APPENDICES

APPENDIX A
STANDARD MEDIUM

Preparation of Standard Culture Medium

Ingredient

1. Corn flour	125	g
2. Sugar	100	g
3. Yeast	50	g
4. Agar	14	g
5. Propionic acid	5	ml
6. Water	1000	ml

Step of preparation of standard medium for *Drosophila melanogaster* stocks.

1. Boil and blend sugar, agar, yeast and corn flour in 1000 ml water until sticky.
2. Add propionic acid.
3. Fill each 125 ml Erlenmeyer flask with 50 ml of the medium.
4. Close off the flask with a plug (made of gauze and cotton cover with aluminum foil).
5. Sterile the flasks in an autoclave microbial contamination that can harm the flies.

APPENDIX B
STATISTICAL CONSIDER ATION

In experiments designed to assess the mutagenicity of a chemical, most often a treatment series were compared with a control series. One might like to decide whether the compound used in the treatment should be considered as mutagenic or non-mutagenic. The formulation of 2 alternative hypotheses allowed one to distinguish among the possibilities of a positive, inconclusive, or negative result of an experiment (Frei and Würgler, 1988).

In the null hypothesis one assumes that there was no difference in the mutation frequency between control and treated series. Rejection of the null hypothesis indicated that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis postulated a priory that the treatment results in an increased mutation frequency compared to the spontaneous frequency. The alternative hypothesis was rejected if the mutation frequency was significantly lower than the postulated increased frequency. Rejection indicates that the treatment did not produce the increase requires to consider the treatment as mutagenic. If neither of the 2 hypotheses was rejected, the results were considered inconclusive as one could not accept at the same time the 2 mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring the mutation frequency in the treated series be *m* times that in the control series and used together with the null hypothesis. It might happen in this case that both hypotheses had to be rejected. This should mean that the treatment was weakly mutagenic, but led to a mutation frequency which was significantly lower than *m* times the control frequency.

Testing against the null hypothesis (H_O) at the level α and against the alternative a hypothesis (H_A) at the level β led to the error probabilities for each of the possible diagnoses: positive, weakly but positive, negative, or inconclusive. The following four decision were possible; 1) accept both hypotheses; these can not be true simultaneously, so no conclusions can be drawn--inconclusive result; 2) accept the

first hypothesis and reject the second hypothesis--negative result; 3) reject the first hypothesis and accept the second hypothesis--positive result; 4) reject both hypotheses--weak effect.

Calculation step by step

Estimation of spot frequencies and confidence limits of m_e Particularly in the case that both hypotheses, H_O as well as H_A, had to be rejected, one might be interested in knowing the confidence interval of m_e, i.e., of the estimated multiple by which the mutation frequency in the experimental series was larger than the spontaneous frequency. The estimated value was

$$m_e = \frac{(n_t/n)/N_c}{(n_c/n)/N_t}$$

Where N_c and N_t represented the respective sample sizes in control and treatment series, n_c and n_t the respective numbers of mutations found, and n the total of mutations in both series together. Exact lower and upper confidence limits p_l and p_u for the proportion n_c/n on one hand, as well as q_l and q_u for the proportion n_t/n on the other hand, may be an easy method to calculate these values using an F-distribution table. To determined q_l and p_u one-sidedly at the level α, and q_u and p_l also one-sidedly at the level β. In this way and in agreement with the foregoing section, a confidence limit $m_1 > 1$ led to rejection of H_O, while a confidence limit $m_u < m$ led to rejection of H_A.

In the first step, F-distribution were used to determine the value $F_{v1, v2}$ at the level $\alpha = 0.05$, where the degrees of freedom (v_1, v_2) were given by the equations

$$v_1 = 2(n - n_t + 1) \text{ and } v_2 = 2n_t$$

In the second step, the F-value so obtained was used to calculate the lower confidence limit (q_l) for the proportion of spots in the experimental series

$$q_l = n_t / [n_t + (n - n_t + 1) F_{v1, v2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control, which was equal to

$$f_{t,l} = q_l n / N_c$$

This was the following complementarily, namely that the lower confidence limit for the number of spots in the experimental series ($q_l n$) plus the upper confidence

limit for the number of spots in the experiment ($p_u n$) was equal to the total number of spots (n) found in experimental and control series together, i.e.,

$$p_u n = (1-q_l)n$$

This gave an upper limit for the frequency of spots per wing for the control, which is

$$f_{c,u} = p_u n / N_c$$

The lower confidence limit m_1 of the multiple m_e was determined as the ratio between the lower confidence limit for the frequency in the treated series and the upper confidence limit for the frequency in the control, i.e.,

$$m_1 = \frac{f_{t,l}}{f_{c,u}} = \frac{q_l n/N_t}{p_u n/N_c}$$

Only in the case that m_1, the lower confidence limit of m_e, was larger than 1.0 would reject H_O. Since this was not the case, H_O remains accepted.

In the same way, the lower confidence limit of the spot frequency may be determined in the control $f_{c,l}$ which will gave $f_{t,u}$, the upper confidence limit of the spot frequency in the experimental series. This is also done one-sidedly, at the level $\beta = 0.05$. The inverse ratio of these values will provide the upper 5% confidence limit m_u for the multiple m_e.

Again, the F-distribution was used and determined the values $F_{v1, v2}$ at the level $\beta = 0.05$, where the degrees of freedom (v_1, v_2) were given by the equations

$$v_1 = 2(n- n_c + 1) \text{ and } v_2 = 2n_c$$

The F-value so obtained was used to calculate the lower confidence limit (p_1) for the proportion of spots in the control

$$P_1 = n_c/ [n_c + (n- n_c + 1) F_{v1, v2}]$$

This gave a lower confidence limit for the frequency of spots per wing in the control, which equal to

$$f_{c,l} = p_l n/N_c$$

Again, there was complementarily, in that the lower confidence limit for the number of spots per wing in the control ($p_1 n$) plus the upper confidence limit for the number of spots per wing in the experiment ($q_u n$) was equal to the total number of spots (n) so that

$$q_u n = (1-p_l)n$$

This gave an upper limit for the frequency of spots per wing in this series, which is

$$f_{t,u} = q_u n/N_t$$

The upper confidence limit m_u of the multiple m_e can be determined as the ratio between the upper confidence limit for the frequency in the treated series and the lower confidence limit for the frequency in the control, i.e.,

$$m_u = \frac{f_{t,u}}{f_{c,l}} = \frac{q_u n/N_t}{p_l n/N_c}$$

H_A was rejected if m_u, the upper confidence limit of m_e was less than m (m=2 for the total of all spots and for the small single spots, and m=5 for the large single spots as well as for the twin spots). Substitution of m_e by m_l or m_u in the above formulas provided the respective exact upper and lower confidence limit for the frequencies estimated.

APPENDIX C
PREPARATION OF REAGENT ANTIOXIDANT ASSAY

DPPH Reagent:

Chemicals

1. 150 μM DPPH (2, 2- diphenyl-1-picrylhydrazl) in 80% methanol
2. 1.28 mM Trolox in 80% methanol

Standard Trolox was run in triplicate using several concentrations (1.28, 0.64, 0.32, 0.16 and 0.08 mM)

FRAP Reagent:

Chemicals

1. 300 mM Acetate buffer (pH 3.6)

(3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) plus 16 ml glacial acetic acid and made up to 1 L with distilled water)

2. 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl
3. 20 mM $FeCl_3.6H_2O$

Mixing the reagent from 1-3 before use and heated to 37^oC

300 mM Acetate buffer: 10 mM TPTZ solution: 20 mM $FeCl_3.6H_2O$ (ratio 10:1:1)

4. 1000 μM $FeSO_4.7H_2O$

Standard $FeSO_4.7H_2O$ was run in triplicate using several concentrations (1000, 500, 250, 125 and 62.5 μM)

Phenolic Reagent:

Chemicals

1. Folin-Ciocalteu reagent
2. Saturated sodium carbonate solution
3. 800 mg/l Gallic acid

Standard Gallic acid was run in triplicate using several concentrations (800, 400, 200, 100, 50 and 25 mg/l)