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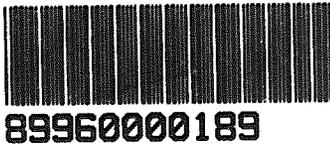
Attention: Section 8(e) Coordinator

This is a follow up to a March 5, 1996 notice submitted to EPA under provisions of TSCA Section 8(e). In the notice, Hercules reported preliminary data showing estrogenicity from low molecular weight polystyrene (LPS).

Enclosed is a copy of the final report for your records.

Sincerely,

Gary L. McCallister  
Corporate Manager  
Regulatory Affairs & Toxicology



GLM:cj  
Enclosure

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TNO-report V96.160

Determination of oestrogenic activity of F2L5250 in the Tiecco test with rats

Author:  
M.K. Prinsen

At the request of:  
Hercules B.V., Rijswijk, The Netherlands

TNO Project number:  
450595

Study assay number:  
range-finding test: 1794  
main test: 1795

Study director:  
M.K. Prinsen

Date and status:  
May 1996; final

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## Summary

1. A sample of **F2L5250** was examined for oestrogenic (uterotrophic) activity by means of the Tiecco test with weanling, female rats, fed 0, 10, 20, 40, 80 and 160 ppm **F2L5250** in their standard diet during a 4-day period. The various dose-levels in the diet were selected on the basis of the results of a range-finding Tiecco test with 0, 10, 100 and 1000 ppm **F2L5250**. To verify the sensitivity of the Tiecco test and for quantitative comparison, three groups of rats were fed the standard diet supplemented with 5, 10, and 20 ppb diethylstilbestrol (DES). The mean absolute and relative uterus weights of the various treatment groups were used for qualitative and quantitative assessment of possible oestrogenic (uterotrophic) activity.
2. No significant differences in mean absolute and relative uterus weights were observed between the control group and the 0, 10, 20, 40 and 80 ppm **F2L5250** groups. Significant and dose-related increases in mean absolute and relative uterus weights occurred in the groups fed with 160 ppm **F2L5250** and 5, 10 and 20 ppb DES.
3. The results of the main test showed that the highest no-effect-level for oestrogenic activity was 80 ppm in the diet, corresponding to a daily intake of 13.3 mg **F2L5250** per kg body weight. The high sensitivity of the Tiecco assay was clearly demonstrated by the 5 ppb DES dose-level which still induced a statistically significant uterotrophic effect. The 100 ppm dose-level of **F2L5250** showed the same level of oestrogenic activity as the 5 ppb DES dose level. Therefore it could be concluded that the potency of **F2L5250** with respect to oestrogenic activity is a factor 20,000 less than that of DES.

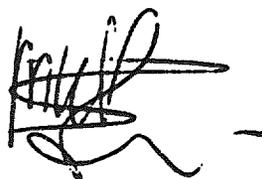
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### Statement of GLP compliance

We, the undersigned, hereby declare that this report constitutes a true and complete representation of the procedures followed and of the results obtained in this study by TNO Nutrition and Food Research Institute, and that the study was carried out under our supervision.

The study was carried out in accordance with the OECD Principles of Good Laboratory Practice.



M.K. Prinsen  
(Study director)

Date 22 May 1996



Dr R.A. Woutersen  
(Management)

Date 22 May 1996

### Quality Assurance Statement

On : Determination of oestrogenic activity of F2L5250 in the Tiecco test with rats  
Report Number : V96.160  
Date : May 1996

The experimental phase of this study was inspected by the Quality Assurance Unit of TNO Nutrition and Food Research Institute as follows:

Date of inspection:	Date of report:
8 February 1996	8 February 1996
9 February 1996	9 February 1996
13 February 1996	13 February 1996

The protocol was inspected on 30 January 1996 and 8 February 1996 (amendment I)

This report was audited as follows:

Dates of audit:	Date of report:
22/23 February 1996	23 February 1996
23 May 1996	23 May 1996

I, the undersigned, hereby declare that this report provides an accurate record of the procedures employed and the results obtained in this study; all inspections were reported to the study director and the management on the dates indicated.

  
Ms. M.W. van Marwijk  
(Quality Assurance Officer)

Date: 24 May 1996

## Testing facility

The toxicity study was conducted by:

TNO Nutrition and Food Research Institute

Toxicology Division

P.O. Box 360, 3700 AJ ZEIST, the Netherlands

Telephone +31 30 6944144

Telefax +31 30 6960264

Visitors address: Utrechtseweg 48, Zeist, the Netherlands

## Contributors

Study Director	:	M.K. Prinsen, Toxicology Division, TNO Nutrition and Food Research Institute.
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Senior Biotechnician	:	G. de Kruijff
Responsible for archives	:	R. Dekker
Responsible for histotechnique	:	E.C.M. van Oostrum
Responsible for pathology and management	:	Dr R.A. Woutersen
Quality Assurance Manager	:	P.B. Davis B.A.
Archives	:	R. Dekker

## 1 Introduction

At the request of Hercules B.V., Rijswijk, The Netherlands, a sample of **F2L5250** was examined by means of a bioassay with rats to provide data on the oestrogenic activity of the test substance. In this test, groups of female rats, aged 18-20 days at the onset of treatment, were fed with different levels of the test substance in a standard laboratory rodent diet during a 4-day feeding period. Subsequently, the weight of the uterus was recorded and compared to the uterus weight of controls to detect possible oestrogenic (uterotrophic) activity of the test compound. A dose-range

finding test and different dose-levels in the main test were used to determine a no-oestrogenic-effect level. Diethylstilbestrol (DES, Sigma, batch no. D4628, arrival 20/6/89) was used as a positive control substance. There are no international guidelines for this kind of testing. The method, as used in this Institute for many years, is based on a publication by G. Tiecco (Veterinaria Italiana 12 (1961) 447-460).

## 2 Procedure

### 2.1 Test substance

Date of receipt	: 9 January 1996
Name of the test substances	: F2L5250
Quantity	: ca 250 ml
Package	: tin can
Labeling	: F2L5250
Purity	: 100%
TNO internal reference no.	: 960005
General appearance	: viscous liquid
Storage conditions	: ambient

### 2.2 Animals and housing conditions

Species	: Wistar outbred rats
Strain	: HsdCpb:WU
Supplier	: CPB Harlan, Austerlitz, The Netherlands
Sex and age	: females, 17 ± 1 day old upon arrival
Identification	: within each group, the animals were individually marked by one of ten different V-shaped earmarks
Acclimatization period	: 2 days
Caging	: five animals per cage (stainless steel cages, 45x32x18 cm, fitted with wire-screen floor and front)
Lighting	: 12 hours light/12 hours dark cycle

Temperature during testing	:	22 ± 3°C
Humidity during testing	:	30%-70%
Ventilation	:	ca 10 air changes/hour.

Upon arrival on 24 January 1996 (30 females; no sisters) and on 7 February 1996 (105 females; littermates were marked by the supplier), the animals were transported to the test room (no. 15.15) and checked for sex, overt signs of ill health and anomalies. In the range-finding test, 25 females were randomly divided over five groups of 5 rats; in the main test 90 females were divided over nine groups of 10 rats (littermates were placed in different groups). As a consequence, randomization in the main test was not performed by a computer program. Since the Tiecco test is performed with very young animals, they were acclimatized to the laboratory conditions for 2 days only. During the acclimatization period the animals were identified with a temporary ink mark on their tail and weighed (nominal day -1; not reported but kept as raw data in the files). On the next day when treatment was started (nominal day 0), they were weighed again. In the main test, one female (C45) was exchanged with a reserve animal because of an outlying high body weight. The remaining animals were kept as reserves.

### 2.3 Basal diet and drinking water

Following the arrival of the rats till the initiation of the experimental period, the animals were fed *ad libitum* with a cereal-based rodent diet (see Annex 1 for composition) obtained from SDS (Special Diets Services), Witham, England. Each batch of this diet is analyzed by the supplier for the nutrients and contaminants listed in Annex 1. The certificate of analysis pertaining to the batch used is also given in Annex 1.

The drinking water was presented *ad libitum* in polypropylene bottles which were refilled when necessary. Potable water for human consumption (quality guidelines according to Dutch legislation based on the EEC Council Directive 80/778/EEC, see Annex 2) was supplied by N.V. Waterleidingbedrijf Midden-Nederland (WMN). Results of the routine physical, chemical and microbial examination of the drinking water as conducted by the supplier are made available to TNO Nutrition and Food Research Institute. In addition, WMN periodically analyses water samples taken on the premises of TNO in Zeist for a limited number of components. The results of the most

recent analyses are presented in Annex 2.

## 2.4 Time schedule

### Range-finding test:

- a. Arrival of the animals : 24 January 1996.
- b. Experimental starting date : 26 January 1996.
- c. Autopsy date : 30 January 1996.

### Main test:

- a. Arrival of the animals : 7 February 1996.
- b. Experimental starting date : 9 February 1996.
- c. Autopsy date : 13 February 1996.

## 2.5 Experimental design

The study was carried out at the testing facilities of the TNO Nutrition and Food Research Institute, Utrechtseweg 48, 3704 HE Zeist, the Netherlands, according to protocol P 450595 with one amendment, approved by the study director on 10 January 1996 and 5 February 1996, respectively.

## 2.6 Test design and dose levels

A range-finding test (study assay number 1794) was performed with four different dose-levels of the test substance in the basal diet, i.e. 1, 10, 100, and 1000 ppm and a negative control group. The range-finding groups consisted of 5 females each. The results of this preliminary experiment were used to select the appropriate dose-levels for the main test (study assay number 1795) in such a way that a no-oestrogenic-effect level could be established.

The design of the main test conducted with 9 groups of 10 females is given in the scheme below.

Group	Colour code	Test substance	Number of females
A (negative control)	white	basal diet	10
B	blue	F2L5250: 10 ppm	10
C	green	F2L5250: 20 ppm	10
D	red	F2L5250: 40 ppm	10
E	yellow	F2L5250: 80 ppm	10
F	orange	F2L5250: 160 ppm	10
G (positive control)	purple	DES: 5 ppb	10
H (positive control)	light-blue	DES: 10 ppb	10
I (positive control)	grey	DES: 20 ppb	10

## 2.7 Administration of the test substance

The diets for the range-finding test and for the main test were prepared only once for each test, viz. on 25 January 1996 and 8 February 1996, respectively. Dilutions in acetone of the test substance and of the positive control substance were prepared and mixed in appropriate amounts with the basal diet. After mixing in a mechanical blender, the acetone was allowed to evaporate for one day. The control diet was treated with acetone only in the same amount as used for the test diets. Records of all preparations were maintained and are filed with the other raw data of this test. In addition, samples of the test diets were stored at -20°C for a period of a least one year for possible future analysis. The diets were provided to the animals once in amounts sufficient for the 4-day feeding period as meal in stainless steel cans. The feed was covered by a perforated stainless steel plate which prevented spillage.

## **2.8 Observations, analyses and measurements**

### **2.8.1 Body weight and food consumption**

The body weight of each animal was recorded just prior to the start of treatment and at the end of the range-finding test and of the main test. The amount of food consumed during the 4-day feeding period was also recorded.

### **2.8.2 Clinical signs**

The general condition and behaviour of all animals in the range-finding test and in the main test were checked daily.

### **2.8.3 Pathology**

At the end of the 4-day observation period of the range-finding test and of the main test, the animals were killed in such a sequence that, on the average, time of killing was approximately the same for each group. The animals were killed by decapitation. Next, the uteri were carefully removed without puncturing (to prevent leakage of any intrauterine liquid), using a standardized procedure, and weighed.

## **2.9 Statistical analysis and evaluation of the results**

The statistical procedures used in the evaluation of data were as follows:

- for body weights: analysis of covariance followed by Dunnett's multiple comparison tests.
- for food consumption: one-way analysis of variance (anova) followed by L.S.D. tests.
- for uterus weight: the Mann-Whitney U-test.

The differences in relative uterus weights between the control group and the test groups was compared in order to assess possible oestrogenic activity of the test materials. The sensitivity of the test method was assessed by comparing the relative uterus weights of the control group with those of the positive control (DES) groups.

## 2.10 Retention of records

A reference sample of the test substance is retained for 10 years if its nature allows this. All raw data and the master copy of this report are filed in the archives of the TNO Nutrition and Food Research Institute and will be retained in the archives for a period of at least fifteen years after reporting of the study. Unless otherwise agreed, remaining test substance will be retained for at least six months after submission of the report.

## 2.11 Deviations from the protocol

Allocation of the animals to the various groups of the main test was not performed by computer randomization. The positive control substance DES was diluted in acetone instead of ethanol. No other deviations occurred.

## 3 Results

### 3.1 Clinical signs

#### Range-finding test

No clinical signs were observed in the animals during the 4-day experimental period.

#### Main test

On days 0 through 2 of the test, no clinical signs were observed in the animals.

On day 3 of the test, female C57 of the 20 ppm F2L5250 dose group was found dead. Because of cannibalism by the other rats in the cage, a probable cause for this death could not be found. The death of this animal is not attributed to the test substance because no mortality occurred at higher feeding levels. Female H149 (10 ppb DES) showed weakness, thinness, hunched posture and a perineum soiled with urine. The latter observation was also seen in female H157 and females I161 and I163 (20 ppb DES). Female I179 showed thinness.

At autopsy on day 4 of the test, female H149 still showed thinness and a perineum soiled with urine, the latter observation was also observed in females H157, I161 and I163. Female I179 still showed thinness.

### 3.2 Body weight and food consumption (Tables 1 and 2; Appendices 1-3)

#### Range-finding test

All animals gained weight during the 4-day experimental period. The 1000 ppm group, however, showed a somewhat lesser body weight gain and lower food consumption compared to the other groups.

#### Main test

All animals, except H149 and I179, gained weight during the 4-day experimental period. The 20 ppm F2L5250 group and the 20 ppb DES group showed a somewhat lesser body weight gain and lower food consumption compared to the other groups. The lower food consumption in the 20 ppm F2L5250 group was related to the death of female C57. Since a dose-relationship with the other F2L5250 dose-levels was not present, this finding was not attributed to the test substance. The food consumption of the 5 ppb DES group was slightly, but statistically significantly higher compared to the control group. No toxicological significance was attached to this finding.

The mean total amounts of F2L5250 ingested by the 10, 20, 40, 80 and 160 ppm dose groups were calculated to be 1.6, 2.9, 6.9, 13.3 and 27.2 mg F2L5250/kg b.w./day, respectively.

The mean total amounts of DES ingested by the 5, 10 and 20 ppb dose groups were calculated to be 0.00089, 0.0015 and 0.003 mg DES/kg b.w./day, respectively.

### 3.3 Uterus weights (Tables 1 and 2; Appendices 1-3)

#### Range-finding test

At autopsy, the mean absolute uterus weight showed a significant and dose-related increase in the 100 and 1000 ppm F2L5250 groups ( $P < 0.05$  and  $P < 0.002$ , respectively) when compared to the negative control group. The mean relative uterus weights showed significant and dose-related increases in the 100 and 1000 ppm F2L5250 groups ( $P < 0.02$  and  $P < 0.002$ , respectively).

Macroscopical examination at autopsy only revealed swollen uteri (also containing clear liquid) in four out of the five 1000 ppm F2L5250 animals, i.e. animal nos. E41, E45, E47 and E49.

### Main test

At autopsy, the mean absolute and relative uterus weights showed a statistically significant increase in the 160 ppm **F2L5250** group only ( $P < 0.002$ ) when compared to the negative control group. The mean absolute and relative uterus weights also tended to be higher in the 80 ppm **F2L5250** group, but the increases were not statistically significant.

The mean absolute and relative uterus weights showed statistically significant and dose-related increases in the DES groups ( $P < 0.05$ - $P < 0.002$ ).

Macroscopical examination at autopsy only revealed swollen or partly swollen uteri (in most instances also containing clear liquid) in females F105, F109, and F119 of the 180 ppm **F2L5250** group, in females H141, H155 of the 10 ppb DES group, and in females I163-I177 of the 20 ppb DES group. In addition, female H149 showed a swollen cervix and enlarged kidneys (left kidney; pelvic space filled with yellow exudate) and female H157 a swollen bladder-wall.

## 4 Discussion and Conclusions

From the results of the range-finding test it was clear that the level without uterotrophic effects of **F2L5250** was between 10 and 100 ppm in the diet. The results of the main test showed that the highest no-effect-level for oestrogenic activity was 80 ppm in the diet, which corresponds to a daily intake of 13.3 mg **F2L5250** per kg body weight.

The high sensitivity of the Tiecco assay was clearly demonstrated at the 5 ppb DES dose-level which still induced a statistically significant uterotrophic effect.

The 100 ppm dose-level of **F2L5250** examined during the range-finding test showed oestrogenic activity comparable with 5 ppb DES. Therefore it can be concluded that in this study the potency of **F2L5250** with respect to oestrogenic activity was circa a factor 20,000 less than that of DES.

Table 1 - Oestrogenic activity (Tiecco test) of F2L5250 in rats; mean body weights, food consumed and absolute and relative uterus weights obtained in the range-finding test (assay no. 1794)

Treatment group	Animal No.	Day 0	Day 4	Day 0-4	Day 4	Day 4
		Body Wgt g	Body Wgt g	ConsFood# g	Uterus g	Uterus g/kg BW
Neg. Control	Mean	33.3	50.3	7.9	0.066	1.32
	sem	0.6	0.9	0.0	0.004	0.07
	n	5	5	5	5	5
1 PPM	Mean	33.3	49.4	7.1	0.068	1.38
	sem	0.5	0.4	0.0	0.005	0.10
	n	5	5	5	5	5
10 PPM	Mean	33.2	50.3	7.6	0.066	1.32
	sem	0.7	1.1	0.0	0.008	0.15
	n	5	5	5	5	5
100 PPM	Mean	33.2	49.9	7.4	0.086*	1.72**
	sem	0.5	1.0	0.0	0.005	0.08
	n	5	5	5	5	5
1000 PPM	Mean	33.6	47.3**	6.6	0.231**	4.89**
	sem	0.7	1.2	0.0	0.026	0.55
	n	5	5	5	5	5

Statistics

Body weight: Covar + Dunnett's tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 Uterus weight: Mann/Whitney u-test (two-sided) : \* P<0.05; \*\* P<0.02; \*\*\* P<0.002

# Mean food consumption per rat per day: not statistically evaluated because only one cage per treatment was used

Table 2 - Oestrogenic activity (Tiecco test) of F2LS250 and DES in rats; mean body weights, food consumed and absolute and relative uterus weights obtained in the main test (assay no. 1795)

Treatment group	Animal No.	Day 0 Body Wgt g	Day 4 Body Wgt g	Day 0-4 ConsFood# g	Day 4 Uterus g	Day 4 Uterus g/kg BW
BASAL DIET	Mean	33.6	48.4	6.3	0.059	1.22
	sem	0.7	1.4	0.1	0.004	0.07
	n	10	10	10	10	10
10 PPM	Mean	31.8	47.3	6.4	0.056	1.18
	sem	0.8	1.1	0.0	0.003	0.06
	n	10	10	10	10	10
20 PPM	Mean	32.4	45.7	5.7	0.059	1.29
	sem	1.1	1.1	0.2	0.003	0.06
	n	10	9	9	9	9
40 PPM	Mean	33.1	48.9	7.0	0.063	1.29
	sem	0.8	1.1	0.1	0.003	0.05
	n	10	10	10	10	10
80 PPM	Mean	34.9	50.2	7.0	0.073	1.45
	sem	0.9	1.3	0.2	0.004	0.08
	n	10	10	10	10	10
160 PPM	Mean	33.8	48.9	7.0	0.101***	2.08***
	sem	0.8	1.7	0.1	0.007	0.18
	n	10	10	10	10	10
DES 5 ppb	Mean	33.6	49.9	7.4*	0.075*	1.52*
	sem	0.8	1.2	0.1	0.004	0.10
	n	10	10	10	10	10
DES 10 ppb	Mean	33.5	46.9	6.0	0.121***	2.57***
	sem	0.9	2.5	0.1	0.012	0.20
	n	10	10	10	10	10
DES 20 ppb	Mean	32.6	44.5	5.7	0.211***	4.63***
	sem	0.6	2.4	0.0	0.024	0.36
	n	10	10	10	10	10

Statistics

Body weight: Covar + Dunnett's tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 Uterus weight: Mann/Whitney u-test (two-sided) : \* P<0.05; \*\* P<0.02; \*\*\* P<0.002  
 Food consumption: Anova + L.S.D. tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001

# Mean food consumption per rat per day

Appendix 1 - Oestrogenic activity (Tiecco test) of F2L5250 in rats; individual and mean body weights, food consumed and absolute and relative uterus weights obtained in the range-finding test (assay no. 1794)

Treatment group	Animal No.	Day 0 Body Wgt g	Day 4 Body Wgt g	Day 0-4 ConsFood# g	Day 4 Uterus g	Day 4 Uterus g/kg BW
Neg.Control	A 1	33.3	49.3	7.9	0.063	1.28
	A 3	33.0	50.5	7.9	0.079	1.56
	A 5	35.2	53.4	7.9	0.072	1.35
	A 7	31.4	47.7	7.9	0.056	1.17
	A 9	33.8	50.6	7.9	0.062	1.23
Neg.Control	Mean	33.3	50.3	7.9	0.066	1.32
	sem	0.6	0.9	0.0	0.004	0.07
	n	5	5	5	5	5
1 PPM	B 11	33.4	49.9	7.1	0.058	1.16
	B 13	33.0	49.5	7.1	0.069	1.39
	B 15	34.6	48.6	7.1	0.055	1.13
	B 17	31.7	48.3	7.1	0.077	1.59
	B 19	33.7	50.6	7.1	0.082	1.62
1 PPM	Mean	33.3	49.4	7.1	0.068	1.38
	sem	0.5	0.4	0.0	0.005	0.10
	n	5	5	5	5	5
10 PPM	C 21	33.1	49.0	7.6	0.066	1.35
	C 23	32.7	49.5	7.6	0.053	1.07
	C 25	35.4	53.4	7.6	0.054	1.01
	C 27	31.0	47.4	7.6	0.063	1.33
	C 29	33.6	52.1	7.6	0.096	1.84
10 PPM	Mean	33.2	50.3	7.6	0.066	1.32
	sem	0.7	1.1	0.0	0.008	
	n	5	5	5	5	
100 PPM	D 31	33.5	49.1	7.4	0.095	1.53
	D 33	32.6	50.2	7.4	0.085	1.69
	D 35	34.2	52.2	7.4	0.096	1.84
	D 37	31.7	46.6	7.4	0.067	1.44
	D 39	34.2	51.5	7.4	0.088	1.71
100 PPM	Mean	33.2	49.9	7.4	0.086*	1.72**
	sem	0.5	1.0	0.0	0.005	0.08
	n	5	5	5	5	5
1000 PPM	E 41	33.3	49.7	6.6	0.247	4.97
	E 43	32.5	46.2	6.6	0.130	2.81
	E 45	35.9	50.0	6.6	0.266	5.32
	E 47	32.3	43.2	6.6	0.265	6.13
	E 49	34.1	47.3	6.6	0.247	5.22
1000 PPM	Mean	33.6	47.3	6.6	0.231***	4.89***
	sem	0.7	1.2	0.0	0.026	0.55
	n	5	5	5	5	5

## Statistics

Body weight: Covar + Dunnett's tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 Uterus weight: Mann/Whitney u-test (two-sided) : \* P<0.05; \*\* P<0.02; \*\*\* P<0.002

# Mean food consumption per rat per day; not statistically evaluated because only one cage per treatment was used

Appendix 2 - Oestrogenic activity (Tiecco test) of F2L5250 in rats; individual and mean body weights, food consumed and absolute and relative uterus weights obtained in the main test (assay no. 1794)

Treatment group	Animal No.	Day 0 Body Wgt g	Day 4 Body Wgt g	Day 0-4 ConaFood# g	Day 4 Uterus g	Day 4 Uterus g/kg BW
BASAL DIET	A 1	33.4	48.6	6.6	0.047	0.97
	A 3	35.3	49.2	6.6	0.055	1.12
	A 5	36.7	52.1	6.6	0.068	1.31
	A 7	35.2	52.9	6.6	0.072	1.36
	A 9	31.4	43.4	6.6	0.060	1.38
	A 11	33.2	48.8	6.0	0.057	1.17
	A 13	31.0	41.1	6.0	0.034	0.83
	A 15	32.0	48.4	6.0	0.073	1.51
	A 17	36.2	54.6	6.0	0.075	1.37
	A 19	31.8	44.6	6.0	0.051	1.14
BASAL DIET	Mean	33.6	48.4	6.3	0.059	1.22
	sem	0.7	1.4	0.1	0.004	0.07
	n	10	10	10	10	10
10 PPM	B 21	35.9	51.7	6.3	0.053	1.03
	B 23	30.9	45.9	6.3	0.053	1.15
	B 25	33.3	50.3	6.3	0.055	1.09
	B 27	26.6	40.5	6.3	0.058	1.43
	B 29	32.3	48.9	6.3	0.066	1.35
	B 31	33.3	49.2	6.4	0.071	1.44
	B 33	30.5	43.8	6.4	0.039	0.89
	B 35	32.0	48.4	6.4	0.066	1.36
	B 37	34.0	45.7	6.4	0.049	1.07
	B 39	29.0	48.7	6.4	0.048	0.99
10 PPM	Mean	31.8	47.3	6.4	0.056	1.18
	sem	0.8	1.1	0.0	0.003	0.06
	n	10	10	10	10	10
20 PPM	C 41	33.4	48.2	6.1	0.054	1.12
	C 43	32.0	47.7	6.1	0.079	1.66
	C 45	30.7	41.9	6.1	0.054	1.29
	C 47	28.3	41.9	6.1	0.057	1.36
	C 49	30.3	44.8	6.1	0.056	1.25
	C 51	36.7	49.8	5.1	0.052	1.04
	C 53	36.2	49.1	5.1	0.064	1.30
	C 55	32.5	41.3	5.1	0.051	1.23
	C 57	27.2	† 12 February 1996			
	C 59	36.2	46.6	5.1	0.064	1.37
20 PPM	Mean	32.4	45.7	5.7	0.059	1.29
	sem	1.1	1.1	0.2	0.003	0.06
	n	10	9	9	9	9
40 PPM	D 61	32.5	48.1	7.4	0.061	1.27
	D 63	36.8	52.9	7.4	0.079	1.49
	D 65	34.5	51.0	7.4	0.057	1.12
	D 67	32.8	48.7	7.4	0.058	1.19
	D 69	36.0	52.5	7.4	0.071	1.35
	D 71	30.2	42.9	6.6	0.056	1.31
	D 73	29.9	45.8	6.6	0.054	1.18
	D 75	32.6	48.6	6.6	0.077	1.58
	D 77	34.8	52.3	6.6	0.062	1.19
	D 79	30.6	45.9	6.6	0.054	1.18
40 PPM	Mean	33.1	48.9	7.0	0.063	1.29
	sem	0.8	1.1	0.1	0.003	0.05
	n	10	10	10	10	10

## Statistics

Body weight: Covar + Dunnett's tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 Uterus weight: Mann/Whitney u-test (two-sided) : \* P<0.05; \*\* P<0.02; \*\*\* P<0.002  
 Food consumption: Anova + L.S.D. tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 # Mean food consumption per rat per day

Appendix 2 - Oestrogenic activity (Tiecco test) of F2L5250 in rats; individual and mean body weights, food consumed and absolute and relative uterus weights obtained in the main test (assay no. 1795); continued

Treatment group	Animal No.	Day 0 Body Wgt g	Day 4 Body Wgt g	Day 0-4 ConsFood# g	Day 4 Uterus g	Day 4 Uterus g/kg BW
80 PPM	E 81	37.8	53.2	7.5	0.087	1.64
	E 83	36.9	53.3	7.5	0.065	1.22
	E 85	38.4	54.7	7.5	0.087	1.59
	E 87	33.0	46.6	7.5	0.090	1.93
	E 89	36.4	50.8	7.5	0.067	1.32
	E 91	32.4	46.7	6.6	0.056	1.20
	E 93	34.0	49.5	6.6	0.079	1.60
	E 95	31.8	47.2	6.6	0.077	1.63
	E 97	37.9	55.9	6.6	0.069	1.23
	E 99	29.9	44.3	6.6	0.051	1.15
80 PPM	Mean	34.9	50.2	7.0	0.073	1.45
	sem	0.9	1.3	0.2	0.004	0.08
	n	10	10	10	10	10
160 PPM	F 101	34.2	51.1	6.8	0.108	2.11
	F 103	32.9	49.2	6.8	0.101	2.05
	F 105	35.5	54.0	6.8	0.132	2.44
	F 107	30.5	38.1	6.8	0.084	2.20
	F 109	31.9	46.4	6.8	0.112	2.41
	F 111	38.2	56.1	7.2	0.080	1.43
	F 113	34.3	46.9	7.2	0.097	2.07
	F 115	33.4	49.1	7.2	0.068	1.38
	F 117	36.4	54.2	7.2	0.081	1.49
	F 119	30.5	44.1	7.2	0.142	3.22
160 PPM	Mean	33.8	48.9	7.0	0.101***	2.08***
	sem	0.8	1.7	0.1	0.007	0.18
	n	10	10	10	10	10

Statistics

Body weight: Covar + Dunnett's tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 Uterus weight: Mann/Whitney u-test (two-sided) : \* P<0.05; \*\* P<0.02; \*\*\* P<0.002  
 Food consumption: Anova + L.S.D. tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 # Mean food consumption per rat per day

Appendix 3 - Oestrogenic activity (Tiecco test) of DES in rats; individual and mean body weights, food consumed and absolute and relative uterus weights obtained in the main test (assay no. 1795)

Treatment group	Animal No.	Day 0 Body Wgt g	Day 4 Body Wgt g	Day 0-4 ConsFood# g	Day 4 Uterus g	Day 4 Uterus g/kg BW
DES 5 ppb	G 121	36.1	52.0	7.1	0.073	1.40
	G 123	32.3	46.4	7.1	0.094	2.03
	G 125	32.8	46.1	7.1	0.081	1.76
	G 127	34.0	50.3	7.1	0.070	1.39
	G 129	30.9	46.2	7.1	0.069	1.49
	G 131	35.1	51.9	7.6	0.073	1.41
	G 133	30.5	46.7	7.6	0.056	1.20
	G 135	37.0	55.0	7.6	0.075	1.36
	G 137	36.4	56.1	7.6	0.062	1.11
	G 139	30.4	48.1	7.6	0.100	2.08
DES 5 ppb	Mean	33.6	49.9	7.4*	0.075*	1.52*
	sem	0.8	1.2	0.1	0.004	0.10
	n	10	10	10	10	10
DES 10 ppb	H 141	33.1	49.4	6.2	0.120	2.43
	H 143	37.8	55.3	6.2	0.120	2.17
	H 145	33.6	50.0	6.2	0.175	3.50
	H 147	35.0	49.4	6.2	0.145	2.94
	H 149	32.4	27.7	6.2	0.070	2.53
	H 151	33.8	48.4	5.8	0.073	1.51
	H 153	33.2	48.3	5.8	0.089	1.84
	H 155	36.8	54.9	5.8	0.185	3.37
	H 157	31.5	44.1	5.8	0.105	2.38
	H 159	27.5	41.3	5.8	0.126	3.05
DES 10 ppb	Mean	33.5	46.9	6.0	0.121***	2.57***
	sem	0.9	2.5	0.1	0.012	0.20
	n	10	10	10	10	10
DES 20 ppb	I 161	28.4	37.8	5.6	0.090	2.38
	I 163	32.3	41.8	5.6	0.134	3.21
	I 165	31.7	47.4	5.6	0.283	5.97
	I 167	32.9	49.2	5.6	0.288	5.85
	I 169	33.4	47.5	5.6	0.225	4.74
	I 171	33.8	48.5	5.7	0.230	4.74
	I 173	32.9	45.7	5.7	0.222	4.86
	I 175	35.0	50.9	5.7	0.250	4.91
	I 177	34.4	49.9	5.7	0.282	5.65
	I 179	31.2	26.5	5.7	0.106	4.00
DES 20 ppb	Mean	32.6	44.5	5.7	0.211***	4.63***
	sem	0.6	2.4	0.0	0.024	0.36
	n	10	10	10	10	10

Statistics

Body weight: Covar + Dunnett's tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 Uterus weight: Mann/Whitney u-test (two-sided) : \* P<0.05; \*\* P<0.02; \*\*\* P<0.002  
 Food consumption: Anova + L.S.D. tests (two-sided) : \* P<0.05; \*\* P<0.01; \*\*\* P<0.001  
 # Mean food consumption per rat per day

## ANNEX 1.1 Diet specification part I (of two parts)

## Composition of the TNO-rodent diet

Ingredient	Origin	Percentage enclosure
Defatted soya 45% crude protein	Brazil/USA	11.0
Fish meal 66% crude protein	UK	7.0
Meat meal	UK	4.0
Wheat	EEC	38.5
Maize	EEC/USA	26.0
Lucerne	UK	3.0
Soya oil	Brazil/USA	3.0
Whey powder, delactosed	EEC	2.0
Yeast, unextracted, dry	EEC	3.0
Premix	*	2.5

## \* Premix composition in terms of contribution to the diet

Nutrient	Units per kg diet	
Vitamin A (retinol)	6400	IU
Vitamin D3 (cholecalciferol)	2100	iU
Vitamin E (dl- $\alpha$ -tocopherol acetate)	45	mg
Vitamin K3 (menadione sodium bisulphite)	3.0	mg
Vitamin B1 (thiamine)	2.5	mg
Vitamin B2 (riboflavin)	3.0	mg
Vitamin B6 (pyridoxine)	10.0	mg
Vitamin B12 (cyanocobalamin)	35	$\mu$ g
Folic acid	0.5	mg
Biotin	150	$\mu$ g
Nicotinic acid	12.5	mg
Calcium pantothenate	7.5	mg
Iron	25	mg
Cobalt	0.7	mg
Manganese	41	mg
Copper	8	mg
Zinc	12	mg
Iodine	1.5	mg
Sodium chloride	1800	mg
Calcium	2900	mg

## ANNEX 1.2 Diet specification part II (of two parts)

## Limits of nutrients and contaminants analyzed in the freshly prepared TNO-rodent diet

Nutrient	Unit	Lower Limit	Upper Limit
Moisture	%	9.0	12.5
Crude fat	%	5.0	7.5
Crude protein	%	18.0	23.0
Crude fibre	%	1.5	4.0
Ash	%	4.5	7.5
Calcium	%	0.7	1.1 (Ca/P should be higher than 1)
Phosphorus	%	0.5	0.9
Sodium	%	0.15	0.40
Chloride	%	0.15	0.60
Potassium	%	0.61	0.90
Magnesium	%	0.10	0.25
Fluoride	mg/kg	2.0	40
Iron	mg/kg	140	350
Copper	mg/kg	10	30
Manganese	mg/kg	50	150
Zinc	mg/kg	30	90
Selenium	mg/kg	0.1	0.5
Vitamin E	mg/kg	40	100
Vitamin A	IU/kg	5000	10000
Contaminant	Unit	Detection limit	Maximum Limit
Nitrate as NaNO	mg/kg	1.0	100
Nitrite as NaNO	mg/kg	1.0	5.0
Lead	mg/kg	0.25	1.0
Arsenic	mg/kg	0.2	0.5
Cadmium	mg/kg	0.05	0.15
Mercury	mg/kg	0.01	0.05
Aflatoxin (total)	µg/kg	1.0	5.0
P.C.B. (total)	µg/kg	10.0	50
D.D.T. (total)	µg/kg	1.0	20
Dieldrin	µg/kg	1.0	20
Lindane	µg/kg	1.0	20
Heptachlor	µg/kg	1.0	20
Malathion	µg/kg	20.0	500
Total viable organisms x 1000	#/g	1000/g	2x10
Mesophilic spores x 100	#/g	100/g	2x10
Salmonella species	#/g	absent/20 g	none/20 g
Presumptive E. coli	#/g	absent/20 g	none/20 g
E. coli type I	#/g	absent/20 g	none/20 g
Fungal units	#/g	absent/20 g	200/g
Antibiotic activity			none/1 g

Detection limits were specified by SDS. Contaminant maxima were derived from "Guidelines for the Manufacture and Supply of GLP Animal Diets", British Association of Research Quality Assurance, 1992; Institute of Laboratory Animal Resources, Vol. 19, No. 4, pp. L18-L20, 1976; National Toxicology Program (NTP), National Institute of Health Sciences, Attachment VI, Section II, pp. F1-F3, 1984; Dutch legislation on animal feeds (Diervoederwetgeving, Produktschap voor Veevoeder, 1992).

## ANNEX 1.3 Diet specification of batch 2020

*Special Quality Control  
Certificate of Analysis*

PRODUCT: TNO RODENT SQC GROUND 5MM

BATCH NO: 2020

PREMIX BATCH NO: 76

DATE OF MANUFACTURE: 09-OCT-95

Nutrient	Found Analysis <sup>a</sup>	Contaminant	Found Analysis	Limit of Detection
Moisture	10.0 %	Fluoride	31 mg/kg	1.0 mg/kg
Crude Fat	5.6 %	Nitrate as NaNO <sub>3</sub>	16 mg/kg	1.0 mg/kg
Crude Protein	20.3 %	Nitrite as NaNO <sub>2</sub>	Non Detected mg/kg	1.0 mg/kg
Crude Fibre	2.7 %	Lead	0.29 mg/kg	0.25 mg/kg
Ash	5.8 %	Arsenic	0.20 mg/kg	0.2 mg/kg
Calcium	1.08 %	Cadmium	0.14 mg/kg	0.05 mg/kg
Phosphorus	0.75 %	Mercury	Non Detected mg/kg	0.01 mg/kg
Sodium	0.26 %	Selenium	0.11 mg/kg	0.05 mg/kg
Chloride	0.27 %			
Potassium	0.95 %			
Magnesium	0.15 %	Total Aflatoxins	Non Detected mcg/kg	1 mcg/kg each of B1, B2, G1, G2
Iron	189 mg/kg			
Copper	13 mg/kg	Total P.C.B	Non Detected mcg/kg	10.0 mcg/kg
Manganese	73 mg/kg	Total D.D.T	Non Detected mcg/kg	1.0 mcg/kg
Zinc	55 mg/kg	Dieldrin	Non Detected mcg/kg	1.0 mcg/kg
		Lindane	Non Detected mcg/kg	1.0 mcg/kg
		Heptachlor	Non Detected mcg/kg	1.0 mcg/kg
		Malathion	Non Detected mcg/kg	20.0 mcg/kg
Vitamin A	9.3 iu/g	Total Viable Organisms x 1000	7.25 per gram	1000/g
Vitamin E	63 mg/kg			
Vitamin C	mg/kg	Mesophilic Spores x 100	95.00 per gram	100/g
		Salmonellae Species	Non Detected per gram	Absent in 20 gram
		Presumptive E.coli	Non Detected per gram	Absent in 20 gram
		E.coli Type 1	Non Detected per gram	Absent in 20 gram
		Fungal Units	25 per gram	Absent in 20 gram
		Antibiotic Activity	Non Detected	

Signed *rs F.B. 01*Dated *30/10/95*

## ANNEX 2.1 Parameters checked in drinking water



**N.V. WATERLEIDINGBEDRIJF MIDDEN-NEDERLAND**

Reactorweg 47 - Utrecht - Tel. 030-487211 - Postbus 2124 3500 GC Utrecht - Postadres: 65000 - Fax 030-414855

Utrecht, May 8, 1995

Zeist pumping station supplies Zeist with drinking water which is currently checked for the following parameters according to the Dutch Water Supply Act:

Table 1: Parameters checked in drinking water leaving the pumping station

Parameter		acceptable concentrations
* pH		7.0 < pH < 9.5
* Hydrogencarbonate	HCO <sub>3</sub> <sup>-</sup>	min. 30 mg/l
* Carbonate	CO <sub>3</sub> <sup>-2</sup>	-
* Carbondioxide	CO <sub>2</sub>	-
* Electrical conductivity		max. 125 mS/m
* Oxygen	O <sub>2</sub>	min. 2 mg/l
* Temperature		max. 25°C
Taste (dilution factor)		max. 3 at 25°C
Odour (dilution factor)		max. 3 at 25°C
Clarity		clear
Turbidity		max. 4 FTU
Color		max. 20 mg Pt/l
Saturation index		
* Ammonium	NH <sub>4</sub> <sup>+</sup>	max. 0.16 mg N/l
* Nitrite	NO <sub>2</sub> <sup>-</sup>	max. 0.03 mg N/l
* Nitrate	NO <sub>3</sub> <sup>-2</sup>	max. 11.3 mg N/l
* Sulfate	SO <sub>4</sub> <sup>-2</sup>	max. 150 mg/l
* Chloride	Cl <sup>-</sup>	max. 150 mg/l
* Calcium	Ca	max. 150 mg/l
* Magnesium	Mg	max. 50 mg/l
* Hardness	Ca + Mg	min. 1.5 mmol/l
* Sodium	Na	max. 120 mg/l
* Potassium	K	max. 12 mg/l
* Iron	Fe	max. 0.20 mg/l
* Manganese	Mn	max. 0.05 mg/l
Silicate	SiO <sub>2</sub>	-
Fluoride	F <sup>-</sup>	max. 1.1 mg/l
* Orthophosphate	PO <sub>4</sub> <sup>-3</sup>	max. 2 mg P/l
* Potassiumpermanganate consumption		max. 5 mg O <sub>2</sub> /l
* Volatile chlorinated hydrocarbons		< 1 µg/l (each)
* Coli bacteria (37°C)		< 1/300 ml
* Thermotolerant coli bacteria (44°C)		< 1/300 ml
Platecount 37°C		-
Platecount 22°C		-

- = no regulatory standard
- \* = also checked in unpurified groundwater



ingeschreven in het STERLAB register voor afname onder nr. 41 voor geboden afname in de omgeving.

N.V. Waterleidingbedrijf Midden-Nederland heeft is ingeschreven in het Handelsregister van de KvK te Utrecht onder nummer 67311.

## ANNEX 2.2 Parameters checked in drinking water (continued)



**N.V. WATERLEIDINGBEDRIJF MIDDEN-NEDERLAND**  
 Reactorweg 47 - Utrecht - Tel. 030-487211 - Postbus 2124 3800 GC Utrecht - Postbureau 68000 - Fax 030-414955

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Table 2: Parameters checked in unpurified groundwater in addition to the parameters listed in table 1 marked with \*\*\*

Parameter		maximum acceptable concentrations (in purified water)
Total cyanide	CN	50 µg/l
Sulfide	S	not organoleptically detectable
Aluminium	Al	0.2 mg/l
Arsenic	As	50 µg/l
Boron	B	1 mg/l
Barium	Ba	500 µg/l
Silver	Ag	10 µg/l
Cobalt	Co	-
Cadmium	Cd	5 µg/l
Mercury	Hg	1 µg/l
Lead	Pb	50 µg/l
Nickel	Ni	50 µg/l
Chromium	Cr	50 µg/l
Copper	Cu	0.1 mg/l
Antimony	Sb	10 µg/l
Selenium	Se	10 µg/l
Zinc	Zn	0.1 mg/l
Mineral oil		10 µg/l
Pesticides		0.1 µg/l (each) 0.5 µg/l (total)
Polyaromatic hydrocarbons		0.2 µg/l
Volatile aromatic hydrocarbons		-
Phenol		0.5 mg/l (total)
Total β activity		-

- = no regulatory standard



Ingeschreven in het STEPLAS register voor esters en ether  
nr 41 voor geboden geschreven in de omgeving.

N.V. Waterleidingbedrijf Midden-Nederland heeft aange-  
schreven in het handelsregister van de R.v.K. te Utrecht  
onder nummer 87311.

## ANNEX 2.3 Parameters checked in drinking water (continued)



**N.V. WATERLEIDINGBEDRIJF MIDDEN-NEDERLAND**

Reactorweg 47 - Utrecht - Tel. 030-4872111 - Postbus 2124 3500 GC Utrecht - Postadres 68000 - Fax 030-414855

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Table 3: Parameters checked in drinking water collected on the consumer's premises

Parameters		maximum acceptable concentrations
Odour		odourless
Taste		tasteless
Clarity		clear
Temperature		25°C
Electrical conductivity		125 mS/m
Potassiumpermanganate consumption		5 mg O <sub>2</sub> /l
Iron	Fe	0.20 mg/l
Manganese	Mn	0.05 mg/l
Ammonium	NH <sub>4</sub> <sup>+</sup>	0.16 mg N/l
Nitrite	NO <sub>2</sub> <sup>-</sup>	0.03 mg N/l
Nitrate	NO <sub>3</sub> <sup>-</sup>	11.3 mg N/l
Cadmium	Cd	5 µg/l
Copper	Cu	-
Lead	Pb	-
Coli bacteria (37°C)		<1/100 ml
Thermolerant coli bacteria (44°C)		<1/100 ml
Platecount 37°C		<10/ ml #
Platecount 22°C		<100/ ml #
Aeromonas bacteria		-

# = geometric annual average  
- = no regulatory standard

Drs. B.B. Hoogcarspel  
Laboratory Manager



**ANNEX 2.4 Results of periodical analyses in drinking water collected on the premises of TNO Nutrition and Food Research Institute in Zeist**

This is a translation of the Analysis Report of N.V. Waterleidingbedrijf Midden-Nederland, dated 3 January 1996. The analyses were conducted in a sample taken on 27 November 1995 in room number 15.12 at TNO Nutrition and Food Research Institute, Utrechtseweg 48, Zeist.

Parameter	Unit	Measured
Odour (qualitative)		odourless
Clarity (qualitative)		clear
Oxygen	mg/l	9.84
pH		8.02
Taste (qualitative)		good
Temperature	°C	14
Potassium permanganate consumption	mg O <sub>2</sub> /l	0.11
Iron	mg/l	0.036
Electrical conductivity	mS/m	24.6
Manganese	mg/l	<0.001
Ammonia	mg N/l	<0.03
Nitrite	mg N/l	<0.002
Nitrate	mg N/l	1.86
Cadmium	µg/l	<0.02
Copper	µg/l	34
Lead	µg/l	<0.4
Aeromonas bacteria	#/100 ml	1
Coli bacteria (37°C)	#/100 ml	<1
Plate count 22°C	#/ml	2
Plate count 37°C	#/ml	<1

**Conclusion:**

The above parameters meet the requirements of the Dutch Water Supply Act (based on the EEC council directive relating to water for human consumption, Directive 80/778/EEC).

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