIS#1)

Parameter	Reference Substance*	Substance Structure
Compound Name	β-Estradiol-16,16,17-d_3	
CAS #	79037-37-9	
Central File No.	2063-1	
Initial Receipt Date	07/16/2003	
Expiration Date	07/16/2005 (set by CR)	
Manufacturer	Aldrich	
Lot Number	07223KA	
Purity per supplier	99%	
Battelle Study #	WA 2-17	
Method	SOP, MSL-O-018-00	

*Used as Surrogate internal standard (SIS#2)

Parameter	Reference Substance*	Substance Structure
Compound Name	Phenanthrene-d_{10}	
CAS #	1517-22-2	
Central File No.	NA	
Initial Receipt Date	09/20/2000	
Expiration Date	10/2008	
Manufacturer	Aldrich	
Lot Number	14906Mu	
Purity per supplier	99%	
Battelle Study #	WA 2-17	
Method	SOP, MSL-O-018-00	

* Used as Recovery internal standard (RIS)

EDSP Chemical Request Form

For EPA WA:2-17

Study Director:
>   Name: Crystal Driver
>   Affiliation: PNNL/BNW
>   Location: PSL/1507 Richland, Washington
>   Telephone number: 509-375-2721

Bioassay Information:

Proposed Bioassay: Avian dosing study.

Test Chemical: 17beta-estradiol

Carrier(s): vegetable oil suspension (cottonseed oil has been used in the literature); or ethanol or other suitable volatile solvent

Concentrations/Dilution Series: To be determined by range finding. Assume a logarithmic dilutions series from a high concentration of a consumed dose of 1000 micrograms/bird/per day   (assume a bird eats about 15 grams of food per day) to a low dose of 0.01 microgram/bird/day.

Test concentrations are likely to be in the 1 to 100 or 500 ug/bird/day range.

>   *Consider if analysis method detection limit which may be determined in
>   Purity analysis is above or below desired test concentrations?

In vitro or *in vivo* tests? in vivo

Organism to be tested: Japanese quail

Method of test solution administration: In feed. The compound may have a short half-life under the light conditions in the animal room (10 lux up to 17 hours per day, 72- 76 F, 40-50% humidity). Also, there is some concern about particulate exposure for animal and feed handlers. Not sure which of the two feed incorporation methods will result in less fines or crystals of the compound that could become airborne.

Planned/proposed test duration: 40-44 weeks. However, nutrient stability in feed allows for a maximum of 120 days post mill date (i.e., 120 day shelf-life).

Chemical Information:

Chemical Name: 17beta-estradiol

CAS:

Any known purity information: may refer to attached documentation

Any known stability information: may refer to attached documentation

Desired purity (%) for test? As pure as possible, >99%

Is there a known calibration standard available? (I think you have this from fish studies??)
> Name:
> CAS:
> Manufacturer:

Figure 1. EDSP Chemical Request Form for Estradiol

6.2   TEST SUBSTANCE PURITY

Test substance purity was verified by high performance liquid chromatographic analysis. No statistical analyses were performed for the verification of test substance purity. Purity verification was conducted by making a solution of estradiol in acetonitrile at about 1 µg/mL. This solution was then analyzed on an HPLC with a UV/Vis detector. A blank was also analyzed on the system. The purity was determined by comparing the area of the peak associated with the substance of interest with the total area of all the peaks in the chromatogram. The percentage associated with the largest peak represented the purity of the test substance. This result was compared to the manufacturer's certificate of analysis/purity (Appendix B). The HPLC was set up with an autosampler and a 250X4.6 mm reversed-phase HPLC column. The HPLC system was run isocratically, with a 60% acetonitrile/40% water eluent, the detector was set to a wavelength of 254 nm. One replicate was analyzed.

6.3   STABILITY TESTING PLAN DESIGN AND DETAIL

A general study plan for stability testing based on the WA 2-17 Chemical Request from Crystal Driver (Figure 1) was developed as the *EDSP Stability Analysis and Purity Testing Plan* and is presented in Appendix C. The study was designed to assess the stability of estriadiol measured and blended into bird feed. The stability of the feed dosed with estradiol was evaluated under three different scenarios: 1) dosed feed stored under refrigeration, 2) dosed feed kept in bird troughs, and 3) dosed feed stored in a sealed container in the laboratory. The feed was spiked and blended to 333 ng/g at Battelle Richland, with a portion immediately shipped to MSL Sequim for testing. The remaining 333 ng/g dosed feed was maintained under two different storage conditions for 7 days: 1) feed troughs in cages, 2) bucket for 'in-room' ambient storage conditions. Following the 7 day exposure period, the feed from the trough and the bucket were placed in separate glass containers and shipped to MSL Sequim for analysis. All samples were analyzed in triplicate for calculation of a mean concentration and relative standard deviation (RSD). The feed stored under refrigeration for 28 days was collected twice a week (0, 7, 9, 14, 16, 21, 23, & 28 days) and was extracted in triplicate in accordance with SOP O-018-00. Feed stored in the trough and sealed laboratory containers were sampled at 0 and 7 days, and sent to MSL for extraction and analysis in accordance with SOP O-018-00. The sample extracts were analyzed using GC/MS.

Table 3. Stock Matrix Composition for Stability Testing

Study, Duration	Test Substance	Target Conc.	Stock Matrix
WA 2-17, 28 days	17β-Estradiol	333 ng/g	Dissolved in acetone and blended with feed

6.4   ANALYTICAL CHEMISTRY FOR STABILITY TESTING

Game bird feed and acetone containing a specified concentration of 17β-estradiol were mixed in a large capacity mixer to formulate each test diet. Acetone was used as the solvent vehicle to transport the estradiol into the feed and was then evaporated off.

Test substance stability was evaluated under storage conditions and matrix specifications as requested by the participating laboratory. At initiation and at each time period throughout the duration of the test, the test substance concentration was determined by chromatographic analysis. Triplicate aliquots were tested. The frequency of determinations and the duration of testing were determined by the requesting principal investigator and the chemists based on *a priori* knowledge about test substance stability.

The following is a brief description of the sample extraction and prep for analysis – a detailed description of this procedure is provided in MSL SOP O-018-00: A measured quantity of estradiol spiked bird feed was extracted with an acetone and sodium sulfate solution with ethynyl-estradiol, (EE2) added as an internal standard (surrogate internal standard #1 = SIS#1). The extracted sample was then centrifuged, filtered, and dried. At this point a second internal standard, β-estradiol-16,16,17-d₃ (surrogate internal standard #2= SIS#2) is added and the sample is derivatized. Following derivization, phenanthrene-D10, a third internal standard (recovery internal standard = RIS), is added and the sample is analyzed by GC/MS (all per MSL SOP O-018-00). Data collected in method development indicated that sample analysis within 40 days of extraction was acceptable when extracted samples were stored at ≤ -6 °C. In addition to a 5 point calibration curve, continuing calibration verification (CCV) samples were analyzed to demonstrate on-going calibration accuracy. Data are normalized to the recovery internal standard and then corrected for the efficiency of the derivitization step based on the percent recovery of SIS#2. Finally, data are corrected to account for the total extract volume and the intial amount of the sample extracted.

Table 4. GC/MS Conditions

GC System	Agilent 5890 (Palo Alto, CA)
Column	J+W DB-5, 30 m x 0.25 mm (ID) x 0.25 μm film thickness (Bellefonte, PA)
Oven Temperature Program	120°C held for 2 minutes, increase 15°C/minute to 300°C, hold 5 minutes
Helium Carrier Gas/ Flow Rate	Helium at 1 mL/minute
Injection Volume/Mode	2 μL/ Splitless
Detector Type	Mass Spectrometer (MS)
Detector Mode	SIM (Selective Ion Monitoring)
Transfer Line Temperature	280°C
Ion Source Temperature	150°C
Detector Temperature	300°C
Injector Inlet Temperature	250°C
Run Time	19 minutes

6.5 STATISTICAL ANALYSIS OF STABILITY

Log linear degradation curves were fit to the data to describe the test substance concentration over time and their dependence on storage conditions and solvent matrix. Lack of fit and residual plots were evaluated to determine the form of the regression. Power calculations based on the observed variability were used to determine the sensitivity of the test to detect degraded concentrations. General methods are documented in SOP EDSP.D-012-01.

7.0   RESULTS

7.1 TEST SUBSTANCE PURITY

Battelle-Sequim conducted an HPLC-UV/Vis purity scan on the estradiol. The chromatogram showed only one large peak that had the appropriate retention time for estradiol with several smaller peaks. The area of the estradiol peak was 96.17% of the total area of all peaks in the chromatogram. Test substance purity of estradiol determined by the manufacturer via HPLC was 100% (Appendix B).

7.2 ANALYTICAL CHEMISTRY FOR STABILITY TESTING

 Test substance stability testing was initiated on 07/29/03. Test substance concentrations for refrigerated samples were determined 8 times over a period of 28 days. The analytical and

quality control (QC) results are presented in Appendix D. CCV results ranged from 105.1% to 105.2%. All QC data were within acceptance criteria. The MDL was determined to be 36.7 ng/g.

7.3   STATISTICAL RESULTS OF STABILITY TRIAL

A plot of estradiol with a target concentration of 333 ng/g against time suggested very little test substance degradation during the study (Figure 2) though there was a fair amount of scatter in the measurements and the Day 0 concentration was only 86.1% of the original target concentration. For the 28 day study, only one mean value (Day 7) fell below the 90% average of the Day 0 concentration (Day 7 average was at 87.6% of day zero average). The average Day 0 concentration was used to evaluate stability through time. Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of estradiol was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 19 days (Table 5). Thus, this stock solution was considered stable for the 3-week testing period. The complete statistical analysis is presented in Appendix E.

For the trough study, both observations at Day 7 fell below the 90% average of the Day 0 concentration (Day 7 average was at 234 ng/g which is 81.7% of the Day 0 average). Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of estradiol was expected to stay greater than or equal to 80% of the Day 0 concentration for up to an estimated 4 days (Table 5). The complete statistical analysis is presented in Appendix E.

For the bucket study, none of the observations at Day 7 fell below the 90% average of the Day 0 concentration (Day 7 average was at 267 ng/g which is 93% of the Day 0 average). Based on the final regression model and the lower 95% confidence limit of the slope, the concentration of estradiol was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 5 days (Table 5). The complete statistical analysis is presented in Appendix E.
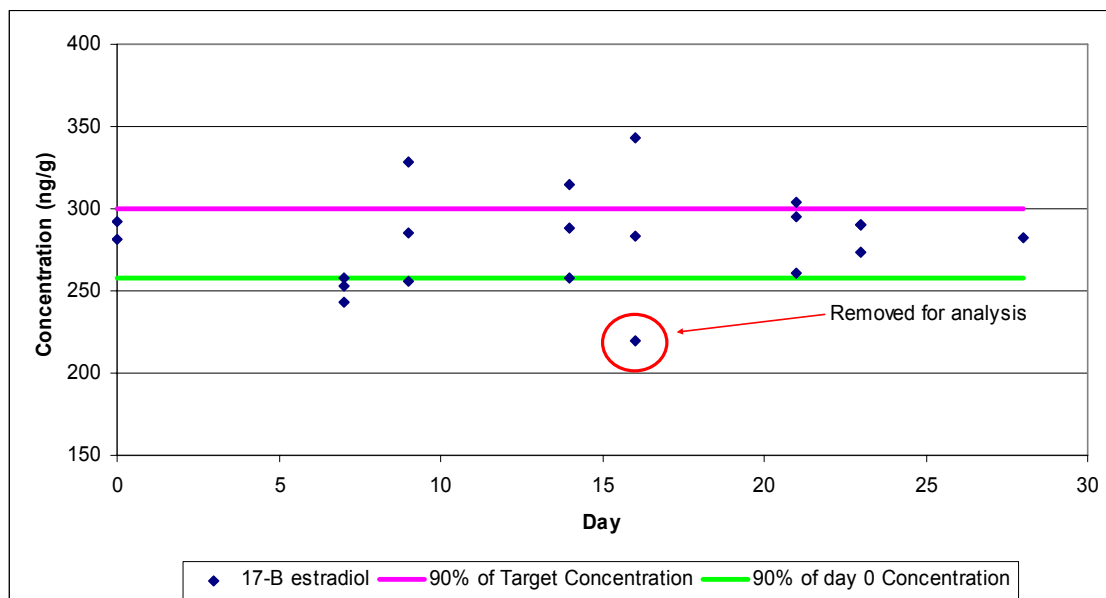

Figure 2. Observed concentration of Estradiol in Bird Feed with a target concentration of 333 ng/g against time

Table 5. Summary of Statistical Results for Estradiol in Bird Feed

Central File Number	2065.2	Trough	Laboratory Bucket
	17-B estradiol	17-B estradiol	17-B estradiol
Average Day 0 Concentration (ng/g)	287	287	287
Number of determinations	1	1	1
Number of days tested	28	7	7
Number of replicates per day	3	3	3
Number of outliers removed	0	0	0
Number of observations removed	4	2	1
Overall Mean Concentration	284	260	275
95% Upper CL	294	297	287
Degrees of freedom	18	3	4
1-Sample t-test of Ho: μ >= Target	NS [a]	NS	S
Estimated intercept of ln(concentration) against time	5.6151	5.6582	5.6582
Estimated slope of ln(concentration) against time	0.0021	-0.0290	-0.0105
Standard error of slope	0.0026	0.0057	0.0036
Error degrees of freedom	18	2	3
Significance test of lack-of-fit for final model	NS	--	--
Significance test of Ho: β = 0 vs. H1: β = 0	NS	S [b]	NS
Lower 95% CL of β	-0.003	-0.053	-0.022
Upper 95% CL of β	0.008	-0.005	0.001
Maximum Percent Loss over 8 days (using LCL)	2.6%	34.7%	16.0%
Mean Percent Loss (using bhat)	-1.7%	20.7%	8.0%
LN(90% of Target)	5.5530	5.4353	5.5530
Number of days until at 90% of Target (using LCL)	19	4	5
Conclusion using Target Concentration:	**Stable for 19 days**	**Stable for 4 days using 20% loss**	**Stable for 5 days**

[a] Not Significant at α = 0.05
[b] Significant at α = 0.05
Note: Statistical Analysis conducted by Valerie Cullinan Using Minitab Version 13.32, Minitab Inc., 1999.

8.0 CONCLUSIONS

Test substance purity was verified at 96.17% versus a supplier claim of 100%. The PI requested a purity of >99% if possible. Since the substance CR determined purity was <97% per the CR QAPP, a QAPP deviation was generated, deviation number EDSP.217-D1.

The statistician reported that the concentration of estradiol was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 19 days when refrigerated. Though the statistical analysis suggests a stability of 19 days, the data clearly indicates there is no degradation over the 28 day evaluation period. In speaking to the statistician about the issue, she indicated the low result for day 7 biased the regression downward, and use of a conservative 95% confidence interval resulted in the conclusion of 19 days stability. The day 7 result is just shy of being 3 standard deviations from the mean of all 8 stability determinations. When comparing the individual stability results to the day zero results, day 7 varies by a negative 12.4%, whereas the remaining 6 determinations are all within 1.7% of day zero. Therefore, in spite of the statistical conclusion, the feed is considered stable for a minimum of 28 days. For the trough study, the concentration of estradiol was expected to stay greater than or equal to 80% of the Day 0 concentration for up to an estimated 4 days. For the bucket study, the concentration of estradiol was expected to stay greater than or equal to 90% of the Day 0 concentration for up to an estimated 5 days.

APPENDIX A

MANUFACTURER'S STATEMENT ON EXPIRATION DATE AND SHELF-LIFE

Sent: Monday, 6/21/2004, 1:39 PM

Dear Michael Cobb:

Thank you for contacting Sigma-Aldrich Technical Service.

Lot number 052K1370 of this product was sold out, so no further testing was performed or will be performed on this lot. However, please keep in mind that a shelf-life is not an expiration date. I have pasted some information below into this e-mail that may better explain the nature of a shelf-life.

EXPIRATION DATES AND SHELF-LIFE

Since most Sigma products are intended for research use, it is impractical and inappropriate to assign formal expiration dates.

Sigma does have general guidelines concerning the shelf-life of our products when they are stored unopened at the temperature recommended (indicated both on the label and in the general catalog). Please be aware that there certainly are exceptions to these guidelines, particularly if products are sensitive to moisture, air or light. Some items may degrade more quickly; others will be stable for years if kept cool, dry and in the dark.

If the recommendation is to store an item at room temperature, then the item is not temperature-sensitive and will usually have a shelf-life of at least five years. When stored properly, items recommended for storage at 2-8 deg. C will have a shelf-life of 2-3 years. Items recommended for storage at temperatures below 0 deg. C usually have a shelf-life of about 1 year. Again, the recommendation is to re-evaluate for suitability in a given application, and to extend the "shelf-life" if a given product has shown no changes in properties.

Regards,
Charles Krueger, Ph.D.
Sigma-Aldrich Technical Service

APPENDIX B

MANUFACTURER'S CERTIFICATE OF ANALYSIS/PURITY

SIGMA-ALDRICH

Certificate of Analysis

Product Name	β-Estradiol
Product Number	E8875
Product Brand	SIGMA
CAS Number	50-28-2
Molecular Formula	$C_{18}H_{24}O_2$
Molecular Weight	272.38

TEST	SPECIFICATION	LOT 052K1370 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 200 MG PLUS 4 ML OF ETHANOL	CONFORMS
PURITY BY HPLC	98% MINIMUM	100%
SHELF LIFE SOP QC-12-006	2 YEARS	JANUARY 2004
QC ACCEPTANCE DATE		JANUARY 2002

Lori Schulz, Manager
Analytical Services

APPENDIX C

PURITY AND STABILITY TESTING PLAN

Prepared by W. Thorsen 10/29/03   Distribution: Tim Fortman, project file
                                                                        Deborah Coffey, QA
                                                                        Val Cullinan, Statistical Testing
                                                                        Whitney Hansen, Data Management

EDSP Purity Analysis and Stability Testing Plan WA 2-17

Chemical Name: Estradiol (MSL CF number 1932)

CAS Number: 50-28-2

Lot Number: 052k1370 250mg bottles stored at RT in MSL5, Rm 219

Expiration date: 1/04

Manufacturer's Purity Information: 97%

Manufacturer's Stability Information: stable

MSL Purity Results:
        Purity (%)   96.17% as determined by HPLC/UV

        MDL 0.377 ug/g

Calibration Standard available;

        Name: Estradiol

        CAS: 50-28-2

Bioassay Information: Steriod, endocrine disruptor, see MSDS

Study Director
        Name: Eric Crecelius
        Affiliation: Battelle
        Location: MSL 5/306
        Telephone number: 360-681-3604

Stability Test of Estradiol

A study to determine the stability of estradiol in bird feed will be conducted. The stability of feed dosed with estradiol will be assessed for three different scenarios: 1) dosed feed stored under refrigeration, 2) dosed feed kept in bird troughs, and 3) dosed feed stored in a sealed container in the laboratory. The samples stored under refrigeration will be located at MSL 5/223, while the feed stored in the troughs and sealed container will be located at PNNL-Richland (laboratory of C. Driver). Feed will be dosed at 0.333 µg/g (lowest exposure concentration as of 07/03). The

Prepared by W. Thorsen 10/29/03   Distribution: Tim Fortman, project file
                                                                       Deborah Coffey, QA
                                                                       Val Cullinan, Statistical Testing
                                                                       Whitney Hansen, Data Management

feed stored under refrigeration for 28 days will be collected twice a week (0, 7, 9, 14, 16, 21, 23, 28 days) and will be extracted in triplicate in accordance with SOP 0-018-00.

Feed stored in the trough and sealed laboratory containers will be sampled at 0 and 7 days, and sent to MSL 5 for extraction and analysis in accordance with SOP 0-0180-00. Sample extracts will be analyzed using GC/MS.

Personal Protective Equipment:

All personnel must wear a lab coat, eye protection and nitrile gloves when handling estradiol or feed spiked with estradiol. Disposal of all wastewater must comply with the wastewater treatment plan prepared for WA 2-18.

Records:
- date sample received;
- date(s) sample analyzed;
- sample matrix;
- electronic file identification codes (when applicable to identify instrument data files);
- data summary reports;
    - Chemical repository confirmatory test results of chemical identity and purity;
    - Chemical repository test results of lot-to-lot variation in chemical purity;
    - Chemical repository periodic assessment results of changes in purity of stock solutions and dilutions and generation of degradation products
- QC data reports;
- data qualifying flags; and
- dilution factor(s).

APPENDIX D

ANALYTICAL RESULTS OF STABILITY TESTING

Table D1. Estradiol concentration in Bird Feed (ng/g)

Target Conc.	Sample ID	Date Prepared	Date Extracted	Date Analyzed[1]	17-β Estradiol	Qualifier*	Average	% Recovery
333 ng/g	2065.2a	7/28/2003	7/29/2003	9/14/2003	342.5	Q	-	-
333 ng/g	2065.2b	7/28/2003	7/29/2003	9/14/2003	292.0		286.7	86.1
333 ng/g	2065.2c	7/28/2003	7/29/2003	9/14/2003	281.4			
333 ng/g[†]	2065.2d	7/28/2003	8/5/2003	9/14/2003	252.7			
333 ng/g[†]	2065.2e	7/28/2003	8/5/2003	9/14/2003	257.4		251.1	75.4
333 ng/g[†]	2065.2f	7/28/2003	8/5/2003	9/14/2003	243.2			
333 ng/g[†]	2065.2g	7/28/2003	8/7/2003	9/14/2003	285.3			
333 ng/g[†]	2065.2h	7/28/2003	8/7/2003	9/14/2003	256.1		290.1	87.1
333 ng/g[†]	2065.2i	7/28/2003	8/7/2003	9/14/2003	328.9			
333 ng/g[†]	2065.2j	7/28/2003	8/12/2003	9/14/2003	314.8			
333 ng/g[†]	2065.2k	7/28/2003	8/12/2003	9/14/2003	287.9		286.8	86.1
333 ng/g[†]	2065.2l	7/28/2003	8/12/2003	9/14/2003	257.7			
333 ng/g[†]	2065.2m	7/28/2003	8/14/2003	9/14/2003	219.6			
333 ng/g[†]	2065.2n	7/28/2003	8/14/2003	9/14/2003	342.9		281.8	84.6
333 ng/g[†]	2065.2o	7/28/2003	8/14/2003	9/14/2003	282.9			
333 ng/g[†]	2065.2q	7/28/2003	8/19/2003	9/14/2003	260.8			
333 ng/g[†]	2065.2r	7/28/2003	8/19/2003	9/14/2003	303.6		286.6	86.1
333 ng/g[†]	2065.2s	7/28/2003	8/19/2003	9/14/2003	295.3			
333 ng/g[†]	2065.2t	7/28/2003	8/21/2003	9/14/2003	289.9			
333 ng/g[†]	2065.2u	7/28/2003	8/21/2003	9/14/2003	273.7		284.5	85.4
333 ng/g[†]	2065.2v	7/28/2003	8/21/2003	9/14/2003	289.9			
333 ng/g[†]	2065.2w	7/28/2003	8/26/2003	9/14/2003	282.1		282.1	84.7
333 ng/g[†]	2065.2x	7/28/2003	8/26/2003	9/14/2003	303.5	Q	-	-
333 ng/g[†]	2065.2y	7/28/2003	8/26/2003	9/14/2003	343.5	Q	-	-
Trough								
333 ng/g	2065.2a	7/28/2003	7/29/2003	9/14/2003	342.5	Q	-	-
333 ng/g	2065.2b	7/28/2003	7/29/2003	9/14/2003	292.0		286.7	86.1
333 ng/g	2065.2c	7/28/2003	7/29/2003	9/14/2003	281.4			
333 ng/g	2065.3a	7/28/2003	8/5/2003	9/14/2003	242.4			
333 ng/g	2065.3b	7/28/2003	8/5/2003	9/14/2003	226.0		234.2	70.3
333 ng/g	2065.3c	7/28/2003	8/5/2003	9/14/2003	235.6	Q		
Laboratory Bucket								
333 ng/g	2065.2a	7/28/2003	7/29/2003	9/14/2003	342.5	Q	-	-
333 ng/g	2065.2b	7/28/2003	7/29/2003	9/14/2003	292.0		286.7	86.1
333 ng/g	2065.2c	7/28/2003	7/29/2003	9/14/2003	281.4			
333 ng/g	2065.4a	7/28/2003	8/5/2003	9/14/2003	272.8			
333 ng/g	2065.4b	7/28/2003	8/5/2003	9/14/2003	258.4		266.5	80.0
333 ng/g	2065.4c	7/28/2003	8/5/2003	9/14/2003	268.2			

[1] Analysis date is specified as date run started – some latter runs were processed by the instrument on 09/15/03.

Qualifier = Q = Data had an unacceptable IS recovery and was qualified as questionable.

* Q data not included in calculations

[†] These sample were stored refrigerated

Table D2. CCV Data for Estradiol in Bird Feed

Analysis Date[1]	Sample Name	Expected 17-β Estradiol (ng/mL)	Measured 17-β Estradiol (ng/mL)	Recovery
9/14/2003	CCV	202.4	213.0	105.2%
9/14/2003	CCV	202.4	212.8	105.1%
9/14/2003	CCV	202.4	212.9	105.2%

[1] Analysis date is specified as date run started.

Table D3. Internal Standards Data for Estradiol in Bird Feed

Sample Name	Extraction Date	Deuterated Estradiol (DE2) Recovery
Refrigerator sample		
2065.2a	7/29/2003	50.1%
2065.2b	7/29/2003	66.6%
2065.2c	7/29/2003	109.9%
2065.2d	8/05/2003	105.3%
2065.2e	8/05/2003	112.5%
2065.2f	8/05/2003	108.0%
2065.2g	8/07/2003	95.8%
2065.2h	8/07/2003	102.2%
2065.2i	8/07/2003	93.9%
2065.2j	8/12/2003	107.5%
2065.2k	8/12/2003	100.6%
2065.2l	8/12/2003	113.0%
2065.2m	8/14/2003	110.6%
2065.2n	8/14/2003	106.3%
2065.2o	8/14/2003	112.6%
2065.2q	8/19/2003	114.8%
2065.2r	8/19/2003	108.0%
2065.2s	8/19/2003	103.0%
2065.2t	8/21/2003	108.3%
2065.2u	8/21/2003	111.1%
2065.2v	8/21/2003	108.5%
2065.2w	8/26/2003	114.6%
2065.2x	8/26/2003	135.3%
2065.2y	8/26/2003	123.3%
Trough		
2065.3a	8/05/03	91.5%
2065.3b	8/05/03	111.8%
2065.3c	8/05/03	127.0%
Laboratory bucket		
2065.4a	8/05/03	103.9%
2065.4b	8/05/03	115.5%
2065.4c	8/05/03	110.5%

*Not added to this sample

APPENDIX E

STATISTICAL REPORT

WA-2-17-03-01
Statistical Analysis conducted by Valerie Cullinan
Using Minitab Version 13.32, Minitab Inc., 1999.

──────── **9/25/2003 2:47:35 PM** ──────────────────────────

Analysis-2 of Estradiol (ng/g) in feed
Storage in Refrigerator

Analysis of stability using new clean-up procedure and correction to CCV without Q-flagged data

- Test to determine if the data are from a population with mean of 287 ng/g (the mean of reps 2-3 of day 0)

```
Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X    3
DATA> 287
```

One-Sample T: 17-B estradiol

```
Test of mu = 287 vs mu < 287

Variable          N      Mean     StDev   SE Mean
17-B estradi     21    280.87     28.73      6.27

Variable      95.0% Upper Bound      T      P
17-B estradi            291.68   -0.98  0.170        NS
* NOTE * N missing = 3
```
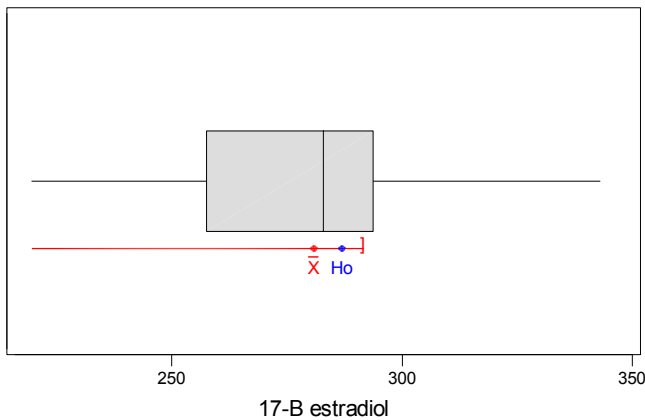
t Boxplot of 17-B estradiol

Boxplot of 17-B estradiol
(with Ho and 95% t-confidence bound for the mean)


```
Outliers are < Median-3*IQD OR > Median+3*IQD
Boundary for outliers are values <    174.500  and >    391.384
```

```
No outliers
```
- Transform data to natural logarithm and conduct regression analysis.

```
    Day     Rep Ln(Concentration)

      0       1            *
      0       2        5.6769
      0       3        5.6396
      7       1        5.5323
      7       2        5.5507
      7       3        5.4941
      9       1        5.6536
      9       2        5.5455
      9       3        5.7957
     14       1        5.7520
     14       2        5.6625
     14       3        5.5516
     16       1        5.3920
     16       2        5.8375
     16       3        5.6452
     21       1        5.5637
     21       2        5.7156
     21       3        5.6881
     23       1        5.6696
     23       2        5.6119
     23       3        5.6697
     28       1        5.6423
     28       2            *
     28       3            *
```

```
Conducts Simple Linear Regression
```

Regression Analysis: 17-B estradiol versus Day

```
The regression equation is
17-B estradiol = 5.61 + 0.00173 Day

21 cases used 3 cases contain missing values

Predictor         Coef      SE Coef          T         P
Constant       5.60828      0.04847     115.71     0.000
Day           0.001732     0.003011       0.58     0.572         NS


S = 0.1048      R-Sq = 1.7%      R-Sq(adj) = 0.0%

Analysis of Variance

Source              DF          SS          MS          F         P
Regression           1     0.00363     0.00363       0.33     0.572
Residual Error      19     0.20870     0.01098
  Lack of Fit        6     0.03950     0.00658       0.51     0.793    NS
  Pure Error        13     0.16920     0.01302
Total               20     0.21233


1 rows with no replicates


Unusual Observations
Obs        Day   17-B est         Fit      SE Fit      Residual      St Resid
 13       16.0     5.3920      5.6360      0.0235       -0.2440        -2.39R
R denotes an observation with a large standardized residual
```
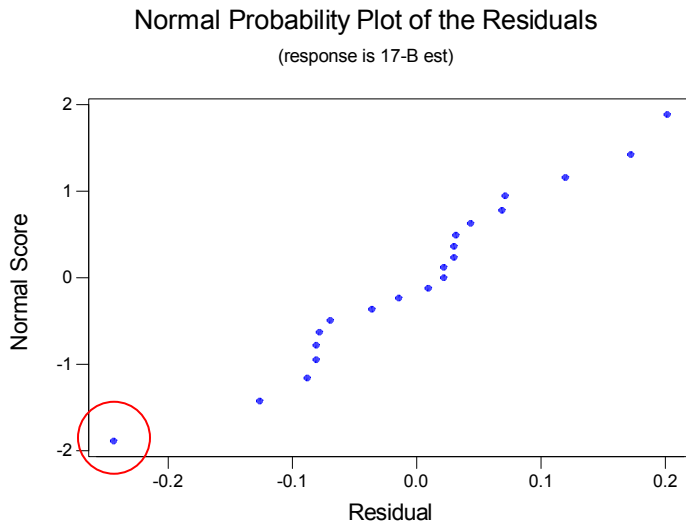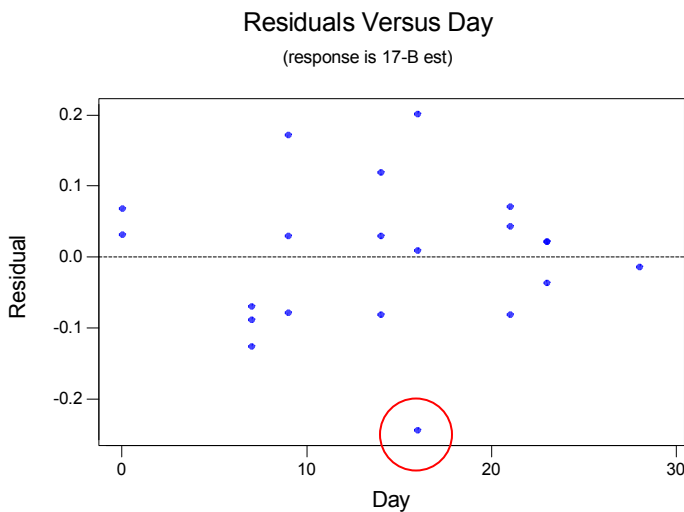
Normplot of Residuals for 17-B est

Normal Probability Plot of the Residuals
(response is 17-B est)

Residuals from 17-B est vs Day

Residuals Versus Day
(response is 17-B est)

- Power analysis for t-test of slope less than zero

## Power and Sample Size

```
1-Sample t Test

Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05  Sigma = 0.104806

Sample
  Size   Power   Difference
    19   0.9900     -0.0994
```

- That means we would detect a mean of 260 as significantly less than 287 = 9.4% loss.
- Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: 17-B estradi versus Day

Regression Plot

17-B estradi = 5.60828 + 0.0017320 Day

S = 0.104806   R-Sq = 1.7 %   R-Sq(adj) = 0.0 %
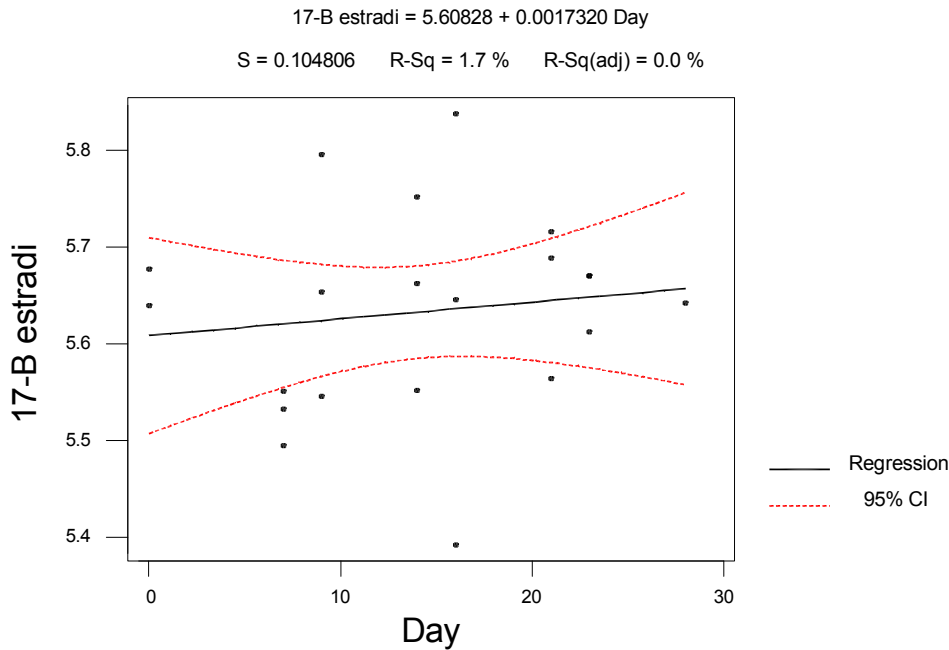
- **Conclusion: Redo without rep 1 day 16.**

───────── **9/26/2003 10:19:55 AM** ──────────────────────────────────

Analysis of stability using new clean-up procedure and correction to CCV without Q-flagged data and day 16 rep 1

- Test to determine if the data are from a population with mean of 287 ng/g (the mean of reps 2-3 of day 0)

```
Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X    3
DATA> 287
```
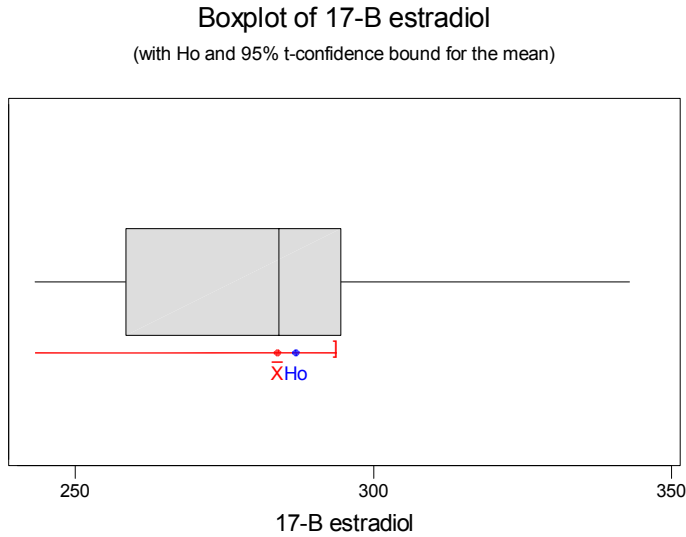
One-Sample T: 17-B estradiol

```
Test of mu = 287 vs mu < 287

Variable          N      Mean     StDev   SE Mean
17-B estradi     20     283.93    25.73      5.75
Variable      95.0% Upper Bound      T       P
17-B estradi          293.88    -0.53   0.300      NS
* NOTE * N missing = 4
```

t Boxplot of 17-B estradiol

Boxplot of 17-B estradiol
(with Ho and 95% t-confidence bound for the mean)

17-B estradiol

Outliers are < Median-3*IQD OR > Median+3*IQD

Boundary for outliers are values <   175.944 and >   392.323

No outliers

- Transform data to natural logarithm and conduct regression analysis.

```
Day      Rep Ln(Concentration)

  0        1            *
  0        2        5.6769
  0        3        5.6396
  7        1        5.5323
  7        2        5.5507
  7        3        5.4941
  9        1        5.6536
  9        2        5.5455
  9        3        5.7957
 14        1        5.7520
 14        2        5.6625
 14        3        5.5516
 16        1            *
 16        2        5.8375
 16        3        5.6452
 21        1        5.5637
 21        2        5.7156
 21        3        5.6881
 23        1        5.6696
 23        2        5.6119
 23        3        5.6697
 28        1        5.6423
 28        2            *
 28        3            *
```

Conducts Simple Linear Regression

Regression Analysis: 17-B estradiol versus Day

```
The regression equation is
17-B estradiol = 5.62 + 0.00212 Day

20 cases used 4 cases contain missing values

Predictor         Coef      SE Coef           T          P
Constant       5.61507      0.04172      134.59      0.000
Day           0.002116     0.002591        0.82      0.425        NS

S = 0.09006      R-Sq = 3.6%       R-Sq(adj) = 0.0%

Analysis of Variance

Source            DF           SS           MS           F          P
Regression         1     0.005407     0.005407        0.67      0.425
Residual Error    18     0.145999     0.008111
  Lack of Fit      6     0.058182     0.009697        1.33      0.319        NS
  Pure Error      12     0.087816     0.007318
Total             19     0.151406

1 rows with no replicates

Unusual Observations
Obs       Day    17-B est          Fit       SE Fit      Residual      St Resid
 14      16.0      5.8375       5.6489       0.0207        0.1886         2.15R

R denotes an observation with a large standardized residual
```
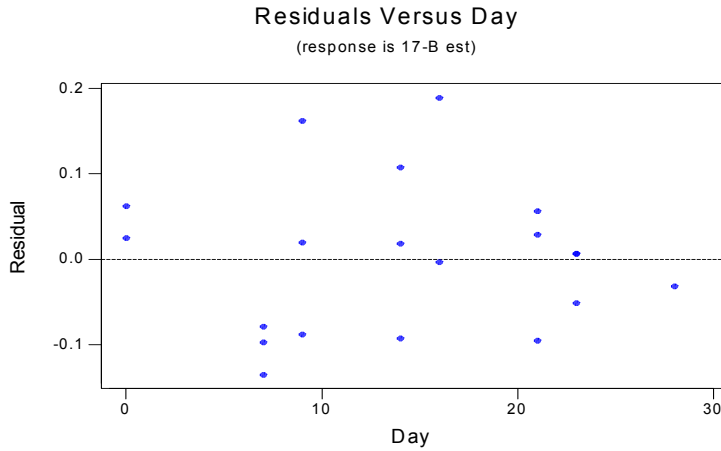
Normplot of Residuals for 17-B est

Normal Probability Plot of the Residuals
(response is 17-B est)

Residuals from 17-B est vs Day
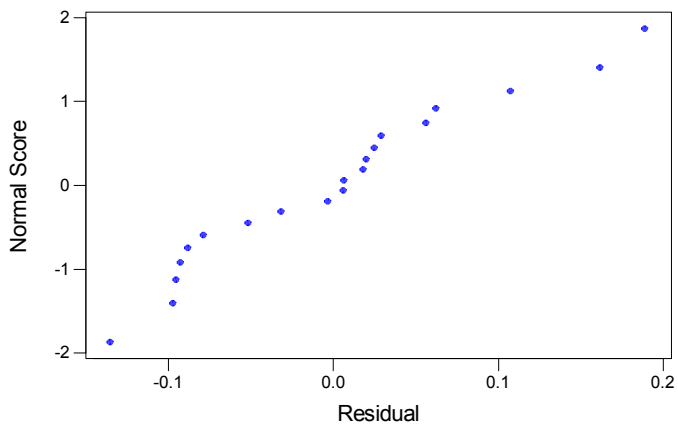
Residuals Versus Day
(response is 17-B est)

- Power analysis for t-test of slope less than zero

**Power and Sample Size**

```
1-Sample t Test

Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05   Sigma = 0.0900613

Sample
  Size    Power   Difference
    18   0.9900     -0.0880
```

- That means we would detect a mean of 263 as significantly less than 287 = 8.4% loss.
- Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: 17-B estradi versus Day

Regression Plot
17-B estradi = 5.61507 + 0.0021159 Day

S = 0.0900613   R-Sq = 3.6 %   R-Sq(adj) = 0.0 %
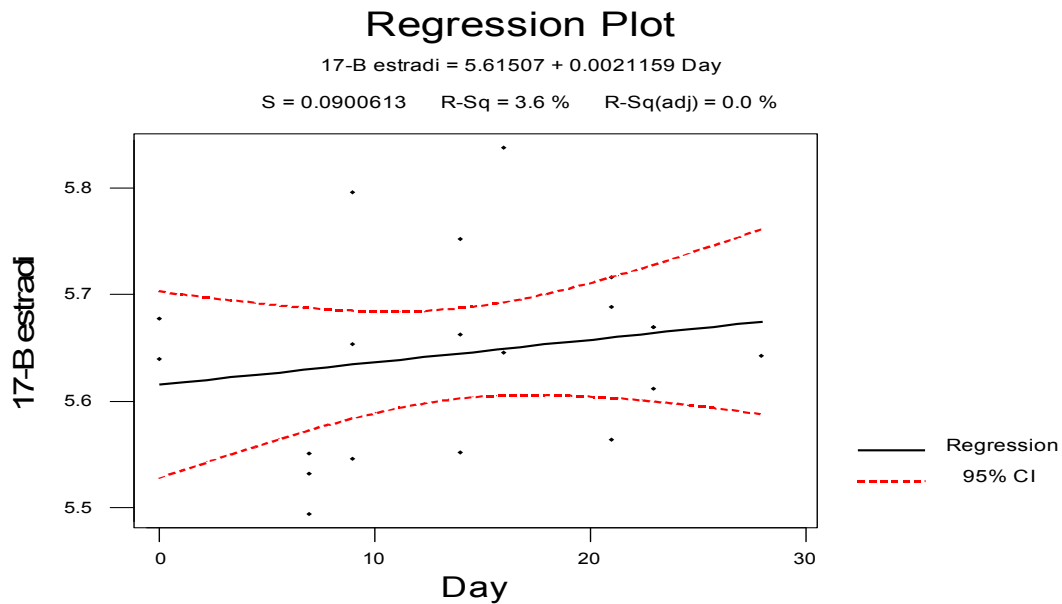
Conclusion: Stable for 19 days.

─────── **9/17/2003 1:02 PM** ───────────────────

Results for Analysis-1: Trough removing only rep 1 day zero as an outlier. (Note, all other Q flagged data are used in this analysis.**)**

- Test to determine if the data are from a population with mean of 287 ng/g (the mean of reps 2-3 of day 0)

```
Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X    3
DATA> 287
```
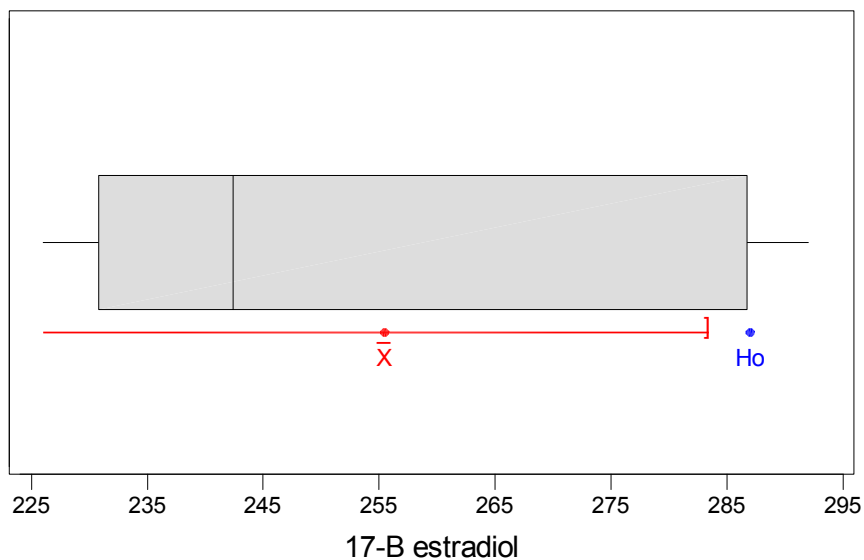
One-Sample T: 17-B estradiol

```
Test of mu = 287 vs mu < 287

Variable          N      Mean     StDev   SE Mean
17-B estradi      5     255.5      29.3      13.1

Variable     95.0% Upper Bound         T      P
17-B estradi               283.4    -2.40  0.037
* NOTE * N missing = 1
```

t Boxplot of 17-B estradiol

Boxplot of 17-B estradiol

(with Ho and 95% t-confidence bound for the mean)


```
Outliers are < Median-3*IQD OR > Median+3*IQD

Boundary for outliers are values <    74.7202  and >    410.034

No outliers
```

- Transform data to natural logarithm and conduct regression analysis.

```
    Day     Rep Ln(Concentration)

     0       1           *
     0       2        5.6769
     0       3        5.6396
     7       1        5.4905
     7       2        5.4206
     7       3        5.4621
```

```
Conducts Simple Linear Regression
```

Regression Analysis: 17-B estradiol versus Day

```
The regression equation is
17-B estradiol = 5.66 - 0.0286 Day

5 cases used 1 cases contain missing values

Predictor        Coef     SE Coef          T        P
Constant      5.65824     0.02297     246.32    0.000
Day         -0.028642    0.004236      -6.76    0.007


S = 0.03249     R-Sq = 93.8%     R-Sq(adj) = 91.8%

Analysis of Variance

Source            DF          SS          MS        F        P
Regression         1    0.048238    0.048238    45.71    0.007
Residual Error     3    0.003166    0.001055
Total              4    0.051404

The number of distinct predictor combinations equals the
number of parameters. No degrees of freedom for lack of fit.

Cannot do pure error test.
```
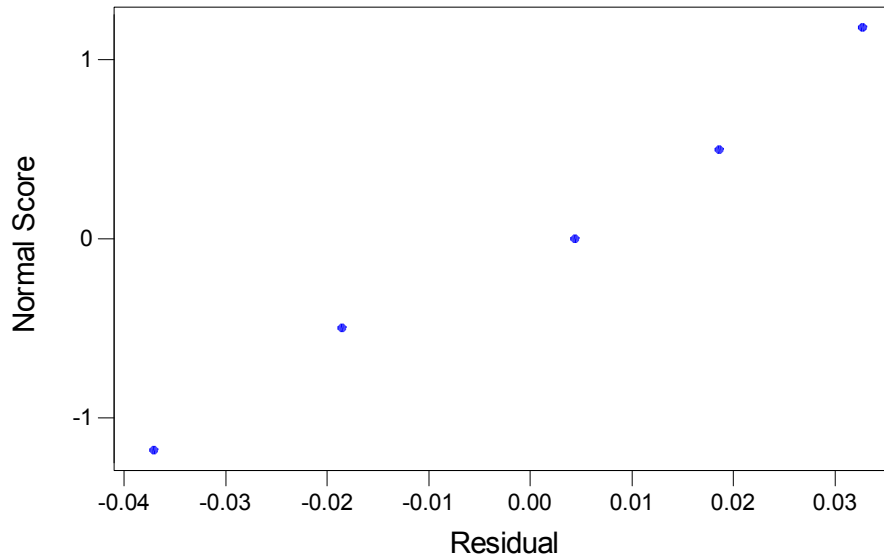
Normplot of Residuals for 17-B est

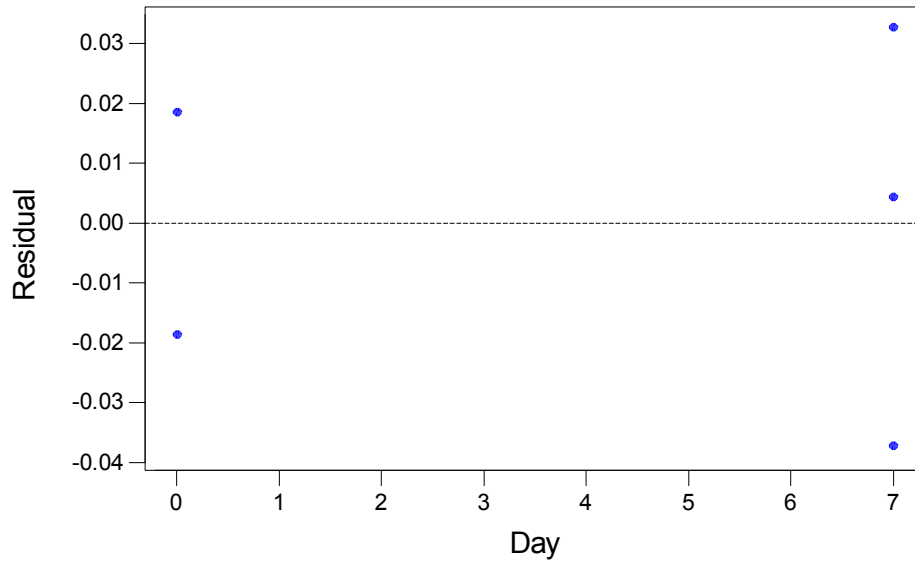## Normal Probability Plot of the Residuals

(response is 17-B est)

Residuals from 17-B est vs Day

Residuals Versus Day

(response is 17-B est)

- Power analysis for t-test of slope less than zero

Power and Sample Size

```
1-Sample t Test

Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05  Sigma = 0.0324856

Sample
  Size   Power   Difference
     3  0.9900     -0.1291
```
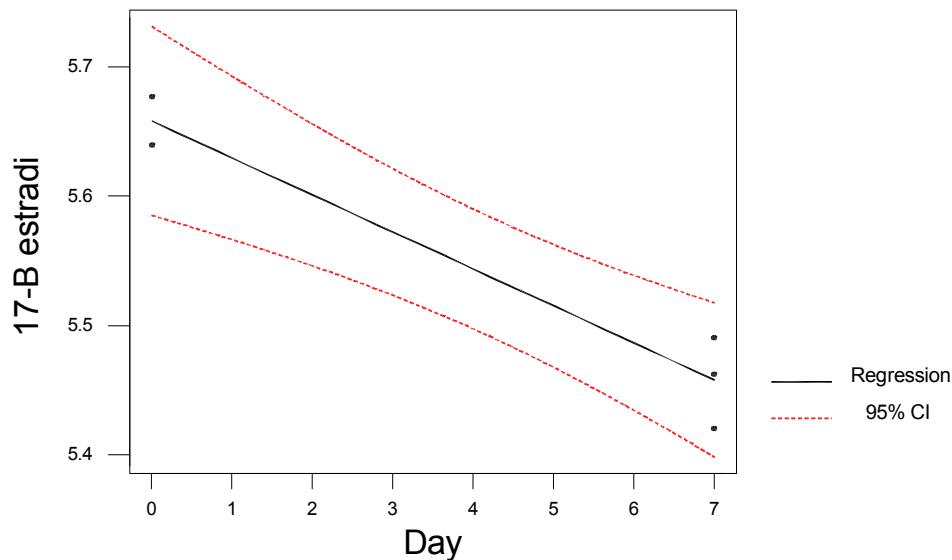
- That means we would detect a mean of 252 as significantly less than 287 = 12% loss.

- Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: 17-B estradi versus Day

Regression Plot

17-B estradi = 5.65824 - 0.0286421 Day

S = 0.0324856   R-Sq = 93.8 %   R-Sq(adj) = 91.8 %

- **Conclusion: stable for 2 days using a 10% loss criteria and 5 days using a 20% loss criteria.**

──────── **9/26/2003 10:50 AM** ───────────────────────

Results for Analysis-2: Trough removing all Q-flagged data.

- Test to determine if the data are from a population with mean of 287 ng/g (the mean of reps 2-3 of day 0)

```
Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X    3
DATA> 287
```

One-Sample T: 17-B estradiol

```
Test of mu = 287 vs mu < 287

Variable          N       Mean     StDev   SE Mean
17-B estradi      4      260.4      31.3      15.7

Variable      95.0% Upper Bound        T      P
17-B estradi              297.3     -1.69  0.094      NS
* NOTE * N missing = 2
```
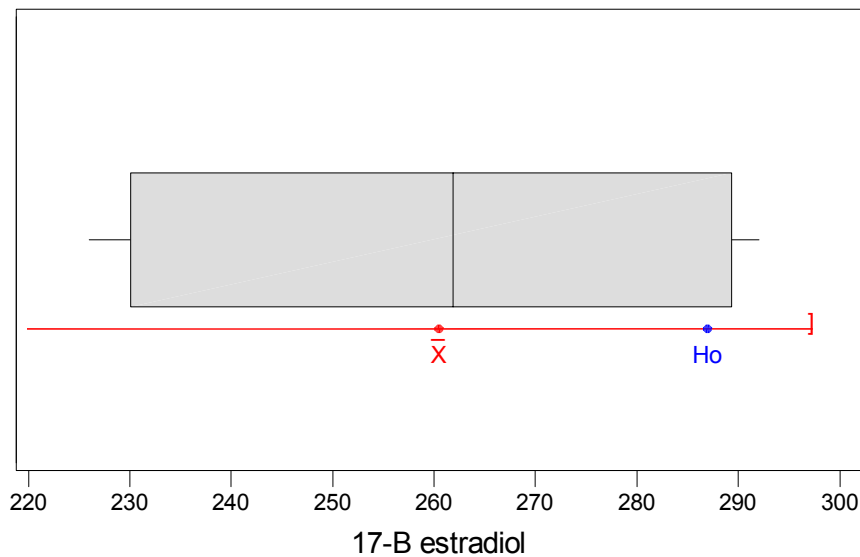
t Boxplot of 17-B estradiol

Boxplot of 17-B estradiol

(with Ho and 95% t-confidence bound for the mean)


```
Outliers are < Median-3*IQD OR > Median+3*IQD

Boundary for outliers are values <    84.0928  and >    439.639

No outliers
```

- Transform data to natural logarithm and conduct regression analysis.

```
    Day     Rep Ln(Concentration)

     0       1            *
     0       2        5.6769
     0       3        5.6396
     7       1        5.4905
     7       2        5.4206
     7       3            *

Conducts Simple Linear Regression
```

Regression Analysis: 17-B estradiol versus Day

```
The regression equation is
17-B estradiol = 5.66 - 0.0290 Day

4 cases used 2 cases contain missing values

Predictor        Coef      SE Coef          T        P
Constant      5.65824      0.02800     202.05    0.000
Day          -0.028956     0.005658      -5.12    0.036


S = 0.03960     R-Sq = 92.9%     R-Sq(adj) = 89.4%

Analysis of Variance

Source            DF          SS          MS          F         P
Regression         1    0.041085    0.041085      26.19    0.036
Residual Error     2    0.003137    0.001568
Total              3    0.044222

The number of distinct predictor combinations equals the
number of parameters. No degrees of freedom for lack of fit.

Cannot do pure error test.
```
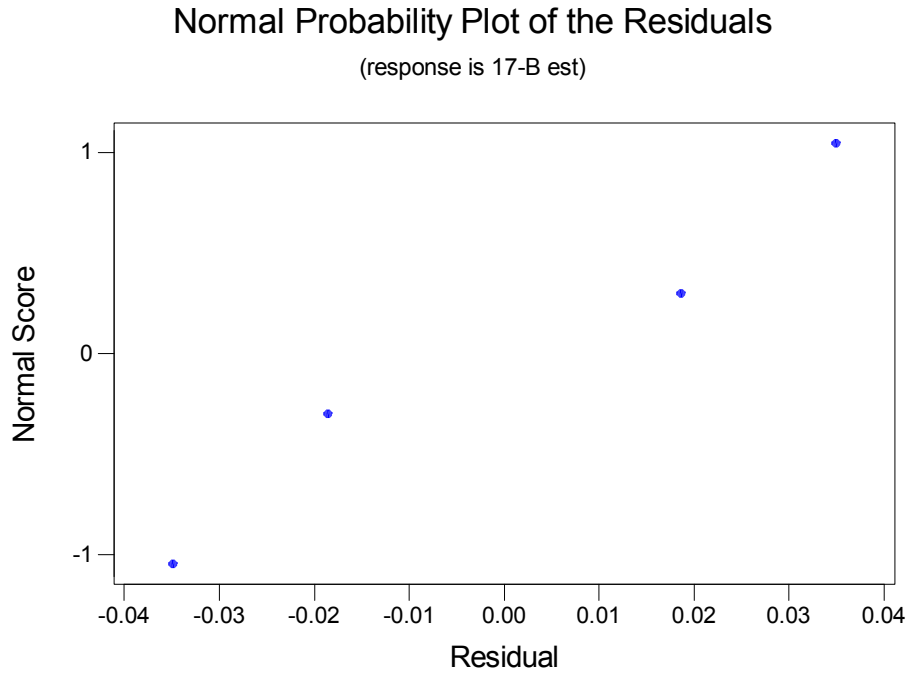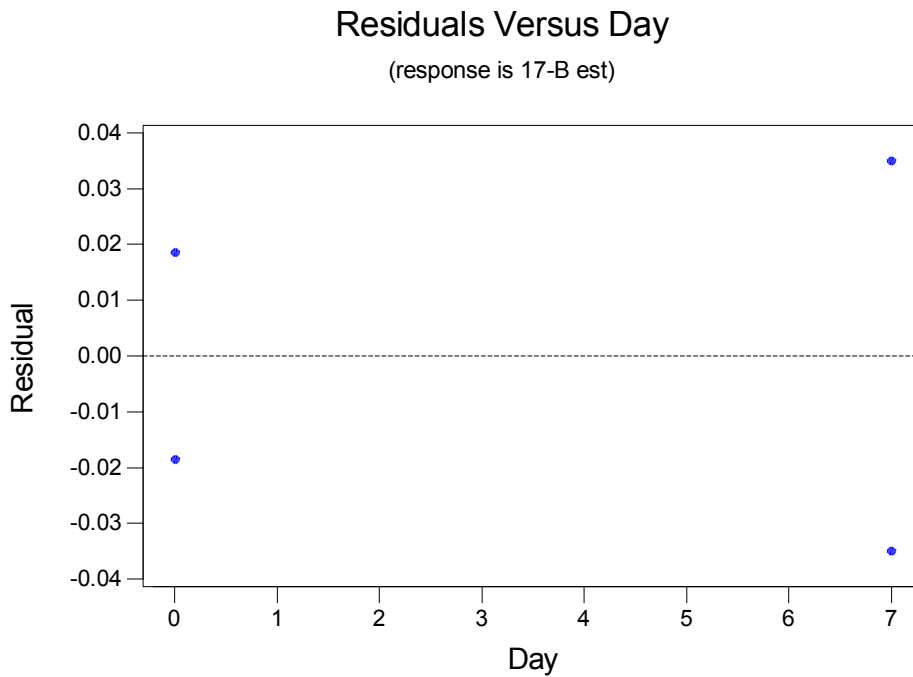
Normplot of Residuals for 17-B est

Normal Probability Plot of the Residuals

(response is 17-B est)

Residuals from 17-B est vs Day

Residuals Versus Day

(response is 17-B est)

- Power analysis for t-test of slope less than zero

**Power and Sample Size**

```
1-Sample t Test

Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05  Sigma = 0.0396037

Sample
  Size   Power  Difference
     2  0.9900     -0.4611
```

- That means we would detect a mean of 181 as significantly less than 287 = 37% loss.

- Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: 17-B estradi versus Day

Regression Plot

17-B estradi = 5.65824 - 0.0289564 Day

S = 0.0396037   R-Sq = 92.9 %   R-Sq(adj) = 89.4 %
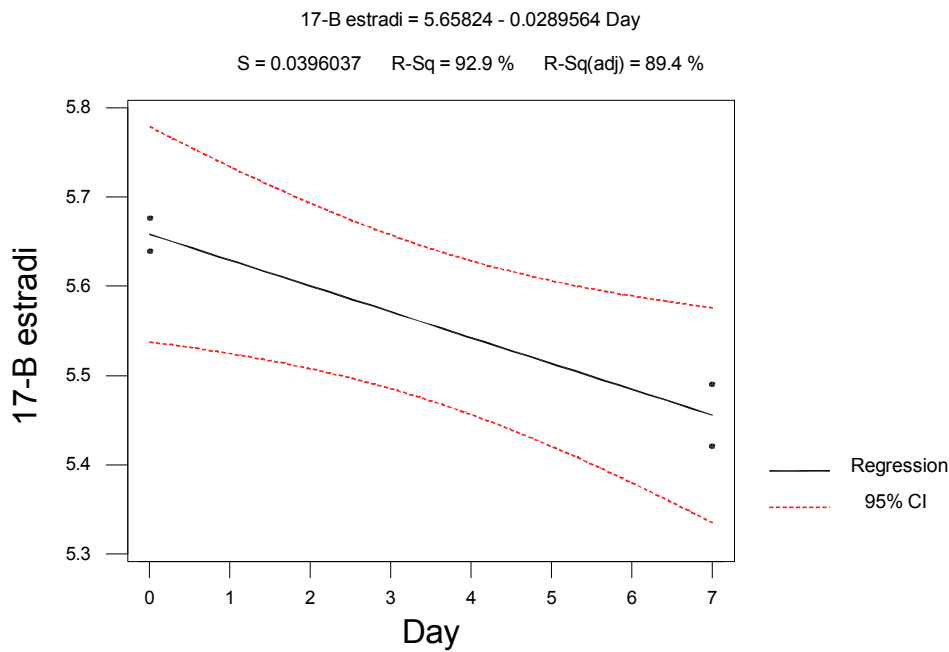
- **Conclusion: stable for 2 days using a 10% loss criteria and 4 days using a 20% loss criteria.**

──────── 9/17/2003 11:41:46 AM ────────────────────

Results for: Laboratory Bucket removing only rep 1 day zero as an outlier. (Note: this was the only Q flagged data point in this data set so Analysis-1 and Analysis-2 are the same.)

- Test to determine if the data are from a population with mean of 287 ng/g (the mean of reps 2-3 of day 0)

```
Macro performs a one-sample t-test for mu less than TARGET &
What is the target value for X    3
DATA> 287
```

One-Sample T: 17-B estradiol

```
Test of mu = 287 vs mu < 287

Variable          N       Mean      StDev   SE Mean
17-B estradi      5     274.56      12.80      5.72

Variable     95.0% Upper Bound        T       P
17-B estradi            286.77     -2.17   0.048
* NOTE * N missing = 1
```
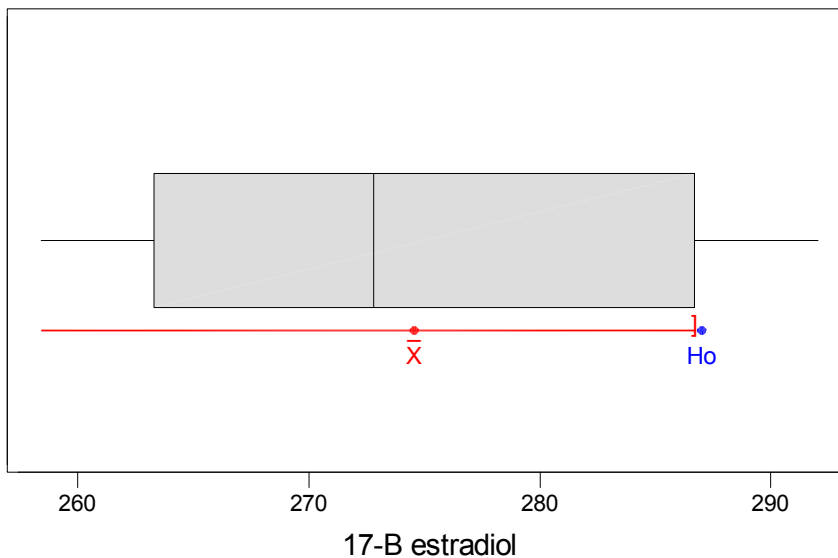
t Boxplot of 17-B estradiol

Boxplot of 17-B estradiol
(with Ho and 95% t-confidence bound for the mean)

17-B estradiol

```
Outliers are < Median-3*IQD OR > Median+3*IQD

Boundary for outliers are values <    202.670  and >    342.956

No outliers
```

- Transform data to natural logarithm and conduct regression analysis.

```
    Day    Rep Ln(Concentration)
```

```
       0        1              *
       0        2         5.6769
       0        3         5.6396
       7        1         5.6088
       7        2         5.5546
       7        3         5.5917
```

```
Conducts Simple Linear Regression
```

Regression Analysis: 17-B estradiol versus Day

```
The regression equation is
17-B estradiol = 5.66 - 0.0105 Day

5 cases used 1 cases contain missing values

Predictor        Coef      SE Coef          T          P
Constant       5.65824      0.01927     293.57      0.000
Day          -0.010457     0.003555      -2.94      0.060        NS


S = 0.02726     R-Sq = 74.3%     R-Sq(adj) = 65.7%

Analysis of Variance

Source            DF          SS          MS          F          P
Regression         1   0.0064294   0.0064294       8.65      0.060
Residual Error     3   0.0022289   0.0007430
Total              4   0.0086583


The number of distinct predictor combinations equals the
number of parameters. No degrees of freedom for lack of fit.

Cannot do pure error test.
```
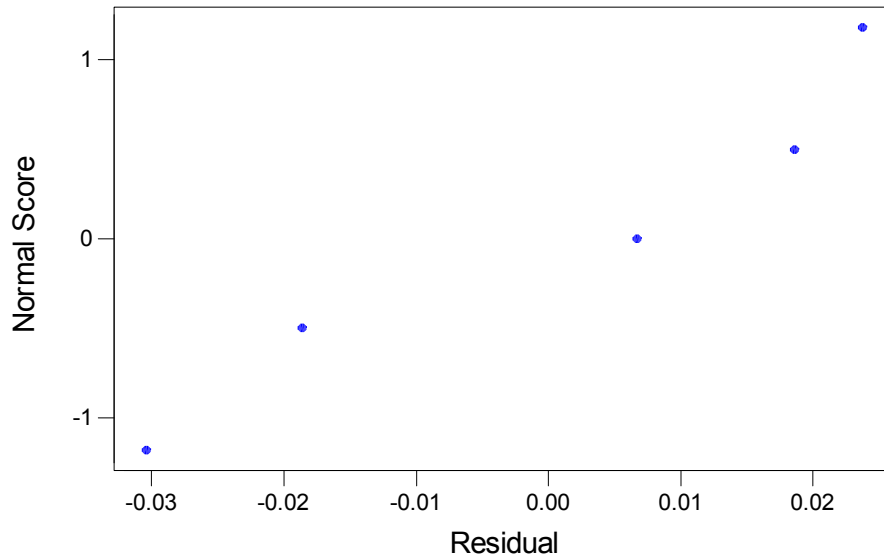
Normplot of Residuals for 17-B est
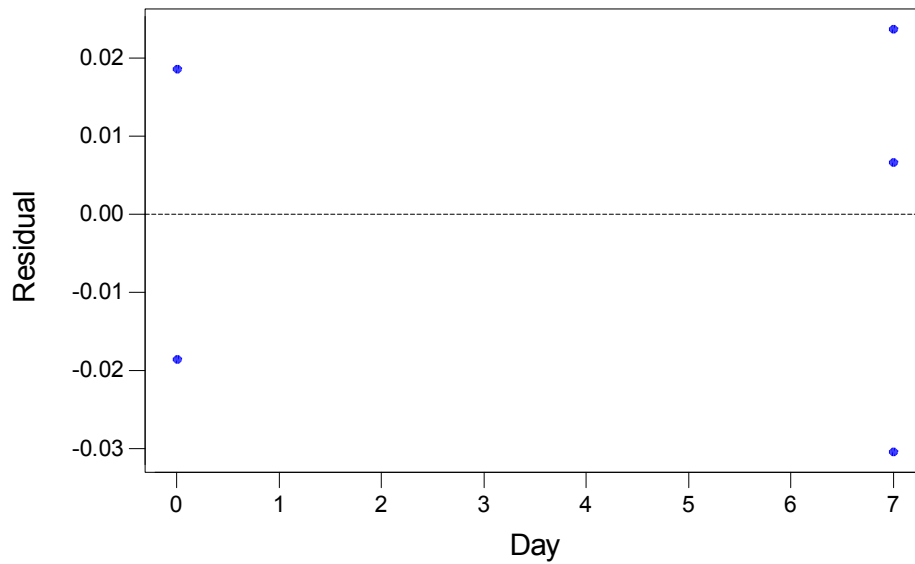
Normal Probability Plot of the Residuals

(response is 17-B est)

Residuals from 17-B est vs Day

Residuals Versus Day

(response is 17-B est)

- Power analysis for t-test of slope less than zero

Power and Sample Size

```
1-Sample t Test

Testing mean = null (versus < null)
Calculating power for mean = null + difference
Alpha = 0.05  Sigma = 0.0272574

Sample
  Size   Power  Difference
     3  0.9900     -0.1083
```
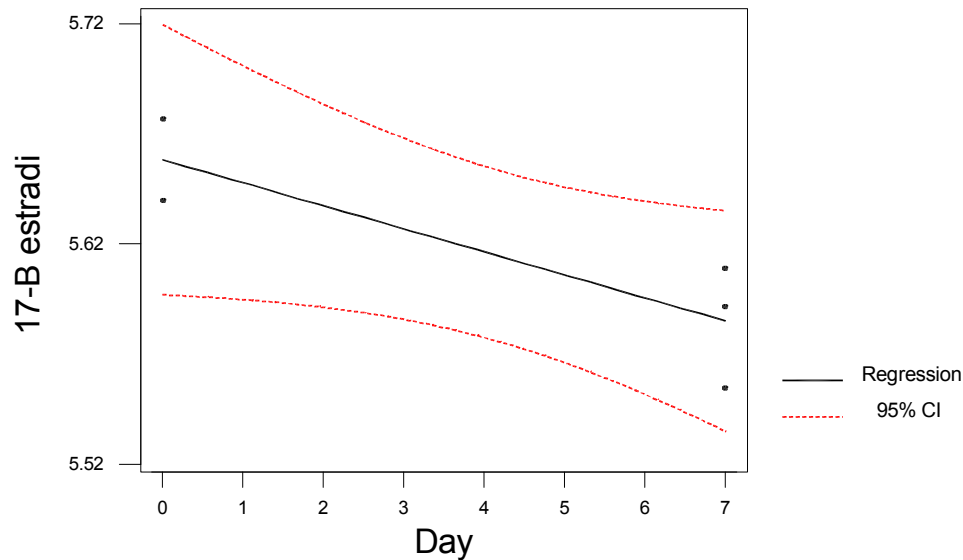
- That means we would detect a mean of 258 as significantly less than 287 = 10% loss.

- Fit 95% confidence bands about the fitted simple linear model

Fitted Line Plot: 17-B estradi versus Day

Regression Plot

17-B estradi = 5.65824 - 0.0104567 Day

S = 0.0272574   R-Sq = 74.3 %   R-Sq(adj) = 65.7 %

- **Conclusion: stable for 5 days.**