Mixture Design and Multivariate Analysis in Mixture Research

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Mixture design has been used to identify possible interactions between mutagens in a mixture. In this paper the use of mixture design in multidimensional isobolographic studies is introduced. Mutagenicity of individual nitro-polycyclic aromatic hydrocarbons (PAH) was evaluated in an organic extract of diesel exhaust particles (DEPs). The particles were extracted with dichloromethane (DCM). After replacing DCM with dimethyl sulfoxide, the extract was spiked with three individual nitro-PAH: 1-nitropyrene, 2-nitrofluorene, and 1,8-dinitropyrene. The nitro-PAH were added separately and in various combinations to the extract to determine the effects of each variable and to identify possible interactions between the individual nitro-PAH and between the nitro-PAH and the extract. The composition of the mixtures was determined by mixture design (linear axial normal) with four variables (the DEP extract and the three nitro-PAH), giving 8 different mixtures plus a triplicate centerpoint, i.e., a total of 11. The design supports a model with linear and interaction (product) terms. Two different approaches were used: traditional mixture design within a well-defined range on the linear part of the dose–response curves and an isobolographic mixture design with equipotent doses of each variable. The mixtures were tested for mutagenicity in the Ames assay using the TA98 strain of Salmonella typhimurium. The data were analyzed with projections to latent structures (PLS). The three individual nitro-PAH and the DEP extract acted additively in the Ames test. The use of mixture design either within a well-defined range of the linear part on the dose–response curve or with equipotent doses saves experiments and reduces the possibility of false interaction terms in situations with dose additivity or response additivity.

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Different strategies have been described for the toxicologic evaluation of mixtures: integrative (studying the mixture as a whole), dissection (dissecting or fractionating a mixture to determine causative constituents), and synthetic (studying interactions between agents in simple combinations) (1). In a recent study fractionation of organic extracts followed by recombination of the fractions was introduced as a strategy for toxicologic evaluation of mixtures (2). Spiking has been used to evaluate the mutagenicity of individual polycyclic aromatic hydrocarbons (PAH) in a complex mixture (3,4).

In studies using either the synthetic approach, fractionation/recombination, or spiking, well-defined variables may be combined differently to obtain the effect of each variable and possible interactions between them. The composition of the new mixtures may be determined by means of statistical experimental design, which is increasingly used in mixture research. Most commonly factorial designs are used (4–7). However, factorial designs require orthogonality, which is not always the case in liquid mixtures (8,9). As an alternative, mixture design has been used in the evaluation of exhaust emissions of fuels (3,10), in inhalation studies with vapor mixtures of hydrocarbons (11), and to recombine the fractions of organic extracts of diesel exhaust particles (DEPs) (2).

In this study, mixture design was used to evaluate the mutagenicity of individual nitro-PAH in a DEP extract, which is a complex mixture of organic substances such as aliphatic hydrocarbons, PAH, nitro-PAH, and polar compounds (12). DEP extracts are mutagenic in the Ames assay primarily because of the direct-acting nitro-PAH. In this study, the DEP extract was spiked with three different nitro-PAH: 1-nitropyrene, 2-nitrofluorene, and 1,8-dinitropyrene. The nitro-PAH were added separately and in various combinations to the extract to identify possible interactions between the individual nitro-PAH and between the nitro-PAH and the DEP extract. The mixtures were tested for mutagenicity in the Ames assay. The results were analyzed with projections to latent structures (PLS). The models obtained are empirical, and interaction (product) terms are indications of possible synergism or antagonism and should be evaluated with respect to dose additivity and response additivity (13). As an alternative to traditional mixture design, a mixture design with equipotent doses was used in a multidimensional isobolographic study.

Materials and Methods

Extraction of Diesel Exhaust Particles

Diesel exhaust particles (Standard Reference Material 1650) were obtained from U.S. National Institute of Standards and Technology (Gaithersburg, Maryland). Extraction of DEPs was carried out as described by Alsberg et al. (14) with a few modifications as described below. A sample of 500 mg DEPs was collected in filters (Schleicher & Schuell no. 595, Dassel, Germany) and placed in a 250-ml Soxhlet extractor. (Brand, Wertheim, Germany). Approximately 250 ml of dichloromethane (DCM) (Merck, Darmstadt, Germany, >99.8% pure) was used as a solvent, giving a siphoning rate of 20 min. The extraction proceeded for 16 hr. The extract was evaporated to approximately 5 ml in rotovap and to approximately 1 ml under dry nitrogen and dissolved in dimethyl sulfoxide (DMSO) (EMS, Fort Washington, Pennsylvania, >99.9%).
Nitro-Polycyclic Aromatic Hydrocarbons

1-Nitropyrene and 2-nitrofluorene were obtained from TCI (Toyko, Japan), and 1,8-dinitropyrene from Aldrich (Milwaukee, Wisconsin). The individual PAHs were dissolved in DMSO. A volume of 100 μl test solution was added to each plate.

Ames Salmonella Assay

The standard plate incorporation assay as described by Maron and Ames (15) was used for mutagenicity testing. However, the doses were determined by the statistical experimental design. The Salmonella typhimurium strain TA98 was obtained from B.N. Ames (University of California, Berkeley, California).

Statistical Experimental Design

The study was designed as a mixture (linear axial normal) design (9) with four variables (the extract and the three nitro-PAH), giving eight blends plus a triplicate centerpoint, i.e., a total of 11 mixtures. The design supports a model with linear and interaction (product) terms. The centerpoint is important to verify linearity. The replicates are used to evaluate reproducibility (pure error). The blend matrix is shown in Table 1. The doses are between 0 and 1, meaning 0 or 100% of each variable, respectively. The high level (1 in the matrix) was chosen from the linear part of the dose–response curves determined in introductory dose–response studies for each variable (not shown). The high level of DEPs corresponds to 200 μg of particles per plate. The high levels of the nitro-PAH correspond to 0.40 μg 1-nitropyrene per plate, 3.00 μg 2-nitrofluorene per plate, and 0.00150 μg 1,8-dinitropyrene per plate.

The study was also performed as an isobolographic mixture design applying equipotent doses of each variable. Two different response levels for TA98 were chosen (300 and 600 revertants per plate). The design at each level supports a model with linear and interaction (product) terms. The isobolographic blend matrix is shown in Table 2. The equipotent dose of each variable was determined by linear regression analysis of the dose–response curves determined introductory for each variable (not shown). The doses of the variables anticipated to correspond to 300 revertants per plate were 0.250 μg 1-nitropyrene per plate, 1.57 μg 2-nitrofluorene per plate, 0.00127 μg 1,8-dinitropyrene per plate, and 166 μg DEPs per plate. The doses of the variables anticipated to correspond to 600 revertants per plate were 0.487 μg 1-nitropyrene per plate, 3.10 μg 2-nitrofluorene per plate, 0.00250 μg 1,8-dinitropyrene per plate, and 344 μg DEPs per plate.

Multivariate Data Analysis

Multivariate data analysis and modeling were performed with PLS using Modde 4.0 for Windows (Umetri AB, Umeå, Sweden). The nonisobioic and isobolic data were analyzed separately. Before the analysis the blend matrix was augmented with interaction terms to give a full blend model (X matrix). The data were scaled to unit variance before the PLS analysis. The models were evaluated by means of unscaled PLS regression coefficients. To evaluate the significance of the interaction terms, the following approach was used (11). First, a full model with all interaction terms was calculated. In each full model the interaction term with the lowest variable influence was eliminated one at a time. This procedure was repeated until the best model was obtained, both with respect to correlation coefficients \( r^2 \) and prediction coefficients \( Q^2 \). The latter is obtained after cross-validation and is important to avoid overfit. In addition, the significance of the remaining interaction terms was evaluated on the basis of 95% confidence intervals.

Results

Table 1 shows the blend matrix (first four columns) for the traditional (nonisobolic) mixture design and the response representing mutagenicity in S. typhimurium strain TA98.

A full model with five variables gives six possible binary interactions. First, a full model with all interaction (product) terms was calculated. However, all interaction terms were insignificant, although the best model in terms of high \( r^2 \) and highest possible \( Q^2 \) contained one insignificant interaction term. Figure 1 shows the unscaled PLS regression coefficients with 95% confidence intervals. These PLS regression coefficients are not orthogonal because the starting point was a mixture and should therefore

Table 1. The traditional (nonisobolic) blend matrix and the response (revertants per plate) in TA98.

| Mixture no. | NP | NF | DNP | DEPs | Response  |
|-------------|----|----|-----|------|-----------|
| 1           | 1  | 0  | 0   | 0    | 433       |
| 2           | 0  | 1  | 0   | 0    | 698       |
| 3           | 0  | 0  | 1   | 0    | 373       |
| 4           | 0  | 0  | 0   | 1    | 388       |
| 5           | 0.625 | 0.125 | 0.125 | 0.125 | 483       |
| 6           | 0.125 | 0.125 | 0.125 | 0.125 | 576       |
| 7           | 0.125 | 0.125 | 0.125 | 0.625 | 423       |
| 8           | 0.125 | 0.125 | 0.125 | 0.625 | 373       |
| 9           | 0.25 | 0.25 | 0.25 | 0.25 | 506       |
| 10          | 0.25 | 0.25 | 0.25 | 0.25 | 461       |
| 11          | 0.25 | 0.25 | 0.25 | 0.25 | 480       |

Abbreviations: DNP, 1,8-dinitropyrene; NF, 2-nitrofluorene; NP, 1-nitropyrene. *The highest dose (11) corresponds to 0.40, 3.00, 0.00150, and 200 μg/plate of NP, NF, DNP, and DEPs, respectively. Each response is the mean of three parallels.

Table 2. The isobolic blend matrix and the responses (revertants per plate) in TA98 at two response levels.

| Mixture no. | NP | NF | DNP | DEPs | TA98 high | TA98 low  |
|-------------|----|----|-----|------|-----------|-----------|
| 1           | 1  | 0  | 0   | 0    | 598       | 271       |
| 2           | 0  | 1  | 0   | 0    | 625       | 333       |
| 3           | 0  | 0  | 1   | 0    | 717       | 321       |
| 4           | 0  | 0  | 0   | 1    | 677       | 320       |
| 5           | 0.625 | 0.125 | 0.125 | 0.125 | 574       | 260       |
| 6           | 0.125 | 0.125 | 0.125 | 0.125 | 562       | 335       |
| 7           | 0.125 | 0.125 | 0.125 | 0.625 | 602       | 292       |
| 8           | 0.125 | 0.125 | 0.125 | 0.625 | 611       | 295       |
| 9           | 0.25 | 0.25 | 0.25 | 0.25 | 594       | 259       |
| 10          | 0.25 | 0.25 | 0.25 | 0.25 | 564       | 298       |
| 11          | 0.25 | 0.25 | 0.25 | 0.25 | 620       | 276       |

The high dose (1) corresponds to 0.487, 3.10, 0.00250, and 344 μg/plate of NP, NF, DNP, and DEPs, respectively, at the high response level. The high dose (1) corresponds to 0.250, 1.57, 0.00127, and 166 μg/plate of NP, NF, DNP, and DEPs, respectively, at the low response level. Each response is the mean of three parallels.
be interpreted the same way as regression coefficients that come from an evaluation of a Cox mixture model (16). The coefficients are proportional to the change in response when a factor is changed from the centroid to its maximum and the proportions of all other factors are kept constant. As a consequence some PLS regression coefficients representing main effects will be negative.

Table 2 shows the blend matrix (first four columns) for the isobolic mixture design and the two responses representing mutagenicity at two different response levels in S. typhimurium strain TA98. The design matrices in Tables 1 and 2 are identical; the numbers refer to the proportions of each variable in each mixture. These proportion numbers represent different doses as described in “Materials and Methods” and in the table legends.

The PLS analysis of the isobolic data revealed no significant terms—neither linear (main effects) nor interaction (product) terms. Figure 2 shows the unscaled PLS regression coefficient for the four variables and one interaction term in TA98 at the higher response level. A similar picture is obtained for the low response level (not shown). Because the responses are independent on the variables, $r^2$ and $Q^2$ become low. The presence of significant interaction terms would improve the models because the responses would be dependent on the combination of some variables.

**Discussion**

According to terminology frequently used in mixture research, interactions may be synergistic or antagonistic, whereas no interaction is referred to as additivity (17). Multivariate data analysis gives empirical models after a mathematical adaptation to the experimental data and should be interpreted with care. It is important to avoid overfit. The software used for multivariate data analyses can in principle model any swarm of data points by using a complicated mathematical equation containing lower and higher order interaction terms and quadratic and even cubic terms. This gives high correlation coefficients. However, these models may have poor prediction properties, and many of the terms in the equation are simply due to the variability (noise) in the data set. Therefore, cross-validation (giving the prediction coefficient $Q^2$ and confidence intervals are important (2,4,11,18). Further evaluation of the models may be performed by predictions and verifications by new experiments. When removing insignificant terms from the model, $r^2$ decreases whereas $Q^2$ increases. However, this procedure gives the best model in terms of being as good as the data permit.

The traditional mixture design used in this study supports an empirical model with linear and interaction (product) terms. The design may be expanded to support models with quadratic and even higher order terms. This requires a higher number of mixtures (combinations of variables) to be tested. Furthermore, because interactions indicate a kind of curvature, the regression technique may not be able to distinguish between quadratic and interaction terms. In addition, in mutagenicity testing in the Ames assay, toxicity may occur at high doses of test substances, reducing the mutagenic response. In mixtures, this kind of curvature may be misinterpreted as genotoxic interactions.

Hence, studying interactions within a range of the dose–response curve that can be described only with linear and product terms reduces the number of required mixtures and makes the interpretation of interaction terms easier. In fact, interaction terms in situations with dose additivity are in principle avoided. Hence, positive interaction terms are probably due to synergism. Negative interaction terms may be due to antagonism or response additivity and may be further evaluated with respect to response addition in isobolographic studies.

With a high number of variables in factorial designs, the span between the combined dose with all variables at low levels and the combined dose with all variables at high levels becomes large. In mixture design, when some variables are at high levels the others are at correspondingly low levels. As a consequence, mixture design makes it possible to keep the total response at acceptable levels.

It should, however, be emphasized that factorial design is generally easier to handle than mixture design, primarily because of the orthogonality. Factorial design is suitable for fractionated designs (8). Mixture design is as a general rule required for the generation of blend matrices of liquid mixtures (e.g., fuels). Mixture design is also convenient when the mixture is constrained (9), as demonstrated in fuel testing (10).

In this study no significant interactions were observed between the different nitro-PAH or between the nitro-PAH and the DEP extract, implying additivity between the four variables. However, there were indications of interactions between 1-nitrofluorene and the DEP extract. This possible interaction was insignificant compared to the variability (noise) in the data. Interactions between primary mutagens and between PAH and primary mutagens in binary mixtures have, however, been reported previously (19–21). On the other hand, additivity was reported between individual PAH and between PAH and DEP extracts (4).

Both approaches—the traditional and the isobolographic mixture design—make it possible to study many variables with a limited number of mixtures and revealed essentially the same result. A major drawback with the isobolic mixture design is that it is difficult to hit the same response of all variables in the designed experiments in spite of much introductory work to find the equipotent doses of each variable. The isobolic approach is designed for identifying interactions only and gives poor models when there are no significant product terms because the response is independent of the variables. The main advantage with isobolographic studies in general is that false interaction terms in situations with dose additivity and response additivity are avoided (13).
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