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The Response Surface Methodology as an Approach of Choice to Modeling and Analyzing Combined Toxicity: Theoretical Premises, the Most Important Inferences, Experimental Justification

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ABSTRACT

Identification of the types of joint action of factors are considered on the basis of the response surface theory. On the example of the model of the full factorial experiment 22, the main effects model with interaction was investigated. It is shown that, within the framework of this model, there is an equivalence of the paradigms of dose additivity and effect additivity. The types of joint action possible in this model are considered. Examples of specific toxicological studies with the help of the main effects model with interaction are given.

Introduction

Studying the combined action of biologically active agents is becoming increasingly important in various branches of biomedical research, toxicology included.

Human health risks due to exposure to toxic agents are associated, as a rule, with multiple factors. Such technologies as steel making (alloyed steels especially), electric arc welding, pyrometallurgy of heavy nonferrous metals (particularly copper smelting and refining), and electroplating bring about multicomponent (polymetallic) pollution of workroom and ambient air and of other compartments of the environment including foodstuffs produced in contaminated areas. Nevertheless, risk assessment experts have tended to focus or are still focusing on certain isolated risks from exposure to hazardous metals that are considered (on more or less serious grounds) as a priority in a specific industrial or environmental context. The typical examples are studies conducted to assess the adverse effects of environmental pollution with lead on children's health and development in areas around a copper smelter or the toxic impact of manganese on the central nervous system in arc welders.

At the same time, it is well known that these environments are actually contaminated with other elements as well (in particular, arsenic, copper, cadmium and zinc in copper smelting, or chromium, nickel, iron and silicon in arc welding). This may not seem as creating any particular problems since the generally accepted methodology implies the possibility of assessing health risks individually for each of the factors operating in a combination with

subsequent summation of risks of the same type. However, does the science of toxicology indeed provide sufficiently reliable and uniformly understood grounds for such a seemingly simple solution? As an analysis of the state of the art carried out by our group a few years ago [1] showed, answering this question is a challenge, and the answer itself is more likely to be negative.

Generally speaking, modern toxicology usually characterizes combined toxicity using the term "additivity" along with two other terms to describe some departure from it: superadditivity (or "synergism"), and subadditivity (or "antagonism"). The exact meaning of each of these terms can, however, vary broadly depending on which paradigm of combined adverse action is meant, even if not explicitly, by a researcher [2-6] or, by an Agency (e.g. the US EPA or the ACGIH). We will only be using the terms "additivity", "subadditivity" and "superadditivity" assuming that any use of the terms "antagonism" and "synergism" should be reserved for the case of a proven toxicological explanation for the mechanism that causes a corresponding formal departure from additivity.

The so-called independence paradigm assumes that a similar effect of two or more substances is due to their action at different biological sites, and so the net effect of one chemical is independent of the presence of another chemical. The best known mathematical expression of this paradigm for the case of exposure to two toxicants is the so-called Bliss independence assumption [7], which, however, is strictly applicable only to indices that have the meaning of probability of a certain event.

For estimating the type of combined impact in cases where its result is estimated by a quantitative shift in this or that index for the status of the organism compared with the baseline or the control value (this approach dominates in experimental toxicology), the central assumption is that of additivity of effects.

For a combination of two toxicants at the doses d_A and d_B, this assumption is expressed by the equation:

$$y_{11} - y_{00} = [y_{10} - y_{00}] + [y_{01} - y_{00}], \quad (1)$$

Where $y11 = Y(d_A, d_B)$ is the value of an index Y under exposure to a combined effect of two toxicants A and B; $y_{10} = Y(d_A, 0)$ and $y_{01} = Y(0, d_B)$ are the values of the same index in response to the effect of one of the toxicants alone; and $y_{00} = Y(0,0)$ is the baseline or control value of the same index in the absence of both toxicants [3].

The differences $[y_{10} - y_{00}]$ and $[y_{01} - y_{00}]$ in the right-hand part of the equation (1) show a change in the value of the index Y under the effect of one agent only, i.e. they represent the single-factor effects of the acting agents $E(d_A)$ and $E(d_B)$, while the difference $y_{11} - y_{00}$ represents the value of the two-factor effect $E(d_A, d_B)$. Thus, the equation (1) expresses a combined action of agents for which the two-factor effect is equal to the sum of the single-factor ones

$$E(d_A, d_B) = E(d_A) + E(d_B). \quad (2)$$

If the actually observed effect of a combination (A + B) is higher or lower than the expected effect $E(d_A, d_B)$, this may be due to a non-zero interaction between the effects, and thus we deal with either "superadditivity" or "subadditivity", respectively. These notions may be correctly applied only where the single-factor effects $E(d_A)$ and $E(d_B)$ display the same direction, i.e. where these effects have the same sign. Note also that in an experiment with several observations of the response Y for each combination of the exposure levels exhibited by the agents A and B, the value of the response Y to a given combination will be the mean value of Y over all observations with this combination of exposure levels. In this case, the validity of the equation (2) is verified as a statistical hypothesis by means of ANOVA: the zero hypothesis of the equality (1) or (2) being met is rejected if the cross term in the two-way ANOVA model is statistically significantly different from zero.

An alternative paradigm, the so-called "Loewe additivity", assumes that two or more chemicals impact on the same biological site by the same mechanisms of action, being different in their potency only [8]. Thus A and B assumingly act as one and the same substance and, consequently, do not enter into any interaction between them. If D_A and D_B are isoeffective doses of these chemicals, one and the same effect of their combination in actual doses d_A and d_B can be obtained only where

$$d_A/D_A + d_B/D_B = 1 \quad (3)$$

Where this sum is > 1 or < 1, it points to subadditivity or superadditivity, respectively. It is very popular to represent this paradigm with a graphic analogue called Loewe isobole or isobologram.

The official definitions of the terms «additive», «more than additive (potentiation, synergy) » and «less than additive (antagonism) » for combined action developed by a special Expert Committee [9], fully complied with the paradigm of

effect additivity. However, later on the so-called Saariselkä Agreement recommended the use of both (effect additivity and dose additivity) models [10]. More recently, the report of a WHO/IPCS International workshop on "Assessment of combined exposures to chemicals" [11] virtually repeated this duality, also reproducing the widespread concept of a fundamental mechanistic difference between these two models.

Meantime, some researchers have demonstrated that the conformability of experimental data with this or that mathematical model of combined toxicity based on different paradigms depends essentially on the shape of the dose–effect curve for the isolated effect of each substance and on which segment of this curve the added effect of the second substance is considered [4,6,12]. Moreover, the type of combined toxicity may essentially differ depending on which of the components prevails in the combination quantitatively. In particular, this dependence gives biphasic Loewe isoboles, an example of which (for combined LD50 of sodium fluoride and manganese chloride in both mice and rats) was presented in [4,5]. In this case, the combination proved subadditive where fluoride prevailed but superadditive where manganese did.

Tajima et al., [13] also came to the conclusion that the type of combined action of two toxicants depends on their dose ratio.

Rozman et al., [3] evaluated the complex interaction between different doses and time–response using equations showing a sigmoid dose–response at a constant time and a sigmoid time–response at a constant dose.

It was also postulated that the type of combined action can depend on the organ or the system of the organism to which the effect considered pertains, as well as on the character of the effect [4]. The same conclusion was made in the Agency for Toxic Substances and Disease Registry overview document [14] stating that "the predicted direction of interaction for the effects of these mixtures (Pb–As and Pb–Cd) is not consistent across endpoints. This observation is most striking for the effects of cadmium on the toxicity of lead. The predicted direction is greater than additive for the neurological effects (the critical effect) and testicular effects (a less sensitive effect), less than additive for renal and hematological effects, and additive for cardiovascular effects."

Analysis of epidemiological data on combined cadmium–lead nephrotoxicity for children dwelling in industrially polluted areas led us, for the first time, to the conclusion that effect additivity vs. dose additivity should be regarded as two methods for estimating combined toxicity rather than two fundamentally different types of the latter [5].

This hypothesis was in conformity with the theoretical conclusion of [10] who had proved analytically that any variant of combined action could be well described by isobolograms or, if this approach were to be generalized in order to take into account different dose levels, by response surfaces.

We should underline in this connection that practical applications of the issue of combined toxicity (or "mixture toxicology") to health risk assessment and to permissible exposure level setting, have to be, and are indeed straightforward and unavoidably simplified circumventing all the above-mentioned uncertainties of the theory. Nevertheless, we maintain that such a practical approach (which we discuss in detail in the concluding part of this paper) would not be merely a simplification; rather, it would be deceptive if inferred from an oversimplified and uncertain theory, especially if the latter is formulated muddily and is not understood uniformly.

This general statement served as a starting point for a new series of experimenting and mathematical modeling ventures [15-20], the main methodological issues of which we propose to synthesize here.

An analysis of our own reciprocally corroborating experimental results has led us to the following principal conclusions:

(1) The widely recognized paradigms of dose additivity and effect additivity are virtually interchangeable, and so they might be regarded as different approaches to modeling the combined toxicity mathematically rather than as concepts reflecting fundamentally differing processes.

(2) Within both approaches, there exist not merely three traditionally recognized types of combined toxicity (additivity, subadditivity and superadditivity) but at least 10 variants of these types depending on exactly which effect is considered and what its level is, as well as on dose levels and their ratios. Moreover, when the adverse action of one and the same combination of toxics is assessed by many different outcomes (especially but not exclusively where these outcomes characterize the responses of different organs or systems) the type of combined toxicity always proves to be multivarious and so cannot be unambiguously described by a single deterministic term.

(3) Of special interest are some peculiarities of mathematical description and important terminological difficulties associated with the contra-directional action of combined toxics. As we have found it from our studies, where one deals with multi-outcome characterization of subchronic combined intoxications, one and the same pair of toxics may be found to act both unidirectionally and oppositely in relation to even one and the same effect but at different dose or effect levels. The Response Surface Methodology enables one to circumvent these difficulties and to describe a combined toxicity irrespective of the directions in which the combined toxics act. That is why we now preferably use this methodology.

Response Surface Methodology

As a result of an experiment, the toxicologist obtains a set of response values for different doses of the toxic agents. To characterize the type of the combined toxicity produced by these agents, the experimental data obtained need to be compared to the above-mentioned paradigms of effect additivity or dose additivity. Since the experimenter does not know in advance whether the toxicants impact on one or various organs, both paradigms have to be checked. The effect additivity equality (1) may be checked by the Analysis of Variance (ANOVA) methods. If the processing of experimental data by the ANOVA methods reveals a statistically significant cross term, the hypothesis of equality (1) should be rejected (for a given level of significance). Further verification of the experimental data will show a departure from additivity either towards superadditivity or towards subadditivity.

The paradigm of dose additivity can formally be checked by formula (3). Such a check would, however, require knowing the isoeffective doses DA and DB; determining which in an experiment presents a separate and rather difficult problem, particularly where it is essential to have isoeffective doses for several response values.

At present, there are several methods that may be used for defining combined toxicity types [1,10,21-23]. We believe the response surface methodology to be one of the most effective tools [24-27]. Below we consider in more detail one of the basic response surface models with reference to the combined toxicity characterizing problem. But first let us briefly consider the general framework of the response surface theory.

Generally, the statement of the problem looks as follows (for n acting factors $x_1, x_2,\ldots, x_n$): choose a certain function $f(x_1, x_2,\ldots, x_n)$ and find the parameters of the function $f(x_1,x_2,\ldots,x_n)$ such that it would describe the obtained experimental material as best as possible. This means that the error between the values of this function for these experimental values of the factors $x_1, x_2,\ldots, x_n$ and corresponding observed (experimental) values of the response should be minimal. The minimization criterion of choice is, as a rule, the sum of squared errors.

The choice of shape for the dependence of the response on factors $x_1, x_2,\ldots, x_n$ is determined both by experimental data and corresponding theoretical premises and by the possibility of precise or rough analysis of the formalized model.

The problem of finding the parameters of the function $f(x_1, x_2,\ldots, x_n)$ is obviously directly related to regression analysis and, thus, factors $x_1, x_2,\ldots, x_n$ are called predictors or regressors while the geometrical representation of the function $f(x_1, x_2,\ldots, x_n)$ is called 'response surface' [24,25]. It is important to note that the Response Surface methods were from the outset of this theory [28] associated with the Design of Experiment theory - with factorial experiment or fractional factorial design in particular. For simplifying computations and for ensuring important model properties (such as orthogonality, rotatability, etc.), the values of the natural variables $x_1, x_2,\ldots, x_n$ are coded so that each level of factor x_i is attributed a certain integer value. For instance, in a type 2^2 experiment factor levels are coded with values

– 1 and 1, while the midpoint, if added, has a corresponding code value of 0; the resulting design of the experiment is called 'central composite design' with midpoints. Specifically, a type 2^2 experiment will have an orthogonal design which enables efficient experimental data analysis to be carried out with correct interpretation. As a rule, the response model $y = f(x_1, x_2,\ldots, x_n)$ is conjectured just in relation to such coded variables, and in what follows we will be assuming the same.

For the general form of the function $f(x_1, x_2,\ldots, x_n)$, the solution to the problem of finding its parameters could be too complicated or display some pathological features (for instance, it could be unstable in relation to a small change in the input data). Needless complexity of the model also poses difficulties for a meaningful interpretation of the inferences obtained with its help. Therefore, the choice of the model function for the

response f (x_1, x_2, …, x_n) is typically a simple function that features sufficient flexibility at the same time.

Thus, the classical monographs [24,25] consider in detail and recommend using two basic models for the model function of the response f (x_1, x_2, …, x_n), which were also thoroughly investigated and recommended in the original work on the response surface theory [29]:

1) A first order model, or main effects model, for which

$$f(x_1, x_2,\ldots,x_n) = b_0 + b_{1x1} + \ldots + b_{nxn}, \quad (4)$$

i.e. a model which is fully linear in predictors;

2) A second order model, for which the function $f(x_1, x_2,\ldots,x_n)$ is a quadratic function of its arguments. For instance, for two predictors x_1, x_2 it is given by

$$F(x_1, x_2) = b_0 + b_{1x1} + b_{2x2} + b_{11x12} + b_{22x22} + b_{12x1x2}. \quad (5)$$

An important special case of model (5) follows for $b_{11} = b_{22} = 0$, i.e. in the absence of quadratic terms. This model is called main effects model with interaction, or first-order model with interaction

$$F(x_1,x_2) = b_0 + b_{1x1} + b_{2x2} + b_{12x1x2}. \quad (6)$$

Note that a type 22 experiment may have only two possible polynomial models: a linear model of main effects (4) and a model of main effects with interaction (6). In so doing it is obvious that the linear model (4) cannot be used for constructing a combined action model because it does not allow for a possible combined effect of the factors. This defines the exclusive importance of the main effects model with interaction (6) for analyzing experiments of type 2^2 or type 2^2 with midpoint. Since the surface described by the equation (6) presents a hyperbolic paraboloid, in what follows we will refer to the model (6) of main effects with interaction as 'hyperbolic paraboloid model'. Note also that although this model naturally arises in type 2^2 experiments, it may also be constructed based on data with a greater range of factor gradations.

It should be pointed out that in the majority of toxicological experiments dose levels are chosen and fixed with the help of certain discrete values. If the response surface model $y = f(x_1, x_2,\ldots, x_n)$ approximates the experimental data well, it may expected to provide a satisfactory interpolation to all intermediate dose values as well. Such a prediction could be made beyond the limits of the experimental dose range (extrapolation), although with a greater uncertainty than under interpolation.

Once a suitable approximation of the response surface $y = f(x_1, x_2, \ldots, x_n)$ has been obtained, the character of the predictors' combined action may be examined in two ways:

1- Testing the effect additivity hypothesis using the response surface for obtaining estimates for response values used in the formula (1).

2- Testing the dose additivity hypothesis by analyzing the Y = const surfaces, i.e. contour plots of the model response surface (formula (3) not being used here).

For the general function $y = f(x_1, x_2, \ldots, x_n)$, the constant response surfaces are $(n - 1)$-dimensional surfaces in an n-dimensional space. For a two-dimensional ($n = 2$) or three-dimensional ($n = 3$) case, these are level lines on a plane or two-dimensional level surfaces in a three-dimensional space. It is important that where the recommended first-order or second-order models are used, these geometrical objects are quadrics or second-order surfaces, which makes it possible to fully investigate the dependence of the response on the factors.

It should be noted that obtaining a constant level surface presents geometrically the problem of constructing a section of the response surface $y = f(x_1, x_2, \ldots, x_n)$ by the plane Y = const. ANOVA considers implicitly similar sections, made, however, by constant dose value planes, i.e. x_i = const planes.

Let us now consider in more detail the features of the main effects model with interaction (the hyperbolic paraboloid model) with reference to the problem of combined action type determination.

1. The hyperbolic paraboloid model

Consider first the case of full factorial experiment 2^2. We have a set of response values Y (i,j), i, j = –1, 1, using which we will construct a response surface model of type (6) so as to minimize the magnitude of the mean-square divergence between theoretical and experimental data. In a case of the same number of observations in the experimental groups Y (i,j), the coefficients b_0, b_1 , b_2 and b_{12} may be obtained directly from the group-average values of the response Y using the following formulae (for convenience, the average values of the response in groups (–1,–1), (–1, 1), (1, –1) and (1, 1) are designated as y_{00}, y_{01}, y_{10} and y_{11}, respectively)

$$\begin{cases} b_0 = \frac{1}{4}\left(y_{00} + y_{01} + y_{10} + y_{11}\right) \\ b_1 = \frac{1}{4}\left(-y_{00} - y_{01} + y_{10} + y_{11}\right) \\ b_2 = \frac{1}{4}\left(-y_{00} + y_{01} - y_{10} + y_{11}\right) \\ b_{12} = \frac{1}{4}\left(y_{00} - y_{01} - y_{10} + y_{11}\right) \end{cases} \quad (7)$$

Thus, in the case of full factorial experiment 2^2, the coefficients of the response surface (6) are uniquely determined by the average values of the response in the experimental groups. Moreover, the hyperbolic paraboloid surface given by the equation (6) passes precisely through the points (i, j, y_{ij}). In other cases, (not a 2^2 experiment but, for instance, an experiment with a midpoint) where model (6) is also used, the response surface passes, as a rule, near these points rather than exactly through them.

2. Isoboles for the Classical Types of Combined Action

The effect additivity condition (1) leads to equality $b_{12} = 0$. Hence, in the hyperbolic paraboloid model for type 2^2 experiment, effect additivity is equivalent to model (6) being reduced to the fully linear main effects model (4). In this case, the response surface (6) takes on form of a plane and constant effect lines, i.e. the response surface cut by Y = const planes, present parallel straight lines (i.e. the isoboles for this case will be parallel straight lines). These straight lines may have two possible positions as shown in Figure 1 (a), (b).

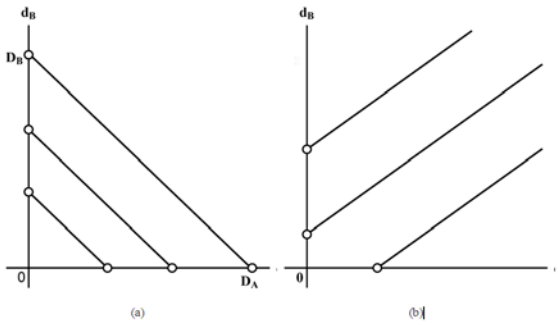

Figure 1: Isoboles for a case of equality of effect additivity (1): (a) isoboles for a unidirectional action of two toxicants; (b) isoboles for oppositely directed actions of the toxicants.

Obviously, in the case of Figure 1(a), the coefficients b_1 and b_2 will be of the same sign, while in that of Figure 1(b) they will have opposite signs. The result is that for Figure 1(a) there are values of D_A and D_B such that the constant-level straight lines cross the coordinate axes. This means that the value of the response Y at the points (D_A, 0) and (0, D_B) is the same, i.e. Y(D_A, 0) = Y(0, D_B). Such values of the doses D_A and D_B of isolated factors are called isoeffective doses and they fully determine the corresponding straight line (the line of the additive isobole). As is known from the course of geometry, in this case the equation of a straight line passing through the points (D_A, 0) and (0, D_B) may be represented as equation (3)

$$\frac{d_A}{D_A} + \frac{d_B}{D_B} = 1$$

where d_A, d_B are the coordinates of any point on the additive isobole straight line; the doses dA and dB satisfy the equality Y(d_A,d_B) = Y(D_A, 0) = Y(0, D_B).

On the contrary, for Figure 1(b) the direction of the straight line is such that the isobole crosses one axis only (either the axis of the toxicant A or of the toxicant B), hence it makes no sense to regard the doses of toxicants A and B as isoeffective. This draws an essential difference between these cases, the former being a case of classical additivity and the latter that of oppositely directed single factor actions (strictly speaking, it should not be referred to the type of classical cases accepted for unidirectional action).

Let us consider the product of coefficients b_1b_2 and replace coefficients b_1, b_2 in it by the right-hand terms of the corresponding equalities (7). Allowing for the additivity equality (1), we obtain

$$b_1 \cdot b_2 = \frac{1}{4}\left(y_{10} - y_{00}\right) \cdot \left(y_{01} - y_{00}\right) \quad (8)$$

Given the fact that the coefficients b_1 b_2 have the same sign, we have b1b2 > 0; hence, the right-hand part of the relation (8) is also positive. The right-hand part of (8) contains a product of single-factor effects of the first and second toxicants, which may be positive only in the case of unidirectional single-factor effects.

When considering superadditivity and subadditivity from the perspective of the effect additivity paradigm (1), we noted the need for an unidirectional action of the factors, i.e. either both of these actions should be positive or both should be negative. Thus,

in the case of superadditivity, the following inequalities should hold

$$\begin{cases} y_{11} - y_{10} > y_{01} - y_{00} \\ y_{01} > y_{00} \\ y_{10} > y_{00} \end{cases} \quad (9a)$$

or

$$\begin{cases} y_{11} - y_{10} < y_{01} - y_{00} \\ y_{01} < y_{00} \\ y_{10} < y_{00} \end{cases} \quad (9b)$$

It is not hard to check that the sets of inequalities (9a) and (9b) are equivalent to the following sets of inequalities

$$\begin{cases} b_{12} > 0 \\ b_2 > b_{12} \\ b_1 > b_{12} \end{cases} \text{ and } \begin{cases} b_{12} < 0 \\ b_2 < b_{12} \\ b_1 < b_{12} \end{cases}, \text{ respectively.}$$

As has already been noted, the response surface model (6) presents a hyperbolic paraboloid which has the so-called saddle point with coordinates where

$$x_1^0 = -\frac{b_2}{b_{12}},\ x_2^0 = -\frac{b_1}{b_{12}} \quad (10)$$

From the standpoint of the relation (1) the conditions of superadditivity are equivalent to the satisfaction of the inequalities

$$x_1^0 < -1,\ x_2^0 < -1 \quad (11)$$

Thus, the traditional superadditivity of the unidirectional action in terms of the theoretical response surface as being represented by the hyperbolic paraboloid corresponds to the position of this surface in relation to the range of admissible doses (in our case, allowing for the above-described coding of the variables, this is a square [–1; 1]□[–1; 1]) such that the saddle point occurs outside the range of doses, the inequalities (11) being met. As is easy to verify, in this case the response level surfaces (isoboles) are given by the shapes shown in Figure 2 (a).

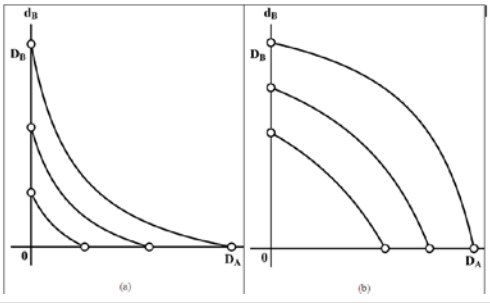

Figure 2: Isoboles for (a) superadditivity and (b) subadditivity in the hyperbolic paraboloid model.

It is significant that in this case isoeffective doses are also available for all admissible effect levels.

A similar analysis for the case of subadditivity leads to the conclusion that subadditivity occurs if and only if the following inequalities are fulfilled:

$$\begin{cases} b_{12} > 0 \\ b_2 < -b_{12} \\ b_1 < -b_{12} \end{cases} \text{ or } \begin{cases} b_{12} > 0 \\ b_2 > -b_{12} \\ b_1 > -b_{12} \end{cases},$$

which may also be represented in the form of a condition for the saddle point coordinates

$$x_1^0 > 1,\ x_2^0 > 1.$$

Allowing for these inequalities, it is easy to check that the sections of the response surface produced by constant effect planes (i.e. isoboles) are given by shapes in Figure 2 (b).

Thus, all classical types of interaction considered from the standpoint of the effect additivity paradigm (1) feature one-to-one correspondence to the isoboles in Figure 1(a), 2 in the hyperbolic paraboloid model. Hence, within the framework of the model (6), the paradigms of effect additivity (i.e. a result of using ANOVA) and dose additivity (i.e. analysis with the help of the response surface (6) are equipotential and interchangeable. Possibly, if a more complex response surface model were chosen (for instance, a full quadratic model), the above would not hold true. It is sufficiently obvious that the effect additivity paradigm may not be fulfilled if the single-factor 'dose-response' functions are non-linear; for example, in this case the relation (1) is not fulfilled for two similar toxicants (the so called sham interaction effect (2). At the same time, the dose additivity paradigm in the case of two similar toxicants is fulfilled precisely because the equality (3) is fulfilled and the isoboles are straight lines as shown in Figure 1(a).

3. Isoboles for non-classical types of combined action

As we have seen, the conditions that determine the type of combined action may be formulated in the form of some inequalities relative to the saddle point coordinates of the hyperbolic paraboloid (6)

- There is no saddle point, i.e. $b_{12} = 0$. Then the surface (6) degenerates into a plane and the isoboles are given by the straight lines shown in Figure 1.
- The coordinates of the saddle point meet the inequalities $x_1^0 < -1,\ x_2^0 < -1$. In this case, the

combined action features superadditivity and the isoboles are represented by concave curves.

- The coordinates of the saddle point meet the inequalities $x_1^0 > 1, x_2^0 > 1$. In this case, the combined action features subadditivity and the isoboles are represented by convex curves.

Since these conditions have a one-to-one correspondence, all the other cases (as well as the case of Figure 1(b) should be referred to some special type of combined action which do not fit into the classical triad of unidirectional action (additivity, subadditivity, superadditivity). Below we consider these cases and continue comparing the isoboles obtained for them with the effect additivity condition (1).

First consider cases where none of the coordinates of the saddle point falls within the range of doses [–1; 1]□[–1; 1]. If the inequalities $x_1^0 < -1, x_2^0 > 1$ or $x_1^0 > 1, x_2^0 < -1$ hold, the single factor effects are seen to have opposite signs, i.e. the factors act contra-directionally. For example, the inequalities $x_1^0 < -1, x_2^0 > 1$ are equipotential to the following conditions which are analogous to the inequalities (9)

$$\begin{cases} y_{11} - y_{10} - y_{01} + y_{00} > 0 \\ y_{01} > y_{00} \\ y_{11} < y_{01} \end{cases}$$ or

$$\begin{cases} y_{11} - y_{10} - y_{01} + y_{00} < 0 \\ y_{01} < y_{00} \\ y_{11} > y_{01} \end{cases},$$

from which it follows that $y_{10} - y_{00} < 0$ (for the first set of inequalities) or $y_{10} - y_{00} > 0$ (for the second set).

In this case, we obtain the following isoboles (see Figure 3).

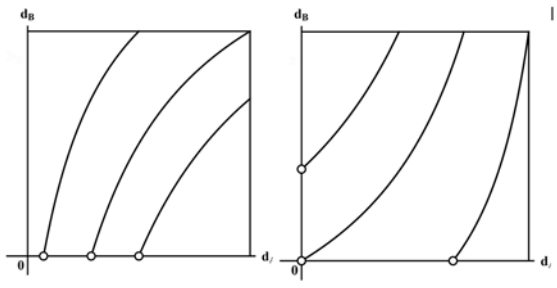

Figure 3: Isoboles for the cases (a); (b) for the position of the saddle point relative to the range [–1; 1]□[–1; 1] of experimental doses.

As follows from Fig. 3, the opposite action shows itself on the isoboles in different positions of the level lines compared to the isoboles in Figure 1(a) and Figure 2. It may be stated that for a contra-directional action an increase in the values of one predictor leads to an increase in the values of the other provided that the value of the combined effect of these predictors is maintained constant. Formally, this may be expressed by the inequality

$$\left.\frac{dx_2}{dx_1}\right|_{Y=const} = -\frac{b_1 + b_{12}x_2}{b_2 + b_{12}x_1} < 0$$ for unidirectional action

and

$$\left.\frac{dx_2}{dx_1}\right|_{Y=const} = -\frac{b_1 + b_{12}x_2}{b_2 + b_{12}x_1} > 0$$ for contra-directional action.

Allowing for the equalities (10), these conditions may be re-written in the form (provided $b_{12} \neq 0$)

$$\frac{x_2 - x_2^0}{x_1 - x_1^0} < 0 \text{ or } \frac{x_2 - x_2^0}{x_1 - x_1^0} > 0 \text{ .} \quad (12)$$

Figure 4 shows cases where the saddle point of the hyperbolic paraboloid falls outside of the range of experimental doses (i.e. in the coded variables, the coordinates and are outside the interval (–1, +1). Figure 4(a) and 4(b) show examples where one coordinate of the saddle point falls within the range of experimental doses. In this case, one of the coordinate axes is divided by an asymptote towards either side of which there are different types of combined action. Both plots on Fig. 4 have a vertical asymptote, although a horizontal one is possible, too.

Besides the cases of Figure 4 (where only one of the coordinates x_1^0 and x_2^0 falls within the range of experimental doses, with one asymptote available), there may be a case where both coordinates of the saddle point occur within the range of experimental doses, i.e. the conditions $x_1^0 \in (-1;1), x_2^0 \in (-1;1)$ are met. This case may be obtained by putting together the plots shown in Figure 4 and similar plots in relation to the horizontal asymptote (see Figure 5). In this case, the dose range splits into 4 parts each of which displays one of the above-described types of combined action, and one and the same value of the effect will be simultaneously

represented by two isoboles located in different dose range regions.

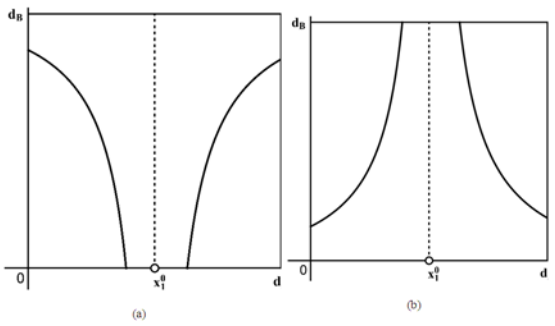

Figure 4: Isoboles for (a) and (b) . Dashed line denotes the asymptote passing through the saddle point within the range of doses under consideration.

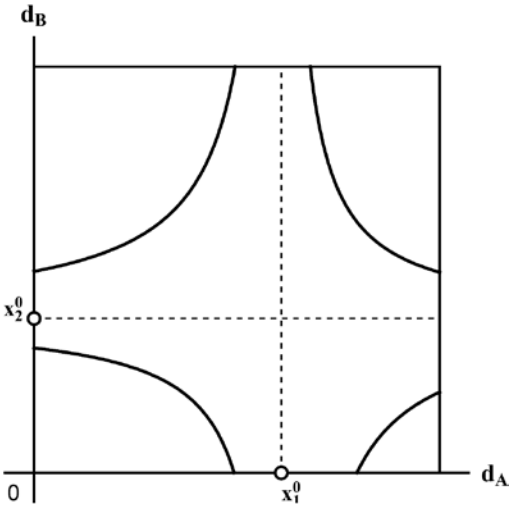

Figure 5: Isoboles in a case where the conditions are met. The dashed lines show two asymptotes.

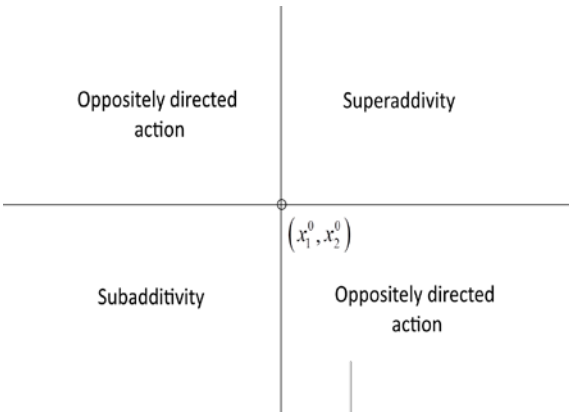

Figure 6: The character of combined action depending on the position of the isobole relative to the saddle point.

It follows from the previous analysis and inequalities (12) that the type of a combined action may be determined depending on the position of the isobole in relation to the saddle point using the following chart (Figure 6)

This enables us to obtain a complete description of all types of combined action without the need to carry out detailed computations.

Some Applications of the Hyperbolic Paraboloid Model to the Combined Toxicity Characterization Problem

Below we consider some experimental results of using the model (6) for describing and representing combined toxicity types. It should be noted preliminarily that practically in each experiment the model (6) proved to represent well the experimental data for the majority of the indices studied. Moreover, in the experiments all of the indices studied displayed the entire variety of the possible types of combined action permitted by the model (6). Thus, experimental practice should assume as typical the existence of a multitude of possible types of combined action rather than just the traditionally accepted additivity, subadditivity and superadditivity. We emphasize it that the occurrence of these additional types is always associated with the occurrence of a region of oppositely directed actions within the range of experimental doses. Such regions can be effectively identified by means of the hyperbolic paraboloid model (6). Certainly, more precise determination of the boundaries of the regions in which agents acts in this or that direction requires additional experiments and corresponding processing of their results.

1. Traditional isoboles of unidirectional action

Below we present only the isobole plots that were obtained in corresponding experiments. A more detailed description of the experiments and conclusions derived from the isobole plots can be found in the cited references.

We provide as a typical example of the additive combined action of toxicants the subchronic intoxication experiment with a combined action *in vivo* of sodium fluoride and lead acetate [16]. Note that additivity in the model (6) can result not only from exact equality of the coefficient b_{12} to zero but also where its value is small. Since the latter situation occurs more often, the conclusion as to how significant this coefficient is, i.e. whether it could be taken as equal to zero, should be drawn on the basis

of both expert opinions and statistical estimates and testing of the corresponding statistical hypothesis. In the case of Figure 7(a), such a test shows that we could assume that $b_{12} = 0$.

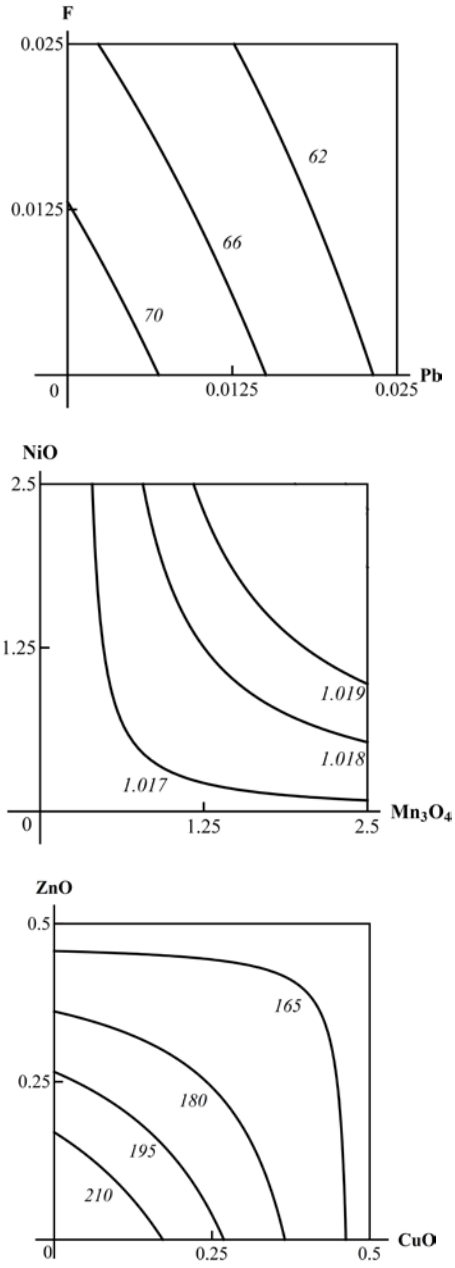

Figure 7: Traditional isoboles for unidirectional effects: (a) the additivity of P and F combined action for the number of lymphocytes [16]. The doses of lead and fluoride are given in LD50 units. (b) the superaddivity of Mn3O4 and NiO combined action for urine density [17]. The doses of Mn3O4 and NiO are plotted on the axes in mg per rat. (c) the subadditivity of CuO and ZnO combined action for Alkaline Phosphatase (AF) in blood serum (20). The doses of CuO and ZnO are plotted on the axes in mg per rat. The numbers at the isobole lines show the value of the effect Y (for example, values 60, 65 and 70 in (a) show the number of lymphocytes for which the isobole has been constructed; AF in IU/L).

2. Isoboles for the presence of contra-directional action

As has been noted, the presence of regions of doses with a contra-directional isolated action of the factors results in isoboles the shape of which cannot be described in unidirectional action terms as in the previous section. Nevertheless, they can be quite correctly interpreted if we divide all the range of dose combinations into regions, each of which displays this or that type of combined action. This makes it necessary, in particular, to highlight cases of pure contra-directional action in all the range of doses (Figure 8).

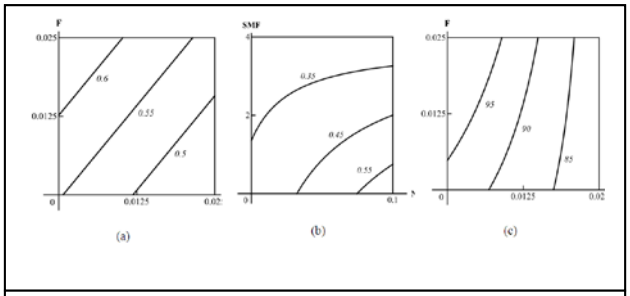

Figure 8: Isoboles with oppositely directed actions of toxicants across the range of experimental doses: (a) for the index of ALT activity (in mM/h L) from [16]; (b) for spleen mass (g per 100 g body mass) from [29]. On the axes, the fluoride doses are plotted in fractions of LD50 and SMF (Static Magnetic Field) doses – in hours of daily exposure. (c) for hemoglobin (g/L) from (Panov et al., 2015). The numbers at the isobole lines show the value of the effect Y.

The type of contra-directional action shown in Figure 8(a) is similar to the one shown above in Figure 1(b). Since the condition $b12 = 0$ must be fulfilled here as well, the notes made in the previous section concerning the methods for checking this equality also hold true.

Although we have so far been considering the toxicological applications of the model (6), all conclusions concerning the applicability of this model and diversity of types of combined action which it can represent hold true for other experimental conditions as well. As an example of this, Figure 8 (b) shows isoboles of combined action produced by sodium fluoride and Static Magnetic Field (SMF) on spleen mass [28].

Research experiments often encounter situations where the condition of unidirectional action of the factors across the entire range of experimental doses is not fulfilled. However, even if one index displays a unidirectional action, this condition may be violated by the other index. Since the design of experiments *in vivo* implies that physiological indices will be obtained for various systems of the organism, situations with different variants of both directionality of action and types of combined action for various indices should recognized typical. In particular, it is not

infrequent that the range of doses is divided by asymptotes into 2 or 4 parts each of which demonstrates a specific type of combined action (Figure 4, 5). Geometrically this corresponds to one or both co-ordinates of the saddle point (10) falling within the range of experimental doses.

The range of experimental doses may be divided into 2 parts by a horizontal or vertical asymptote, the positioning of the branches of the constant effect line hyperbolas being also possible in two ways according to Figure 4 for the vertical asymptote. Since each of these cases corresponds to a special combination of regions of unidirectional and contra-directional action, each of them may be considered as a separate type of combined action. For example, in Figure 4(a) we deal with subadditivity to the left of the point, and contra-directional action to the right of it. In Figure 4(b), the pattern is different – contra-directional action to the left of, and superadditive action to the right of it. Similar combinations of contra-directional action and sub- or superadditivity take place where the range of experimental doses is divided by a horizontal asymptote. In this case, however, there will be other corresponding regions with the same type of combined action.

Finally, there may be a case where the range of doses is divided into 4 parts each of which demonstrates the same type of combined action. This corresponds to both co-ordinates x_1^0 and x_2^0 of the saddle point occurring within the range of experimental doses. In this case, the interpretation of the types of combined action in each region is unambiguous and can be obtained in accordance with the diagram in Figure 5.

Thus, if we consider all the above cases of combined action as special and separate types, the hyperbolic paraboloid model (6) enables us to represent, describe and analyze 11 types of combined toxicity in addition to a possible case of purely one-factor action expressed by a horizontal or vertical straight line.

Conclusions

The foregoing analysis suggests the possibility of a one-to-one correspondence of isobole types in Figure 1–5 and all possible types of interaction plots used in ANOVA. In particular, for a type 2^2 full factorial experiment, the traditional concepts of the theory of combined toxicity (additivity, subadditivity, superadditivity) if analyzed by means of ANOVA (effect additivity paradigm) have a one-to-one correspondence to the isoboles in Figure 1(a), 2. Hence, within the framework of the models considered, the effect additivity paradigm (realized by means of ANOVA) and the dose additivity paradigm (realized by means of RSM) are equivalent.

On the other hand, the model (6) can be constructed for any data, not just for a type 2^2 full factorial experiment. Then in interpreting the isoboles of the model (6) for these data we can rely on the correspondences obtained above. For general data (for example, continuous predictors), the construction of any variants of interaction plots in ANOVA is knowingly impossible, and thus in this case only would RSM be a correct and effective method for characterizing the type of combined operation displayed by factors.

Note that the elementary model (6) demonstrates the possibility of not only above-mentioned classical types of combined action but also of types which cannot be interpreted within the framework of this triad. It is important that all these additional types are associated with the presence of contra-directional actions of two factors across the entire range of doses as in (Figure 3) or in any of its parts (Figure 4, 5). Since in designing an experiment it is, as a rule, impossible to foresee whether the factors will maintain the unidirectionality of action across the range of doses, it is essential to have a means of detecting such a phenomenon (i.e. a suitable means of data analysis which provides for such possibility – in our case it is the model (6) and examples of correct interpretation of mathematical analysis outcomes.

Although in the above case of type 2^2 full factorial experiment the RSM and ANOVA approaches to characterizing combined action types are equivalent, the response surface methodology is generally more preferable. Indeed, the model (6) can be constructed for others experiment designs or for observational studies. Data that are used for analysis of combined action types in ANOVA are contained in the constructed response surface (in a more or less exact form; the more exact, the better the response surface model represents the data). In this sense, it may be stated that the response surface methodology combines both the effect additivity paradigm and the dose additivity paradigm.

There is an important circumstance which is essential for correct interpretation of ANOVA results - one-factor effects should have the same direction. If this condition is not met in ANOVA, the researcher would only be able to state the presence of contra-directional action but would not be able to tell whether it is

present across the board (i.e. in which regions of the dose combinations it is available). RSM enables a detailed analysis of this problem as shown above by the examples in Figure 6-10. Knowledge of such regions of unidirectional action may help design subsequent experiments.

The response surface methodology also makes it easier to construct isoboles in general situations where preliminary experiments for determining the isoeffective doses of the acting agents have not been carried out. In this case, the researcher can use available data for constructing the response surface model which, if cut with constant response value planes, provides a preliminary or sufficiently accurate idea of the real isoboles – the more accurate it is, the better the chosen model represents the data.

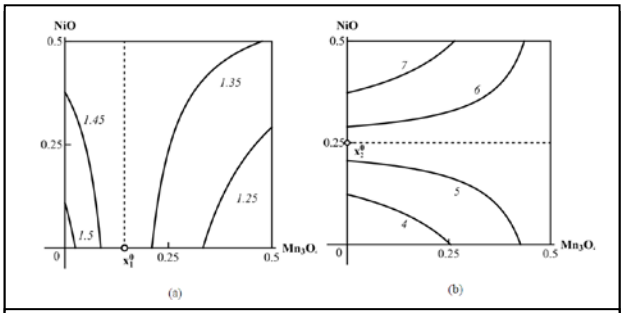

Figure 9: Isoboles for metallic Nanoparticles (NPs) intoxication: (a) and (b) – the ranges of toxicant doses are divided by an asymptote passing through the coordinates or of the saddle point (the asymptotes are shown as dotted lines). (a) A/G index (albumin/globulin ratio); to the left of the asymptote there is subadditivity of the combined action, and to the right – oppositely directed action; (b) number of akaryotic hepatocytes (per 100 liver cells); subadditivity occurs below the asymptote, and oppositely directed action is above it. The doses of Mn3O4-NPs and NiO-NPs are given in mg per rat. The number at the isobole line shows the value of the effect Y, i.e. A/G ratio for (a), and number of akaryotic hepatocytes for (b) [17].

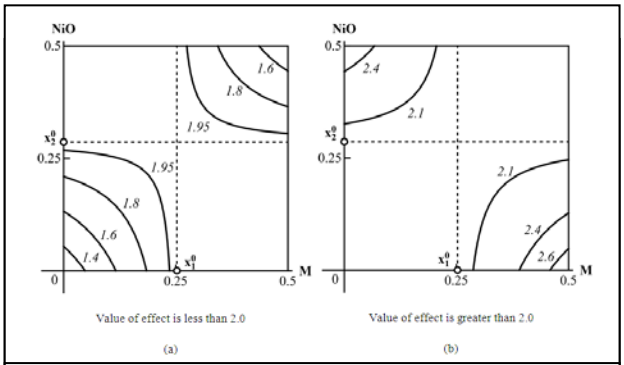

Figure 10: Isoboles for the combined Mn3O4-NPs and NiO-NPs intoxication (Katsnelson et al., 2015b): (a) and (b) – the ranges of toxicant doses are divided by two asymptotes passing through the saddle point (the asymptotes are shown as dotted lines). Both plots (a) and (b) represent the isobologram for relative percentage of granulocytes in blood serum for different effect values. (a) Granulocytes, % is less than 2.0; subadditivity occurs in the lower left corner and superadditivity in the upper right corner of the dose's combination square. (b) Granulocytes, % is greater than 2.0; oppositely directed joint action of NPs is in both cases. The doses of Mn3O4-NPs and NiO-NPs are given in mg per rat. The number at the isobole line shows the value of the effect Y (Katsnelson et al., 2015b).

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