

Data Evaluation Report on the Acute Toxicity Effects of Trifluralin Metabolite TR-4 on Earthworms

PMRA Submission #: {.....}

EPA MRID #: 47807010

Data Requirement:	PMRA Data Code	{.....}
	EPA DP Barcode	367525
	OECD Data Point	{.....}
	EPA MRID	{.....}
	EPA Guideline	850.6200; OECD 207

Test material: Trifluralin Metabolite TR-4 Purity: 100% w/w

Common name

Chemical name: IUPAC Not reported
CAS name N2,N2-D1-(N-propyl)-3-nitro-5-trifluoromethyl-O-phenylenediamine
CAS No. Not reported
Synonyms Not reported

Primary Reviewer: Moncie Wright
Staff Scientist, Cambridge Environmental

Signature:

Date: 11/4/09

Secondary Reviewer: Teri S. Myers
Senior Scientist, Cambridge Environmental

Signature:

Date: 12/07/09

Primary Reviewer: Christine Hartless
{EPA/OPP/EFED/ ERB 1}

Date: 4/30/10

Secondary Reviewer(s): {.....}
{EPA/OECD/PMRA}

Date: {.....}

Reference/Submission No. {.....}

Company Code {.....} [For PMRA]
Active Code {.....} [For PMRA]
EPA PC Code 036101

Date Evaluation Completed: 4/30/10

CITATION: Hayward, J.C. 2003. Trifluralin Metabolite TR-4: 14 Day Soil Exposure Acute Toxicity Test in the Earthworm, *Eisenia foetida*. Unpublished study performed by CEM Analytical Services Limited (CEMAS), Berkshire, United Kingdom. Laboratory study no.: CEMS-2082. Study sponsored by The Dow Chemical Company, Midland, Michigan, for Dow AgroSciences LLC, Indianapolis, Indiana. Sponsor project no.: 031065. Study completed October 9, 2003.



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I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: The study was conducted following OECD Guideline 207 for Testing of Chemicals, "Earthworm, acute toxicity tests" (1984). The test was only conducted for 14 days, but the reviewer used the guidance for a 28-day sub-chronic test to note deviations in acclimation and environmental conditions. The following deviations from OPPTS Guideline 850.6200 were noted:

1. Only a solvent control was tested with the treatment groups in this study, which is in accordance with OECD guidelines. However, OPPTS guidelines suggest that a negative control be tested along with a solvent control to determine whether the solvent might have had an undue effect on the test results. In the solvent control group, % weight loss was 25%, and the reviewer could not determine if any subsequent weight losses in the treatment groups were caused by the solvent, test material, or a combination of the two.
2. The % organic carbon content of the soil was not reported.
3. Only the pH of the soil at test initiation and test termination was reported; OPPTS guidelines suggest that daily pH values be reported.
4. The amount of soil used for each replicate was not officially reported in the study, but was referenced in the Study Plan.
5. The light intensity ranged from 592 to 745 lux; OPPTS guidelines suggest a light intensity of 400 lux.
6. The concentrations of the test mixtures were not analyzed; OPPTS guidelines suggest that test concentrations be measured daily.
7. The concentration of the solvent used for testing was not reported.
8. The pretest health of the earthworms was not reported.
9. The study author did not report quarantining and observing the earthworms; OPPTS guidelines suggest this should occur for at least 14 days prior to testing.

These deviations do impact the acceptability of the study.

COMPLIANCE:

Signed and dated No Data Confidentiality, Quality Assurance and GLP statements were provided. This study was conducted in compliance with U.S. EPA Title 40 Code of Federal Regulations Part 160 (1989), and with OECD principles (EN/MC/CHEM(98)17; 1998).

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A. MATERIALS:

1. Test Material Trifluralin Metabolite TR-4

Description: Liquid

Lot No./Batch No. : TSN104126 (lot no.)

Purity: 100% w/w

Stability of compound under test conditions: Analytical verification was not performed.
(OECD recommends water solubility, stability in water and light, pKa, Pow, vapor pressure of test compound)

Storage conditions of test chemicals: The test material was stored frozen.

Physicochemical properties of Trifluralin Metabolite TR-4.

Parameter	Values	Comments
Water solubility at 20EC	Not reported	
Vapor pressure	Not reported	
UV absorption	Not reported	
pKa	Not reported	
Kow	Not reported	

2. Test organism:

Species: Earthworm (*Eisenia foetida* Michaelson)
(EPA and OECD recommend *Eisenia fetida andrei* (Bouche). The earthworms should weigh 300-600 mg at the beginning of the test.)

Age at test initiation: 6 to 7 months

Weight at study initiation: 430-471 mg (based on group mean values at study initiation)

Source: Cultures from CEMAS (performing laboratory); culture no. EF/01/04/03

B. STUDY DESIGN:

1. Experimental Conditions

a. Range-finding Study: A range-finding study was conducted with a control and nominal test concentrations of 10, 30, 100, 300, and 1000 mg ai/kg dw soil for 14 days. Mortality was 0% in the negative control and the 10, 30, and 100 mg ai/kg dw soil treatments. In the 300 and 1000 mg ai/kg dw soil treatments, mortality was 100%. The % weight loss was 13.6% in the negative control, and was 11.6, 11.5, and 23.1% in the 10, 30, and 100 mg ai/kg dw soil treatments. Complete mortality in the two highest treatment levels precluded the ability to measure body weight change.

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b. Definitive Study

1. Soil

Table 1: Physicochemical Properties of Natural Soil

Property	Value	Remarks
		Criteria
For natural soil: Texture: % sand % silt % clay Textural classification For artificial substrate (provide composition):	Artificial soil used in the definitive tests was comprised of 10% sphagnum peat (finely ground, sieved to 2 mm and dried to known moisture content), 20% Kaolin clay, and 70% industrial fine quartz sand (>50% of particles between 50 and 200 µm).	<i>Recommended testing medium is artificial soil consisting of a mixture of 68% of No. 70 mesh silica sand, 20% kaolin clay, 10 sphagnum peat moss, and 2% calcium carbonate, mixed and moistened to 35% by weight with deionized/distilled water.</i>
pH (___ : ___ soil:water)	Calcium carbonate was added to the soil mixture to obtain a pH range of 5.5 to 6.5.	
Organic carbon (%)	The % organic carbon was not reported.	
Moisture (%)	The initial soil moisture content was 35% w/w.	

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Table 2: Experimental Design

Parameter	Detail	Remarks
		<i>Criteria</i>
Acclimation: duration: conditions (state if same as the test conditions): health:	2 days Same as test Not reported	<i>Earthworms should be acclimated at test temperature for 7 days.</i>
Soil [fresh or stored]	Freshly prepared	
Test Container material size amount of soil/substrate	Glass 1000 mL Not reported in official report; 750 g wet weight according to Study Plan	
No. of replicates: per treatment group: per control:	4 4	<i>Recommended number of replicates include at least 3 and a control.</i>
No. of earthworms per treatment	40 earthworms per treatment and the control	<i>Recommended number of earthworms per treatment include a minimum of 30 plus a control; 10 per each of three replicates and a control.</i>
Solvents used or not (if yes report the name and concentration)	Acetone; concentration not reported	
Rates of application: nominal: measured:	0 (solvent control), 32, 56, 100, 180, and 320 mg ai/kg dw soil N/A	<i>Earthworms should be exposed to at least five test concentrations, in geometric series, in which the ratio is between 1.5 and 2.0 mg of test chemical per kg (air-dry weight) of artificial soil.</i>
Reference chemical (if used) name: concentration:	2-chloroacetamide Not reported	
Test conditions:		

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Parameter	Detail	Remarks
		<i>Criteria</i>
temperature Lighting conditions Moisture	initiation. After 14 days, moisture content was 31.3% of the soil dry weight.	<i>Recommended temperature: 22 + 2°C Recommended lighting: Continuous illumination, with a light intensity of 400 lux Recommended relative humidity: above 85%</i>
Duration of the study	14 days	<i>Recommended duration of study is 28 days.</i>

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2. Observations:

Table 3: Observations

Parameters	Details	Remarks
		<i>Criteria</i>
Observation intervals	Mortality, weights of the worms as replicate and group mean weights of worms, and behavioral abnormalities and pathological signs were assessed on days 7 and 14. (weight only on day 14)	<i>Recommended observation intervals are days 7, 14, 21, and 28.</i>
Parameters measured including the sublethal effects/toxicity symptoms	-Mortality -Weight of surviving earthworms (weight at days 0, and 14) -Sub-Lethal Effects (behavior and pathological signs)	<i>The test is usually not acceptable if more than 20% of control earthworms die or the total mean weight of control earthworms lose 20% or more of body weight.</i>
Were raw data included?	Yes	
Other observations, if any	None	

II. RESULTS AND DISCUSSIONS

A. MORTALITY:

After 14 days of exposure, mortality was 0% in the solvent control and 32, 56, and 100 mg ai/kg dw soil treatment groups. Mortality was 45 and 100% in the 180 and 320 mg ai/kg dw soil treatment levels, respectively. The study author reported 14-day NOAEC and LC₅₀ values of 100 and 186 mg ai/kg dw soil, respectively.

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Table 4: Effect of Trifluralin Metabolite TR-4 on Mortality of *Eisenia foetida*

Nominal Concentrations (mg ai/kg soil)	Observation period			
	Day 7		Day 14	
	No Dead	% mortality	No Dead	% mortality
Solvent Control	0	0	0	0
32	0	0	0	0
56	0	0	0	0
100	0	0	0	0
180	0	0	18	45
320	39	98	40	100
NOAEC	100 mg ai/kg dw soil			
LOAEC	180 mg ai/kg dw soil			
LC ₅₀ (95% confidence limits)	242 (238-245) mg ai/kg dw soil		186 (166-209) mg ai/kg dw soil	
Reference chemical % mortality: LC ₅₀ (95% confidence limits)	47.9 (43.8-51.9) mg/kg dw soil			

B. SUB-LETHAL TOXICITY ENDPOINTS:

Mean % weight loss was 25.2% in the negative control, and was 23.9, 24.4, 28.5, and 50.3% in the nominal 32, 56, 100, and 180 mg ai/kg dw soil treatment groups, respectively. Complete mortality in the highest treatment level precluded the ability to measure weight change. The 14-day NOAEC value was 100 mg ai/kg dw soil.

On day 14, no sublethal effects were observed in the control, 32, 56, or 100 mg ai/kg dw soil treatment levels. In the 180 mg ai/kg dw soil treatment, 86% of the surviving worms had yellow secretion, 64% were emaciated, and 32% were truncated.

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Table 5: Sub-lethal Effect of Trifluralin Metabolite TR-4 on *Eisenia foetida*. Average weights were used

Nominal Concentrations (mg ai/kg soil)	Observation period			
	Day 0		Day 14	
	weight	% gain	weight	% gain
Solvent Control	456	N/A	341	-25.2
32	440	N/A	334	-23.9
56	471	N/A	356	-24.4
100	430	N/A	308	-28.5
180	450	N/A	224	-50.3
320	458	N/A	--	--
NOAEC	100 mg ai/kg dw soil			
LOAEC	180 mg ai/kg dw soil			
EC ₅₀ (95% confidence limits)	NA			
Reference chemical % mortality: LC ₅₀ (95% confidence limits)	47.9 (43.8-51.9) mg/kg dw soil			

-- complete mortality occurred

C. REPORTED STATISTICS:

The percent biomass change and mortality data were tested for normality using the Chi-squared and Shapiro-Wilks tests and for homogeneity of variance using the Hartley and Bartlett's tests in TOXSTAT 3.4. The biomass change NOAEC and LOAEC were determined using Dunnett's two-tailed test (p=0.05). The mortality NOAEC and LOAEC were determined using Steel's Many-One Rank test (one-tailed, p=0.05). The 7 and 14-day LC₅₀ values were determined using the Trimmed Spearman-Kärber test (trim = 10%) in ToxCalc 5.0.

D. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method: The reviewer tested the percent weight loss gain for normality using the Chi-square and Shapiro-Wilks tests and for homogeneity of variance using the Hartley and Bartlett's tests via Toxstat statistical software. The data met the assumptions of ANOVA, thus the NOAEC value was determined using the parametric Dunnett's and Williams tests via Toxstat statistical software. The IC₅₀ was visually determined as no test concentration resulted in weight losses greater than 50% of the weight loss in the control.

Mortality data were analyzed using Toxanal2009. The confidence limits could not be calculated with the binomial method, while neither the moving average nor probit method could give statistically sound results due to the presence of less than two concentrations at which the percent mortality was between 0 and 100. The reviewer used the LC₅₀ value determined based on the binomial method and visually determined the confidence interval. Further,

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because only two test levels were affected, the reviewer was able to visually determine the NOAEC value. The study author did not include a negative control in this study; therefore, the reviewer conducted all analyses using the solvent control.

Mortality*

LC₅₀: 186 mg ai/kg dw soil 95% C.I.: (100, 320) mg ai/kg dw soil

NOAEC: 100 mg ai/kg dw soil

LOAEC: 180 mg ai/kg dw soil

*LC₅₀ determined via binomial method, CI determined visually

Percent weight gain

IC₅₀: >320 mg ai/kg-dw soil 95% C.I.: N/A

NOAEC: 100 mg ai/kg dw soil

LOAEC: 180 mg ai/kg dw soil

E. STUDY DEFICIENCIES:

Only a solvent control was tested with the treatment groups in this study, which is in accordance with OECD guidelines. However, OPPTS guidelines suggest that a negative control be tested along with a solvent control to determine whether the solvent might have had an undue effect on the test results. In the solvent control group, % weight loss was 25%, and the reviewer could not determine if any subsequent weight losses in the treatment groups were caused by the solvent, the test material, or a combination of the two.

F. REVIEWER'S COMMENTS:

The reviewer's and study author's results were generally in agreement. The study author's results for the LC₅₀ value and the reviewer's results for the EC₅₀ are reported in the Executive Summary and Conclusions sections of this DER.

No OPPTS guidance exists for a 14-day acute earthworm toxicity test, thereby making this a non-guideline test. Therefore, the reviewer used the guidance for a 28-day sub-chronic test to note deviations in acclimation and environmental conditions.

The % organic carbon content of the soil was not reported.

Only the pH of the soil at test initiation and test termination was reported; OPPTS guidelines suggest that daily pH values be reported.

The amount of soil used for each replicate was not officially reported in the study, but was referenced in the Study Plan.

The light intensity ranged from 592 to 745 lux; OPPTS guidelines suggest a light intensity of 400 lux.

The concentrations of the test mixtures were not analyzed; OPPTS guidelines suggest that test concentrations be measured daily.

The concentration of the solvent used for testing was not reported.

The pretest health of the earthworms was not reported.

The study author did not report quarantining and observing the earthworms; OPPTS guidelines suggest this should

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occur for at least 14 days prior to testing.

The complete experimental period was started July 17 and ended September 17, 2003.

G. CONCLUSIONS:

This study is scientifically sound and classified as a Supplemental non-guideline study (although it follows OECD 207, EPA does not have a guideline for a 14-day earthworm test).

Percent survival was affected, yielding NOAEC and LC₅₀ values of 100 and 186 mg ai/kg dw soil, respectively. Body weight change was only affected at the 180 mg ai/kg dw soil test level, yielding NOAEC and IC₅₀ values of 100 and >180 mg ai/kg dw soil, respectively.

Mortality*

LC₅₀: 186 mg ai/kg dw soil 95% C.I.: (100, 320) mg ai/kg dw soil

NOAEC: 100 mg ai/kg dw soil

LOAEC: 180 mg ai/kg dw soil

*LC₅₀ determined via binomial method, CI determined visually

Percent weight gain

IC₅₀: >180 mg ai/kg dw soil 95% C.I.: N/A

NOAEC: 100 mg ai/kg dw soil

LOAEC: 180 mg ai/kg dw soil

Endpoint(s) Affected: Mortality, % weight loss, and sublethal effects such as yellow secretion, emaciation, and truncation

III. REFERENCES:

OECD (1984) Earthworm, Acute Toxicity Test. Test Guideline Number 207. OECD Guidelines for Testing of Chemicals. Adopted 04 April 1984.

ISO 10390 (1994) Soil quality – Determination of pH.

ISO 11268-1 (1993) Soil quality – Effects of pollutants on earthworms (*Eisenia fetida*). Part 1: Determination of acute toxicity using artificial soil substrate.

ISO 11465 (1993) Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method.

ToxCalc (1999) Windows version 5.0.23. M A Ives and Tidepool Scientific Software (1994-1999). A comprehensive toxicity data analysis and database software package for Microsoft Excel under Microsoft Windows 3.1, Microsoft Windows 95 or Apple Macintosh.

West Inc and Gulley D (1994) TOXSTAT Version 3.4, Western Ecosystems Technology, Inc. 1402 South Greeley Highway, Cheyenne, WY, 82007-3031.

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APPENDIX I. OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

Moncie Wright TR-4 LC50

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*****
CONC.    NUMBER    NUMBER    PERCENT    BINOMIAL
         EXPOSED    DEAD      DEAD      PROB. (PERCENT)
320      40          40        100       0
180      40          18        45        0
100      40          0         0         0
56       40          0         0         0
32       40          0         0         0
*****
```

BECAUSE THE NUMBER OF ORGANISMS USED WAS SO LARGE, THE 95 PERCENT CONFIDENCE INTERVALS CALCULATED FROM THE BINOMIAL PROBABILITY ARE UNRELIABLE. USE THE INTERVALS CALCULATED BY THE OTHER TESTS.

AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 186.8225

WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE NOR THE PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.

TR-4 & earthworm 14-day % body weight gain; mg ai/kg
File: 7010w Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

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INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	1.340	4.840	7.640	4.840	1.340
OBSERVED	0	6	8	6	0

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Calculated Chi-Square goodness of fit test statistic = 3.2530
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.

TR-4 & earthworm 14-day % body weight gain; mg ai/kg
File: 7010w Transform: NO TRANSFORMATION

Shapiro Wilks test for normality

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D = 93.735

W = 0.970

Critical W (P = 0.05) (n = 20) = 0.905
Critical W (P = 0.01) (n = 20) = 0.868

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Data PASS normality test at P=0.01 level. Continue analysis.

TR-4 & earthworm 14-day % body weight gain; mg ai/kg
File: 7010w Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance

Calculated H statistic (max Var/min Var) = 9.79
Closest, conservative, Table H statistic = 151.0 (alpha = 0.01)

Used for Table H ==> R (# groups) = 5, df (# reps-1) = 3
Actual values ==> R (# groups) = 5, df (# avg reps-1) = 3.00

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal but do not differ greatly, the Hartley test may still be used as an approximate test (average df are used).

TR-4 & earthworm 14-day % body weight gain; mg ai/kg
File: 7010w Transform: NO TRANSFORMATION

Bartlett's test for homogeneity of variance

Calculated B statistic = 3.86
Table Chi-square value = 13.28 (alpha = 0.01)
Table Chi-square value = 9.49 (alpha = 0.05)

Average df used in calculation ==> df (avg n - 1) = 3.00
Used for Chi-square table value ==> df (#groups-1) = 4

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is used to calculate the B statistic (see above).

Title: TR-4 & earthworm 14-day % body weight gain; mg ai/kg
File: TR4-WORM.TXT Transform: NO TRANSFORMATION

ANOVA Table

SOURCE	DF	SS	MS	F
Between	4	2003.5730	500.8933	80.1557
Within (Error)	15	93.7350	6.2490	
Total	19	2097.3080		

(p-value = 0.0000)

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Critical F = 4.8932 (alpha = 0.01, df = 4,15)
 = 3.0556 (alpha = 0.05, df = 4,15)

Since F > Critical F REJECT Ho: All equal (alpha = 0.05)

Title: TR-4 & earthworm 14-day % body weight gain; mg ai/kg
 File: TR4-WORM.TXT Transform: NO TRANSFORMATION

Dunnett's Test - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	Solv control	-25.2500	-25.2500		
2	32	-23.9000	-23.9000	-0.7637	
3	56	-24.4500	-24.4500	-0.4526	
4	100	-28.4750	-28.4750	1.8245	
5	180	-50.2250	-50.2250	14.1291	*

Dunnett critical value = 2.3600 (1 Tailed, alpha = 0.05, df = 4,15)

Title: TR-4 & earthworm 14-day % body weight gain; mg ai/kg
 File: TR4-WORM.TXT Transform: NO TRANSFORMATION

Dunnett's Test - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	MIN SIG DIFF (IN ORIG. UNITS)	% OF CONTROL	DIFFERENCE FROM CONTROL
1	Solv control	4			
2	32	4	4.1716	-16.5	-1.3500
3	56	4	4.1716	-16.5	-0.8000
4	100	4	4.1716	-16.5	3.2250
5	180	4	4.1716	-16.5	24.9750

Title: TR-4 & earthworm 14-day % body weight gain; mg ai/kg
 File: TR4-WORM.TXT Transform: NO TRANSFORMATION

William's Test - TABLE 1 OF 2 Ho: Control<Treatment

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	Solv control	4	-25.2500	-25.2500	-24.5333
2	32	4	-23.9000	-23.9000	-24.5333
3	56	4	-24.4500	-24.4500	-24.5333
4	100	4	-28.4750	-28.4750	-28.4750
5	180	4	-50.2250	-50.2250	-50.2250

Title: TR-4 & earthworm 14-day % body weight gain; mg ai/kg
 File: TR4-WORM.TXT Transform: NO TRANSFORMATION

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William's Test - TABLE 2 OF 2 Ho: Control<Treatment

IDENTIFICATION	COMPARED MEANS	CALC. WILLIAMS	SIG 0.05	TABLE WILLIAMS	DEGREES OF FREEDOM USED
Solv control	-25.2500				
32	-24.5333	-0.4054		1.7500	k= 1, v=15
56	-24.5333	-0.4054		1.8400	k= 2, v=15
100	-28.4750	1.8245		1.8700	k= 3, v=15
180	-50.2250	14.1291	*	1.8800	k= 4, v=15

s = 2.4998

WARNING: Procedure has used isotonized means which differ from original (transformed) means.