TOXICOLOGICAL SCIENCES **88(2)**, 287–297 (2005)
doi:10.1093/toxsci/kfi275
Advance Access publication August 4, 2005

A Unifying Concept for Assessing Toxicological Interactions: Changes in Slope

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Received May 5, 2005; accepted July 12, 2005

Robust statistical methods are important to the evaluation of toxicological interactions (i.e., departures from additivity) among chemicals in a mixture. However, different concepts of joint toxic action as applied to the statistical analysis of chemical mixture toxicology data or as used in environmental risk assessment often appear to conflict with one another. A unifying approach for application of statistical methodology in chemical mixture toxicology research is based on consideration of change(s) in slope. If the slope of the dose-response curve of one chemical does not change in the presence of other chemicals, then there is no interaction between the first chemical and the others. Conversely, if the rate of change in the response with respect to dose of the first chemical changes in the presence of the other chemicals, then an interaction is said to exist. This concept of zero interaction is equivalent to the usual approach taken in additivity models in the statistical literature. In these additivity models, the rate of change in the response as a function of the i^{th} chemical does not change in the presence of other chemicals in a mixture. It is important to note that Berenbaum's (1985, *J. Theor. Biol.* 114, 413–431) general and fundamental definition of additivity does not require the chemicals in the mixture to have a common toxic mode of action nor to have similarly shaped dose response curves. We show an algebraic equivalence between these statistical additivity models and the definition of additivity given by Berenbaum.

Key Words: **additivity; synergy; nonadditivity; interaction index; isobologram.**

Bailar and Bailer (1999) describe hypothetical data of a mixture of two chemicals A and B. Using common methods of analysis for testing for interaction available in the statistical, epidemiology, and toxicology literatures, different conclusions from the same data were made including synergy, independence, and antagonism, respectively. They point out that these are conceptual differences across the three disciplines, and they conclude that these differences have ''clear implications for risk assessment, an interdisciplinary exercise of serious societal impact.'' In a peer-reviewed publication written by a Society of Toxicology expert panel, Teuschler *et al.* (2002) point out that ''development of more generalized approaches for describing additivity and departure from additivity of mixtures of chemicals with particular emphasis on low-dose regions would be useful.''

The objective of this article is to describe a generalized approach for modeling additivity for toxicological mixture studies. This method is developed for a ''data rich'' scenario—reproducible dose-response information are assumed available on the mixture components, presumably on the same health endpoint in the same animal species, strain/stock, age, and gender under the same exposure conditions (route, duration, gavage vehicle, volume, etc.). In other more ''data poor'' situations such as is often the case in environmental risk assessment, simplifying assumptions may be necessary in order to proceed. The most widely used methods for assessing risks from low-level exposures to chemical mixtures are based on additivity concepts (ATSDR, 2004; U.S. EPA, 2000). These include *dose-addition* risk assessment methods which assume the same toxic mechanism-of-action across the mixture's components and *response-addition* risk assessment methods which assume independence of toxic action across the mixture components. These approaches are chosen based either on real data on the mode of action by which toxicity is produced for each mixture component (which may be scarce), or by using expert judgment regarding mode of action. Dose-addition methods sum the exposure levels of similar components in a mixture (after scaling for relative potency among components) and estimating the effect (or risk) of the mixture directly from the summed dose. In response addition, the probabilistic risk of an effect for each individual chemical in the mixture is estimated and then these individual risks are summed. Consequently, the two methodologies may yield quite different estimates of risk, which is problematic when the mode of action data used to choose between them are uncertain. These approaches are useful in data poor situations where toxicological dose-response information for all of the chemicals in a mixture may not be available. As data availability increases, however, environmental risk assessment may be improved by

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developing methods that rely less on assumptions of toxic mechanism. Such methods can be used to harmonize approaches to environmental risk assessment and increase consistency in data uses and interpretation. The goal of this article is to describe statistical models of additivity and departures from additivity which provide a high level of statistical rigor for testing biological hypotheses. A unifying approach compatible with both forms of additivity is suggested, using a statistical modeling approach based on the fundamental definition of additivity developed by Berenbaum (1985).

A basic concept of an interaction is that the slope (i.e., steepness) of a dose-response curve of a chemical changes in the presence of one or more other components in a mixture. Conversely, if the slope of the dose-response curve of a chemical is not altered in the presence of another chemical then the chemicals are said to exhibit no interaction (see Fig. 1), or that they are said to combine *additively* (i.e., zero interaction) (Teuschler *et al.*, 2002). This concept of interaction as a change in slope is well grounded in the pharmacology literature. The earliest designations of receptors (receptive substance) and drug-receptor interactions occurred in the late nineteenth century when Langley suggested that these interactions were based on the law of mass action (Goodman and Gilman, 2001). This hypothesis was extended theoretically and experimentally by A. J. Clark in the 1920s, Ariëns and Beld (1977), and many others (Goodman and Gilman, 2001). The equation that was used to describe this interaction between a drug and its receptor was that of a simple rectangular hyperbola (e.g., Michaelis-Menton). It is from these initial studies that terms for drugs with different activities (e.g., potency, partial agonist, antagonist) were coined and various types of drug interactions (e.g., ''additivity'' as no interaction) were described (Goodman and Gilman, 2001). A good compilation of how these concepts have been developed and used in different applications and case studies is contained in the book edited by Yang (1994).

A definition of additivity, which is often used to test for interactions among components in a mixture, is given by Berenbaum (e.g., 1985) and is based on the classical isobologram for the combination of two chemicals (e.g., Loewe, 1953; Loewe and Muischnek, 1926). In fact, Berenbaum (1985, 1989) refers to this definition as a ''general solution'' which is ''mechanism-free'' with the advantage of being based on empirical information. In a combination of c chemicals, let E_i represent the concentration/dose of the i^{th} component alone that yields a fixed response, and let x_i represent the concentration/dose of the i^{th} component in combination with the c agents that yields the same response. According to this definition of additivity if the substances combine with *zero interaction*, then

$$\sum_{i=1}^{c} \frac{x_i}{E_i} = 1. \tag{1}$$

If the left-hand side of Equation 1 is less than 1, then a greater than additive response (i.e., *synergism*) can be claimed at the combination of interest. If the left-hand side of Equation 1 is greater than 1, then a less than additive response (i.e., *antagonism*) can be claimed at the combination. As Equation 1 is the equation of a plane in c dimensions, this definition of additivity implies that under additivity contours of constant response are planar. It is important to note that Berenbaum's general definition as given in Equation 1 places no constraint on the single chemical slopes, and the mixture may include active and inactive compounds. Further, the chemicals in the mixture do not need to have similarly shaped dose response curves, a requirement for applications of dose addition that use an index chemical to estimate risk. An example of the use of an index chemical to assses mixture risk is the Toxic Equivalency Factor (TEF) approach to dose addition for dioxins which assumes common slopes across the chemicals under study (e.g., Safe, 1998; U.S. EPA, 1989); Berenbaum's definition of additivity in Equation 1 does not require such an assumption.

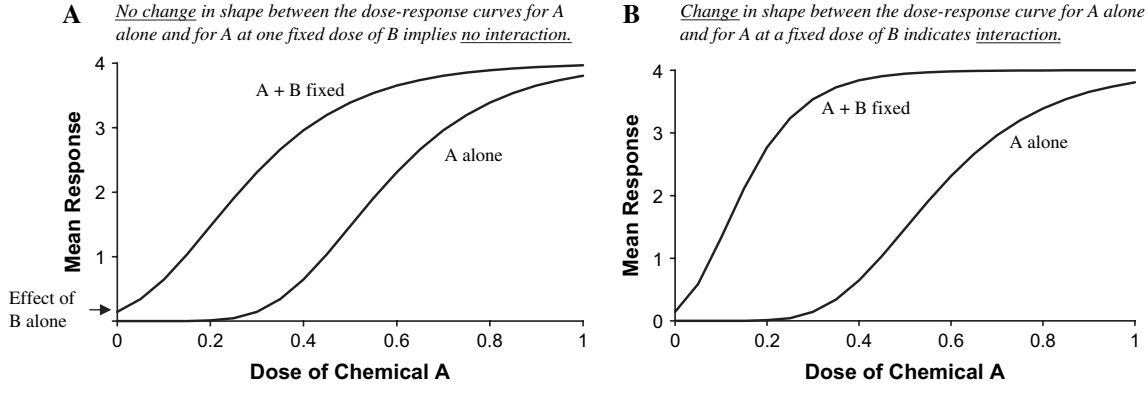

FIG. 1. Hypothetical dose-response curves of chemical A alone and at one fixed dose of chemical B when (A) the shapes are similar which is the case when the chemicals do not interact, and (B) when the shape changes which is the case when the two chemicals interact. Statistical tests are available that account for biological variability when testing for changes in shape. (A) No change in shape between the dose-response curves for A alone and for A at one fixed dose of B implies no interaction. (B) Change in shape between the dose-response curve for A alone and for A at a fixed dose of B indicates interaction.

In what follows we will show an algebraic equivalence of Berenbaum's definition of additivity given in Equation 1 to statistical additivity models. We will also demonstrate that these statistical additivity models satisfy the fundamental concept of zero interaction for additivity. We begin with a general discussion of the justification for using empirical statistical models, which approximate an underlying relationship; these models are not based on mechanistic assumptions/knowledge. Later, we describe an approach that relates the mean of the response variable to a set of covariates that is applicable to many different types of responses, dose-response shapes and distributional assumptions. That is, the methods may be applied to continuous response values, proportional responses, count data, etc. We demonstrate that these are additivity models when they are parameterized to include an intercept and linear terms only. Otherwise, higher-order cross product terms are associated with interactions according to Berenbaum's definition. In each of these sections

- we demonstrate the algebraic equivalence of the additivity model to the definition of additivity given in Equation 1; and,
- we demonstrate using the additivity model that the slope of the i^{th} chemical does not change in the presence of other chemicals in the mixture.

Thus, the additivity models satisfy the fundamental notion of additivity and Bernbaum's definition given in Equation 1. We conclude with a discussion on the advantages of additivity models in the analysis of chemical mixtures, where the number of chemicals in the mixture is large, with implications for optimal experimental designs and finish with some summary statements. An advantage of this framework is that the important hypotheses of additivity can be tested with statistical rigor.

Justification of Statistical Models

To define the notation, let $g(\mu)$ be a known function of the mean response of interest in an analysis of a mixture of c components. Examples of commonly used transformations, $g(\mu)$, include the probit transformation for proportional data, μ (or a power of μ) for continuous data, and $\log(\mu)$ for count data. We justify the common parameterization of statistical models based on two assumptions through a Taylor series argument[2]: (1) $g(\mu)$ is a function of the exposure concentrations of the c mixture components, i.e., the response changes with exposure. It is usually the case that the algebraic form of this underlying relationship is unknown. (2) Although the underlying relationship is unknown, we assume that the relationship is smooth (differentiable) and continuous. When these two assumptions are met, it follows that the unknown relationship can be expanded in a Taylor series (see Appendix) which motivates general parameterization of a response surface using linear and cross-product terms. As such, a first-degree model with only linear terms is an additivity model. Models with cross-product terms allow for interactions among the components in the mixture.

For this model development to be useful, it is necessary to demonstrate the adequacy of the approximation. Since the observed data contain information about the underlying dose-response relationship, comparison of these data to the predictions of the model are important in assessing the model. Such comparisons can be accomplished with varying levels of statistical rigor. Often simple plots of observed and predicted results are sufficient. In other cases, it may be necessary to test the null hypothesis of model adequacy. While testing model adequacy is an activity that can only occur after the data collection and analysis phases of a study, it should be noted that experimental designs have been developed to maximize the power of the test of model adequacy (e.g., Atkinson and Donev, 1992). For models that provide an adequate representation of the data, the appropriateness of Box's (1979) observation, ''all models are wrong, but some are useful,'' is readily understood.

Generalized Linear Models as Additivity Models

The class of statistical models that we consider in this section is known as *generalized linear models* (e.g., McCullagh and Nelder, 1989). They are general in the sense that they can be used for different types of data including continuous, proportional, and count data. The mean of the response of interest is modeled as a function of covariates of interest (e.g., doses/concentrations) through what is termed the *link function*, a user specified smooth and monotone function. Commonly used link functions include the logit link (i.e., $\log(\mu/(1-\mu))$), probit or others for modeling binary data (e.g., loss of righting reflex); a log link is often used in modeling count data (e.g., motor activity); and a power link (i.e., μ^{λ}) is often used with continuous data (e.g., serum enzyme levels). The choice of the link function depends on the type of response variable (i.e., binary, count, continuous) and the analyst's choice for ease of interpretation.

Algebraic equivalence of the additivity model with Berenbaum's definition of additivity. A statistical additivity model (e.g., Neter *et al.*, 1996) for a combination of two chemicals is

$$g(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \qquad (2)$$

where

μ is the mean response and $g(\mu)$ is a specified transformation of the mean known as the *link function*,
x_1 is the dose of chemical A,
x_2 is the dose of chemical B,
β_0 is an unknown parameter associated with the intercept,

[2] A Taylor series can be used under general conditions to approximate a function by including a finite number of terms. Further explanation is provided in the Appendix.

β_1 is an unknown parameter associated with the slope for chemical A, and

β_2 is an unknown parameter associated with the slope of chemical B.

For convenience, the additivity model in Equation 2 is expressed for the case of two chemicals. The results retained in the following sections for the case of two chemicals are readily generalized to additivity models for c chemicals where $g(\mu) = \beta_0 + \sum_{i=1}^{c} \beta_i x_i$.

In the statistical literature this model is known as a *generalized linear model* due to the link between a transformation of the mean, g(μ), and a set of covariates (e.g., McCullagh and Nelder, 1989). It is also an additivity model in the statistical sense (Neter *et al.*, 1996). (Interestingly, the model in Equation 2 is equivalent to the slope ratio model described by Finney [1971] when $g(\mu) = \mu$.) It can be shown algebraically that the additivity model given in Equation 2 also satisfies the definition of additivity as given by Berenbaum in Equation 1. Consider the model in Equation 2 at a specified response μ_0, such that the transformed mean $g(\mu_0) = \beta_0 + \beta_1 x_1 + \beta_2 x_2$. For example, to work with an ED_{50} contour, $\mu_0 = 0.5$ and using a logit link, $g(\mu_0) = \log(\mu_0/(1 - \mu_0)) = \log(0.5/(1 - 0.5))$. From Equation 2, the dose associated with μ_0 for each of the single chemicals alone is $E_i = \frac{g(\mu_0)-\beta_0}{\beta_i}$. Then,

$$\begin{aligned} g(\mu_0) - \beta_0 &= \beta_1 x_1 + \beta_2 x_2 \\ 1 &= \frac{x_1}{(g(\mu_0) - \beta_0)/\beta_1} + \frac{x_2}{(g(\mu_0) - \beta_0)/\beta_2} \\ 1 &= \frac{x_1}{E_1} + \frac{x_2}{E_2}, \end{aligned}$$

which is the equation of a line, indicating that the contours of constant response are linear for two chemicals and planar in the general case of c chemicals. Thus, the additivity model in Equation 2 is algebraically equivalent to the definition of additivity given in Equation 1.

Following Carter *et al.* (1988), if the model is parameterized to include a cross-product term as motivated by a Taylor series argument, i.e., $g(\mu_0) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2$, then Equation 1 becomes

$$\frac{x_1}{(g(\mu_0) - \beta_0)/\beta_1} + \frac{x_2}{(g(\mu_0) - \beta_0)/\beta_2} = 1 - \frac{\beta_{12} x_1 x_2}{g(\mu_0) - \beta_0}.$$

When $\beta_{12} = 0$, i.e., no interaction, then $(1 - \frac{\beta_{12} x_1 x_2}{g(\mu_0)-\beta_0}) = 1$, and additivity is the case. For $g(\mu_0) > \beta_0$ (i.e., for responses above background) and for increasing dose-response curves, if $\beta_{12} > 0$ then a greater than additive response (*synergism*) can be claimed since $(1 - \frac{\beta_{12} x_1 x_2}{g(\mu_0)-\beta_0}) < 1$; if $\beta_{12} < 0$ then a less than additive response (*antagonism*) can be claimed since $(1 - \frac{\beta_{12} x_1 x_2}{g(\mu_0)-\beta_0}) > 1$. This is important because it relates the idea of a departure from additivity to a parameter in a statistical model. Thus, the hypothesis of additivity can be expressed as H_0: $\beta_{12} = 0$ and statistical methodology for testing this hypothesis exists (e.g., Neter *et al.*, 1996).

Equivalence of the additivity model with fundamental notion of zero interaction. The model in Equation 2 is an additivity model in that it satisfies Berenbaum's definition of additivity (i.e., planar contours of constant response). It also satisfies the fundamental concept of an interaction as a change in the slope of one chemical in the presence of another chemical. That is, for $g(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2$, the slope of $g(\mu)$ with respect to x_1 is β_1; the slope of $g(\mu)$ with respect to x_2 is β_2. For comparison, consider the model with a cross-product term given by

$$g(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2,$$

which is rewritten as

$$g(\mu) = \beta_0 + \beta_1 x_1 + (\beta_2 + \beta_{12} x_1) x_2,$$

which demonstrates that the slope of the dose-response curve of chemical 2 is changed in the presence of chemical 1. If $\beta_{12} = 0$ then the level of x_1 is not involved in the "slope" for x_2 and vice versa. It is important to note that the results are in terms of $g(\mu)$. Typically the biological user of this methodology is interested in the relationship between a biological response (which has mean μ) and dose, as opposed to a transformed mean, $g(\mu)$, and dose. Use of the chain rule from calculus (e.g., Munem and Foulis, 1978) permits us to show that for a two chemical mixture, chemical 2 affects the dose-response relationship of chemical 1 when combined, and vice versa, on the μ scale. This can be shown symbolically. For ease of notation, define $g(\mu) = w = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2$ so that $\mu = g^{-1}(w)$ where w is the linear predictor. In the following, we show that the "slope" or rate of change in the mean response as a function of the dose of each chemical (as specified by a partial derivative) depends on the dose of the other chemical:

$$\frac{\partial \mu(x_1, x_2)}{\partial x_1} = \frac{\partial \mu}{\partial w}\frac{\partial w}{\partial x_1} = \frac{\partial \mu}{\partial w}(\beta_1 + \beta_{12} x_2) \text{ since } \frac{\partial w}{\partial x_1} = \beta_1 + \beta_{12} x_2$$

and

$$\frac{\partial \mu(x_1, x_2)}{\partial x_2} = \frac{\partial \mu}{\partial w}\frac{\partial w}{\partial x_2} = \frac{\partial \mu}{\partial w}(\beta_2 + \beta_{12} x_1) \text{ since } \frac{\partial w}{\partial x_2} = \beta_2 + \beta_{12} x_1,$$

where the notation $\mu(x_1, x_2)$ signifies that the mean is a function the dose of both chemicals. The value of the slope in terms of the mean μ is expressed as the product of two terms—one that has to do with the rate of change of the mean with respect to the linear predictor, w, (i.e., $\frac{\partial \mu}{\partial w}$), which only depends on the mean; and a term that comes from differentiating the linear predictor with respect to the variables. This second part is a function of the linear and interaction parameters and the dose of the other chemical, and it actually characterizes the slope of the dose-response curve.

For comparison, the rate of change of the mean as a function of the dose of either chemical of the additivity model given in

Equation 2 is characterized by only the corresponding linear parameter. Here, let $w = \beta_0 + \beta_1 x_1 + \beta_2 x_2$, so that

$$\frac{\partial \mu(x_1,x_2)}{\partial x_1} = \frac{\partial \mu}{\partial w}\frac{\partial w}{\partial x_1} = \frac{\partial \mu}{\partial w}\beta_1 \tag{3}$$

and

$$\frac{\partial \mu(x_1,x_2)}{\partial x_2} = \frac{\partial \mu}{\partial w}\frac{\partial w}{\partial x_2} = \frac{\partial \mu}{\partial w}\beta_2.$$

Thus, under additivity, the shape of the dose-response curve of either chemical does not change in the presence of the other chemical.

Use of these derivatives to describe the rate of change in the mean as a function of either chemical elucidates the complexity of working with a nonlinear model. The actual slope depends on the level of response selected (here denoted by w). If straight line or linear approximations are used instead of a nonlinear shape, then comparisons of the slope of the linear functions may lead to an incorrect inference. If the approximations are based on different regions of the dose-response relationship, then presumed differences in slopes may be due to the approximation and not due to an interaction. Figure 2 depicts two nonlinear and parallel dose-response curves. If linear approximations were made in the two locations shown in the figure, then an incorrect conclusion of non-parallelism could be made.

It was noted that Berenbaum's Equation 1 is the equation of a plane, i.e., contours of constant response (isobols) are planar when chemicals combine additively. For two chemicals, the contours are straight lines, which is the "line of additivity" in an isobologram. The contours of constant response associated with the general form of the additivity model in Equation 2 for c chemicals also are planar, i.e., $g(\mu_0) = \beta_0 + \sum_{i=1}^{c} \beta_i x_i$ is a plane. Consider the following example. Suppose we observe proportional data from Chemical A and from Chemical B (Fig. 3A) which are fit with a logistic regression model using the additivity parameterization given in Equation 2 with $g(\mu) = \log(\mu/(1-\mu))$. The resulting three-dimensional response surface (Fig. 3B) has a general sigmoid shape. However, the contours of constant response displayed in Figure 3C are linear. Figure 3 illustrates the connection between the additivity model given in Equation 2 and the definition of additivity given in Equation 1 which is an equation of a plane for any fixed response. So although the dose-response relationship is sigmoidal, the contours are linear.

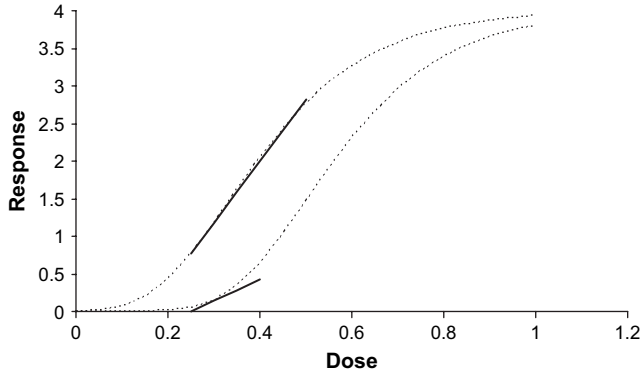

FIG. 2. The slope of a linear approximation to data in different effect regions of two parallel curves incorrectly indicates different slopes for Chemical A and Chemical B.

Nonlinear Models as Additivity Models

In this section we consider a class of flexible nonlinear models that are sigmoid-shaped. A common way of parameterizing these models is to use known functions that are sigmoid in shape but whose responses are contained in the range between 0 and 1. Frequently, cumulative distribution functions, $F(\boldsymbol{\beta}, \mathbf{x})$, from the statistical literature are used. To change the response range to what the data support, additional parameters are included. The general model is then given by $\mu = \alpha + \gamma F(\boldsymbol{\beta}, \mathbf{x})$ which ranges between α and $\alpha + \gamma$ and may be sigmoid-shaped. Figure 4 depicts the construction of this model.

Algebraic equivalence of a nonlinear additivity model with Berenbaum's definition of additivity. The connection between additivity models and Berenbaum's definition of additivity can be made for this class of nonlinear models with an additional condition. Instead of demonstrating this in general notation, we will use a specific nonlinear function which in practice is selected for its flexibility and asymmetric properties; however, the results hold for the general class of nonlinear models. The Gompertz nonlinear model is of the form

$$\mu = f(x) = \alpha + \gamma[\exp(-(\exp(-(\beta_0 + \beta_1 x_1 + \beta_2 x_2)))], \tag{4}$$

where the bracketed part in Equation 4, $[\exp(-(\exp(-(\beta_0 + \beta_1 x_1 + \beta_2 x_2)))]$, is the Gompertz function and the α and γ parameters are the range parameters. Other examples of commonly used functions include the logistic function and the exponential cumulative distribution function. Additivity models built from these other functions maintain similar associations as those shown for the Gompertz model. The model in Equation 4 is linearized by solving for the argument in the exponents, i.e.,

$$-\log\Big(-\log\Big(\frac{f(x)-\alpha}{\gamma}\Big)\Big) = \beta_0 + \beta_1 x_1 + \beta_2 x_2.$$

Similar to the generalized linear model, this nonlinear model can be put in the form of Equation 1 indicating it has planar contours of constant response conditional on the values of α and γ.

$$-\log\Big(-\log\Big(\frac{f(x)-\alpha}{\gamma}\Big)\Big) - \beta_0 = \beta_1 x_1 + \beta_2 x_2$$

Rearranging terms yields

$$1 = \frac{x_1}{(-\log(-\log(\frac{f(x)-\alpha}{\gamma})) - \beta_0)/\beta_1} + \frac{x_2}{(-\log(-\log(\frac{f(x)-\alpha}{\gamma})) - \beta_0)/\beta_2}$$

$$1 = \frac{x_1}{E_1} + \frac{x_2}{E_2}$$

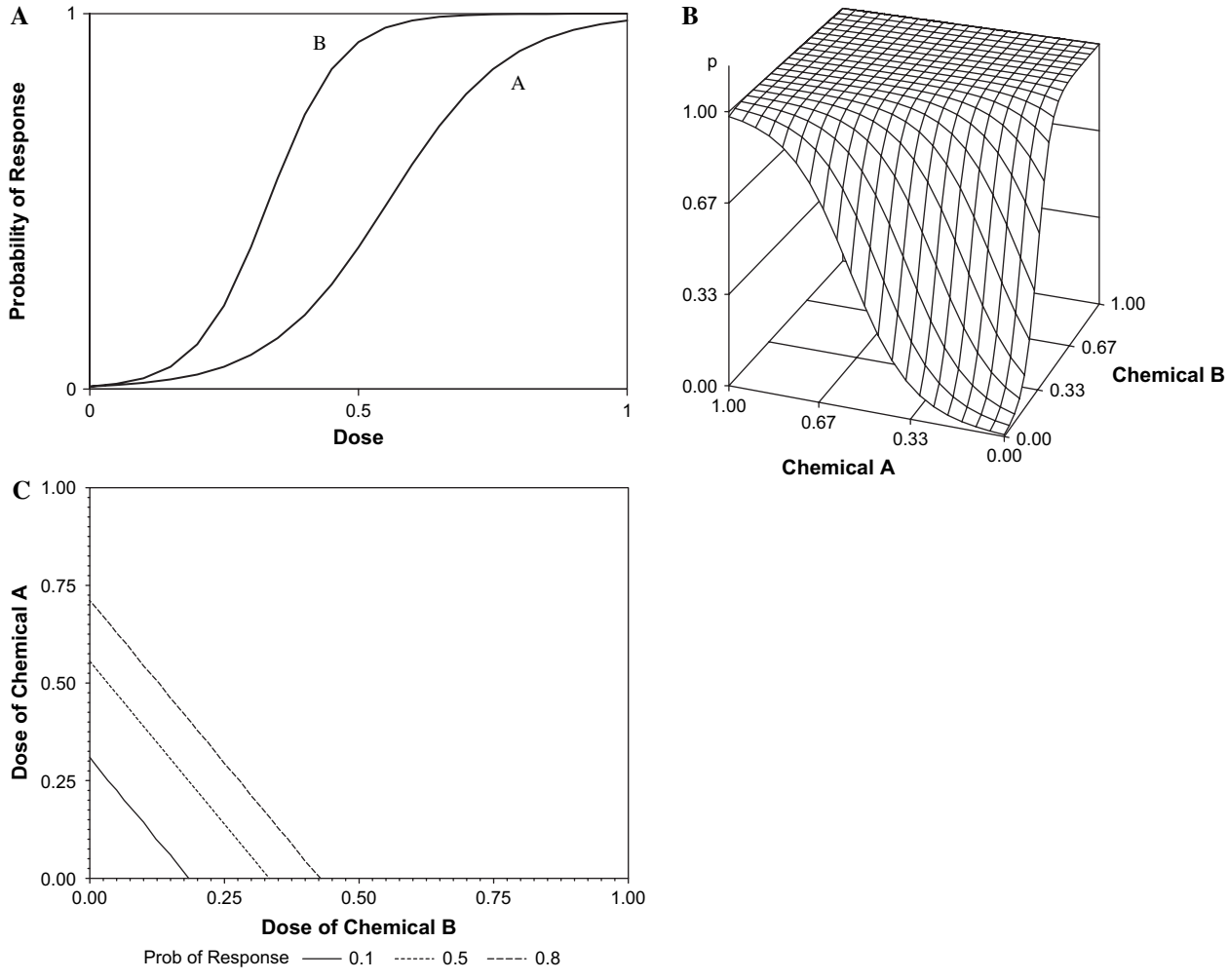

FIG. 3. (A) Dose response curves for chemicals A and B where the observed endpoint is binary and the model is of the probability of response. (B) The corresponding three-dimensional additivity response surface is sigmoid-shaped. (C) However the contours of constant response (i.e., isobols) are linear.

Thus, the nonlinear additivity model given in Equation 4 has planar contours of constant response as specified in Equation 1.

Equivalence of the additivity model with fundamental notion of zero interaction. Similar to the argument that led to Equation 3, the slope of the model in Equation 4 depends on the level of response and the corresponding linear parameter. For ease of notation, let $\mu = \alpha + \gamma F$ where $F = \exp(-\exp(-(w)))$ and $w = \beta_0 + \beta_1 x_1 + \beta_2 x_2$, so that

$$\frac{\partial \mu}{\partial x_i} = \frac{\partial \mu}{\partial F}\frac{\partial F}{\partial w}\frac{\partial w}{\partial x_i} = \gamma \frac{\partial F}{\partial w}\beta_i,$$

As in Equation 3, the slope of the additivity model in Equation 4 depends on the rate of change of the function with respect to the linear predictor, i.e., $\frac{\partial F}{\partial w}$, which is the location of the tangent line to the curve; and the dose-response curve is characterized by the parameter β_i. Thus, the slope of the additivity model associated with the i^{th} chemical does not depend on the other chemicals in the mixture.

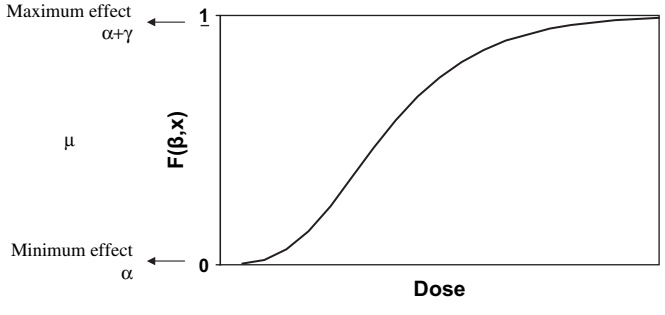

FIG. 4. General class of nonlinear models where the function F(β,x) which is restricted to values between 0 and 1 is adjusted to have response range [α, α + γ].

Threshold Models as Additivity Models

Algebraic equivalence of a threshold additivity model with Berenbaum's definition of additivity. Threshold models are

piecewise models (i.e., connected segmented lines) that allow for a dose range/region associated with a response the same as background, and an increase/decrease in response beyond the ''threshold.'' Consider the following parameterization of an increasing threshold additivity model for a combination of c chemicals,

$$g(\mu) = \left\{ \begin{array}{ll} \beta_0, & \text{if } \sum_{i=1}^{c} \beta_i x_i < \delta \\ \beta_0 + \sum_{i=1}^{c} \beta_i x_i - \delta, & \text{if } \sum_{i=1}^{c} \beta_i x_i \geq \delta \end{array} \right\} \qquad (5)$$

where

the link function $g(\mu)$ is as defined in (2),
β_0 is an unknown parameter associated with background response,
β_i is an unknown parameter associated with the ith chemical, and
δ is an unknown parameter associated with the threshold.

Using this model, the dose threshold associated with the ith chemical is $\delta_i^* = \frac{\delta}{\beta_i}$, $i = 1, \ldots, c$. Figure 5A depicts the dose threshold as the dosage where the background response mean changes to an increasing dose-response relationship. The algebra relating this model to Berenbaum's definition of additivity in Equation 1 is similar to that for the generalized linear model as it would be for response values greater than background, i.e., in the increasing part of the curve. That is, for the increasing part of the curve, i.e., where $\sum_{i=1}^{c} \beta_i x_i \geq \delta$, and the model is given by

$$g(\mu) = \beta_0 + \sum_{i=1}^{c} \beta_i x_i - \delta = \beta_0^* + \sum_{i=1}^{c} \beta_i x_i \qquad (6)$$

where $\beta_0^* = \beta_0 - \delta$. In this region the threshold additivity model is parameterized similarly to the model in Equation 2.

$$g(\mu_0) = \beta_0^* + \sum_{i=1}^{c} \beta_i x_i$$

$$g(\mu_0) - \beta_0^* = \sum_{i=1}^{c} \beta_i x_i$$

$$1 = \sum_{i=1}^{c} \frac{\beta_i x_i}{g(\mu_0) - \beta_0^*}$$

$$1 = \sum_{i=1}^{c} \frac{x_i}{(g(\mu_0) - \beta_0^*)/\beta_i}$$

$$1 = \sum_{i=1}^{c} \frac{x_i}{E_i}$$

Thus, the threshold additivity model also satisfies Berenbaum's definition of additivity.

The ''threshold additivity surface'' is the plane that connects the dose thresholds for each of the chemicals. Figure 5B is a schematic of such a plane for a combination of three chemicals. Based on the threshold additivity model, all doses/concentrations of the mixture that are between this plane and the origin are associated with a background response mean.

Equivalence of the additivity model with notion of zero interaction as lack of slope change. Recognizing the generalization of Equation 6 in the form of Equation 2, it follows from Equation 3 that $\frac{\partial \mu}{\partial x_i} = \frac{\partial \mu}{\partial w} \beta_i$. Thus, using the threshold additivity model, the shape of the dose response curve of the ith chemical does not change in the presence of the other chemicals.

Use of Additivity Models

A general strategy for testing for interactions among chemicals in a mixture is to use an additivity model to define the ''no interaction'' case and to use mixture data to describe the so-called ''unrestricted,'' or general case, as described by the mixture data. Only single chemical dose-response data are necessary to estimate an additivity model. Selection of mixture points in regions of environmental or biological relevance

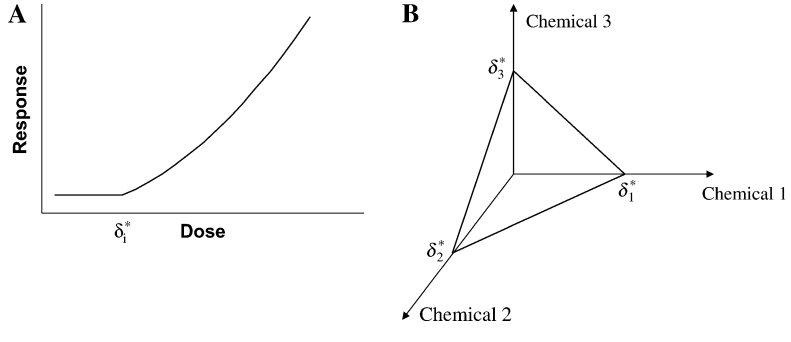

FIG. 5. (A) A schematic of a threshold dose-response curve for a single chemical. (B) When three chemicals are combined in a threshold additivity model as given in Equation 4 the additivity threshold surface is a plane which intersects at the dose thresholds for each of the three chemicals. The response associated with any combination of the three chemicals below this plane (i.e., between the plane and the origin) is the same as the background response.

(defined by fixed-ratio mixtures of the chemicals in the mixture) results in economical and practical designs for use in testing for interactions when the number of components in the mixture is large. Examples of the use of additivity models in testing the hypothesis of additivity follow.

Gennings *et al.* (1997) compared mean responses from an additivity model to that observed at a mixture point of interest. In particular, these authors describe $100(1 - \alpha)\%$ prediction intervals at each mixture point of interest using the additivity model. If the observed sample mean from the mixture point is included outside of the prediction interval, then they conclude evidence of departure from additivity. As the number of mixture points increases, multiple comparison corrections (e.g., Bonferroni corrections) become important. Dawson *et al.* (2000) compared the dose locations at specified responses under an additivity model to that observed using the interaction index. These authors estimate the interaction index at each mixture point of interest and develop a statistical test of whether the index equals one. They used Hochberg corrections for multiple testing.

More recently, several authors used a ray design to compare predicted responses from an additivity model to a mixture model along one or more fixed-ratio mixture rays (Casey *et al.*, 2004, 2005, in press; Gennings *et al.*, 2002; Meadows *et al.*, 2002). Figure 6 depicts a ray design for a combination of two chemicals with two mixture rays. Casey *et al.* (2004) developed methodology for testing the hypothesis of additivity in a mixture of c chemicals and for testing whether subsets of the chemicals interact with the remaining chemicals. Let a_i be the proportion of the i^{th} chemical in the fixed mixture ratio, $i = 1,\ldots, c$, where $\sum_{i=1}^{c} a_i = 1$. Gennings *et al.* (2002) and others have pointed out that the slope in terms of total dose along the fixed-ratio ray under additivity is given by $\theta_{add} = \sum_{i=1}^{c} a_i \beta_i$. These authors develop a test of additivity by testing whether the slope for the dose-response curve of the mixture in terms of total dose is equivalent to θ_{add}. Although this inference is limited to the mixing ratio used in the experiment, it results in experimentally feasible studies of mixtures of many chemicals.

The additivity models described throughout this paper are algebraically equivalent to the definition of additivity given in Equation 1. That is, the models can be algebraically manipulated to demonstrate that the contours of constant response are planar. Gennings *et al.* (2004) used a more general additivity model which was associated with the same definition of additivity. They fit each single chemical and mixture ray with a sufficiently flexible dose-response model that allowed for full and partial agonists in the mixture. To predict along the mixture ray(s) under additivity, they used the single chemical models and imposed the constraint of linear contours. By using such an approach, prediction from the additivity model was only conducted implicitly; however, the advantage is that the single chemical data have a customized model fit. This general approach of a more flexible additivity model has been used in the analysis of the effect of a mixture of 18 chemicals on thyroid function (Crofton *et al.*, in press).

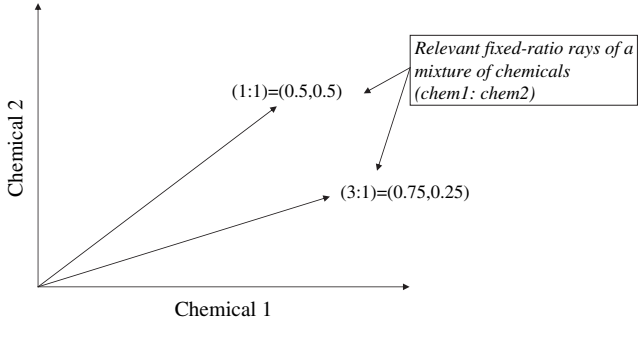

FIG. 6. A schematic of a ray design with single chemical axes and two mixture rays: (1:1) and (3:1).

Experimental Designs

One of the primary advantages to using fixed-ratio ray designs is the savings in terms of experimental resources required to test hypotheses of additivity. In general, the estimation of the additivity model requires only suitable single-chemical dose-response data. Our experience suggests that four to six dose groups spanning the active region of each of the single chemical dose-response curves is sufficient to predict the additivity surface. Similarly, if a single fixed-ratio mixture is of interest, then a target of about six total dose groups along the ray spanning the active part of the dose-response curve is suggested. Thus, with c single chemicals and one mixture ray, such a design includes about $6(c + 1)$ dose groups. By contrast, a factorial design with c chemicals and d dose groups per chemical has d^c dose groups. A further advantage of the use of statistical models is their connection to statistical experimental designs. A vast experimental design literature (e.g., Abdelbasit and Plackett, 1983; Atkinson and Donev, 1992; Kalish, 1990; Minkin, 1987) has developed that can be exploited to provide estimates with desirable properties, such as estimates with minimized variance. Meadows *et al.* (2002) and Casey *et al.* (2005) developed optimal experimental design strategies for tests of interaction using fixed-ratio ray designs. That is, these designs specify dose locations and sample size allocations that are associated with desirable statistical properties of the model parameters. The approach taken by Meadows *et al.* and Casey *et al.* was to determine the experimental designs associated with minimizing the variance of the test statistic associated with the test of additivity. By reducing its variance, the resulting test statistic has increased power for rejecting the hypothesis of additivity.

Summary and Discussion

We have described the justification for using empirical statistical models with a Taylor series argument. The argument begins with an assumption of a functional relationship of unknown form but which is assumed to be continuous and

differentiable. Using a Taylor series, the functional form is approximated by a polynomial function with a finite number of terms. The models that we fit to the observed data are meant to only approximate the underlying unknown dose-response relationship. The approximation should be verified with a goodness-of-fit test before continuing in the analysis with further inference. However, if the model of the mean response, or a transformation of the mean, is parameterized with linear terms, we have demonstrated that these terms may be interpreted as being associated with the rate of change in the mean as a function of the dose/concentration. In the additivity models we have described, the rate of change in the response with respect to the i^{th} chemical does not change in the presence of other chemicals. When cross-product terms are added to the model to construct an interaction model, then the rate of change in the response with respect to the i^{th} chemical does change in the presence of other chemicals. Thus, these additivity models satisfy the condition that the slope of a chemical does not change shape in the presence of other chemicals.

It is indeed the case that statisticians and life scientists (e.g., toxicologists) working independently have each developed a body of knowledge pertinent to the study of interactions. Over time an extensive vocabulary has evolved to characterize interactions, i.e., departure from additivity, which is perhaps unnecessarily confusing and complex. Dose-addition is the fundamental premise behind such risk assessment approaches as the Hazard Index and Toxicity Equivalence Factors (U.S. EPA, 2000). Such approaches assume that any amount of the agent, no matter how small or large, can contribute to the overall toxicity of the mixture because total dose is the unit of concern. Response addition assumes that if the toxicity of the mixture is truly independent among the chemicals, then it is appropriate to add small risks following the law of statistical independence. With response addition, sub-threshold doses of individual chemicals do not contribute to the overall toxicity of the mixture (essentially adding zero responses). Mumtaz *et al.* (1994) states the ''default'' and ''most conservative'' form of response addition ''is equivalent to dose addition, provided the dose-response curves . . . are within the linear range.'' Similar to the default form of response addition is the concept of *effect addition* (e.g., Berenbaum, 1989; Kortenkamp and Altenburger, 1999) where the expected effect of a mixture is the arithmetic sum of a measured toxic effect of the single agents in the mixture. The method of effect addition is applicable for linear (Berenbaum, 1989) or linearizable (when the summation is conducted on the transformed scale, e.g., the logit or probit scale) dose-response curves. In short, dose addition, response addition, and effect addition define three ways of defining additivity—by adding doses, risks, or effects.

The methods shown in this article require single chemical dose-response information on the mixture components, presumably on the same health endpoint in the same animal species, strain/stock, age, and gender under the same exposure conditions (route, duration, gavage vehicle, volume, etc.). The additivity models accommodate these similarities across studies with a common background (intercept) parameter (which may be verified by comparing the means of the vehicle control groups in an analysis of variance model). Further, the single-chemical dose-response curves should either be reproducible over time or be collected at the same time as the mixture data. When such data rich situations occur, the methods described here provide an improvement over simple additivity approaches. That is, the additivity models as described in the previous sections estimated using single chemical dose-response data do not require the simplifying assumptions of other methods (e.g., common mode of action) and thereby may provide improved estimation of risk. They are also useful in providing evidence of the joint toxic action for a group of chemicals; such information may serve to actually support or refute the use of dose addition or response addition methods. Given the plethora of potential chemical combinations and exposure scenarios, default risk assessment methods may always be necessary, particularly for the data poor situations. However, the approach shown in this article contributes to the library of available techniques applicable for the data rich cases.

We have demonstrated that the additivity models we propose not only satisfy the concept of no interaction as no change in slope, but they also satisfy Berenbaum's definition of additivity. Since these models can all be linearized (or at least conditionally linearized in the case of a nonlinear model), they also satisfy the definition of effect additivity where the addition is conducted on the transformed scale. The added effect due to the i^{th} chemical is determined by the term $\beta_i x_i$ on the transformed mean response scale. When sufficient dose-response data are available for this kind of modeling exercise, it may not be necessary to restrict the interpretation of additivity into categories such as dose addition and response addition. The additivity models described here satisfy the concept of no interaction as the condition where chemicals combine in a way that does not affect their individual dose-response relationships. That is, the rate of change in response with respect to the dose/concentration of each chemical does not change in the presence of the other chemicals. It is a theoretical possibility that a shift in a threshold level of a component in the mixture is non-additive. The additivity models considered in the previous sections allow for a shift in the ''threshold'' due to the presence of the other chemicals. If the shift is different from that supported by the additivity model, then an interaction may be said to exist. Such a phenomenon is testable using the additivity model and appropriate statistical tests.

In Bailar and Bailer (1999), the suggested analytical schemes of the hypothetical data in a 3×3 design included logistic regression where the log odds were used in the analysis to suggest additivity; a linear model of the observed responses which resulted in a claim of synergism; and a dose addition model that resulted in a claim of antagonism. Although limited by the design, these data do not seem appropriately modeled with a linear assumption—the data demonstrate curvilinearity.

It is often reasonable to assume that dose-response data are sigmoid in shape. This is particularly the case with proportional data where the range of the response is constrained to be in the interval [0,1]. The general form of the models described in the previous sections is sufficiently flexible to adequately represent the observed data. For example, assuming the data are actually proportional, if the analyst had initially transformed the data to achieve a linearized form (say, using a logit or probit link function) and properly accounted for the variability in the data, then the three analyses would have resulted in the same conclusions—additivity as suggested by a logistic regression analysis. This emphasizes once again that the choice of the model should be based on properties of the data.

In conclusion, we have attempted to bridge the gap between a concept of no interaction and statistical additivity models. When the dose-responsiveness of a chemical in a mixture does not change in the presence of other chemicals, then it is claimed to act additively with the other chemicals. We have shown that this concept of additivity is inherent within the statistical additivity models described herein. These models can be estimated with the support of appropriate experimental single chemical dose-response data. When the zero interaction case is well described with sound statistical rigor, then the likelihood of adequately detecting and characterizing departure from additivity with experimental mixture data is improved.

APPENDIX

For convenience and without loss of generality, we evaluate the Taylor series expansion at zero. Summarizing the above we have $g(\mu) = f(x_1, x_2, \ldots, x_c)$, where the form of $f(\ldots)$ is unknown and unspecified and x_1, x_2, …, x_c are doses/concentrations of the c chemicals in the combination. From the Taylor series expansion of $g(\mu)$ we have

$$\begin{aligned} g(\mu) = {} & f(0,0,\ldots,0) + f_1^{(1)}(0,0,\ldots,0) \\ & + f_2^{(1)}(0,0,\ldots,0) + \cdots f_c^{(1)}(0,0,\ldots,0) \\ & + \sum_{i=1}^{c-1}\sum_{j=i+1}^{c} f_{ij}^{(2)}(0,0,\ldots,0)\frac{x_i x_j}{2!} \\ & + \sum_{i=1}^{c-2}\sum_{j=i+1}^{c-1}\sum_{k=i+2}^{c} f_{ijk}^{(3)}(0,0,\ldots,0)\frac{x_i x_j x_k}{3!} + \cdots \\ & + f_{12\ldots c}^{(c)}(0,0,\ldots,0)\frac{x_1 x_2 \ldots x_c}{c!} + \cdots \end{aligned}$$

where $f^{(*)}_{\text{variables}}(0,0,\ldots,0)$ represents the $*^{\text{th}}$ partial derivative of f with respect to *variables* evaluated at (0,0,. . .,0). Notice that the form of $f(\ldots)$ is unspecified and that the expansion has an infinite number of terms. Let's consider these two concerns separately. First, note that if the function were known its derivatives could be determined and evaluated at the point (0,0,. . ., 0). Thus, each of these terms can be represented as unknown constants, $\beta_{(.)}$, where the subscript (.) denotes the variable(s) involved in the derivative evaluated in each case. This permits reducing the expression to

$$\begin{aligned} g(\mu) = {} & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_c x_c + \sum_{i=1}^{c-1}\sum_{j=i+1}^{c} \beta_{ij} x_i x_j \\ & + \sum_{i=1}^{c-2}\sum_{j=i+1}^{c-1}\sum_{k=i+2}^{c} \beta_{ijk} x_i x_j x_k + \cdots + \beta_{12\ldots c} x_1 x_2 \ldots x_c + \cdots \end{aligned}$$

which is a polynomial with an infinite number of terms and where $\beta_0 = f(0,0,\ldots,0)$, $\beta_1 = f_1^{(1)}(0,0,\ldots,0)$, $\beta_2 = f_2^{(1)}(0,0,\ldots,0)$, …, $\beta_c = f_c^{(1)}(0,0,\ldots,0)$, $\beta_{12} = f_{12}^{(2)}(0,0,\ldots,0)$, etc. Up to this point, the representation of $g(\mu)$ is exact, but requires an infinite number of terms. Truncating the expansion after a finite number of terms provides an approximation to the underlying relationship, which permits us to write

$$\begin{aligned} g(\mu) \approx {} & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_c x_c + \sum_{i=1}^{c-1}\sum_{j=i+1}^{c} \beta_{ij} x_i x_j \\ & + \sum_{i=1}^{c-2}\sum_{i=j+1}^{c-1}\sum_{k=i+2}^{c} \beta_{ijk} x_i x_j x_k + \cdots + \beta_{12\ldots c} x_1 x_2 \ldots x_c. \end{aligned}$$

Now we have approximated the unknown dose-response relationship with a polynomial relationship with a finite number of terms. The terms of the polynomial reflect the slopes of the individual mixture components' dose response and, through the cross-product terms, the effect that a component has on the slopes of the other components' dose response curve when the components are combined together in a mixture. For example, β_{ij} is the effect of the i^{th} component on the slope of the j^{th} component when the two chemicals are combined; and, β_{ijk} can be interpreted as the effect of the i^{th} component on the interaction between the j^{th} and k^{th} components.

DISCLAIMER

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory and the National Center for Environmental Assessment, Office of Research and Development, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

ACKNOWLEDGMENTS

This research was partially supported by the U.S. EPA National Center for Environmental Assessment (Cincinnati, OH) cooperative agreement #CR-827208. The paper has benefited from the thorough review made by Drs. L. Birnbaum, M. DeVito, D. Herr, D. Gaylor, and G. Suter of an earlier version. Conflict of interest: none declared.

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