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DETERMINATION OF EFFECTIVE CONCENTRATION 50% (EC50): CASE OF URANIUM TOXICITY ON CARROT ROOT GROWN IN VITRO CROPPING DEVICE

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I. INTRODUCTION

A. Background of the study

Uranium toxicity

Uranium is a naturally existing heavy metal found in low levels in rocks, soil and water. In soil, the normal concentration of Uranium is 300 μg Kg^{-1} to 11.7 mg Kg^{-1} (Wikipedia 2006). In exceptional situations, Uranium concentrations in soils can reach tens to hundreds of milligrams per kg of soil, mostly because of mining and milling ores activities (Plant et al. 1999). High Uranium concentrations in soil can be toxic and therefore poses danger to the living organisms.

Because of the undesirable effects of chemicals in soil, the evaluation of their toxicity becomes paramount. Toxicological tests are conducted, for instance by measuring the decrease in the rate of soil respiration upon increasing the concentration of heavy metals (Haanstra and Doelman 1985). Another way is by measuring the growth of terrestrial plants at increasing chemical concentrations. The part of plant that is first exposed to the chemical is the root so that in toxicological studies, root length is measured for different chemical exposures at certain points in time after planting.

EC50

The toxicity of chemicals is commonly expressed in terms of dosage which gives 50% effect to the response (such as soil respiration or growth of a plant eg. root length) compared to the control. The effect can be either an increase or a decrease in response. This is called EC50 or Effective Concentration 50. The latter is also termed Effective Dose 50(ED50) or RD50 for dosage causing 50% reduction. In animal systems, it is referred to as LD50, the dosage lethal to 50% of the subjects (Schabenberger et al. 1999).

The EC50 is usually estimated by fitting a log-logistic curve to the data. The model is a sigmoidal relation on a logarithmic scale rather than linear relation. The logistic model

can be applied to dichotomous data such as survival or death and to continuous data for example weight or biomass, and in terms of length for growth. Several studies of dose-response in herbicide application experiments have used the log-logistic function to model dose-response relationships (e.g. Streibig 1980; Laerke and Streibig 1995; Seedfeldt et al. 1995; Hsiao et al. 1996; Sandral et al. 1997 as cited in Schabenberger et al. 1999)

Hormesis

Some studies with growth as response (continuous response) have shown that at some low concentrations of the toxic substance, growth is stimulated instead of being suppressed. This stimulus is called hormesis (from the Greek for 'setting into motion'). A definition of hormesis derived from Stebbing (1982) is low-dose stimulation followed by higher-dose inhibition. The most common form of hormesis follows the widely recognized ß-curve shown in Figure 1. The use of the ß-curve follows principally from the widespread use of growth as a principal end point in hormesis research. Hormetic dose-response relationships are also seen in the form U-shaped curves. U-shaped dose-response curves would most appropriately be applied when the end point relates to a traditional toxicologically based health end point such as cancer incidence (Davis and Svendsgaard 1990) or a response for instance, the proportion of affected fetuses (Hunt and Bowman 2004).

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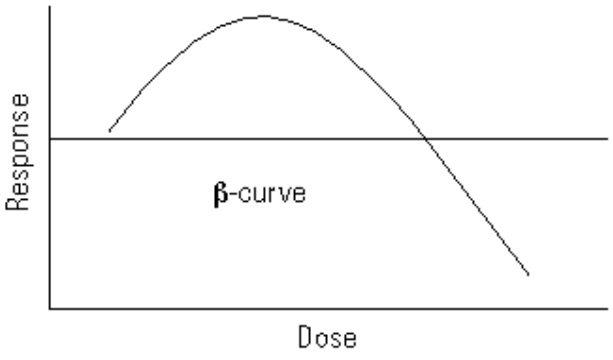

Figure 1. The most common dose-response curve when there is hormesis: the ß-curve

Reference to hormesis can be traced back to Schulz in 1888 who first expressed what is known today as the Arndt-Schultz law that every toxicant is a stimulant at low levels (Schabenberger et al. 1999).Several studies have shown that for low dosages of herbicide, the hormetic effect can occur that raises the average response at low dosages above the control value(Miller et al. 1962,; Freney, 1965; Wiedman and Appleby, 1972 as cited in Schabenberger et al. 1999).

An investigation done by Calabrese and Baldwin in 1998 revealed that chemical hormesis is a reproducible and a relatively common biological phenomenon. Evidence of chemical hormesis was judged to have occurred in approximately 350 of the 4000 studies evaluated. Chemical hormesis was observed in a wide range of taxonomic groups and involved agents representing highly diverse chemical classes, many of which are of potential environmental relevance. Studies with chemical hormesis use different biological endpoints. Growth responses were found to be the most prevalent followed by metabolic effects, longevity, reproductive responses, and survival.

If hormesis occurs, the standard log-logistic model does not fit the data. The usual practice was to still use the log-logistic model or drop part of the data. A solution was proposed in 1989 by Brain and Cousens by extending the log-logistic model. This modification naturally implements hormesis in the log-logistic model (Van Ewijk and Hoekstra 1993).

B. The Data

The experiment for the study was conducted in the laboratory of Radioecology in the Belgian Nuclear Research Center (SCK-CEN) in Mol, Belgium. Hairy carrot roots were grown in an *in vitro* cropping device containing a gel with different Uranium(U) concentrations. Eight replicates of carrot root for each U concentration were grown in the growing medium which contains the following Uranium concentrations: 0, 2.5, 5, 7.5, 10, 15, 20 and 30 mg U per liter. The initial and subsequent days root length (in centimeter) was measured.

The objective of this study is to estimate the EC50 considering the possibility of occurrence of hormesis.

This thesis is organized as follows. The next section discusses the methodology. Results are presented in Section 3, followed by the discussion and conclusion in the last section.

II. METHODOLOGY

In order to estimate the EC50, the given data was analyzed by establishing a dose–response relationship for every time point, that is, at day 0, 2, 6, 9, 13, 16, 20, 27 and 34. The response variable is in terms root length (in centimeters) and the dose is in milligrams of Uranium per liter of the growing medium.

Mathematical non-linear models presented in detail below were fitted to the data by non-linear regression using the procedure PROC NLIN in SAS. Initially, the presence of hormesis was investigated by fitting the Brain-Cousens model. Two equations of Brain-Cousens model were fitted to the data. When results reveal the non-significance of hormesis, the analysis proceeded to fitting the log-logistic models.

The fitted models were verified if the assumption of normality of residuals was met.

Non-linear Regression models

The dose response curves are assumed to follow a non-linear curve specified by the function f, which are known (the Brain Cousens model or the Log-logistic model). The function f is a function of dose and a number of parameters. In general, the non- linear regression models considered in this study can be written as:

$$y_i = f(x_i; \alpha_j) + \varepsilon_i \ , \qquad i = 1,...,n \quad \text{j} = 1,\ldots\text{m}$$

where x_i denotes the ith dose value, α_j are the unknown parameters and ε_i is the error for the response y_i (root length in centimetres in this study). The unknown set of parameters is different depending on the model assumed (see below). The errors ε_i are

assumed mutually independent and normally distributed N(0, σ^2). In particular, all observations have the same variance (homoscedastic).

The parameters are estimated using ordinary least squares (OLS) minimizing

$$\sum_{i=1}^{n}\left(y_i - f(x_i;\alpha)\right)^2$$

with respect to the parameters ($\alpha_1,\ldots,\alpha_m$). Estimations were done using iterative algorithm Levenberg-Marquardt in PROC NLIN in SAS.

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A. The Log-logistic model

Dose-response toxicity data usually follow a sigmoidal curve which can be described by the log-logistic model. This model expresses mean dose response as a sigmoidal, monotonic increasing or decreasing function in ln(dose) that is symmetric about its point of inflection as shown as a solid line in Figure 2.

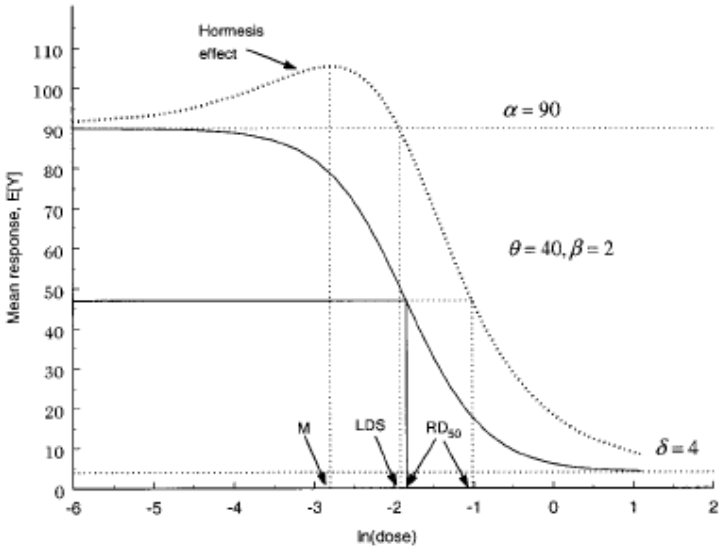

Figure 2. An example of Log-logistic dose-response curve and Brain-Cousens modification with hormesis effect. (Source: Schabenberger et al. 1999)

A common form or parameterization of the log-logistic response function is

$$E[Y \mid x] = \delta + \frac{\alpha - \delta}{1 + \theta \exp[\beta \ln(x)]} \quad [1]$$

Where E[Y|x] is the average response at dosage x and α and δ are the upper and lower asymptote of the response respectively. The parameters θ and β are related to the rate of change and point of inflection of the curve.

An alternative expression (Schabenberger et al.1999) of the log-logistic function is

$$E[Y \mid x] = \delta + \frac{\alpha - \delta}{1 + \exp[\beta \ln(x / RD50)]} \quad [2]$$

where the term RD50 is the effective concentration at which 50% of the total effect is achieved . When referring to Figure 1, this is the dosage producing a response halfway between the upper and lower asymptote or limit, that is (α-δ)/2. The term RD50 is the EC50. The parameter β is the relative slope around RD50. If β > 0, the response trend is monotonically decreasing. The log-logistic function is symmetric around RD50. Equation [2] is a **four-parameter function** with α, β, δ and RD50 as the parameters to be estimated by non linear regression.

A variation of equation [2] is the **three-parameter log-logistic model**. It is arrived at when the lower limit, δ in equation [2] is set to 0, and has the form:

$$E[Y \mid x] = \frac{\alpha}{1 + \exp[\beta \ln(x / RD50)]} \quad [3]$$

The lower limit,δ can be set to zero for response such as growth, which cannot go lower than zero.

The **five-parameter log-logistic model** (Finney 1979 as cited in Ritz and Streibig 2005) is given by the formula

$$E[Y \mid x] = \delta + \frac{\alpha - \delta}{\{1 + \exp[\beta \ln(x / RD50)]\}^f} \quad [4]$$

Setting the parameter f to 1 in equation [4] yields the four-parameter log-logistic model in equation [2].

B. The Brain-Cousens model

In order to allow for hormesis, Brain and Cousens modified the log-logistic model [1] into the following equation:

$$E[Y \mid x] = \delta + \frac{\alpha - \delta + \gamma x}{1 + \theta \exp[\beta \ln(x)]} \quad [5]$$

where γ measures the initial rate of increase at low dosages. γ is the hormesis term.

Equation [5] is expressed in another way, still allowing for hormesis but generalized in order to incorporate any effective dosage as a parameter of the equation. The details of the reparameterization are presented in Schabenberger et al. 1999. The following equation, is one of the reparameterizations which includes RD50 (which is EC50) as a parameter:

$$E[Y \mid x] = \delta + \frac{\alpha - \delta + \gamma x}{1 + \omega \exp[\beta \ln(x / RD50)]} \quad [6]$$

where $\omega = 1 + \frac{2\gamma RD50}{\alpha - \delta}$

Equation [6] reduces to the log-logistic function for $\gamma = 0$. This Brain-Cousens model permits a simple test for hormesis, by fitting the model to the data and obtaining an estimate of the parameter γ. If the 95% confidence interval for the estimate of γ does not cover the value 0, the data exhibit a statistically significant effect of hormesis at the 0.05 probability level (Schabenberger et al. 1999).

A variation of equation [6] when the lower limit, δ is 0 takes the form:

$$E[Y \mid x] = \frac{\alpha + \gamma x}{1 + \omega \exp[\beta \ln(x / RD50)]} \quad [7]$$

where $\omega = 1 + \frac{2\gamma RD50}{\alpha}$

Another parameterization of the Brain-Cousens was proposed earlier by Van Ewijk and Hoekstra (1992). The equation explicitly includes the EC50 as a parameter.

$$E[Y \mid x] = \frac{k(1 + fx)}{1 + (2fx_0 + 1) * (x / x_0)^b} \quad [8]$$

The parameter k stands for the response at x = 0; f stands for hormesis (corresponding to the γ in equation [6]); if f >0, the dose-response curve shows an increase for low doses. The parameter x_0 is the EC50. The parameter b has no simple interpretation.

III. RESULTS

A. Exploratory Data Analysis

Eight dosages were used in the study, and for each dosage, there are eight observations (8 replicates). The dosages are: 0, 2.5, 5, 7.5, 10, 15, 20 and 30 milligrams per liter. Measurement of the root length was done at nine (9) time points: day 0, 2, 6, 9, 13, 16, 20, 27 and 34. The data was noted to have no missing observations.

Plots of dose-response scatter graph for each time point (Figure 3 Appendix) showed a general trend of decreasing root length at increasing dosage except on the earlier days of observation, that is, at day 0 and day 2. This observation in the trend is confirmed by negative correlation of dose and root length in all the days, with day 0 having almost no correlation.

Table 1. Correlation of root length and dose at different time points

Time (days)	**Correlation**
0	- 0.08008
2	- 0.28627
6	- 0.67176
9	- 0.72171
13	- 0.73937
16	- 0.77511
20	- 0.80570
27	- 0.83285
34	- 0.85687

The scatter plots are also indicative of non-linear downward trend of relationship of root length and dose. In some days however, it can be seen from the graphs that there are observations in low doses where the corresponding root length exceed that of the control (at dose 0). These are at days 6, 9, 13, 16, and 20. This leads us to suspect that there might be enhancement of growth at these low doses referred to as the occurrence of hormesis.

B. Non Linear Regression Results

1. Brain-Cousens models

a. Brain-Cousens model: Schabenberger et al.(1999)

The Brain-Cousens model with the parameterization by Schabenberger (1999) in equation [6]

$$E[Y \mid x] = \delta + \frac{\alpha - \delta + \gamma x}{1 + \omega \exp[\beta \ln(x / RD50)]} \quad [6]$$

where $\omega = 1 + \frac{2\gamma RD50}{\alpha - \delta}$

was fitted to the data by non-linear regression, using root length as the response and dose as the independent variable. Unlike linear regression, estimation of parameters in non-linear regression requires the specification of initial parameter values .Thus, initial values of parameters α, β, δ and RD50 have to be supplied.

The choice of the initial values of the parameters may influence the estimation algorithm, in the worst case yielding no convergence and in the best case convergence in few iterations is achieved.

Results of the non-linear regression (for all time points) indicated that the hormesis term, γ and the lower bound, δ were not significant as indicated by the parameter having zero in their 95 percent confidence interval.

It was however noted that eight observations were not used in the model fitting. This arose from dose = 0 being fitted in the equation. (ln 0 is imaginary). In order for the data at dose 0 to be used in the model fitting, a very small number (0.0001) was substituted in place of 0 dose. The non linear regression gave similar results, that is, γ and δ are insignificant. The insignificance of δ leads us to try to fit the data to the following equation:

$$E[Y \mid x] = \frac{\alpha + \gamma x}{1 + \omega \exp[\beta \ln(x / RD50)]} \quad [7]$$

where $\omega = 1 + \frac{2\gamma RD50}{\alpha}$

which is a variation of the previous equation [6], wherein the lower limit term, δ is set to zero, and δ is not a parameter anymore of the model. Fitting the data with dose = 0 substituted with a very small number (0.0001) resulted in all the four parameters α , β, RD50 and the hormesis term, γ being significant in days 13, 16 and 20, while for the rest of the days, the three parameters α, β and RD50 were significant and the hormesis term, γ not significant. When dose = 0 was not substituted by a very small number, the model fitting yielded non meaningful results, that is, α was not significant, implying that the upper bound can be zero which is meaningless for growth data.

Other small values of dose such as $1x10^{-7}$ and $1x10^{-10}$ were substituted to dose = 0 to find out if there is an effect on the estimates. The same results as substituting 0.0001 were arrived at.

Non-linear models for day 13, day 16 and day 20 use equation [7], with values of the parameters presented in the following tables:

Table 2 .Day 13: Parameter Estimates and Confidence Interval for the Brain-Cousens Model parameterized by Schabenberger et al. (1999)

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
Alpha	34.6561	3.5898	27.4753	41.8368
Beta	2.2294	0.2383	1.7527	2.7061
Gamma	9.0330	3.6581	1.7158	16.3503
RD50	16.2268	2.0911	12.0439	20.4097

Table 3.Day 16: Parameter Estimates and Confidence Interval for the Brain-Cousens Model parameterized by Schabenberger et al. (1999)

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
Alpha	51.1482	4.7680	41.6108	60.6856
Beta	2.4816	0.2846	1.9122	3.0509
Gamma	9.5347	4.0297	1.4740	17.5954
RD50	13.4195	1.3208	10.7775	16.0615

Table 4. Day 20: Parameter Estimates and Confidence Interval for the Brain-Cousens Model parameterized by Schabenberger et al. (1999)

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
Alpha	80.5505	6.4831	67.5824	93.5186
Beta	2.8228	0.3551	2.1126	3.5330
Gamma	9.4613	4.4831	0.4938	18.4288
RD50	11.6488	0.8681	9.9123	13.3852

b. Brain-Cousens model: Van Ewijk and Hoekstra (1992)

Fitting the Brain-Cousens model with the parameterization done by Van Ewijk and Hoekstra (1992) with the following equation

$$\mathrm{E[Y \mid x]} = \frac{k(1+fx)}{1+(2fx_0+1)*(x/x_0)^b} \quad [8]$$

where x_0 is the EC50, resulted with the hormesis term, f to be not significant. However, when dose = 0 was substituted with a small number near zero (0.0001), hormesis was significant in days 13 and 16 which parameter estimates are presented in Tables 5 and 6:

Table 5. Day 13: Parameter Estimates and Confidence Interval for the Brain-Cousens Model parameterized by Van Ewijk and Hoekstra (1992)

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
k	34.6561	3.5898	27.4753	41.8368
b	2.2294	0.2383	1.7527	2.7061
EC50	16.2268	2.0911	12.0439	20.4097
f	0.2606	0.1216	0.0174	0.5039

Table 6. Day 16: Parameter Estimates and Confidence Interval for the Brain-Cousens Model parameterized by Van Ewijk and Hoekstra (1992)

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
k	51.1481	4.7680	41.6107	60.6856
b	2.4816	0.2846	1.9122	3.0509
EC50	13.4195	1.3208	10.7775	16.0614
f	0.1864	0.0897	0.00696	0.3659

2. Log-logistic models

The data in those days where hormesis was not significant (after fitting the Brain-Cousens model) were fitted with the log-logistic models. These included data from day 0, 2, 6, 9, 27 and 30.

a. 5-parameter log-logistic model

None of the data fitted the five-parameter log-logistic model. Results of the non-linear regression with this model showed either the f term to be not significant or cannot be estimated.

b. 4-parameter log-logistic model

Only one data set, that is, Day 9 fitted the four-parameter log-logistic model. Taking the form of equation [2], the model for Day 9 is:

$$E[Y \mid x] = \delta + \frac{\alpha - \delta}{1 + \exp[\beta \ln(x / RD50)]} \quad [2]$$

where the parameter estimates are in Table 7:

Table 7. Day 9: Parameter Estimates and Confidence Intervals using four-parameter log-logistic model

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
alpha	27.5435	1.5173	24.5086	30.5785
delta	8.0027	2.9376	2.1265	13.8788
beta	3.9448	1.8102	0.3239	7.5658
RD50	11.9733	1.7801	8.4126	15.5340

c. 3-parameter log-logistic model

Four data sets: Day 6, Day 9, Day 27 and Day 34 fitted into the three-parameter log-logistic model, with the following equation:

$$E[Y \mid x] = \frac{\alpha}{1 + \exp[\beta \ln(x / RD50)]} \quad [3]$$

where the parameter estimates are in Tables 8 , 9 , 10 and 11 :

Table 8. Day 34: Parameter Estimates and Confidence Interval using the three-parameter log-logistic model

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
alpha	239.0	8.7193	221.5	256.4
beta	4.1299	0.6776	2.7751	5.4848
RD50	9.5749	0.4337	8.7076	10.4422

Table 9. Day 6. Parameter Estimates and Confidence Interval using the three-parameter log-logistic model

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
alpha	17.9316	1.1011	15.7298	20.1335
beta	1.9375	0.5411	0.8555	3.0195
RD50	20.4727	2.5696	15.3345	25.6109

Table 10: Day 27: Parameter Estimates and Confidence Interval using the three-parameter log-logistic model

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
alpha	162.2	6.9322	148.4	176.1
beta	3.9345	0.7275	2.4799	5.3891
RD50	9.8947	0.5454	8.8040	10.9854

Table 11: Day 9: Parameter Estimates and Confidence Interval using the three-parameter log-logistic model

Parameter	Estimate	Approx Std Error	Approximate 95% Confidence Limits	
alpha	27.8477	1.6698	24.5087	31.1867
beta	2.2842	0.5682	1.1481	3.4204
RD50	16.8273	1.8477	13.1325	20.5220

Fitting the data of days 0 and day 2 yielded a confidence interval of β (which is the slope at point RD50) that included zero in it, implying that the curve for these sets of data is flat.

Day 9 data was fitted in both four-parameter and three-parameter log-logistic models.

Graphs of the models with their confidence intervals are presented in Figure 4 in the Appendix.

Results of Diagnostics

The normal probability plots of residuals for each day-model are shown in Figures 7 to 14 in the Appendix. No serious departures from normality can be seen from the graphs.

Comparing the normal probability plots for the two models for day 9, the three-parameter model appears to be better than the four-parameter model. The three-parameter model for day 9 is therefore adapted as the final model.

IV. DISCUSSION AND CONCLUSION

The study investigates whether there was an occurrence of growth stimulation at low dosage (called hormesis) of Uranium and estimate the EC50. To answer these research questions, the data was analized for each time point(day) by establishing a dose-response relationship for each time point with the use of non linear regression.

Fitting the Brain-Cousens model into the data was deemed appropriate because this allows for simultaneous investigation of the statistical significance of hormesis, and the estimation of EC50 (and its confidence interval), because these two are included as parameters of the equation. Disregarding hormesis may lead to erroneous calculation of the EC50.

There were two parameterizations of the Brain-Cousens models tried in this study in order to validate if the same conclusions regarding the occurrence of hormesis and the EC50 estimates are arrived at. It attempts to verify whether different parameterizations would result to the same conclusions. In this study, same conclusions are arrived at for most of the time points (days), but different conclusion at one time point, that is at day 20.The use of the Brain-Cousens model parameterized by Schabenberger, et al. for Day 20 data led to a conclusion of significant hormesis but using the Brain-Cousens model parameterized by Van Ewijk and Hoekstra resulted to the opposite conclusion (ie. not significant hormesis).

This raises the question whether different parameterization may lead to differing conclusions. The estimates of EC50 however are equal even at day 20.

In the course of doing the non linear regression in this study, it was experienced that the starting values supplied for the parameters can affect convergence, at one time may not converge and on other times may converge after just a few iterations. Therefore, caution in supplying the starting values of the parameters is suggested.

It was also noticed that when some values at the lower doses, particularly at dose 0 were not included in the modelling, lead to the conclusion of no hormesis. When dose 0 was substituted with a very small number (therefore including the observations in the analysis), lead to the conclusion of significant hormesis at some time points, particularly at days 13, 16 and 20. It can be seen from this that the observations at the control (dose 0) are very important in the analysis. As a recomendation, it would be helpful to include some smaller doses in the design of the experiment to be able to detect hormesis.

From the results, it can be seen that there was no hormesis in the earlier days (day 0, day 6 and day 9) and in the later days (day 27 and 34). Hormesis is observed in days 13, 16 and 20. It implies that it takes a few days to pass before hormesis is observed, then hormesis ceases after sometime.

A summary of the EC50 and its 95% confidence interval at each time point is in Table 12.

Table 12. EC50 and its 95 Percent Confidence Interval at different time points in days

Day	EC50	EC50 95% Confidence interval	
		lower limits	upper limits
0	-	-	-
2	-	-	-
6	20.47	15.33	25.61
9	16.83	13.13	20.52
13	16.23	12.04	20.41
16	13.42	10.78	16.06
20	11.65	9.91	13.39
27	9.89	8.80	10.99
34	9.57	8.71	10.44

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APPENDIX

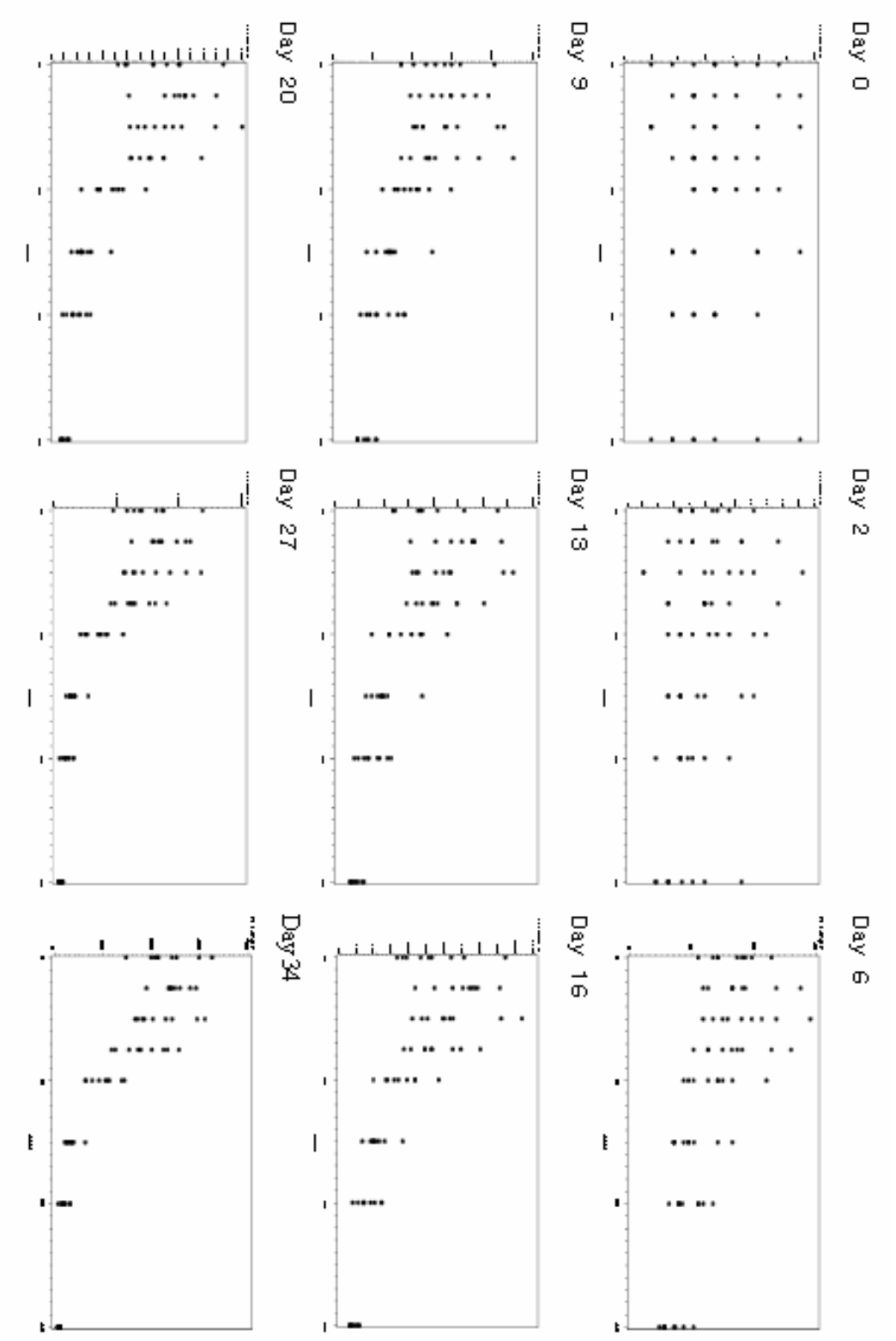
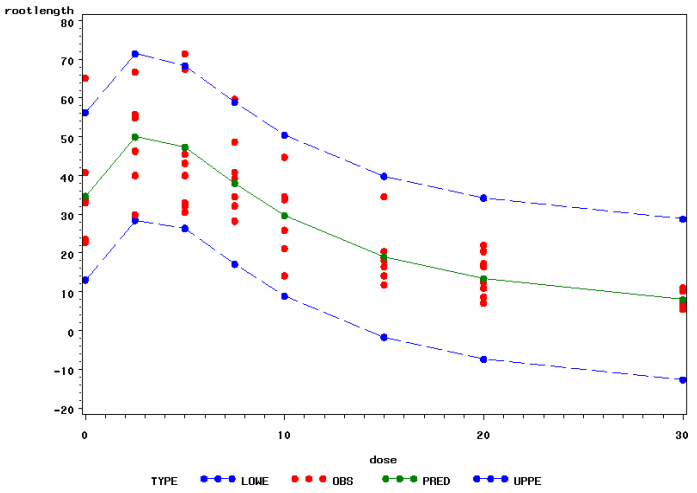
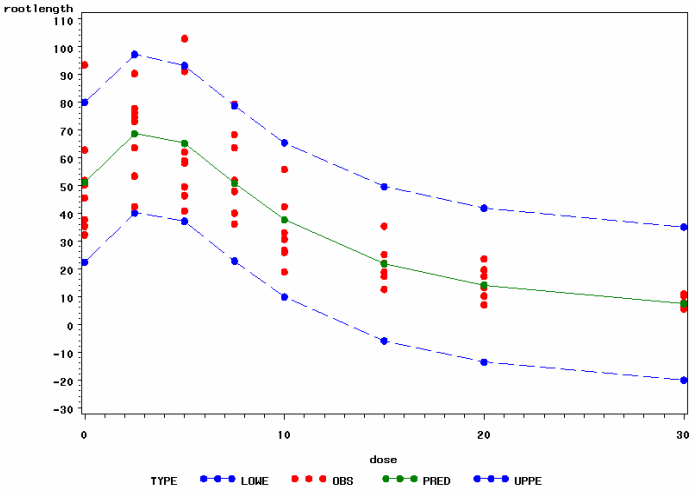


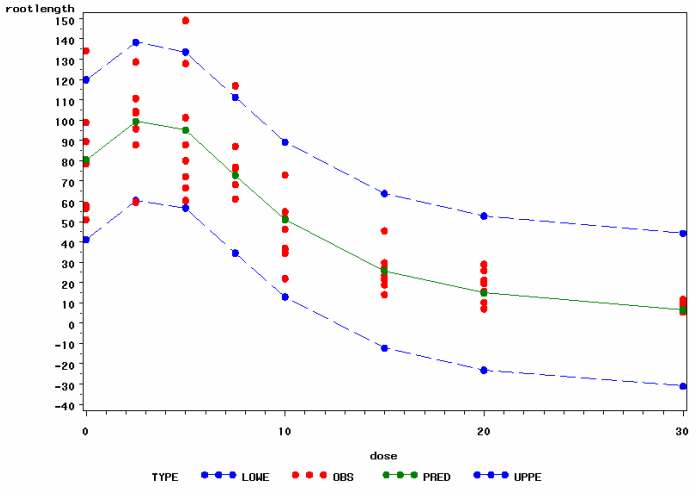
Figure 3. Scatter Graph of Root Length vs Dose



Day 13

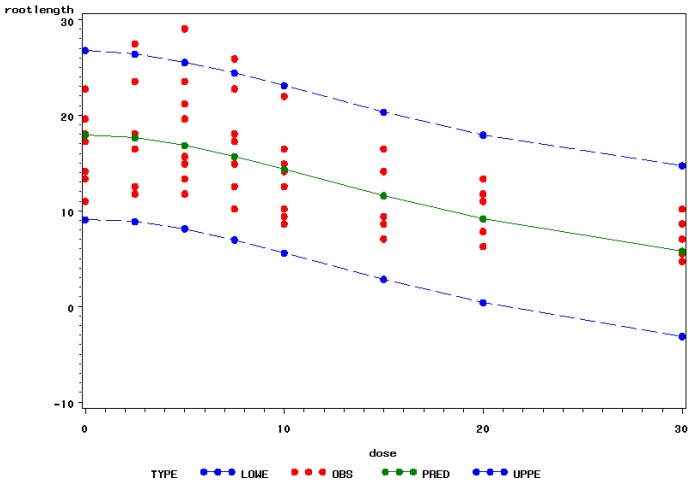


Day 16

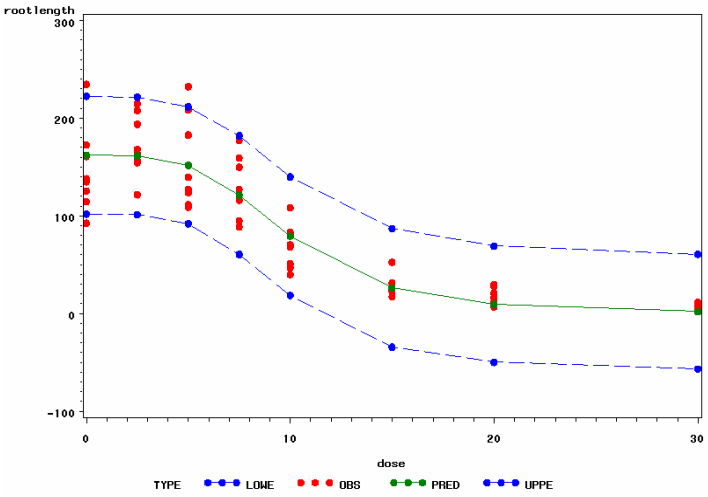


Day 20

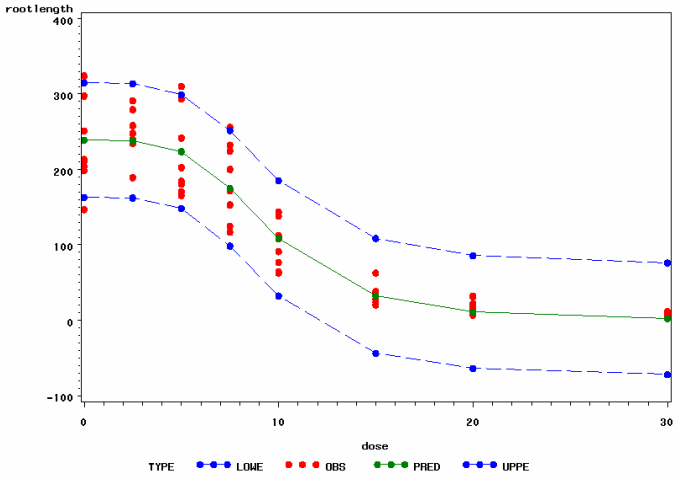
Figure 4. Graphs of the Brain-Cousens model (Schabenberger, et. al parameterization) with significant hormesis, for days 13,16 and 20



Day 6



Day 27



Day 34

Figure 5. Graphs of the 3- parameter logistic models for days 6, 27 and 34

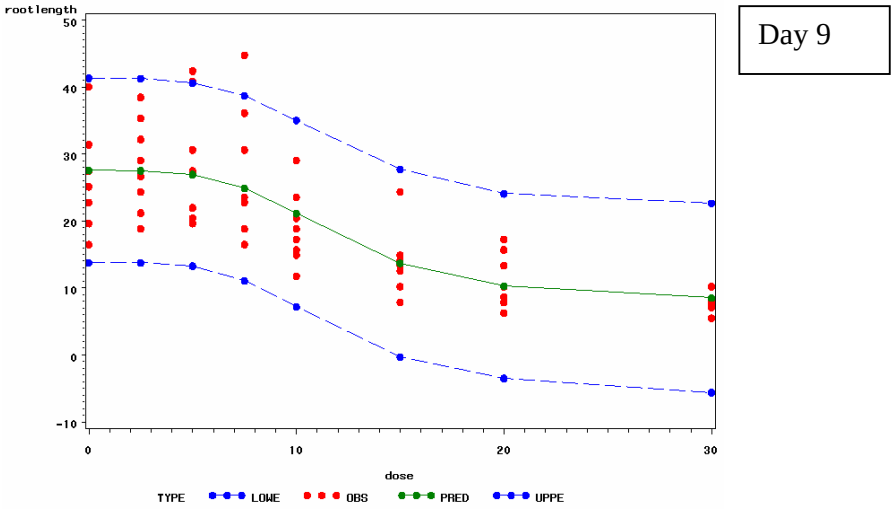


Figure 5. Graph of the four- parameter logistic model for day 9

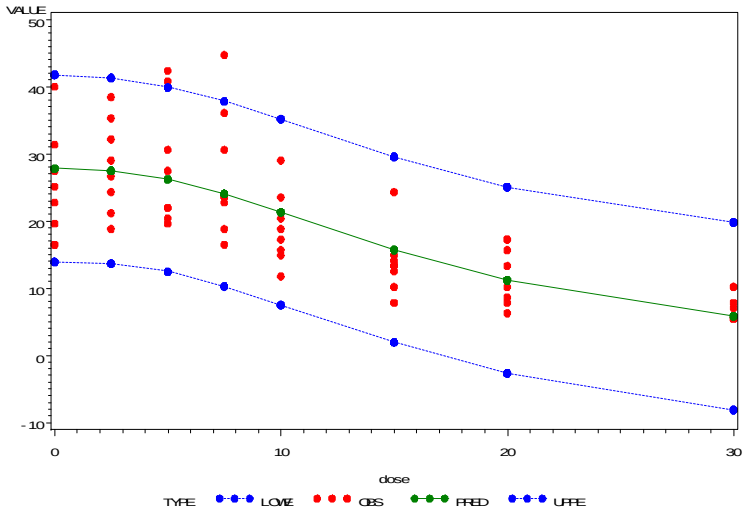


Figure 6. Graph of the three- parameter logistic model for day 9

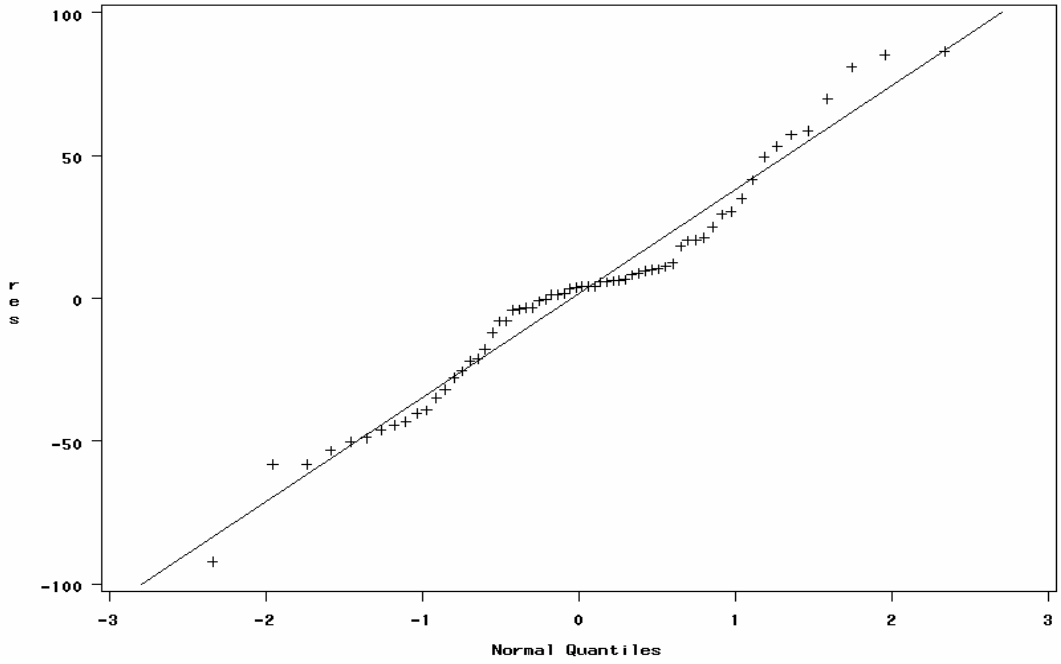


Figure 7. Day 34 Normal Probability Plot of Residual

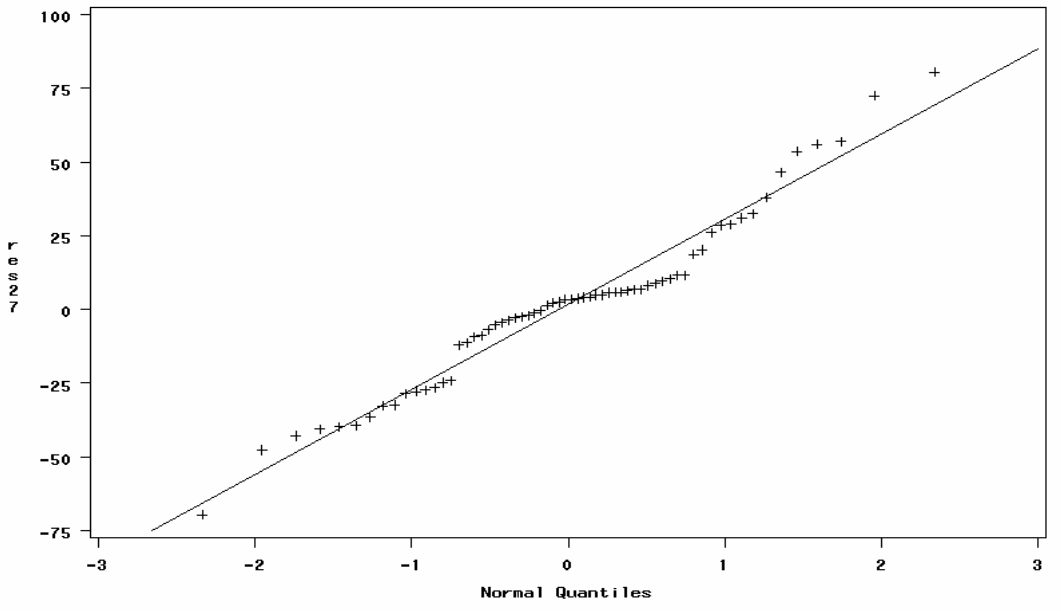


Figure 8. Day 27 Normal Probability Plot of Residual

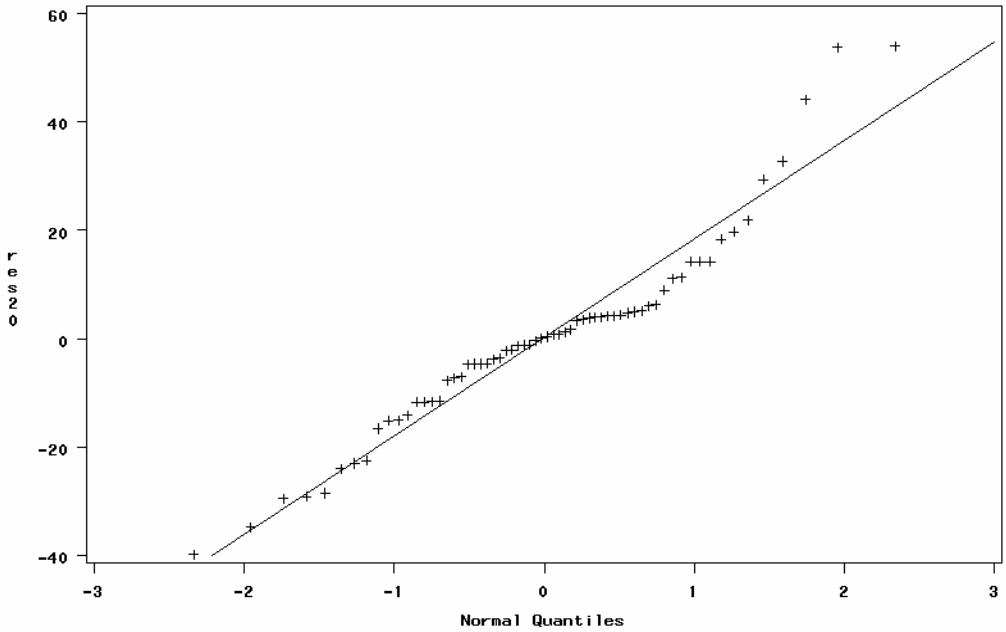


Figure 9. Day 20 Normal Probability Plot of Residual

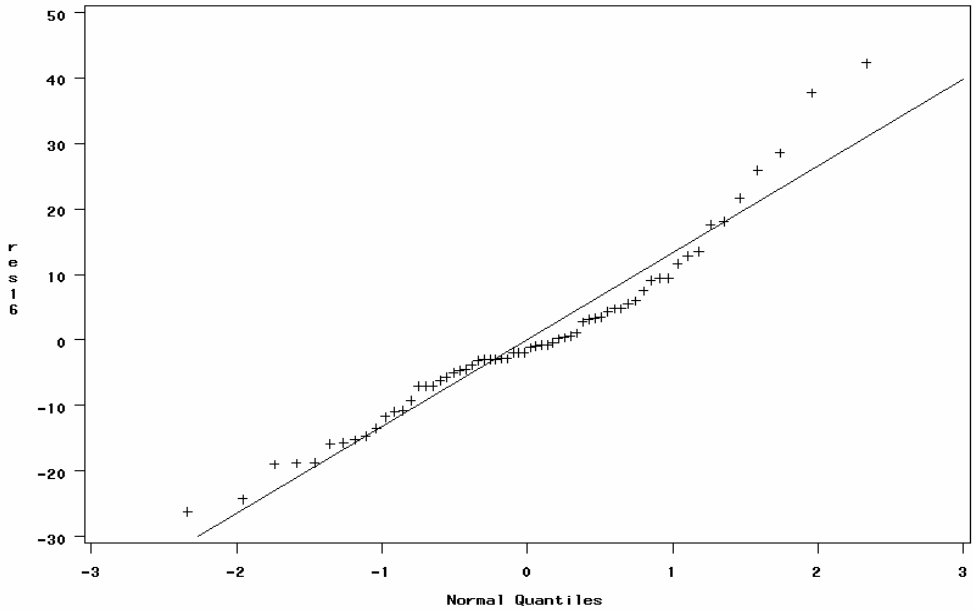


Figure 10. Day 16 Normal Probability Plot of Residual

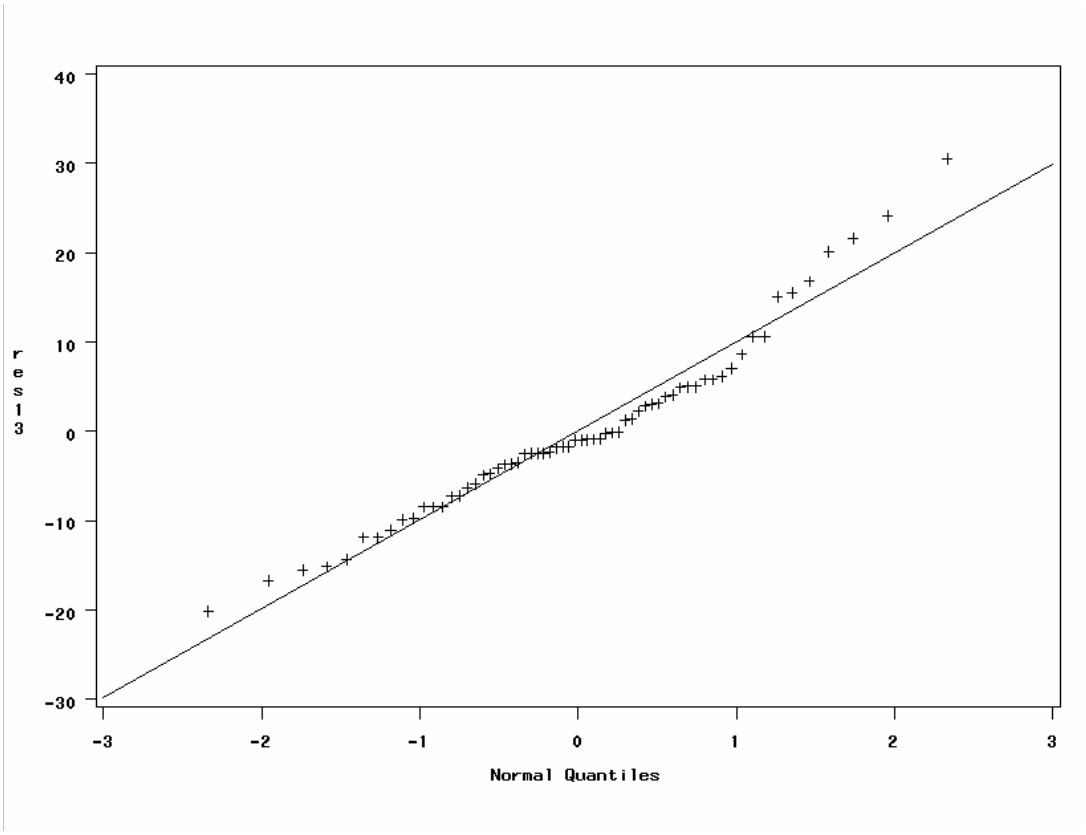


Figure 11. Day 13 Normal Probability Plot of Residual

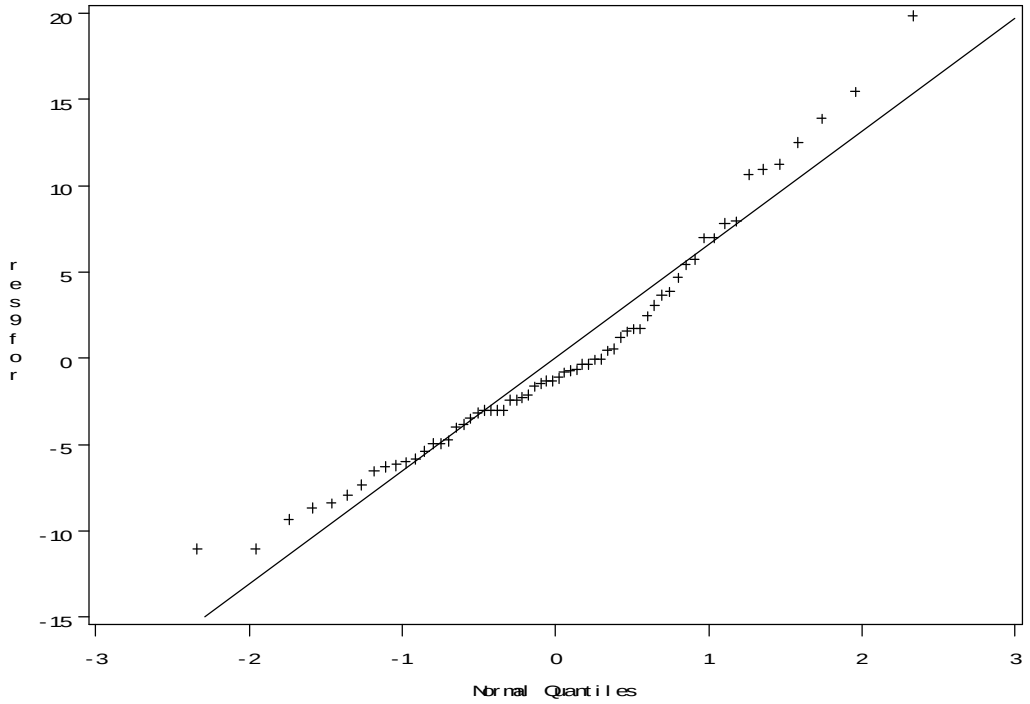


Figure 12. Day 9 Normal Probability Plot of Residual (four-parameter log-logistic model)

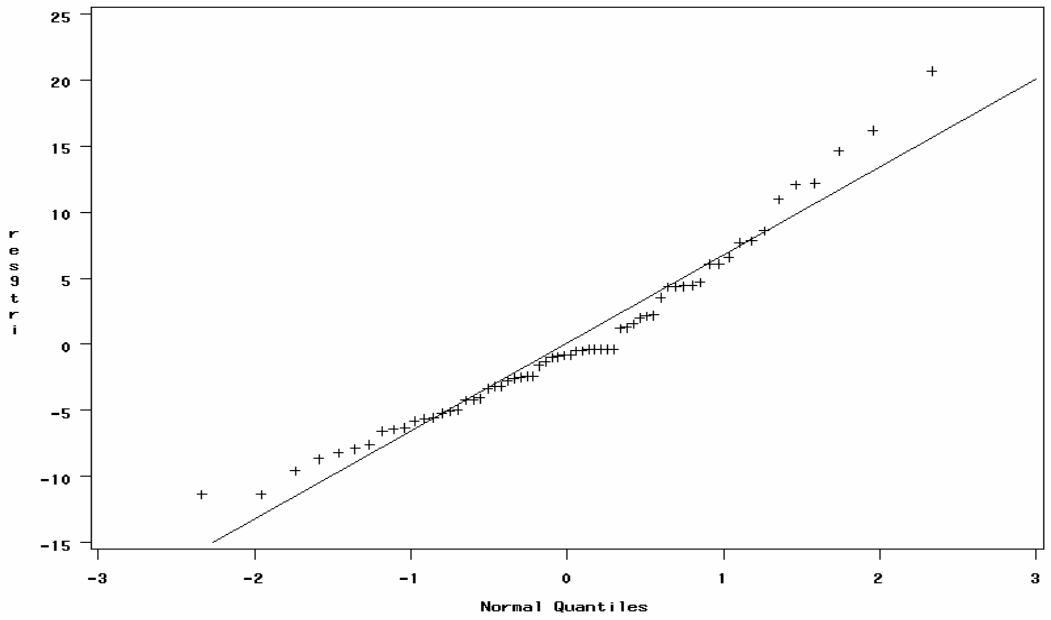


Figure 13. Day 9 Normal Probability Plot of Residual (three-parameter log logistic model)

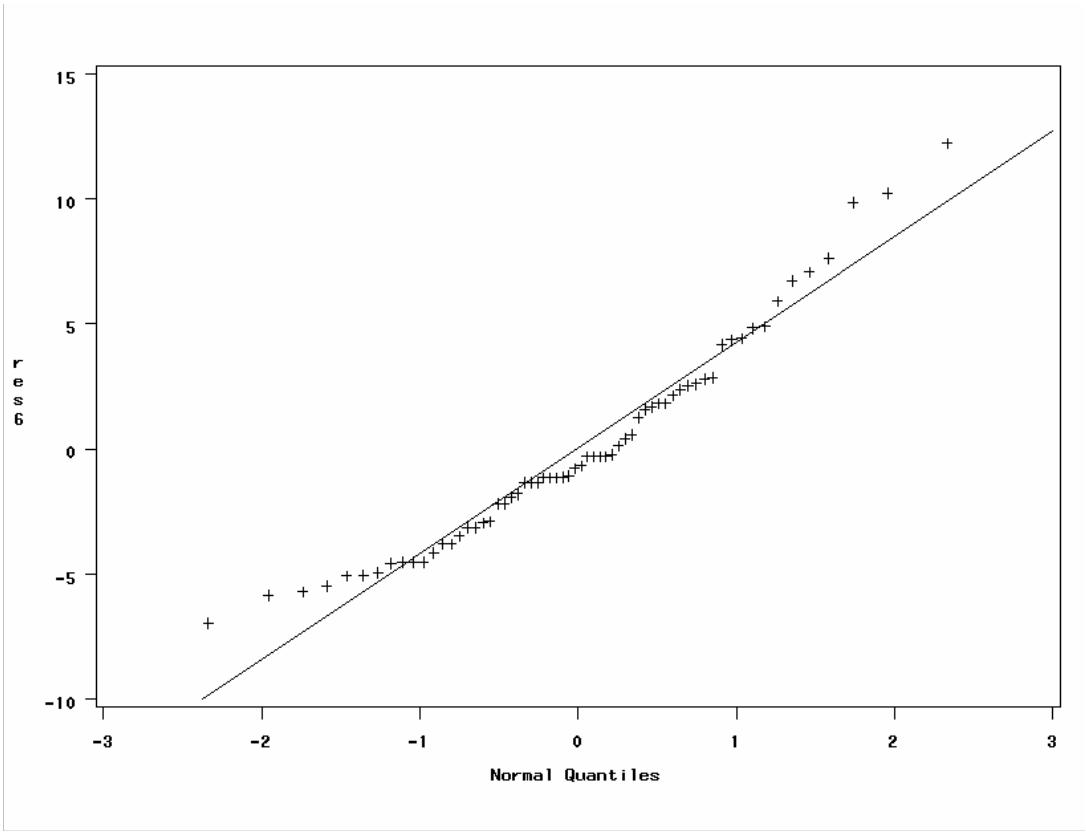


Figure 14. Day 6 Normal Probability Plot of Residual

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Datum: **06.11.2006**