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QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS (QSAR) FOR BINARY MIXTURES AT NON-EQUITOXIC RATIOS BASED ON TOXIC RATIOS-EFFECTS CURVES

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□ The present study proposed a QSAR model to predict joint effects at non-equitoxic ratios for binary mixtures containing reactive toxicants, cyanogenic compounds and aldehydes. Toxicity of single and binary mixtures was measured by quantifying the decrease in light emission from the *Photobacterium phosphoreum* for 15 min. The joint effects of binary mixtures (TU_{sum}) can thus be obtained. The results showed that the relationships between toxic ratios of the individual chemicals and their joint effects can be described by normal distribution function. Based on normal distribution equations, the joint effects of binary mixtures at non-equitoxic ratios ($TU_{sum}^{n:m}$) can be predicted quantitatively using the joint effects at equitoxic ratios ($TU_{sum}^{1:1}$). Combined with a QSAR model of $TU_{sum}^{1:1}$ in our previous work, a novel QSAR model can be proposed to predict the joint effects of mixtures at non-equitoxic ratios ($TU_{sum}^{n:m}$). The proposed model has been validated using additional mixtures other than the one used for the development of the model. Predicted and observed results were similar ($p>0.05$). This study provides an approach to the prediction of joint effects for binary mixtures at non-equitoxic ratios.

Keywords: joint effect, binary mixture, non-equitoxic, QSAR, toxic ratio-effect curve

INTRODUCTION

Since Bliss proposed the fundamental theory for mixture toxicity and defined basic modes of action for mixtures in 1939 (Bliss 1939), research assessing the joint effects of mixtures has increased substantially in the past several decades (Ra *et al.* 2006). Environmental contaminants are frequently encountered as mixtures rather than single chemicals. Moreover, interactions of components in a mixture might cause complex and substantial changes in the apparent properties of its constituents (LeBlanc and Wang 2006), which pose a potential threat to human health and environmental systems. It is therefore necessary to assess and predict the joint effects of mixtures, especially those mixtures with interactions.

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A variety of indices have been derived to assess the joint toxic effects of mixtures, including the sum of toxic units ($TU_{sum}=\sum TU_i$) (Sprague and Ramsay 1965), the additivity index (AI) (Lloyd 1961), the mixture toxicity index (MTI) (Konemann 1981), the similarity parameter (λ) (Christensen and Chen 1989) and so on. Among these indices, TU_{sum} is one of the most widely applied indices to assess the joint effects (Lin 2009; Parvez *et al.* 2009; Mauffret *et al.* 2010); concentration addition are characterized by $TU_{sum}=1.00\pm0.20$, $TU_{sum}<0.80$ represents synergistic effects and $TU_{sum}>1.20$ indicates antagonistic effects. TU_{sum} can be obtained using the following equation,

$$TU_{sum} = \frac{C_A}{EC_{50-A}} + \frac{C_B}{EC_{50-B}} \quad (1)$$

where C_A and C_B are concentrations of components A and B in mixtures at median inhibition and can be calculated according to the median effective concentration of the mixture. $EC_{50\text{-}A}$ and $EC_{50\text{-}B}$ are the median effective inhibition concentrations of single chemicals A and B, respectively.

Quantitative structure-activity relationship (QSAR) models have been widely employed to predict toxicities of single chemicals over the past decades, it has also been employed in the fields of mixture toxicity recently (Lin *et al.* 2003a; Castillo-Garit *et al.* 2008). For example, many QSAR models were developed to predict the joint effects of mixtures containing narcotic chemicals such as halogenated benzenes (Cronin and Schultz 1997), alkanols (Wang *et al.* 2006), and phenols (Wang *et al.* 2008). However, the study on reactive chemicals is much fewer than narcotic chemicals because mixture toxicity of reactive chemicals is more complex (Escher and Hermens 2002). Cyanogenic compounds and aldehydes are common reactive chemicals with excess toxicity (If the predicted baseline toxicity is 10 times more than observed toxicity to the same organism, the chemicals are defined as reactive toxicants with excess toxicity) (Verhaar *et al.* 1992). Previous studies have shown that the predicted baseline toxicities of cyanogenic compounds and aldehydes were 10 times greater than observed toxicities to the same organism (Lipnick 1991). Therefore, these chemicals were classified as reactive chemicals with excess toxicity. In the field of organic synthesis, these chemicals have been extensively used as intermediates. In particular, their simultaneous applications are common and they are often simultaneously detected in the wastewater (Shinkai *et al.* 1980; Lin *et al.* 2003b; Li *et al.* 2005), which posed a potential threat on ecological system (Monosson 2005). It is therefore necessary to study their joint effects. Chen and Huang (1996) and Chen and Lu (2002) assessed the joint effects at equitoxic ratios ($TU_{sum}^{1:1}$) of mixtures containing cyanogenic compounds and aldehydes and developed some criteria to qualitatively predict the probability of occurrence of joint

effects in mixtures containing reactive chemicals. Based on the studies by Chen and Huang (1996) and Chen and Lu (2002), our previous study further revealed that joint effects of cyanogenic compounds and aldehydes resulted from intracellular chemical reactions, and the reactions are inherently related to both the reactivity of aldehydes and the release of CN^- from cyanogenic compounds under the action of hydrolysis (Lin *et al.* 2003a). Because the ability of hydrolysis to release cyano group (CN^-) of cyanogenic compounds is different, and the reactivity of aldehydes with CN^- is also different, so their intracellular chemical reactions are different and thus their joint effects are various, even they are all cyanogenic compounds and aldehydes. Based on the toxicological mechanism, we developed a QSAR model to predict $TU_{sum}^{1:1}$ (Tian *et al.* 2012).

$$\begin{cases} TU_{sum}^{1:1} = 1.00 \pm 0.20 & \text{when } (O_{aldehyde} - C_{cyanogenic}) > -0.125 \\ TU_{sum}^{1:1} = -27.6 \times O_{aldehyde} - 5.22 \times C_{cyanogenic} - 6.97 & \text{when } (O_{aldehyde} - C_{cyanogenic}) \le -0.125 \end{cases} \quad (2)$$

$$n=40,\ r=0.887,\ SE=0.195,\ F=140,\ p<0.001,\ q^2_{Loo}=0.748$$

However, Equation 2 can only be utilized to predict the joint effects of mixtures containing reactive toxicants at equitoxic ratios. To date, there is still no QSAR model that has been developed to predict the joint effects of mixtures containing reactive chemicals at non-equitoxic ratios. Mixtures at equitoxic ratios are just an ideal state and contaminants are usually encountered as mixtures at non-equitoxic ratios in the real environment. Consequently, it is necessary to propose a QSAR model to predict the joint effects of mixtures containing reactive chemicals at non-equitoxic ratios.

Dose response curve is an important tool in toxicological research and usually serves as an important reference for the evaluation of chemical toxicity and the determination of permissible exposure levels. One of the most common dose response curves is the S-shaped curve, and this curve can be described using the cumulative normal distribution function (Faust *et al.* 2001; Loureiro *et al.* 2010). For a binary mixture, the dose response curve can describe the relationship between the response and the total concentration of components in this mixture. But it is insufficient to describe the relationship between the response of the mixture and the concentration of individual toxicants in the mixture. That is, it is difficult to distinguish the contribution of individual chemicals in the binary mixture. In this study we take the logarithm of toxic ratios of individual chemicals as the *x* axis:

$$x = \lg\frac{n}{m} = \lg\left[\left(\frac{C_A}{EC_{50-A}}\right)\Big/\left(\frac{C_B}{EC_{50-B}}\right)\right] \quad (3)$$

$$n=C_A/EC_{50\text{-}A} \quad (4)$$

$$m=C_B/EC_{50\text{-}B} \quad (5)$$

where C_A and C_B are concentrations of components A and B in a mixture at median inhibition, n and m are multiples of EC_{50} of individual chemicals A and B in a binary mixture. The term, 'n/m', is defined as a toxic ratio of individual chemicals in the binary mixture. The corresponding joint effects are defined as y ($y=TU_{sum}$). Then a curve can be obtained to describe the relationship between toxic ratios of individual toxicants and their joint effects. Here we named the curve as Toxic Ratio-Effect Curve (TREC).

Therefore, the purposes of this study were: 1) to determine the EC_{50} of single chemicals and toxic units of binary mixtures containing cyanogenic compounds and aldehydes at both equitoxic ($TU_{sum}^{1:1}$) and non-equitoxic ratios ($TU_{sum}^{n:m}$), 2) to quantitatively describe the relationships between toxic ratios of individual chemicals and their joint effects for binary mixtures, 3) to predict the joint effects of binary mixtures at non-equitoxic ratios ($TU_{sum}^{n:m}$) using $TU_{sum}^{1:1}$, and 4) to propose a QSAR model for the prediction of joint effects of binary mixtures at any ratio using the structure descriptors of individual chemicals.

MATERIALS AND METHODS

Materials and instrumentation

Malononitrile and acetonitrile were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). Other chemicals were purchased from Sigma-Aldrich Company (St Louis, USA). All these chemicals were analytical reagent grade or above. The toxicity test instrument (chemiluminescent immunoassay analyzer BH9507) was provided by Beijing Hamamatsu Company (Beijing, China). The freeze-dried marine bacterium, *Photobacterium phosphoreum* (T3 mutation), was supplied by the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China).

Toxicity experiment

Toxicity was measured by quantifying the decrease in light emission from the bacteria as a result of exposure to a 3% NaCl solution of the test chemical for 15 min. The diluted bacteria were cultured at 20°C in yeast-tryptone-salt-gycerol broth for 12-14 h, and then a 100-fold dilution was used as inoculums. The test tube was filled with 800 μL of test aqueous solution and 200 μL of the inoculums. The final diluted bacteria in negative control were about 1×10^7 cells mL^{-1}. The decrease in light emission

was measured at different concentrations and each concentration was tested in triplicate (3 test units per concentration and 1 measured per test unit). Based on the decrease in light emission, EC_{50} was calculated using the probit model ($I = a \times \lg C + b$, I and C denote the inhibition and concentration respectively, 'a' and 'b' denote the slope and intercept).

Binary mixtures, including mixtures at equitoxic ratios (identical fractions of EC_{50}) and non-equitoxic ratios, were tested based on observed EC_{50} values of single toxicants. The test methods for binary mixtures were conducted in a similar manner as the single chemical tests according to our previous study (Lin *et al.* 2003a). Briefly, an initial prepared concentration of a mixture was defined as 100% and the luminous inhibition was measured at different dilutions (e.g., 3.2%, 5.6%, 10%, 18%, 32%, 56% and 100%). Based on the determined concentration-response relationship, the joint effect (TU_{sum}) of this mixture can be obtained. For example, the detailed concentrations of a binary mixture are listed in Table 1. It should be noted that the concentrations in this study are in the form of nominal concentrations. Furthermore, previous studies had demonstrated that the concentrations of cyanogenic compounds and aldehydes were constant in the duration of 15 min (Chen *et al.* 2005).

Toxic ratio-effect curve

The joint effect is described by the sum of toxic unit index at median inhibition (TU_{sum}), and the value of TU_{sum} can be obtained using Equation 1. The joint effect of a mixture at the equitoxic ratio and median inhibition is defined as $TU_{sum}^{1:1}$. The joint effect of a mixture at a non-equitoxic ratio and median inhibition is defined as $TU_{sum}^{n:m}$. All toxicity data, including single toxicants and binary mixtures, refer to that at a median inhibition. Then lg n/m is employed as an independent variable and $TU_{sum}^{n:m}$ is utilized as a dependent variable, a toxic ratio-effect curve for a binary mixture can thus be developed.

TABLE 1. Example of concentrations of binary mixtures at non-equitoxic ratios

n:m ($n\times EC_{50\text{-}A}$:$m\times EC_{50\text{-}B}$)[a]	10:1	3.2:1	1:1	1:3.2	1:10
prepared toxic ratio	0.56: 0.056	0.32: 0.1	0.2: 0.2	0.1: 0.32	0.056:0.56
concentration of A in mixtures	$0.56\times EC_{50\text{-}A}$	$0.32\times EC_{50\text{-}A}$	$0.2\times EC_{50\text{-}A}$	$0.1\times EC_{50\text{-}A}$	$0.056\times EC_{50\text{-}A}$
concentration of B in mixtures	$0.056\times EC_{50\text{-}B}$	$0.1\times EC_{50\text{-}B}$	$0.2\times EC_{50\text{-}B}$	$0.32\times EC_{50\text{-}B}$	$0.56\times EC_{50\text{-}B}$
lg n/m	1.00	0.51	0	-0.51	-1.00

[a] This table illustrates the concentrations of individual components in a mixture by taking malononitrile (A) and acetaldehydes (B) as examples. $EC_{50\text{-}A}$ and $EC_{50\text{-}B}$ represent the median effective inhibition concentrations of malononitrile and acetaldehyde, n and m are multiples of EC_{50} of individual chemicals in a binary mixture. The term, 'n/m', is defined as a toxic ratio of individual chemicals in the binary mixture. In this study, more than 200 binary mixtures were used to develop a QSAR model and other 25 mixtures were used as an external validation set.

Statistical analysis

A normal distribution equation is employed to describe the toxic ratios-effects curves. The normal distribution equation contains four parameters: y_0 is the offset, x_c is the value of the peak at the abscissa, w is the parameter related to full width at half maximum, and A represents the integral area of the curve (Figure 1).

$$y = y_0 + \frac{A}{w \times \sqrt{\pi / 2}} \times e^{-\frac{2(x-x_c)^2}{w^2}} \quad (6)$$

A QSAR model of $TU_{sum}^{1:1}$ in our previous study (Equation 2) was used to develop the QSAR of $TU_{sum}^{n:m}$. In this model, the charge of the carbon atom connected to CN^- in the carbon chain ($C_{cyanogenic}$) is employed to describe the capability of CN^- release from cyanogenic compounds. The charge of oxygen atom in the aldehyde group ($O_{aldehyde}$) is employed to describe the toxicity contribution of aldehydes to joint effects.

Data analysis and linear regression were conducted using SPSS 18.0 software. External validation was carried out to validate the predictive capability of the proposed model. The statistic parameters, including the correlation coefficient (r), the standard error (SE), the Fisher criterion (F) and the significance level (p), were employed to evaluate the quality of equations. Based on a report by Golbraikh and Tropsha (2002), a statistical criterion ($r>0.8$, $p<0.05$) was used to assess whether there was a significant relationship between the dependent and independent variables.

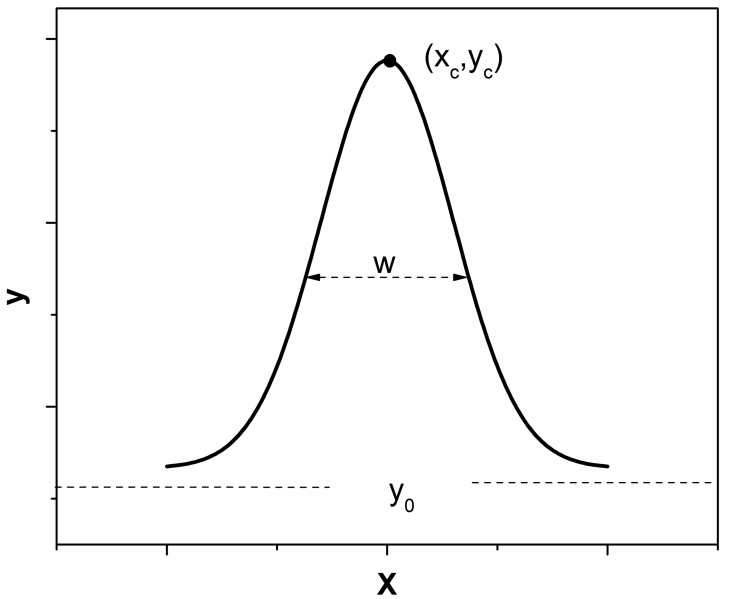

FIGURE 1. Schematic of normal distribution function.

RESULTS AND DISCUSSION

Observed toxic ratios-effects curves for binary mixtures

Before determination of the joint effects between cyanogenic compounds and aldehydes, EC_{50} of individual chemicals to *Photobacterium phosphoreum* was observed and the results are listed as –lg EC_{50} in Table 2.

Based on observed results (EC_{50}) of single chemicals, the joint effects of binary mixtures at equitoxic and non-equitoxic ratios at median inhibition were obtained. Although the joint effects between cyanogenic compounds and aldehydes at equitoxic ratios were various, these mixtures still can be classified as three categories, including mixtures with additive effects at equitoxic ratios, mixtures with antagonism at equitoxic ratios and mixtures with synergism at equitoxic ratios (Lin *et al.* 2005). Specifically, for mixtures that their joint effects at equitoxic ratios are concentration additive, we defined them as mixtures with addition at equitoxic ratios. Similarly, other mixtures were defined as mixtures with synergism (or antagonism) at equitoxic ratios. Then the logarithms of the toxic ratios of individual chemicals (lg n/m) were taken as independent variables and the joint effects of binary mixtures were taken as dependent variables; the curves were obtained in Figure 2-4 based on the above classification.

TABLE 2. Results of the individual toxicity experiment

		Probit Model				
Individual chemical	CAS NO.	-lg EC_{50} [a]	95% CI [b]	a	b	*r*
malononitrile	109-77-3	2.55	2.49-2.61	-0.543	1.88	0.995
acetonitrile	75-05-8	0.77	0.66-0.88	-0.735	1.06	0.994
benzonitrile	100-47-0	3.14	3.06-3.22	-0.551	2.23	0.994
pathalonitrile	91-15-6	3.35	3.28-3.42	-0.472	2.08	0.997
acrylonitrile	107-13-1	1.65	1.51-1.79	-0.722	0.17	0.978
acetaldehyde	75-07-0	2.36	2.26-2.46	-0.621	1.96	0.999
propanal	123-38-6	2.70	2.63-2.77	-0.734	2.46	0.982
butyraldehyde	123-72-8	3.25	3.12-3.38	-1.209	4.42	0.994
valeraldehyde	110-62-3	3.27	3.20-3.34	-0.470	2.05	0.981
heptaldehyde	111-71-7	3.98	3.80-4.16	-0.621	1.96	0.999
benzaldehyde	202-860-4	3.43	3.30-3.56	-1.063	4.16	0.957
p-methylbenzaldehyde	104-87-0	3.82	3.71-3.93	-0.594	2.77	0.991
p-chlorobenzaldehyde	104-88-1	3.97	3.83-4.11	-0.528	2.58	0.995
p-methoxybenzaldehyde	123-11-5	4.03	3.93-4.13	-0.442	2.28	0.993
terephthaldehyde	623-27-8	4.07	3.96-4.18	-0.534	2.68	0.989
p-nitrobenzaldehyde	555-16-8	4.28	4.07-4.49	-0.619	3.13	0.997
p-bromobenzaldehyde	1122-91-4	4.30	4.17-4.43	-0.726	3.64	0.996
p-dimethylaminobenzaldehyde	100-10-7	5.40	5.19-5.60	-0.451	2.92	0.995

[a] Effect concentration at median inhibition in the unit of mol·L^{-1} with a 15 min exposure duration.
[b] 95% Confidence interval

$TU_{sum}^{1:1}$ of these mixtures are less than 0.80 (Figure 2), indicating these mixtures yield synergistic effects at equitoxic ratios. As the toxic ratios vary from the equitoxic point to non-equitoxic ratios, $TU_{sum}^{n:m}$ approaches to 1.00±0.20, indicating their synergistic effects weaken to additive effects. For example, $TU_{sum}^{1:1}$ of malononitrile and acetaldehyde at the equitoxic ratio is 0.12 (Figure 2a), indicating the mixture yields the synergistic effect at the equitoxic ratio. As the increasing non-equitoxic ratios, their joint effects approach to concentration addition ($TU_{sum}^{1000:1}$=1.09, $TU_{sum}^{1:1000}$ =0.92). However, for various mixtures, it is difficult to obtain a uniform threshold that all mixtures start to yield additive effects (Lin *et al.* 2005).

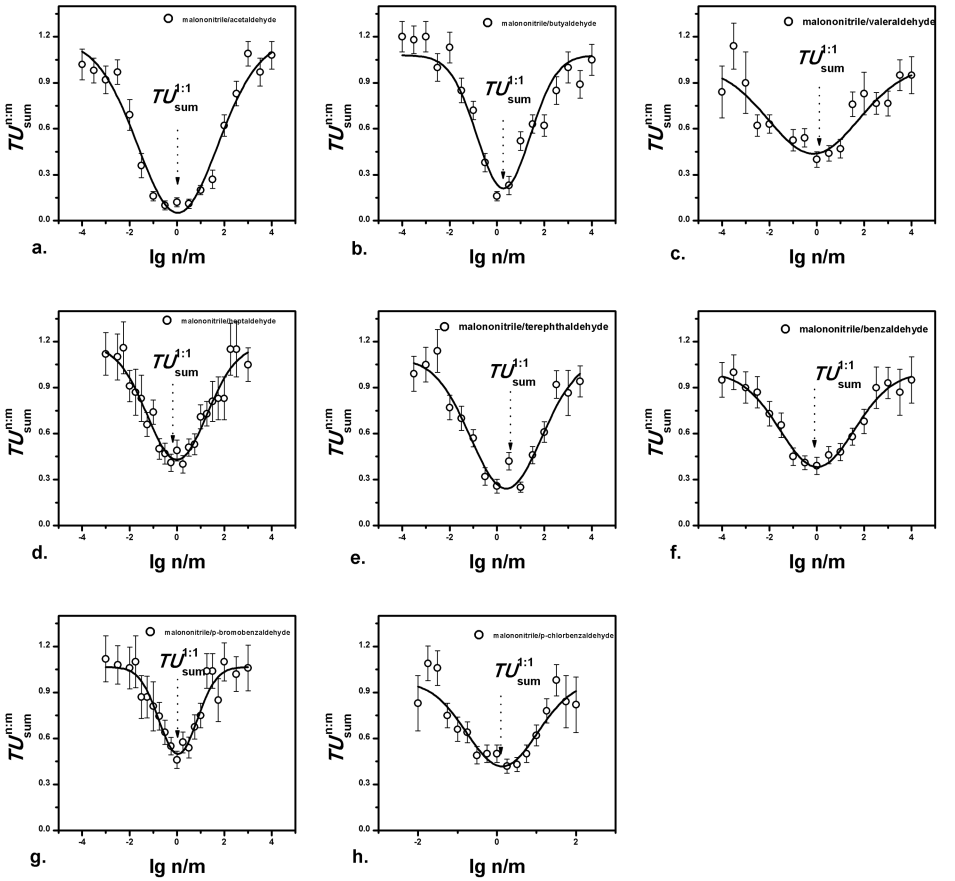

FIGURE 2. Toxic ratios-effects curves of binary mixtures with synergistic effects at equitoxic ratios. Where n and m are multiples of EC_{50} of individual chemicals in a binary mixture, i.e., n=$C_A/EC_{50\text{-}A}$, m=$C_B/EC_{50\text{-}B}$. The term, 'n/m', is defined as a toxic ratio of individual chemicals in the binary mixture. $TU_{sum}^{n:m}$ represents the sum of toxic unit (TU_{sum}) at a non-equitoxic ratio of n/m. $TU_{sum}^{1:1}$ represents the joint effects at the equitoxic ratio. TU_{sum} =1.00±0.20 is defined as a concentration addition. TU_{sum}<0.80 (or TU_{sum}>1.20) is defined as a synergistic (or antagonistic) effect. The dot, '_ open circle', represents the means of the results. Nonlinear fitting is obtained using Equation 6, and the fitted parameters are listed in Table 3.

For mixtures in Figure 3, their $TU_{sum}^{1:1}$ are more than 1.20, indicating their joint effects are antagonistic at equitoxic ratios. As their toxic ratios vary from the equitoxic point to non-equitoxic ratios, $TU_{sum}^{n:m}$ approaches to 1.00±0.20, indicating their antagonistic effects weaken to additive effects. For example, the joint effect of pathalonitrile and *p*-dimethylaminobenzaldehyde at equitoxic ratio is antagonistic ($TU_{sum}^{1:1}$ =1.69, Figure 3a). While their joint effect at 1000:1 is additive ($TU_{sum}^{1000:1}$ =1.04).

For mixtures that their joint effects are additive at equitoxic ratios (TU_{sum}=1.00±0.20), additive effects occur at other non-equitoxic ratios (Figure 4). For example, the joint effect of acetonitrile and *p*-terephthalaldehyde is additive ($TU_{sum}^{1:1}$ =1.08, Figure 4c). While their joint effects at 100:1 and 1000:1 are additive ($TU_{sum}^{100:1}$=1.06, $TU_{sum}^{1000:1}$=1.05, Figure 4c).

It can be seen from Figure 2-4 that these points obey the normal distribution, normal distribution equation can thus be employed to describe the toxic ratios-effects curves. The dependent variable *y* is the toxic unit at any toxic ratio ($y=TU_{sum}^{n:m}$), and the independent variable *x* is the logarithm of toxic ratios of individual chemicals in mixtures (x=lg n/m). This normal equation function is employed to describe the toxic ratios-effects curves of mixtures with synergistic and antagonistic effects at equitoxic ratios (Figure 2 and Figure 3). The fitted results are listed in Table 3.

It can be seen from Table 3 that the fitted correlation coefficients (r) vary from 0.848 to 0.979. The significant correlation indicates that this model can well describe the toxic ratios-effects relationship of binary mixtures.

For binary mixtures with additive joint effects at equitoxic ratios (Figure 4), the value of parameter *A* is equal to zero. As a special normal distribution equation, the normal distribution function of these mixtures can be written as follows,

$$y = y_0 \text{ (Mixtures with additive effects at equitoxic ratios)} \qquad (7)$$

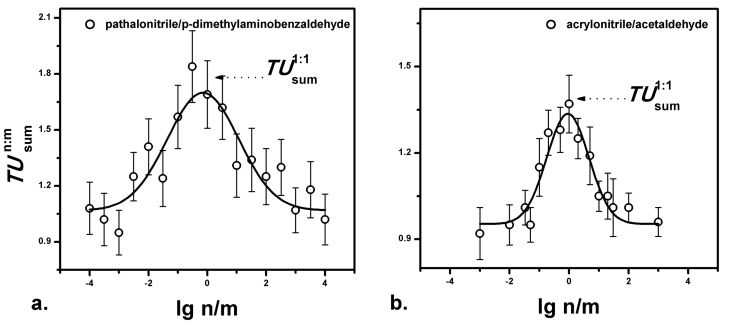

FIGURE 3. Toxic ratios-effects curves of binary mixtures with antagonistic effects at equitoxic ratios.

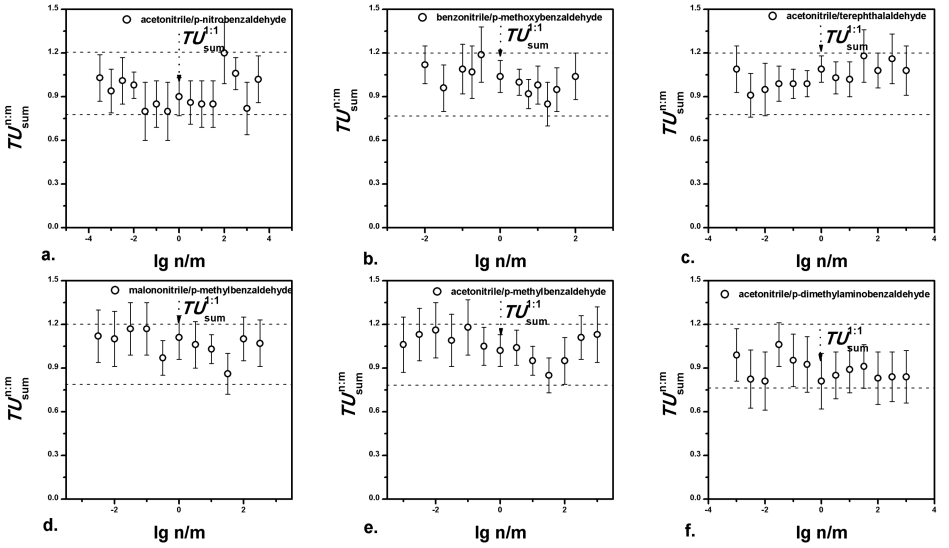

FIGURE 4. Toxic ratios-effects curves of binary mixtures with additive effects at equitoxic ratios.

TABLE 3. Fitted parameters of nominal distribution functions for mixtures in Figure 2 and 3

NO.	Binary mixtures	$TU_{sum}^{1:1}$	y_0	x_c	w	A	r	p^a
malononitrile and								
1	acetaldehyde	0.12	1.12	0.040	3.12	-4.30	0.974	< 0.001
2	butyraldehyde	0.16	1.13	0.402	2.56	-2.80	0.939	< 0.001
3	valeraldehyde	0.40	0.99	-0.166	3.48	-2.39	0.848	< 0.001
4	heptaldehyde	0.47	1.20	-0.001	2.54	-2.44	0.959	< 0.001
5	terephthaldehyde	0.26	1.24	0.343	3.41	-4.14	0.944	< 0.001
6	benzaldehyde	0.38	0.98	0.034	2.98	-2.26	0.979	< 0.001
7	*p*-bromobenzaldehyde	0.46	1.07	0.017	1.62	-1.16	0.941	< 0.001
8	*p*-chlorobenzaldehyde	0.50	0.98	0.118	1.70	-1.21	0.881	< 0.001
pathalonitrile and								
9	*p*-dimethylamino benzaldehyde	1.69	1.09	-0.207	2.25	1.79	0.885	< 0.001
acrylonitrile and								
10	acetaldehyde	1.38	0.95	-0.072	1.43	0.69	0.948	< 0.001
	Mean value		1.08	0.051				
	Theoretical value of the normal distribution function		1.00	0				

[a]The significant level of the fitting equation for each binary mixture.

Deduction of parameters for toxic ratios-effects curves

With the increased deviation from the equitoxic point (lg (n/m)=0), synergistic or antagonistic effects gradually weaken to additive effects (i.e., TU_{sum}=1.00±0.20). This deduction is consistent with the fitted data derived from this experiment. In Table 3, the fitted values of y_0 vary from 0.95 to 1.24 and the average y_0 value is 1.08. The theoretical value of y_0 can be defined as 1.00±0.20.

The other parameter, x_c, is the value of the curve peak at the abscissa. As aforementioned, the synergistic or antagonistic effect is stronger at equitoxic ratios than at any other ratio, i.e., the curve peak is located at the equitoxic point. This result is consistent with the conclusion of the climax hypothesis in our previous study (Lin *et al.* 2005). The climax hypothesis concludes that there is a climax at the equitoxic ratio when plotting the toxic ratios of individual chemicals in mixtures versus their joint effects. Consequently, the theoretical value of x_c should be equal to 0 (x_c=lg (1/1)=0). This deduction is supported by the fitted data derived from the experiment: the average x_c value in Table 3 is equal to 0.051. Consequently, the value of x_c can be defined as zero.

The other parameter, *A*, can also be deduced from the experimental data. Because the integral area of the curve (*A*) is related to half peak width and peak height ($TU_{sum}^{1:1}$) in the normal distribution function, the value of *A* can be mathematically deduced by $TU_{sum}^{1:1}$. Using the corresponding data in Table 3, a linear relationship between *A* and $TU_{sum}^{1:1}$ can be found as follows,

$$A = 3.42 \times TU_{sum}^{1:1} - 3.82 \tag{8}$$

n=10, r=0.928, SE=0.764, F=49.5, P< 0.001

This result shows that there are significant correlations (r=0.928) between *A* and $TU_{sum}^{1:1}$. Therefore, parameter *A* can be deduced using $TU_{sum}^{1:1}$ based on the above Equation.

After the parameters (y_0, *x* and *A*) are obtained, the parameter (w) can be deduced using the coordinate value of equitoxic ratios. Because the coordinate values of *x* and *y* at equitoxic ratios are equal to 0 and $TU_{sum}^{1:1}$ respectively, all deduced parameters, including *x* (x=0), *y* (y= $TU_{sum}^{1:1}$), y_0 (y_0=1.00), x_c (x_c=0) and *A* ($A = 3.42 \times TU_{sum}^{1:1} - 3.82$), are put into the normal distribution equation (Equation 6) and the value of w is thus obtained as follows,

$$TU_{sum}^{1:1} = 1.00 + \frac{3.42 \times TU_{sum}^{1:1} - 3.82}{w \times \sqrt{\pi / 2}}$$
$$\Rightarrow w = \frac{2.73 \times TU_{sum}^{1:1} - 3.05}{TU_{sum}^{1:1} - 1} \tag{9}$$

Because the normal distribution equation can be employed to describe the toxic ratios-effects (Equation 6), the relationship of joint effects between non-equitoxic ratios () and equitoxic ratios ($TU_{\text{sum}}^{1:1}$) can be quantified using the four aforementioned deduced parameters. By substituting the four deduced parameters [y_0=1.00, x_c=0, $w = \frac{2.73 \times TU_{\text{sum}}^{1:1} - 3.05}{TU_{\text{sum}}^{1:1} - 1}$, $A = 3.42 \times TU_{sum}^{1:1} - 3.82$] into the normal distribution equation (Equation 6), the relationship between $TU_{\text{sum}}^{\text{n:m}}$ and $TU_{\text{sum}}^{1:1}$ can be obtained as follows,

$$TU_{\text{sum}}^{\text{n:m}} = y_0 + \frac{A}{w \times \sqrt{\pi/2}} \times e^{-\frac{2(x-x_0)^2}{w^2}} = 1.00 + (TU_{\text{sum}}^{1:1} - 1) \times e^{\frac{-2\times(\lg\frac{n}{m})^2 \times (TU_{\text{sum}}^{1:1}-1)^2}{(2.73\times TU_{\text{sum}}^{1:1} - 3.05)^2}} \quad (10)$$

QSAR of binary mixtures at non-equitoxic ratios

Combining Equation 2 with 10, a model can be obtained to predict the joint effects at non-equitoxic ratios for binary mixtures that their joint effects are synergistic or antagonistic at equitoxic ratios as follows,

$$TU_{\text{sum}}^{\text{n:m}} = 1.00 - (27.6 \times O_{\text{aldehyde}} + 5.22 \times C_{\text{cyanogenic}} + 7.97) \times e^{\frac{-2\times(\lg\frac{n}{m})^2 \times (-27.6\times O_{\text{aldehyde}} - 5.22\times C_{\text{cyanogenic}} - 7.97)^2}{(-75.3\times O_{\text{aldehyde}} - 14.3\times C_{\text{cyanogenic}} - 22.1)^2}} \quad (11)$$

For those binary mixtures with additive joint effects at equitoxic ratios, their parameter *A* can be defined as zero, and the value of y_0 varies from 0.80 to 1.20. Consequently, the prediction equations of these mixtures (Equation 7) can be rewritten as follows,

$$TU_{\text{sum}}^{\text{n:m}} = 1.00 \pm 0.20 \quad (12)$$

A combination of Equation 11 and Equation 12 obtains the total QSAR model:

$$\begin{cases} TU_{\text{sum}}^{\text{n:m}} = 1.00 - (27.6 O_{\text{aldehyde}} + 5.22 C_{\text{cyanogenic}} + 7.97) e^{\frac{-2(\lg\frac{n}{m})^2 (-27.6\times O_{\text{aldehyde}} - 5.22\times C_{\text{cyanogenic}} - 7.97)^2}{(-75.3\times O_{\text{aldehyde}} - 14.3\times C_{\text{cyanogenic}} - 22.1)^2}} & (TU_{\text{sum}}^{1:1} < 0.80 \text{ and } TU_{\text{sum}}^{1:1} > 1.20) \\ TU_{\text{sum}}^{\text{n:m}} = 1.00 \pm 0.20 & (0.80 < TU_{\text{sum}}^{1:1} < 1.20) \end{cases} \quad (13)$$

Based on the above equations, the predicted results of the joint effects for additional mixtures other than the one used for the development of the QSAR were obtained. These predicted joint effects were compared against the observed experimental results, and the corresponding results are shown in Table 4.

$$TU_{\text{sum}}^{\text{observed}} = -0.113 + 1.12 \times TU_{\text{sum}}^{\text{predicted}} \quad (14)$$

n=25, r=0.941, SE=0.109, F=178, P<0.001

The significant correlation between the observed and predicted results (r=0.941) indicates that the predicted results of joint effects for mixtures at non-equitoxic ratios are consistent with the observed results. This predictive capability convinces us that this QSAR model provides a possible approach for the prediction of the joint effects of binary mixtures at non-equitoxic ratios.

TABLE 4. Observed and predicted joint effects of binary mixtures

Binary mixture of malononitrile with	n/m	lg(n/m)	Observed TU_{sum}	Predicted TU_{sum}	Difference[a]
p-nitrobenzaldehyde	56000:1	5.75	1.05	1.00	0.05
p-nitrobenzaldehyde	32000:1	4.51	1.02	0.99	0.03
p-nitrobenzaldehyde	5600:1	3.75	0.92	0.96	-0.04
p-nitrobenzaldehyde	1000:1	3.00	0.86	0.87	-0.01
p-nitrobenzaldehyde	180:1	2.26	0.81	0.72	0.03
p-nitrobenzaldehyde	32:1	1.51	0.57	0.50	0.07
p-nitrobenzaldehyde	5.6:1	0.75	0.24	0.30	-0.06
p-nitrobenzaldehyde	1:1	0	0.15	0.21	-0.06
p-nitrobenzaldehyde	1:5.6	-0.75	0.25	0.30	-0.05
p-nitrobenzaldehyde	1:32	-1.51	0.34	0.50	-0.16
p-nitrobenzaldehyde	1:180	-2.26	0.54	0.72	-0.18
p-nitrobenzaldehyde	1:1000	-3.00	0.60	0.87	-0.27
p-nitrobenzaldehyde	1:5600	-3.75	0.87	1.00	-0.13
p-nitrobenzaldehyde	1:32000	-4.51	0.98	1.00	-0.02
p-nitrobenzaldehyde	1:56000	-5.75	1.04	1.00	0.04
p-methoxybenzaldehyde	1:1	0	1.15	0.96	0.19
p-methoxybenzaldehyde	5.6:1	0.75	1.04	0.97	0.08
propanal	1:5.6	-0.75	0.27	0.43	-0.16
propanal	5.6:1	0.75	0.47	0.43	0.04
propanal	1:10	-1.00	0.49	0.47	0.02
propanal	10:1	1.00	0.27	0.47	-0.20
propanal	1:100	-2.00	0.65	0.70	-0.05
propanal	100:1	2.00	0.71	0.70	0.00
propanal	1:1000	-3.00	0.92	0.89	0.03
propanal	1000:1	3.00	1.04	0.89	0.15

[a] $p > 0.05$ (Here p represents the level of significance for predictive and observed values for each binary mixture, p>0.05 indicates there is no significant difference between the two values).

Discussion on the application of the QSAR model

It should be pointed out that the QSAR was derived from binary mixtures containing cyanogenic compounds and aldehydes; as a result, the model can be applied to similar mixtures. However, for other mixtures, if they have similar toxic ratio-effect curves, the procedure of the development of this QSAR can be used as a reference to propose a new QSAR model for $TU_{sum}^{n:m}$. In other words, this study revealed the relationship between the toxic ratios and the joint effects of binary mixtures using the toxic ratios-effects curves, which provides us with a novel approach to predicting the joint effects of these binary mixtures at non-equitoxic ratios ($TU_{sum}^{n:m}$) based on those at equitoxic ratios ($TU_{sum}^{1:1}$).

The approach in this study has promising applications in risk assessment and environmental pollutant control. For example, effluents coming from chemical plants might contain complex organic chemicals. These mixtures might exert joint effects including antagonistic or synergistic effects. If the concentrations of the main individual chemicals are known, their joint effects at any ratio ($TU_{sum}^{n:m}$ $TU^{u,uu}$) in effluents can be obtained using this approach. Consequently, by adjusting their concentrations, their joint effects can be controlled, and the ecological risk can be minimized, which will reduce work in the fields of environmental pollutant control and ecological risk assessment.

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