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Design and Analysis of Drug Combination Experiments

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Summary

In this paper we present and discuss a novel, simple and easy to implement parametric modeling approach to assess synergy. An extended three parameter log-logistic model is used to analyse the data and calculate confidence intervals of the interaction indices. In addition the model corrects for the bias due to plate-location effects. The analysis is performed with PROC NLMIXED and SAS-code is provided. The approach is illustrated using data coming from an oncology study in which the inhibition effect of a combination of two compounds is studied using 96-well plates and a fixed-ratio design.

Key words: 96-well plates; Antagonism; Interaction index; Drug combination; Drug interaction; Fixed-ratio design; Hill model; Ray design; Synergy; Pharmacology; Plate-location effects.

1 Introduction

When two drugs are applied in combination as a mixture to a biological system the resulting effect can be equal or different as compared to what is expected from the biological activity of the individual compounds. In the latter case, interaction is said to be present while in the first case the mixture is said to be additive (no interaction present). Two excellent review papers on the subject of drug interactions are those from Greco, Brave and Parsons (1995) and Berenbaum (1989). Altough the simple concept of drug interaction seems straightforward, it is a controversial issue. Even in such degree that no uniform agreement on the definitions of drug interaction terms exist. Discussions on different definitions used can be found in Berenbaum (1989), Calabrese (1991), Gessner (1988), Unkelbach and Wolf (1984) and Wampler et al. (1992), among others. In this paper we will use the so called Sarriselkä agreement on terminology. This agreement was the concensus of six scientists who debated concepts and terminology for agent interaction at the Fifth International Conference on the Combined Effects of Environmental Factors in Sarriselkä, Finnish Lapland on September 6 to 10, 1992 (Greco et al., 1992). The two review papers mentioned before describe different reference models to calculate the expected treatment effect for a mixture of two drugs under the assumption of no interaction. Altough lengthy discussions have been held between the advocates of the different reference models, both authors defend the Loewe additivity model as being the most suitable. This model is described by

$$1 = \frac{C_{a,r}}{IC_{X,a}} + \frac{C_{b,r}}{IC_{X,b}}\,, \tag{1}$$

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where in case of an inhibitory drug, X refers to a specific percent inhibition level (e.g. 50%), $C_{a,r}$ and $C_{b,r}$ are the concentration of drug A and B given in a combination of the two drugs and $IC_{X,a}$ and $IC_{X,b}$ are the concentration of drug A and B yielding the same effect level, when administered alone, as the mixture. If the equation holds and the right hand side, also known as the interaction index, I, of Berenbaum (1977), sums to 1 the mixture is said to be additive. When both compounds are active when given alone and I is less then 1 Loewe synergy is concluded and when it is larger then 1 the mixture is said to be Loewe antagonistic.

In recent years the study of drug interactions in pre-clinical drug development is a topic of frequent and growing interest among pharmacologists and biologists. Not surprisingly, many research papers are devoted to the development of statistical techniques for correctly assessing drug interactions (Feng and Kelly, 2004; White et al., 2004; Minto et al., 2000; Machado and Robinson, 1994; among others) when two compounds are mixed. Both parametric as well as non-parametric methods have been developed. One popular approach is fitting a three dimensional response surface. Assessment of interaction can be obtained by graphical comparison to a surface under assumption of additivity or by including an interaction parameter in the surface model which is tested to be different from zero. Another well known method is separately fitting two marginal concentration-response curves to the pure compounds and using Eq. (1) to calculate the expected response for a particular combination which is then compared to the empirical observed response. Our approach can be viewed as an extension of the second method by also fitting concentration-response curves to each of the different mixtures. The different concentration-response curves of the different mixtures and the individual compounds are all jointly fitted and an interaction index based on Eq. (1) is estimated for each mixture, together with a $100(1-\alpha)\%$ Confidence Interval (CI), thus quantifying a possible interaction effect. In addition, our approach adjusts for the presence of plate-location effects that are known to be of significant magnitude in micro-titer experiments (Faessel et al., 1999).

In Section 2, a short description of the fixed-ratio or ray design is given. Section 3 describes an oncology experiment in which different combinations are investigated using the ray design. Section 4 covers the details of the statistical methodology developed to analyse the data. The results are given in Section 5 and finally an overall discussion and conclusion is given in Section 6.

2 Experimental Design

In the fixed-ratio or ray design (Tallarida, 2000, pp. 58–60) of two drugs, the individual compounds are combined together in amounts such that the proportion between them is constant. This is done by preparing a mixture Z according to

$$Z = fA + (1-f)\,B\,, \tag{2}$$

where A and B are preliminary estimates of the concentrations of the individual constituents of the mixture required to obtain a certain effect level. In most cases A and B refer to the individual IC_{50} levels. The symbol f is called the mixture factor and takes values from 0 to 1. For sake of simplicity, we consider each choice of f to correspond to a new compound, of which m concentrations are tested, thus allowing to construct different concentration-response curves. In addition, each series of concentrations for a given f can be regarded to represent a ray, in a so called ray design. The ray design is illustrated in Figure 1 where the different lines correspond to the different rays each with a specific f value and the dots represent the m concentrations within a mixture.

3 Experiment

3.1 Description of the Oncology Experiment

The oncology experiment in question involves an enzymatic reaction in which the enzyme catalyses the transfer of a small molecular moiety from a reagent to a substrate resulting in the transformation of the latter. The purpose of the drug is to inhibit the formation of the catalytic complex, thus prevent-

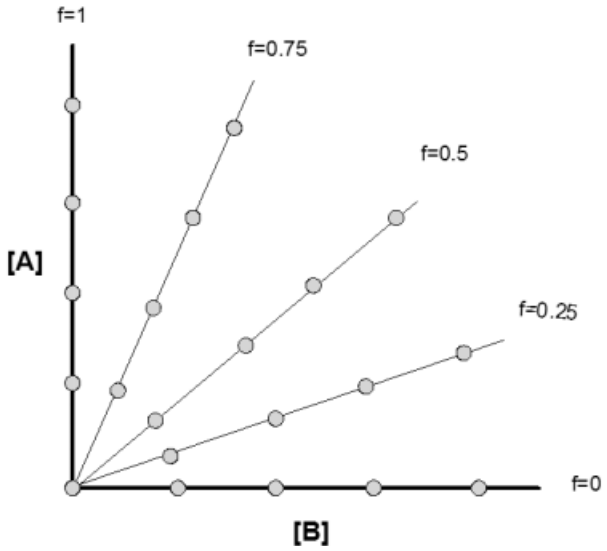

Figure 1 Illustration of the Ray design. The X-axis correspond to the concentration of compound B and the Y-axis to the concentration of compound A. Each line corresponds to a different ray with a specific f value and the dots represent the m concentrations within a ray.

ing the transfer of the chemical moiety. In order to determine the amount of substrate being transformed, this moiety is radioactively labelled. In the experiment we include the mixtures $f = 0$, 0.2, 0.35, 0.5, 0.65, 0.8 and 1. Within each mixture 9 concentrations are prepared by taking 30, 10, 4, 2, 1, 0.7, 0.45, 0.2 and 0.05 times the mixture dose Z (2). The experiment is carried out using 6 independent 96-well plates in which some wells are used to calculate the background radioactivity (negative control) and the maximum of substrate being transformed (positive control). The lay-out of the plate is given in Figure 2. Here zero stands for a positive control, meaning the well contains all the reagents

	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	
R_1	0	0	0	0	0	0	0	0	0	BL	BL	BL	
R_2	0	0	0	30	10	4	2	1	0.7	0.45	0.2	0.05	f=1
R_3	0	0.2	0.05	0	0	30	10	4	2	1	0.7	0.45	f=0.8
R_4	0	0.7	0.45	0.2	0.05	0	0	30	10	4	2	1	f=0.65
R_5	0	2	1	0.7	0.45	0.2	0.05	0	0	30	10	4	f=0.5
R_6	0	10	4	2	1	0.7	0.45	0.2	0.05	0	0	30	f=0.35
R_7	0	0	30	10	4	2	1	0.7	0.45	0.2	0.05	0	f=0.2
R_8	0	30	10	4	2	1	0.7	0.45	0.2	0.05	0	0	f=0

Figure 2 Plate lay-out of a 96-well plate used in the experiment. The plate consists of 12 columns (C1–C12) and 8 rows (R1–R8), together making up 96 wells. One row (except the first one) contains the m concentrations of a ray corresponding to a specific f value. A zero represents a positive control and a BL stands for a blanco or negative control.

but no drugs resulting in a maximal non-inhibited signal. BL stands for a blanco or negative control meaning the well contains all reagents except the enzyme and no drugs. One row (except row 1) correspond to one ray. Half of the six plates have the lay-out as shown in Figure 2 while for the others the order of concentrations is reversed. The mean of the three blanco wells (containing the negative controls) is subtracted as a background correction from the observed data within the same plate.

3.2 Data exploration

The raw data from the experiment are presented by each fraction f in Figure 3.

Note how for each of the fractions a sigmoidal relationship is present between the response variable and the concentration. In addition, the variability in the response variable is proportional to the size of the response. In Figure 4, a 3-D plot is shown for the mean positive control values over all plates in function of the row and column number. Note that positive control values only contain substrate,

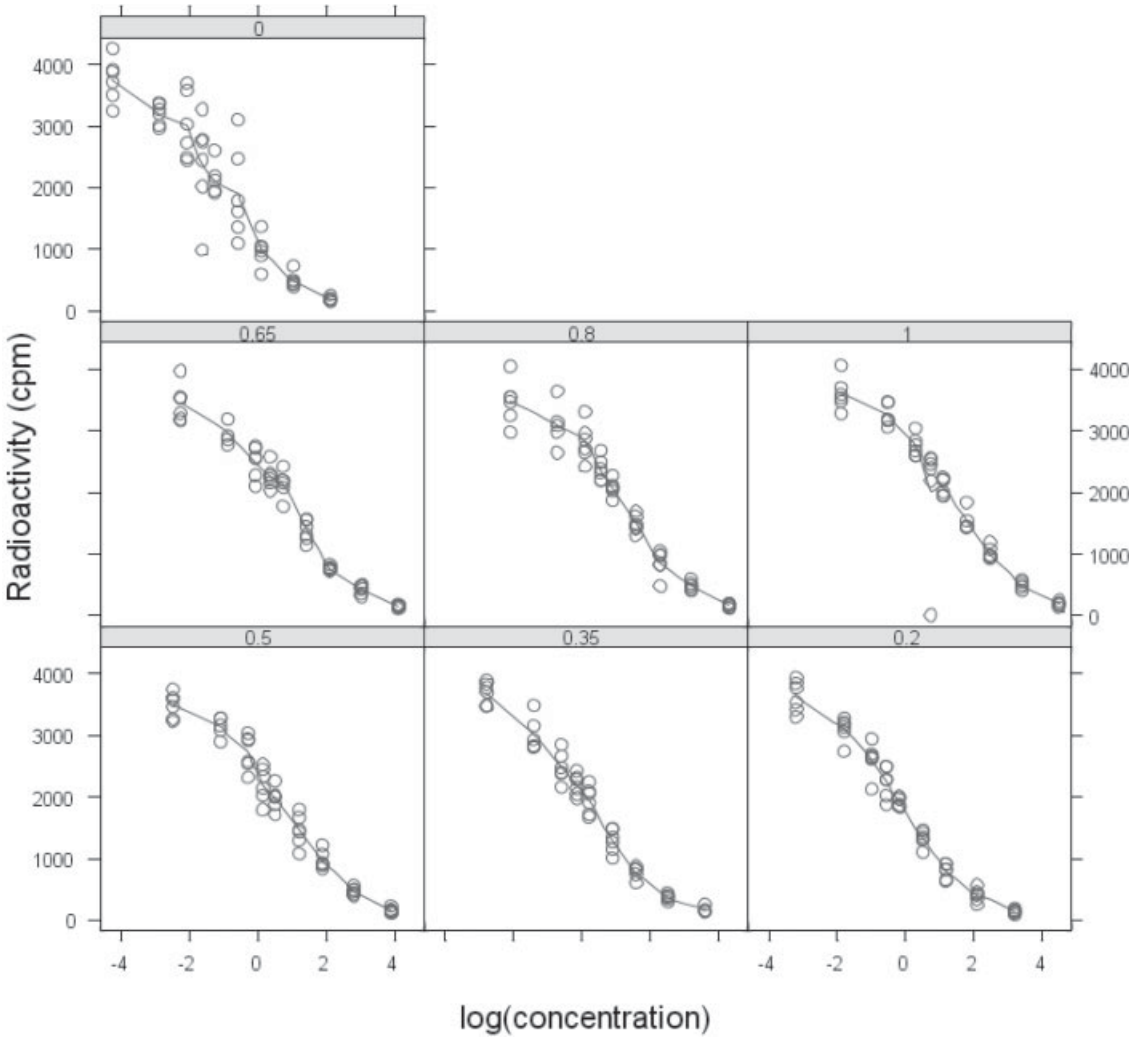

Figure 3 Plot of the raw data by f value. The Y-axis correspond to the measured radioactivity (a measure of amount of substrate transformed) and the X-axis corresponds to the concentration on the log scale. The line represents the arithmetic means by concentration.

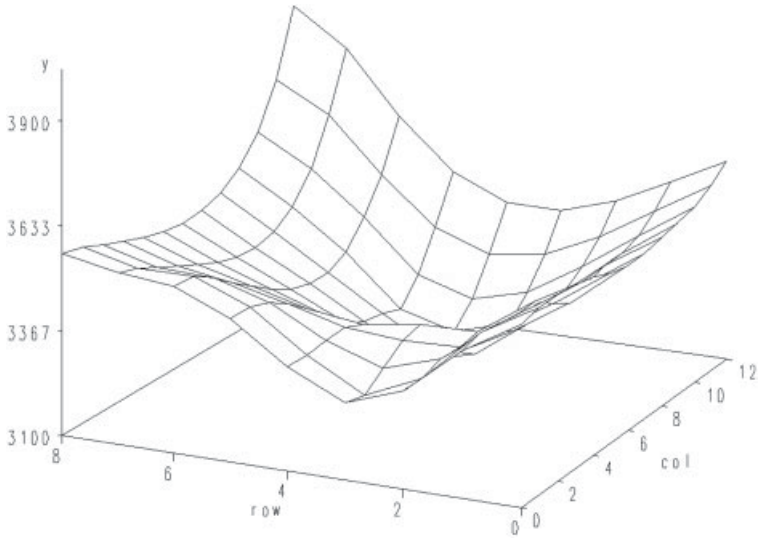

Figure 4 3-D plot of average positive control response values, observed over all plates, versus the row and column number of the corresponding well in which the positive control was present.

reagens and enzyme (all in the same concentration). Therefore these wells are expected to give a similar result and the overall mean by row and column should produce a horizontal plane. In Figure 4 however a clear bowl type response surface can be observed, which indicates that the values within the inside of the plate are the lowest and by going to the outersides of the plate the values increase, indicating a row and column effect. This plate-location effect is typically observed in these 96-well plate experiments and is consistent with what is reported in the literature (Faessel et al., 1999). Therefore, to obtain unbiased results, our analysis has to accommodate for this plate-location effect.

4 Statistical Methodology

4.1 Concentration-response model

The basis of our analysis is to fit a concentration-response curve to each mixture investigated in the experiment. It is assumed that the mean response $E(Y)$ is related to the concentration C by

$$E(Y) = \frac{\theta_1}{1 + \exp\left(\theta_3(\log(C) - \log(IC_{50}))\right)} . \tag{3}$$

Here θ_1 is the expected response at zero concentration level, IC_{50} is the concentration for which half of θ_1 is reached, θ_3 is a slope parameter and C the concentration variable. This model describes a sigmoidally shaped curve for response versus the logarithm of concentration, as was seen in our experiment (see Figure 3). The application of the log-logistic model to concentration-response studies in general dates back to Emmens (Emmens, 1940) and is nowadays still one of the most popular nonlinear models used. In the given parameterization a positive θ_3 value corresponds to a decreasing sigmoidal relationship between the response variable and the concentration. Equation (3) can be simplified to

$$E(Y) = \frac{\theta_1}{1 + \left(\frac{C}{IC_{50}}\right)^{\theta_3}} , \tag{4}$$

the well known Hill model (Hill 1910).

4.2 Separate Ray Model

By using the ray design, it is possible to use a so called separate ray model to jointly fit individual nonlinear curves along each ray (O'Brien, 2004). Equation (4) can be written as

$$y_{rj} = f(\boldsymbol{\theta}_r,\, C_{r,j}) + \varepsilon_{rj}\,, \tag{5}$$

where y_{rj} is the response of ray r at concentration j, f is the nonlinear function, $\boldsymbol{\theta}_r = (\theta_{1,r},\, IC_{50,r},\, \theta_{3,r})$ is a ray specific parameter vector to be estimated, $C_{r,j}$ is the concentration j of ray r and ε_{rj} is the random error which is assumed to be normally distributed with mean zero and variance covariance matrix Σ. Since within a ray the proportion of the two individual compounds is constant, the amount of one of the compounds can be expressed as a linear expression of the other as

$$C_{b,r} = p_r C_{a,r} \tag{6}$$

where $C_{b,r}$ stands for the concentration of compound B in ray $r, C_{a,r}$ stands for the concentration of compound A in ray r and p_r is the constant proportion of the two components within ray r. For each ray r the IC_{50} value, noted as $IC_{50,r}$, consists of an amount $C_{a,r}$ and $C_{b,r}$. By using Eq. (6) we can write

$$IC_{50,r} = C_{a,r} + C_{b,r} = (1 + p_r)\, C_{a,r}\,. \tag{7}$$

By combining Eqs. (1) and (7) the interaction index I for each ray, noted as I_r, can be written as

$$I_r = \frac{IC_{50,r}(IC_{50,b} + p_r IC_{50,a})}{IC_{50,a} IC_{50,b}(1 + p_r)} \tag{8}$$

or equivalently

$$IC_{50,r} = \frac{IC_{50,a} IC_{50,b}(1 + p_r)\, I_r}{IC_{50,b} + p_r IC_{50,a}}\,. \tag{9}$$

Expression (8) and (9) show how for each ray r a combination index can be calculated based on p_r, a known constant, and $IC_{50,a}$, $IC_{50,b}$ and $IC_{50,r}$, parameters obtained from fitting a separate concentration-response model to each of the two pure compounds and the mixtures. In addition to a point estimate we are interested in calculating a $100\,(1-\alpha)\,\%$ *CI* for each I_r parameter. Interaction of the compounds in a specific combination is concluded when the $100(1-\alpha)\,\%$ *CI* does not compromise 1. In order to maintain the experimentwise error rate at its nominal level, the value of α can be adjusted by Bonferroni's inequality resulting in $100\left(1 - \frac{\alpha}{k}\right)\,\%$ *CI*, where k refers to twice the number of mixtures.

Simultaneously, concentration-response curves are fitted for each ray, while Eq. (9) allows to estimate the ray specific combination index I_r. This procedure is implemented using the SAS procedure NLMIXED. An example of the code is given and discussed in more detail in the Appendix. One of the advantages of the NLMIXED procedure is the possibility to specify distributions different from the normal for the response variable. In addition the procedure allows specification of a non-constant variance function, thus allowing for a wide variety of patterns of heteroscedasticity of the response. In our example the variance of the response is modeled as being proportional to a power of the mean, a phenomenon often seen in this type of concentration-response data.

5 Results

The results of the ray specific interaction indices, obtained after fitting the separate ray model using the SAS code presented in the Appendix, are given in Table 1.

From Table 1 we see that all mixtures, except $f = 0.8$, show a moderate ($f = 0.2$ and 0.35) to small point estimate for the interaction index. Notice how for the mixtures $f = 0.2$ and 0.35 the upper limit of the 95% *CI* is smaller than one and hence a statistically significant Loewe synergistic effect is

concluded. No multiplicity correction is applied since these are screening results which serve to detect trends. Formal conclusions will be drawn after a confirmatory experiment.

Finally the adequacy of the used model and the correctness of the model assumptions (e.g. normality) was investigated and concluded to be adequate, using a residual analysis (not shown). An overall idea of the goodness of fit of the model is given by Figure 5, which shows a plot of observed and fitted data for each mixture factor f. The graph indicates that the model fits the data well.

6 Discussion and Conclusion

In the field of oncology, the search for combinations of drugs is widely recognized as being of the utmost importance in the quest for finding successful treatments of cancer. The investigation of combination chemotherapy in clinical trials is very expensive and time-consuming, therefore creating a need for *in vitro* quantification of synergy in a suitable screening model. In order for this screening to be feasible, both the design of the experiment as well as the analysis of the generated data need to be fast, easy and robust. In this paper we show how the fixed-ratio design is used to test a whole series of mixtures for synergy in a single experiment using 96-well plates within an oncology experiment. In this design different mixtures are prepared based on the individual IC_{50} values of the pure compounds A and B. Each mixture is considered as a new compound in which the ratio of the individual amount of drug A and B is kept constant and different concentrations are prepared. In addition, we present a new approach based on a separate ray model in which three parameter log-logistic concentration-response curves are jointly fitted to the individual rays.

While many other techniques have been developed for the assessment of drug interactions, we feel our approach has some important advantages. In the response surface method approach for a mixture of two compounds, often a checkerboard design is used followed by a subsequent modeling of the three dimensional surface of the response in function of the concentration of compound A and B, present in the pure form and in the different mixtures. This approach results in the estimation of one overall interaction parameter. An example of this methodology is given by Machado and Robinson (1994). They describe a response surface model to quantify the interaction of two drugs and the method is illustrated using data from an *in vitro* experiment conducted to evaluate the efficacy of the antiviral drugs AZT and ddI used in combination. In contrast, our procedure produces different interaction indices for mixtures covering the whole spectrum between the two individual compounds. These different interaction indices give a more precise picture of how the type and size of the interaction changes with the mixture composition, a phenomenon that is well known in pharmacology (Tallarida, 2000). Recently some extensions on response surface models are developed which allow estimation of different interaction parameters depending on the ratio of a mixture. Minto et al. (2000) present a response surface model in which quantification of drug interaction over the type of mixture is obtained using an interaction parameter which depends on the ratio of a mixture via a polynomial function. In our method, which also provides information of how interaction can change over mixture type, the response is not modeled as a surface in function of the concentrations of compound A and B over all observed data points. Instead, a two dimensional concentration-response curve along each ray is fitted jointly. For each mixture an interaction index is included based on Eq. (1) which uses the

Table 1 Analysis results.

f-value	Estimate (SE) for I	95% CI for I
0.2	0.64 (0.11)	[0.43; 0.85]
0.35	0.63 (0.11)	[0.41; 0.84]
0.5	0.89 (0.15)	[0.58; 1.19]
0.65	0.82 (0.14)	[0.53; 1.10]
0.8	1.14 (0.20)	[0.76; 1.53]

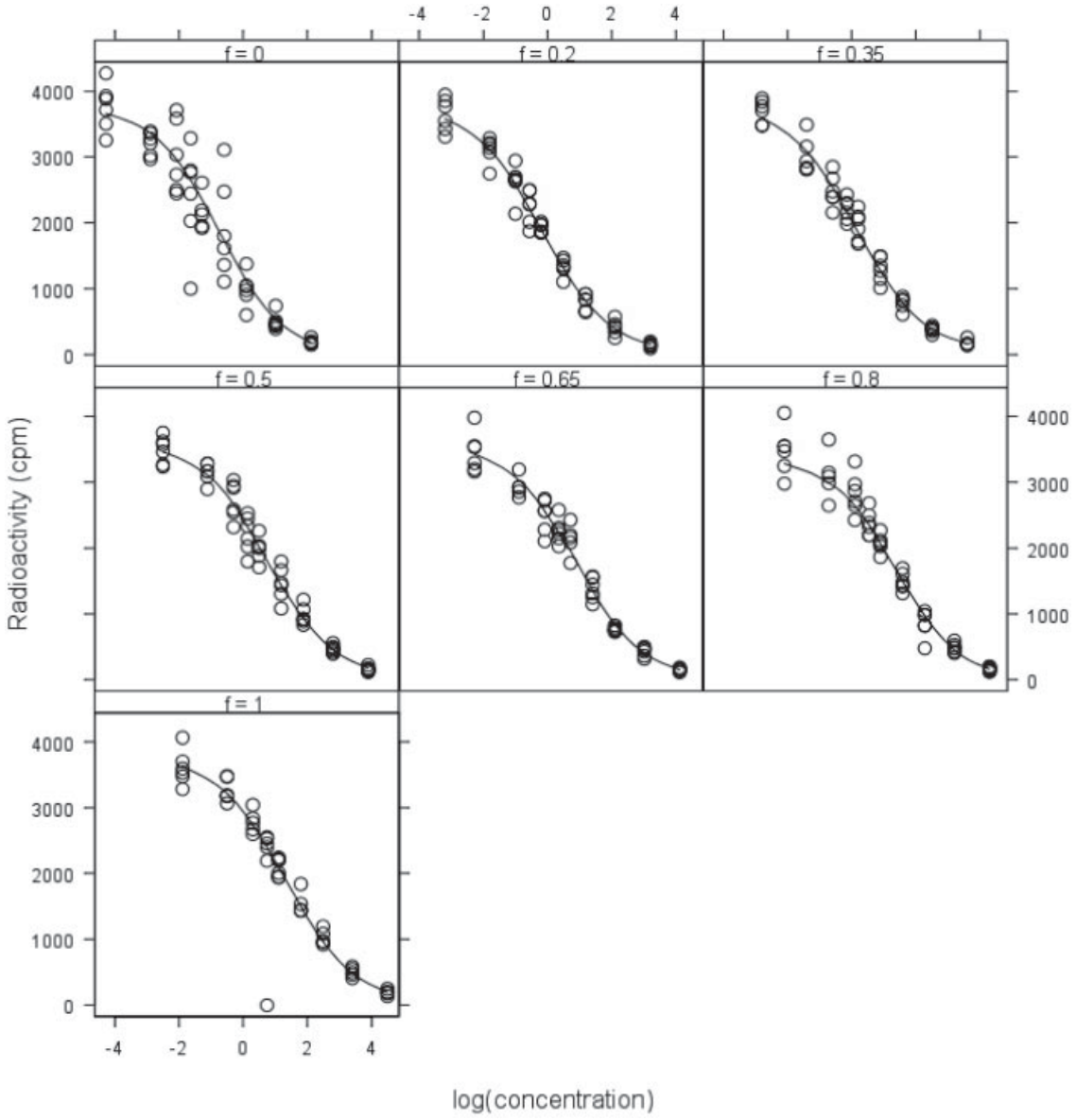

Figure 5 Plot of observed and fitted values by f value. Open circles represent the observed data while the solid lines represent the model predicted mean.

IC_{50} parameter estimates from each mixture and the two pure compounds via Eq. (9). A possible disadvantage of our analysis is that the experiment needs to use a ray design. It can not be used when a factorial or checkerboard design is used. However, a checkerboard design, by its multiplicative nature, often results in a high number of different combinations of concentrations of the compounds. An additional advantage of our approach is that the plate-location effect can be dealt with. Randomization has been advocated as a method to eliminate the plate-location effect (Faessel et al. 1999). Since randomization is based on a probabilistic argument it may not be appropriate when the number of plates in the experiment is small. Joint modeling of the concentration-response curves to both the pure compounds as well as to the mixtures, while adjusting for plate-location effects, as done in our approach overcomes this.

In conclusion, our approach directly estimates the interaction indices and their $100\,(1-\alpha)\,\%$ *CI*'s of the different mixtures while taking into account plate-location effects. In addition it allows for heteroscedasticity and non-normal distributions. The analysis can be implemented using the NLMIXED procedure from the SAS software and an example of code is given.

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Appendix

The SAS program used to analyse the data is shown at the end of the appendix. Notice how θ_{1r}, IC_{50}, r, θ_{3r} (presented as $th1r$, $th2r$ and $th3r$ in the SAS code), c_r and I_r are the three parameters from the log-logistic model, the slope and the interaction index for each of the rays r (with r being 1–7). Table 2 gives the correspondence between the r value and the f value.

The content of the dataset respons is explained in Table 3, where the necessary variables are given in order for the code to work.

The dataset consists of the variables y for the response and *conc* for the applied concentration of the mixture. In addition there are *cc* and *rr*, which respectively contain the mean centered column and row number of the well. The variables *cc*2 and *rr*2 are the square of *cc* and *rr* respectively and are required to fit a quadratic surface.

In the SAS program, the functional model is defined in line [17]. The nonlinear concentration-respons curve (Eq. (4)) is given by the part *th1/den*, while a linear part is added to account for the quadratic row and column effect described in the paragraph on data exploration. The trick for jointly estimating the concentration-respons curves along each ray is obtained by specifying the overal *th*1, *th*2 (which corresponds to the notation IC_{50} in the text) and *th*3 parameters as separate contributions of the different rays (lines [6–8]). The individual I_r parameters are introduced by programming Eq. (9) into the PROC NLMIXED code (lines [10–14]). Notice also how the heteroscedastic variance is accounted for by introducing a power function for the variance. This is implemented by using the $var = sig * sig * conc * rho$ code on line [17].

Table 2 Correspondence between r and f.

r	f
1	1
2	0
3	0.2
4	0.35
5	0.5
6	0.65
7	0.8

Table 3 Content of the SAS data set.

Variable name	Variable description
y	response variable
conc	concentration variable
r	numerical identification of the ray
cc	column number
*cc*2	quadratic column number
rr	row number
*rr*2	quadratic row number

```
[1]PROC NLMIXED data=respons;
[2]  PARMS th11=3800 th12=3800 th13=3800 th14=3800 th15=3600
[3]    th16=3600 th17=3400 th21=3 th22=3 k3=1 k4=1 k5=1 k6=1 k7=1 sig=10 rho=0.5
[4]    th31=1 th32=1 th33=1 th34=1 th35=1 th36=1 th37=1 bcc=0 bcc2=0 brr=0 brr2=0;
[5]
[6]    th1=th11*(r=1)+th12*(r=2)+th13*(r=3)+th14*(r=4)+th15*(r=5)+th16*
       (r=6)+th17*(r=7);
```

```
[7]    th2=th21*(r=1)+th22*(r=2)+th23*(r=3)+th24*(r=4)+th25*(r=5)+th26*
       (r=6)+th27*(r=7);
[8]    th3=th31*(r=1)+th32*(r=2)+th33*(r=3)+th34*(r=4)+th35*(r=5)+th36*
       (r=6)+th37*(r=7);
[9]
[10]    p3=0.368; th23=th21*th22* I3* (1+p3) / (th22+p3*th21) ;
[11]    p4=0.171; th24=th21*th22*I4*(1+p4)/(th22+p4*th21);
[12]    p5=0.092; th25=th21*th22* I5* (1+p5) / (th22+p5*th21) ;
[13]    p6=0.0495; th26=th21*th22* I6* (1+p6) / (th22+p6*th21) ;
[14]    p7=0.0230; th27=th21*th22*I7*(1+p7)/(th22+p7*th21);
[15]
[16]    t=(conc/th2)**th3; den=1+t;
[17]    mean=bcc* cc+bcc2* cc2+brr* rr+brr2* rr2+th1/den; var=sig* sig*mean** rho;
[18]  MODEL y~normal(mean,var);
[19]  PREDICT mean out=twoc;
[20]  RUN;
```

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