# Statistically Designed Experiments to Screen Chemical Mixtures for Possible Interactions

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For the accurate analysis of possible interactive effects of chemicals in a defined mixture, statistical designs are necessary to develop clear and manageable experiments. For instance, factorial designs have been successfully used to detect two-factor interactions. Particularly useful for this purpose are fractionated factorial designs, requiring only a fraction of all possible combinations of a full factorial design. Once the potential interaction has been detected with a fractionated design, a more accurate analysis can be performed for the particular binary mixtures to ensure and characterize these interactions. In this paper this approach is illustrated using an *in vitro* cytotoxicity assay to detect the presence of mixtures of *Fusarium* mycotoxins in contaminated food samples. We have investigated interactions between five mycotoxin species (*Trichothecenes, Fumonisins,* and *Zearalenone*) using the DNA synthesis inhibition assay in L929 fibroblasts. First, a central composite design was applied to identify possible interactive effects between mycotoxins in the mixtures (27 combinations from $5^5$ possible combinations). Then two-factor interactions of particular interest were further analyzed by the use of a full factorial design (5×5 design) to characterize the nature of those interactions more precisely. Results show that combined exposure to several classes of mycotoxins generally results in effect addition with a few minor exceptions indicating synergistic interactions. In general, the nature of the interactions characterized in the full factorial design was similar to the nature of those observed in the central composite design. However, the magnitude of interaction was relatively small in the full factorial design. — *Environ Health Perspect* 106(Suppl 6):1361–1365 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-6/1361-1365groten/abstract.html*

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In the past decade of mixture research a number of test scenarios have been applied to evaluate the effect of a mixture compared to its components. Ideally, one should identify all chemicals in a mixture and determine the toxicity of each of the constituents experimentally or by a review of existing literature. This information is necessary to establish the combined action in subsequent steps. Which test strategy must be followed to detect combined action will largely depend on the number of compounds in a mixture and on the question of whether it is desirable to assess possible interactions between chemicals in a mixture.

One pragmatic approach is to test the toxicity of the mixture without assessing the type of interaction of the chemicals, taking into account that the mixture should be tested at high (effective) concentrations and low (realistic) concentrations. Interactive effects between two or three compounds in a mixture can be identified by physiologically based toxicokinetic modeling or (more empirically) by using isobolographic or response surface analysis. Which model will be used is

often a matter of choice and is dependent on the experimental data set.

The difficulty of studying chemical mixtures arises when one must study the interactive effects of more than three compounds in a mixture. For accurate analysis of interactive effects between chemicals in a defined chemical mixture, statistical designs are necessary to develop the clearest and most manageable toxicologic experiments. Therefore, the combination toxicologist increasingly consults the biostatistician regarding statistical designs for studies.

Full and fractional factorial designs are particularly useful statistical designs. In a full factorial design each of the chemicals in the mixture is studied at all dose levels of the other chemicals. Fractional factorials enable more economy of experimentation because only part of the full factorial is run experimentally (*1*). Another aspect of factorial designs that deserves attention is the fact that the results are often of high precision because for every end point chosen, all data of the experiment are used to calculate a particular effect. For instance, in a recent subacute toxicity study in rats by Groten et al. (*2*), a two-level factorial design was used to describe interactive effects between nine compounds. Instead of the usual comparison of five animals in the test group with five animals in the control group, the effects in that particular study were calculated as the difference of two means of 40 animals each. Therefore, main effects seen in the preliminary studies based on individual dose–response curves appeared to be more pronounced and more easily detectable in the factorial study.

The present study focuses on the economic use of factorial designs in combination with response surface analysis in mixture studies. Multifactor experimental plans designed specifically for exploring response surfaces do not have a factorial structure. They nevertheless yield high precision results because the experimental points have a well-determined spread. There are, however, multifactor experimental models designed specifically for the exploration of response surfaces involving a lower number of experimental points than with a full multifactorial response surface design. The two types of designs frequently used are the central composite design and the Box Behnken design (*3*). With these types of designs it

is possible to screen interactions between more than two compounds in part of the established response surface area. Significant interactions between compounds in a certain dose–response area as found in this first screening can be verified in a follow-up study by using whole-mixture studies, isobolograms, or full factorial designs. This rationale of using a stepwise approach was followed in the present study to efficiently screen interactions between mycotoxins in a bioassay.

## Mycotoxins in Food and Raw Materials: A Case Study of Applied Mixture Research

Mycotoxins belong to an important group of hazardous compounds that occur simultaneously in food or raw materials. In the quality control of foodstuffs, bioassays have been applied to screen for the presence of mycotoxins, especially for Fusarium mycotoxins, because Fusarium fungus species simultaneously produce several toxic secondary metabolites (Fumonisins, Trichothecenes, Zearalone). In fact, a bioassay based on the inhibition of DNA or protein synthesis in the cells has been applied for the screening of Fusarium mycotoxins in contaminated grain samples (4–6). Binary combinations of Trichothecenes increased the effects (i.e., growth inhibition) observed in the bioassay (7,8). However, there is a clear lack of information on effects of exposure to more than two mycotoxins in a mixture, as are present in food products.

In an attempt to develop more generic test strategies for the natural co-occurrence of Fusarium mycotoxins (9,10), we investigated the combined action of five Fusarium mycotoxins by using a DNA-inhibition assay with mammalian cells.

In this paper, emphasis is placed on the use of central composite designs—part of a stepwise approach to test the combined toxicity in a bioassay. The purpose of the studies and all details of the experiments are presented elsewhere (11).

### Design of the Studies

Five mycotoxins were tested: T-2 toxin (T-2), deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), and fumonisin $B_1$ ($FB_1$). The final concentration of dimethyl sulfoxide (DMSO) used as solvent in the culture medium was 0.5%. In preliminary studies it was shown that this is a nontoxic concentration of the solvent. Control medium was prepared with 0.5% DMSO without mycotoxins in the culture medium.

The study was built up in three phases. First, a whole-mixture study was carried out to examine combined action of the five mycotoxins; the mixture was considered a single compound. Second, a central composite design was applied to identify possible interactive effects between the mycotoxins in the mixtures at several dose levels. Finally, two-factor interactions of particular interest were further analyzed by means of a full factorial design.

The dose selection for each mycotoxin was based on the preliminary dose–response studies for single mycotoxins. Five equidistant concentration levels were established such that the maximum effect induced by a single chemical would never exceed 30% DNA synthesis inhibition as compared to control. The exposure levels of each mycotoxin were coded –2, –1, 0, +1, and +2. For more details about the bioassay we refer to Tajima et al. (11).

The central composite design was applied to examine possible two-way interactions in the mixtures of five Fusarium mycotoxins for DNA synthesis inhibition. In total, 27 combinations were chosen from $5^5$ possible combinations of a full factorial design associated with five chemicals each at five concentration levels.

This included 16 dose combinations (cube points) derived from 1/2nd fraction of a $2^5$ factorial design, 1 dose combination (center point) and 10 additional dose combinations (star points), as summarized in Table 1. The 1/2 nd fraction part was made of combinations of each mycotoxin at either –1 or +1 dose level. A center point was a combination of all five chemicals at the middle dose (0 level) among five concentrations. Star points were chosen at the lowest (–2 level) or highest (+2 level) concentration in the presence of all other chemicals at the middle concentrations. The star points were needed to establish curvature in relation with each of the mycotoxins. A general reference for central composite design is Box and Draper (12). Concurrently, the dose–response study for each mycotoxin was also conducted.

The central composite design was intended as a first step to screen possible interactions between compounds in the mixture. Traditionally, fractionated factorial designs are used for screening purposes. They are normally followed by full factorial or central composite designs to obtain more detailed information about the interactions observed. We used a slightly different approach. Our initial central composite design revealed interactions

**Table 1.** Test groups and exposure levels in a bioassay measuring DNA synthesis inhibition in L929 cells exposed to five mycotoxins in combination.

| T-2 | DON | NIV | ZEA | $FB_1$ | |
|---|---|---|---|---|---|
| − | − | − | − | + | Cube points |
| + | − | − | − | − | 1/2nd fraction of $2^5$ design |
| − | + | − | − | − | |
| + | + | − | − | + | |
| − | − | + | − | − | |
| + | − | + | − | + | |
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| + | − | + | + | − | |
| − | + | + | + | − | |
| + | + | + | + | + | |
| −2 | 0 | 0 | 0 | 0 | Star points |
| +2 | 0 | 0 | 0 | 0 | |
| 0 | −2 | 0 | 0 | 0 | |
| 0 | +2 | 0 | 0 | 0 | |
| 0 | 0 | −2 | 0 | 0 | |
| 0 | 0 | +2 | 0 | 0 | |
| 0 | 0 | 0 | −2 | 0 | |
| 0 | 0 | 0 | +2 | 0 | |
| 0 | 0 | 0 | 0 | −2 | |
| 0 | 0 | 0 | 0 | +2 | |
| 0 | 0 | 0 | 0 | 0 | Center points |

between ZEA and NIV and between $FB_1$ and ZEA. The interactions found in the first design would be conclusive if the second-order model commonly used for central composite designs fit the data well. However, this was not the case for all compounds. For instance, a cubic term in $FB_1$ may be real but it may also indicate a complex type of interaction. Therefore, a $5 \times 5$ full factorial design was conducted for ZEA and $FB_1$, for which interaction was detected in the central composite design. The interaction between these factors was the most likely reason for the lack of fit indicated by cubic $FB_1$ and ZEA terms. The interaction between T-2 and NIV was less problematic. However, we also used a full $5 \times 5$ design to study this interaction in more detail. Thus, two $5 \times 5$ designs were carried out in the follow-up study.

To obtain comparable results to those in the central composite design, the same dose levels were used. The other mycotoxins were kept at a constant concentration (0 level). In both designs each combination was replicated four times on separate plates. The wells on each plate were randomly selected for exposure of mixtures to prevent systematic error between the wells.

A dose–response relationship was established for each single mycotoxin, with five dose levels to predict an effect of any combination of mycotoxins. The predicted values were calculated and compared to the data observed in the central composite study.

The radioactivity of the harvested cells was related to the dosage of the mycotoxins using a generalized linear model (GLM) with a variance proportional to the mean and a logarithmic link function (13). Radioactivity (in cpm) measured in the cells treated with mycotoxins was related to the values of the negative controls of the respective plates by using the mean of the logarithms of cpm of these controls as an offset (11).

Central composite design was used to enable the fitting of first- and second-order equations. These have linear and quadratic coefficients for the individual mycotoxins and product terms for the interaction between two mycotoxins. The model for the central composite design was built by using forward selection of cubic terms and backward elimination of interaction terms. Selection and elimination were carried out with large-sample F tests based on log likelihoods. As an example, the following

equation would have to be used for a study when only two compounds are tested:

$$\ln(\% \text{ of control})$$
$$= \ln(100) + d + a1x + a2x2 + b1y$$
$$+ b2y2 + c1xy$$

where $x$ and $y$ are the doses of each compound, e.g., compound A and B, $d$ is the unknown constant parameter, $a1$ and $a2$ are unknown parameters associated with main effect of compound A, $b1$ and $b2$ are unknown parameters associated with the main effect of compound B, and $c1$ is the parameter associated with the interactive effect between compound A and B. If the product term between A and B is not significant, the model can be considered additive. The parameters (coefficients) are estimated under the above GLM. It is also possible to test for lack of fit of these equations by calculating cubic coefficients of the mycotoxins. A particular cubic coefficient is, however, aliased with product terms of the dosage of the corresponding mycotoxin with the squared dosage of the other mycotoxins (12). Thus several equations might be calculated because of the aliases pattern.

## Results

In the central composite design with five mycotoxins, nonlinear regression analyses were carried out for single mycotoxins and their 27 different combinations. The equations associated with the dose response of each of the individual mycotoxins were calculated based on the GLM. For each mycotoxin the effect was converted into a percentage of the response to establish the final equations. A dose-related increase in the inhibition of the DNA synthesis was observed in cells exposed to DON, NIV, T-2, and ZEA. $FB_1$ showed an increase in DNA synthesis at the high concentration level.

Cells exposed to a mixture of mycotoxins showed an inhibition of DNA synthesis ranging between 54 and 28%. The data on DNA synthesis inhibition were used to calculate an equation to describe their dependency on mycotoxin concentrations. Thus we have for each combination of mycotoxins an observed and a predicted value. The discrepancy between observed and predicted data was fairly low (i.e., residuals were all less than 3.8%), demonstrating the accuracy of the equation established in the study. The results of the analysis to describe the main effects of each mycotoxin and the possible interactions are

summarized in the following equation (compounds are expressed in micrograms per milliliter; T-2 is expressed in nanograms per milliliter):

$$\ln(\% \text{ of control}) =$$
$$\ln(100) - 4.7 + 0.443\text{T-2}$$
$$- 0.089\text{T-2}^2 + 2.82\text{ZEA}$$
$$- 0.991\text{ZEA}^2 + 0.1206\text{ZEA}^3$$
$$+ 2.157\text{DON} - 9.41\text{DON}^2$$
$$+ 1.313\text{FB}_1 - 0.354\text{FB}_1^2$$
$$+ 0.0421\text{FB}_1^3 + 2.75\text{NIV}$$
$$- 0.22\text{ZEA} \times \text{FB}_1 - 4.03\text{T-2}$$
$$\times \text{NIV}.$$

In the central composite design, two significant interactions were observed between ZEA and $FB_1$ ($p < 0.001$) and between T-2 and NIV ($p < 0.01$). The interactions between these mycotoxins were further characterized by comparing the slope of the dose–response curves in the presence of the lowest (–2) or the highest (+2) level of another mycotoxin (Figure 1). The effect of ZEA was not clearly indicated in the presence of –2 level of $FB_1$. However, in the presence of +2 level of $FB_1$, the effect of ZEA became apparent, which resulted in decrease of DNA synthesis in a dose-dependent manner. Thus the interaction between ZEA and $FB_1$ could be interpreted as synergistic with respect to the inhibition of DNA synthesis.

The interaction between NIV and T-2 was characterized as synergistic because the effect of T-2 was potentiated in the presence of the higher NIV level (Figure 1B). As indicated by the lack of parallelism (i.e., departure from additivity) of the two lines, the intensity of the interaction increased with concentration of T-2 and reached a maximum when the concentration of T-2 was at the +2 level. Thus in both cases the magnitude of the interactions was dependent on the concentrations of the mycotoxins in the mixture.

In the present study, the central composite design was used as a first screening to detect possible interaction between mycotoxins. The validity of this design to identify or screen possible interactions was evaluated by performing a full factorial design.

In the full factorial design, only those mycotoxins were tested that showed significant interactions in the central composite design. The full factorial design revealed that ZEA decreased the DNA synthesis in a dose-dependent manner and its effect was slightly affected by $FB_1$ in
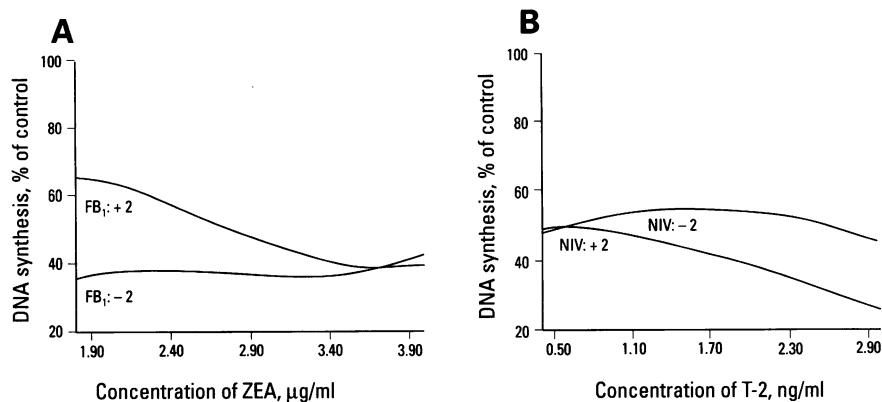
## A



## B



**Figure 1.** Two-factor interactions between (A) ZEA and FB₁ and (B) T-2 and NIV on DNA synthesis inhibition in L929 cells exposed to mixtures of five mycotoxins in a central composite design [modified from Tajima et al. (11)]. Dose–response relationships were calculated with an equation given by the central composite study in Results. Dose–response relationships for ZEA or T-2 were computed in the presence of the highest (+2) or the lowest (−2) level of its interactive counterpart i.e., NIV or FB₁. Dose levels of three other mycotoxins were kept at the middle (0) level. Interactive effects between two compounds are indicated by the absence of parallel curve. If the curves had been parallel, it would have indicated effect additivity.
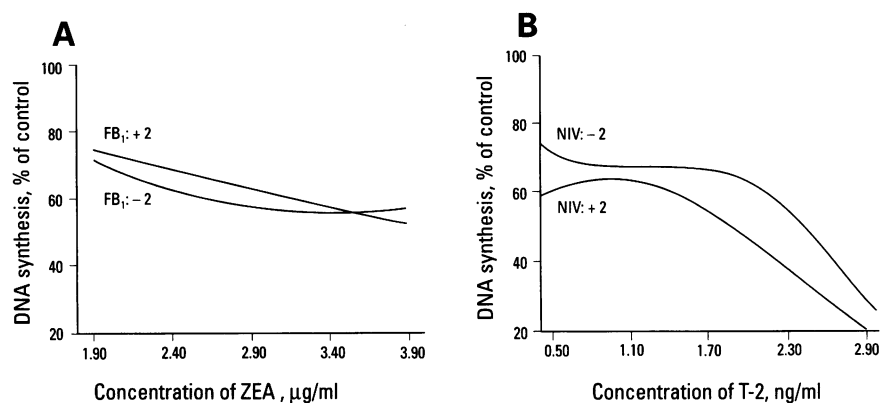
## A



## B



**Figure 2.** Fitted curves representing interactions between (A) ZEA and FB₁ and (B) T-2 and NIV on DNA synthesis inhibition in L929 cells exposed to mixtures of five *Fusarium* mycotoxins. Each of the interactions was studied in a separate 5×5 factorial design in which the three mycotoxins not involved in the interaction were applied at a constant concentration throughout. The figure represents the case in the presence of +2 or −2 level of the interactive mycotoxins and 0 level of three other mycotoxins.

the high-dose range of ZEA (Figure 2A). This implies that in the presence of a high dose of FB₁, the effect of ZEA on DNA synthesis was more pronounced than expected on the basis of additivity. Figure 2B shows the dose-dependent interaction between T-2 and NIV. At the higher levels of T-2, the decreasing DNA synthesis caused by NIV is more pronounced than predicted on the basis of effect additivity. The maximum effect of NIV on DNA synthesis was seen at the middle concentrations of T-2. Thus, overall, the interaction can be considered as a more than additive effect for DNA synthesis inhibition. In both cases of two-factor interactions, the magnitude of the interactions changed depending on the concentration

levels tested. Both in the central composite study as well the factorial design, the interaction profiles observed for T-2 and NIV were more or less similar. For ZEA/FB₁ the observed interaction apparently does not have the same magnitude, but in both designs the interaction was interpreted as synergistic. In general, the nature of interactions characterized in the full factorial design was similar to those observed in the central composite design. However, the magnitude of interaction was smaller, as indicated by small differences in the slope of the curves.

## Discussion

Our study demonstrated that there were two synergistic interactions between the

binary pairs of the mycotoxins in the mixture. The intensity of these interactions appeared to be dose dependent, demonstrating the advantage of this model in describing the change of the interaction with dosages, not merely with the average nature of interactions in an overall dosage. In the central composite design several dose levels are included to depict the dose–response curve as accurately as possible. In particular, both designs (central composite and full factorial) provided a similar dose-related trend of interaction between T-2 and NIV in which the interaction was most evident around the middle concentrations. The interaction between NIV and T-2 seems to follow a general pattern. At a low concentration of T-2, NIV has no appreciable effect, whereas NIV does have an effect when the concentration of T-2 is raised. Further quantification of the interaction, however, revealed that the pattern was not always consistent in the central composite design. A possible reason for this is in the aliasing of first- and second-order interactions in the central composite design. This corroborates the follow-up with a full factorial design.

In contrast to NIV/T-2 interaction, the feature of the interaction between ZEA and FB₁ observed in the full factorial design was not completely similar with that illustrated by the central composite design, although in both cases the interactions point in the direction of synergism. This may be explained by the fact that using cubic terms for a central composite design causes a flexibility of the model prediction (12). In general, the nature of the interactions characterized in both designs was similar for both interactions but the magnitude of interactions seemed to be more emphasized in the central composite design. These phenomena reflect the general thought that a model prediction of the central composite design is best in the central area of the response surface and uncertainty of the prediction may increase with increased distance from the central part of the response surface. Nevertheless the central composite design was satisfactory to our objective, which mainly focused on the screening of any possible interactions in a complex mixture.

It is beyond the scope of our investigation to explain the interactions on the basis of the mechanism of action of the mycotoxins tested. More studies are needed to investigate how these interactions occur— studies that may include the rate of transport of mycotoxins into cells, the change

in affinity for the active binding site, or the metabolism of mycotoxins. The present study suggests that despite a few small and specific interactions, most combinations of Trichothecenes acted additively in terms of inhibition of DNA synthesis for L929 cells. In this paper we have illustrated this additivity by using known mixtures of mycotoxins. Follow-up studies in our lab are currently measuring the DNA synthesis inhibition capacity of unknown samples of mycotoxins. For instance, for barley samples we have shown that this bioassay is able to detect mycotoxin mixtures that were present in a concentration below the detection limit of the gas chromatography analysis (11). The final goal is to incorporate this bioassay in a test strategy to screen combinations of Tricothecenes in contaminated samples of food and raw materials. In this case the bioassay may detect the presence of mixtures of toxins and is complementary to the chemical analysis.

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