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Benesi-Hildebrand 方程的正确性与可靠性

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摘要: Benesi-Hildebrand(B-H)方程现被广泛地应用于各种非键作用体系, 特别是作用比为 1:1 型和 1:2 型的体系. 该方程可以用来确定作用体系的平衡常数以及作用比. 通过计算机模拟, 发现在某些情况下, 对于 1:2 型的作用体系, B-H 方程会给出错误的作用比信息. 无论是弱的作用体系还是强的作用体系, 都可能会出现 1:1 的 B-H 方程曲线呈现出线性, 同时(或者)1:2 的 B-H 方程曲线呈现出非线性的情况. 此外, 本文还研究了体系中两种作用物质的初态浓度比对于 1:1 型作用的平衡常数计算的影响, 发现最小的初态浓度比(r_0)等于 100 是可以确保 B-H 方程近似条件 $C_B^0 \approx C_B$ 成立的安全阈值. 当作用很弱的时候, 比如说作用的平衡常数 K 小于 25 L·mol^{-1} (C_P^0=4×10^{-4} mol·L^{-1})时, 则不需要对最小初态浓度比值r_0进行限制, 就可以满足B-H方程的近似条件. 通过计算机模拟还分析了文献中提出的两个边界条件. 研究表明 $1/(KC_P^0) \geqslant 10$ 可以保证处于平衡状态时的 $C_B/C_B^0 \geqslant 0.91$. 而另一个条件 $KC_B^0>0.1$ 并不是确保 B-H 方程近似条件成立的充分条件.

关键词: Benesi-Hildebrand 方程; 分子间相互作用; 平衡常数; 作用比
中图分类号: O642

Validity and Reliability of Benesi-Hildebrand Method

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Abstract: Benesi-Hildebrand (B-H) method is a widely used approach for determining the stoichiometry and equilibrium constants of nonbonded interactions, particularly 1:1 and 1:2 interactions. Using computer simulation, it was shown that, under certain conditions, the approach could generate inappropriate stoichiometric conclusions for 1:2 interactions. This problem could occur in the cases of both weak and strong interactions, where the 1:1 B-H plots showed a linear feature and the 1:2 B-H plots showed a nonlinear feature. In addition, effect of the initial concentrations on the accurate evaluation of equilibrium constants of 1:1 interactions was investigated. It was found that the minimum safe concentration ratio r_0 between ligand and central species was 100. However, for weak nonbonding interactions, for example K< 25 L·mol^{-1} (C_P^0=4×10^{-4} mol·L^{-1}), the ratio r_0 has no limitation. Two conditions proposed in literatures for the safe application of the B-H method were examined. It was found that the inequation, $1/(KC_P^0) \geqslant 10$, was a condition to secure C_B/C_B^0>91%. The other inequation, KC_B^0>0.1, was not found to be the safe condition to validate the B-H method.

Key Words: Benesi-Hildebrand method; Molecular interaction; Equilibrium constant; Stoichiometry evaluation

Benesi-Hildebrand (B-H) method was initially put forward by Benesi and Hildebrand in 1949[1] and has become a very popular method for determining equilibrium constant K and stoichiometry of binding interactions. It has been widely used in many aspects concerning a variety of quite different systems; data collected from various spectroscopic techniques (UV-Vis, fluorescence, infrared, NMR, etc) can be used in the B-H calculation[2–14].

When absorption spectroscopy is used, let us assume that a

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chromophore-containing molecule P interacts with ligand B to form a 1:1 complex PB:

$$P+B \overset{K}{\rightleftharpoons} PB \quad (1)$$

To be concise, we further assume that the Beer-Lambert law can be expressed in the form of $A=\varepsilon C$. Then, the 1:1 B-H equation is as follows[1],

$$C_P^0/\Delta A=1/[C_B^0 K(\varepsilon_{PB}-\varepsilon_P)]+1/(\varepsilon_{PB}-\varepsilon_P) \quad (2)$$

where ΔA is the change in absorbance during complexation. C_P^0 and C_B^0 are the initial concentrations of P and B, ε_P and ε_{PB} are the absorption coefficients of P and PB, respectively. The approximations used to derive Eq.(2) are $\varepsilon_B=0$ and $C_B^0 >> C_P^0$, the latter is to ensure that the concentration of B at equilibrium state C_B is very close to its initial value. By plotting $C_P^0/\Delta A$ *vs* $1/C_B^0$, a linear relationship can be obtained. Equilibrium constant K can then be calculated from the intercept and slope.

There have been reports on concerns about the accuracy of the B-H method for determining equilibrium constants. Influencing factors that have been examined include concentration range of reactants[11,15–19], the format of the B-H equation[20–24], activity coefficient and concentration type [24–27], and experimental errors[16,18,19,28–30]. Among them, the concentration range is of particular interest to us because it is closely related to the validity of the approximation $C_B^0 \approx C_B$ of the B-H method and, thus to the practical design of experiments. Qureshi *et al*.[15] found that the B-H plot was a random scatter when C_B^0 is as low as C_P^0. This is understandable because low concentration of B cannot guarantee the approximation $C_B^0 \approx C_B$. Clearly, greater C_B^0 and smaller C_P^0 favor the validity of the approximation. Wong and Ng[11] thus suggested that the C_B^0/C_P^0 ratio should be greater than 20 in their experimental investigation. In this case, C_B is at least 95% of C_B^0 . Not limited to these two factors C_B^0 and C_P^0 , however, equilibrium constant K also plays a role in the effect on the approximation $C_B^0 \approx C_B$. Bergeron *et al*.[31] suggested $1/(KC_P^0) \geqslant 10$ from their simulation results, but they did not provide a theoretical reasoning of the inequation. Person[17] proposed an interesting condition $KC_B^0 > 0.1$ based mainly on conceptual reasoning. This was supported by Exner[18] based on his statistical analysis of experimental results. But we found that it is still an arguable issue. In this study, these questions have been discussed by either mathematical analysis or computing simulation.

Another important application of the B-H method is to determine the stoichiometry of binding interactions. Using the criteria of linear B-H plot, 1:1 stoichiometry has been found in the complexation[9,14,31]. In addition to the popular 1:1 binding interactions, other stoichiometric interactions can also be investigated using the extended B-H method. For the general one-step binding interactions in the form of $P+nB \overset{K}{\rightleftharpoons} PB_n$, the extended B-H equation is shown below:

$$C_P^0/\Delta A=1/[(C_B^0)^n K(\varepsilon_{PB_n}-\varepsilon_P)]+1/(\varepsilon_{PB_n}-\varepsilon_P) \quad (3)$$

It is generally believed that the $C_P^0/\Delta A$ *vs* $(1/C_B^0)^n$ plot would yield a straight line for a 1:n complexation. Many researchers assume n=2 in their studies, particularly when cyclodextrins are considered[3,9,14,23,32].

Questions remain unanswered with respect to the 1:2 B-H method that deals with the 1:2 interactions. Due to the fact that any 1:2 reaction, in principle, is a two-step reaction, the one-step assumption could generate misleading conclusions. Can the 1:1 B-H plot reveal a good linear fit and/or can the 1:2 B-H plot be nonlinear if the actual stoichiometry of the interaction is 1:2? Deranleau[33] studied the 1:1 B-H plots of 1:2 binding interactions and found that the plots could show a linear behavior because of the narrow concentration range and lower concentration ratio of the bounded complexes to that of the original unbounded molecules. The simulation, however, was only restricted to conditions of $\varepsilon_P=0$ and $\varepsilon_{PB_2}=2\varepsilon_{PB}$. Pistolis and Malliaris[34] found that differentiation between 1:1 and 1:2 stoichiometries was impossible under a particular condition as described in the following equation, in the case of absorption spectroscopy.

$$K_1/K_2=(\varepsilon_{PB_2}-\varepsilon_P)^2/[(\varepsilon_{PB}-\varepsilon_P)(\varepsilon_{PB_2}-\varepsilon_{PB})] \quad (4)$$

where K_1 and K_2 are the equilibrium constants of the 1:1 and 1:2 interactions, respectively, and ε_P, ε_{PB}, and ε_{PB_2} are the spectroscopic values of free P, 1:1, and 1:2 bounded complexes, respectively[34]. The problem is that, if the equation is not satisfied, which should be the most probable case, can we still use the B-H method unambiguously? In this study, we showed that the misleading conclusion of the B-H method was not restricted to the above equation.

1 Simulation method

The general strategy of the computing simulation in this study is as follows. For 1:1 binding interactions in the form of Eq.(1), exact equilibrium concentrations of all species in the system can be calculated based on the assigned values of the equilibrium constant K and initial concentrations of the reactants B and P. After assigning absorption coefficients to P and BP, the total absorbance at a series of concentrations of the ligand B can be calculated. The B-H method can then be used to evaluate the equilibrium constant $K_{B\text{-}H}$ and to determine the stoichiometry of the 1:1 binding interaction. Similar simulation work can also be performed for interactions involving 1:2 bindings.

1.1 Simulation of 1:1 binding interaction

We set C_P^0 as 4×10^{-4} mol·L^{-1} for all the solutions, which is close to the concentrations used in many previous studies[14,35]. C_B^0 changes over quite a wide range from 4×10^{-4} to 1 mol·L^{-1}, with similar separations between adjacent data points. This gives the range of molar ratio ($r=C_B^0/C_P^0$) from 1 to 2500. Such concentration range is in agreement with that suggested by Exner[18], who recommended that at least one order of magnitude of the range of C_B^0 should be used. A wide range of equilibrium constant K, from 0.1 to 40000 L·mol^{-1}, has been selected, encompassing both weak and strong binding interactions.

On the basis of the hypothetic values K, C_P^0, and each of the C_B^0, the equilibrium concentration C_{PB} can be obtained by solving the following equation:

$$K=C_{PB}/[(C_P^0-C_{PB})(C_B^0-C_{PB})] \quad (5)$$

The next step is to evaluate the equilibrium constant $K_{B\text{-}H}$ by

the B-H method. It is then compared with its original value, measured by Err=$(K-K_{B\text{-}H})/K$. For the purpose of comparison, a simplified form of Eq.(2) can be used after replacing ΔA with $C_{PB}\times(\varepsilon_{PB}-\varepsilon_P)$:

$$C_P^0/C_{PB}=1/(KC_B^0)+1 \quad (6)$$

This equation is independent of the absorption coefficients of the respective substances. But the linear regression using this equation depends on the concentration range of reactants, or alternatively the range of the concentration ratio r. In this part of the work, the end point of r is kept constant at 2500, whereas the starting point r_0 (the minimum value of r) varies.

1.2 Simulation of 1:2 binding interaction

Let us consider a 1:2 binding interaction in the form of P+B$\overset{K_1}{\rightleftharpoons}$PB and PB+B$\overset{K_2}{\rightleftharpoons}PB_2$. Then the expressions of K_1 and K_2 are as follows:

$$K_1=C_{PB}/[(C_P^0-C_{PB}-C_{PB_2})(C_B^0-C_{PB}-2C_{PB_2})] \quad (7)$$

$$K_2=C_{PB_2}/[C_{PB}(C_B^0-C_{PB}-2C_{PB_2})] \quad (8)$$

C_P^0 is still set as 4×10^{-4} mol·L^{-1} for all the solutions, and C_B^0 changes from 1×10^{-4} to 10 mol·L^{-1}, with similar separation between adjacent data points. Weak binding interactions are discussed first. One value of K_1 or K_2 in Eqs. (7, 8) is kept constant at 0.2 or 2 L·mol^{-1}; the other takes a value of 0.2, 0.5, 1.0, 2.0, 4.0, 10, or 20 L·mol^{-1}. For strong interactions such as those associated with the inclusion complexes of cyclodextrins [9,14,30,34], another range of values of K_1 and K_2 is considered. The two parameters are set as 20 and 10, 50 and 20, 100 and 50, 500 and 200, 1000 and 250, or 1000 and 500 L·mol^{-1}, respectively, which are common in the complexation of cyclodextrins [9,14,30,34]. C_{PB2} can be eliminated by combining Eqs.(7, 8), resulting in the following expression:

$$(4(K_2)^2-K_1K_2)(C_{PB})^3+(4K_2+2K_1K_2C_P^0-K_1)(C_{PB})^2+$$
$$(1+K_1C_B^0-2K_1K_2C_P^0C_B^0+K_1K_2(C_B^0)^2+C_P^0K_1)C_{PB}-K_1C_P^0C_B^0=0 \quad (9)$$

Then, C_{PB} can be evaluated with the help of Matlab 6.5.

By combining the Beer-Lambert Law, Eqs. (7, 8), the following equation [36] can be obtained to correlate various parameters of a 1:2 binding interaction:

$$C_P^0/\Delta A=(x^2+K_1x+K_1K_2)/(K_1\Delta\varepsilon_1x+K_1K_2\Delta\varepsilon_2) \quad (10)$$

where $\Delta\varepsilon_1=\varepsilon_{PB}-\varepsilon_P$, $\Delta\varepsilon_2=\varepsilon_{PB_2}-\varepsilon_P$, $x=1/C_B^0$. If any one tries to construct a 1:1 B-H plot ($C_P^0/\Delta A$ *vs* x) to examine an unknown (but actually 1:2 binding) interaction, all the factors such as $\Delta\varepsilon_1$, $\Delta\varepsilon_2$, K_1, and K_2 affect the linearity and thus the conclusion of stoichiometry. It seems that the plot should be nonlinear. But there could exist conditions under which the plot was close to linear. See Section 2.2 for details.

Thus, to evaluate ΔA of binding interactions, we merely need to assign $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$. For weak binding interactions, $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ were assigned with a wide range of values from −80 to −680 L·mol^{-1}, mimicking the value of pyrazine dissolved in heptane[37]. For strong binding interactions, such as the cyclodextrin systems, the values of $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ were taken from −50 to −2000 L·mol^{-1} according to the data used by Yang *et al.*[29]. On the basis of these hypothetic values, the binding stoichiometry could be determined according to the linearity of the B-H plots in the form of either Eq.(2) or Eq.(3), evaluated by the correlation coefficient R^2. By comparing with the real stoichiometry, the validity of the B-H method can be judged.

2 Results and discussion

2.1 Determination of equilibrium constant *K* for 1:1 binding interactions

The reliability in determining equilibrium constant of a 1:1 binding reaction at various hypothetic K and different starting molar ratio of $r_0=(C_B^0/C_P^0)_{min}$ is investigated. Part of the results is listed in Table 1. Clearly, the relative error (Err) in determining K decreases with increasing r_0 and decreasing K, and all the values of K are underestimated by the B-H method. When r_0 is large enough or when K is small enough, the error can be controlled within 1%. More detailed relationship between Err and r_0 is shown in Fig.1. When K is small, for example in the range of 0.1 to 10 L·mol^{-1}, all the values of Err are lower than 1% (Fig.1a). This means that r_0 can be reduced to 1 (equal molar ratio of the two reactants) for weak binding interactions. When K is large, as shown in the example in Fig.1d, Err increases very sharply with decreasing r_0. Err of 70% or even greater can occur. Combining the results shown in both Fig.1 and Table 1, it can be seen that a starting molar ratio (r_0) of 100 is the safe ratio for accurate evaluation of equilibrium constants for both weak and strong binding interactions. This is understandable because, when $C_B^0/C_P^0=100$, only one percent of B at the maximum can be consumed by interaction with P. If Err is reduced to 5%, r_0 merely needed to be greater than 20, regardless of the value of K, as can be seen in Table 1.

We can also examine the effect of r_0 mathematically. For 1:1 binding interactions, a key approximation is $C_B^0\approx C_B$. Combining Eq.(5) with $C_B^0=C_B+C_{PB}$, we have:

$$C_B/C_B^0=1-0.5(1+C_P^0/C_B^0+1/(KC_B^0))+$$
$$[0.25(1+C_P^0/C_B^0+1/(KC_B^0))^2-C_P^0/C_B^0]^{1/2} \quad (11)$$

or by replacing C_B^0/C_P^0 with r

$$C_B/C_B^0=1-0.5(1+1/r+1/(KC_B^0))+[0.25(1+1/r+1/(KC_B^0))^2-1/r]^{1/2} \quad (12)$$

Let us consider a positive threshold value a, when $C_B/C_B^0\geqslant a$ ($a<1$), the approximation $C_B^0\approx C_B$ can be satisfied. Then, we can obtain an inequation as shown below:

$$1-0.5(1+1/r+1/(KC_B^0))+[0.25(1+1/r+1/(KC_B^0))^2-1/r]^{1/2}\geqslant a$$

or

$$[0.25(1+1/r+1/(KC_B^0))^2-1/r]^{1/2}\geqslant a-1+0.5(1+1/r+1/(KC_B^0)) \quad (13)$$

Usually, we take $0.9<a<1$. This means that the expression on the right-hand side of the above inequation takes a positive value. As a result,

$$0.25(1+1/r+1/(KC_B^0))^2-1/r\geqslant[a-1+0.5(1+1/r+1/(KC_B^0))]^2 \quad (14)$$

Simplification of the above inequation gives,

$$r\geqslant 1/(1-a)-1/(aKC_P^0) \quad (15)$$

On the basis of the inequation, the solution for the unlimited value of r is to allow negative values for the expression on the right-hand side of inequation (15), or $1/(KC_P^0)\geqslant a/(1-a)$. If we take a=0.91, then $1/(KC_P^0)\geqslant 10$. This is just the boundary condition proposed by Bergeron and Roberts but has not been explained in their study[31]. Under this condition, C_B is at least 91%

Table 1 The dependence of Err (%) of $K_{B\text{-}H}$ on the starting molar ratio r_0 and equilibrium constant K with final molar ratio (r) of 2500

K/(L·mol^{-1})	r_0											
	1	3	5	7	10	20	30	50	60	70	80	100
10	0.70	0.67	0.66	0.64	0.61	0.56	0.49	0.43	0.41	0.39	0.38	0.35
30	1.86	1.73	1.66	1.60	1.48	1.26	1.04	0.85	0.78	0.73	0.70	0.61
50	2.88	2.61	2.46	2.35	2.14	1.74	1.37	1.08	0.97	0.90	0.83	0.73
100	5.07	4.39	4.04	3.75	3.28	2.48	1.83	1.36	1.21	1.09	0.99	0.86
200	8.64	7.01	6.20	5.36	4.62	3.20	2.22	1.58	1.37	1.23	1.10	0.94
300	11.6	8.94	7.67	6.76	5.42	3.56	2.40	1.66	1.44	1.29	1.15	0.97
500	16.3	11.7	9.62	8.23	6.33	3.92	2.56	1.74	1.50	1.33	1.19	1.00
700	20.1	13.6	11.0	9.12	6.85	4.11	2.65	1.78	1.53	1.36	1.20	1.01
900	23.1	15.0	11.8	9.74	7.19	4.22	2.69	1.80	1.54	1.37	1.21	1.02
1000	24.4	15.6	12.1	9.98	7.32	4.26	2.71	1.81	1.55	1.37	1.22	1.02
3000	40.0	21.1	15.0	11.8	8.22	4.53	2.82	1.86	1.59	1.40	1.23	1.04
5000	47.5	22.9	15.8	12.2	8.44	4.58	2.84	1.87	1.59	1.40	1.24	1.04
7000	52.2	23.8	16.1	12.4	8.54	4.61	2.85	1.87	1.59	1.40	1.24	1.04
9000	55.6	24.4	16.4	12.6	8.59	4.62	2.85	1.87	1.60	1.41	1.24	1.04
10000	57.0	24.6	16.4	12.6	8.61	4.63	2.86	1.87	1.60	1.41	1.24	1.04
20000	65.3	25.6	16.8	12.8	8.70	4.65	2.87	1.87	1.60	1.41	1.24	1.05
30000	69.6	26.0	16.9	12.9	8.73	4.66	2.87	1.88	1.60	1.41	1.24	1.04
40000	72.4	26.2	17.0	12.9	8.75	4.66	2.87	1.88	1.60	1.41	1.24	1.04

of C_B^0.

If we take a=0.99, we have $1/(KC_P^0)\geqslant 99$. Thus, if $C_P^0=4\times10^{-4}$ mol·L^{-1}, an arbitrary assignment in this simulation study, we can get $K<25$ L·mol^{-1}. It means that for as weak an interaction as described by $K<25$ L·mol^{-1}, there would be no limitations for the selection of C_B^0 to satisfy the approximation requirement. The unlimited r_0 provides a comfortable condition for the experiment and it does not affect the accuracy of $K_{B\text{-}H}$ much.

For strong interactions, such as the complexation of micelles[6] and cyclodextrins with various ligands[14], equilibrium constant is usually greater than 100 L·mol^{-1} or even sometimes greater than 10000 L·mol^{-1}. The safe value of r_0 can be obtained from inequation (16),

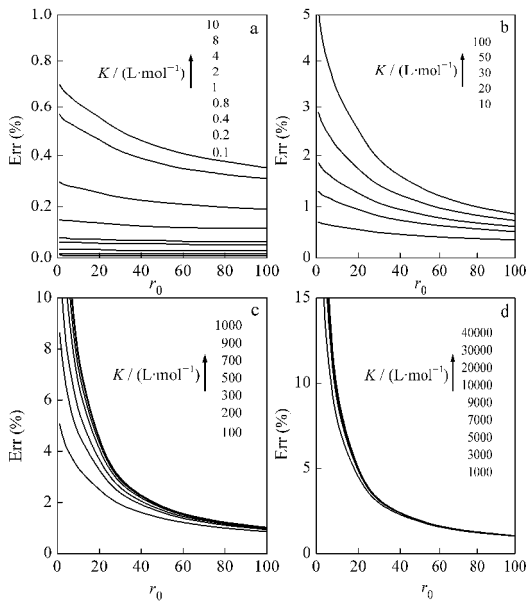

Fig.1 The dependence of Err on r_0 in four ranges of K

$$r_0=1/(1-a)-1/(aKC_P^0) \quad (16)$$

By using the value of $C_P^0=4\times10^{-4}$ mol·L^{-1} and a=0.99, r_0 is determined to be about 75 (when K=100 L·mol^{-1}) and 100 (when K=10000 L·mol^{-1}).

We now discuss the effect of KC_B^0 on C_B/C_B^0. When considered individually, small K and large C_B^0 lead to large C_B/C_B^0, favoring the approximation $C_B^0\approx C_B$. However, it is not straightforward to draw a conclusion on the effect of the product of the two parameters. Nevertheless, negative partial derivative as shown below can be derived from Eq.(12), i.e.,

$$\partial(C_B/C_B^0)/\partial(KC_B^0)\leqslant 0 \quad (17)$$

This suggests that, in principle, smaller KC_B^0 is more favorable. Such conclusion is just opposite to the previous conclusion where $KC_B^0>0.1$[17,18] was recommended for experimental design.

To solve the controversy, we have examined the dependence of C_B/C_B^0 on KC_B^0 under various conditions based on Eq. (12). The range of $r<100$ was selected to perform the analysis, because when $r\geqslant100$ only one percent of B at the maximum can be consumed by interaction with P, implying that $C_B/C_B^0\geqslant 0.99$. The results in the variable ranges of $0\leqslant KC_B^0\leqslant0.1$ are shown in Fig.2a. In this case, $C_B/C_B^0\geqslant0.98$ was found with the range of r from 5 to 100. This shows unambiguously that $KC_B^0\leqslant 0.1$ is not a problem to the application of B-H method, at least in principle. Whereas when $0.1\leqslant KC_B^0\leqslant9$ (a range suggested by Person[17]) C_B/C_B^0 could be much less than 0.9, even less than 0.6 as shown in Fig.2b. Thus, the condition $KC_B^0>0.1$ is not the safe condition to validate the B-H method.

In the study by Person, the condition $KC_B^0>0.1$ was explained as the result of zero slope of the Scott plot, a modified form of the B-H plot. We believe that the slope should be the same as long as the Scott plot is applicable. The examples of both Scott plots and B-H plots when $KC_B^0<0.1$ are shown in Fig. 2(c–f). The hypothetic data satisfy $C_P/C_P^0>0.99$ and $C_B/C_B^0>$

0.9999 in the examples, consistent with the conditions used in the study by Person. Correct equilibrium constants can be evaluated from the slope and intercept, with relative errors less than 0.1%. These results, together with the inequation (17), indicate that the smaller the value of KC_B^0, the better. However, small KC_B^0 means small percentage of the transformation of P to PB. When PB is the only detectable molecule in the examined mixtures, experimental error could cause a serious problem when the concentration of PB is very low. This should be a better explanation to the condition $KC_B^0 > 0.1$ as done by Exner[18]. Whereas if both P and PB are detectable, experimental error could not be a problem. Smaller KC_B^0 should favor the application of B-H method.

2.2 Determination of stoichiometry for binding interactions

For both weak and strong 1∶2 binding interactions, we evaluate the conditions and particularly the concentration ratio ranges for which the 1∶1 B-H plots (Eq.(2)) show linear relationship, generating incorrect information of the stoichiometry for the binding interactions. All the hypothetical conditions listed below do not satisfy the Eq.(4) proposed by Pistolis and Malliaris[34].

2.2.1 Weak binding interactions

For weak binding interactions with equilibrium constants less than 20, we found that both 1∶1 and 1∶2 B-H methods provide correct stoichiometry information under most conditions. Nevertheless, several situations that result in incorrect stoichiometries have been identified.

First, we take K_1=2 L·mol^{-1} and K_2= 1 L·mol^{-1} as representatives of weak interactions. In this case, we can always have $C_B/C_B^0 > 0.999$, meaning that the approximation of B-H method is satisfied. When $1/C_B^0 > 25$ L·mol^{-1} or $r < 100$, all the 1∶1 B-H plots are linear. This is normal as it is found that $C_{PB_2}/C_{PB} < 0.04$, indicating that the second step of the interaction can be ignored and the stoichiometry of 1∶2 interaction can be taken as 1∶1. However, there occurs problem when r is greater than 100. The results are shown in Fig.3. Again, linear portions can be observed in all the curves in the figure. The nonlinear portions of the plots can only be observed when $1/C_B^0$ is extremely small, typically less than 2 L·mol^{-1} ($r > 1250$) as shown in the figure. When r is 1250, C_{PB_2}/C_{PB} was found to be 0.5. Thus, in the molar ratio range from 100 to 1250, C_{PB_2}/C_{PB} changes from 0.04 to 0.5, indicating that the second step of the interaction cannot be ignored. But the 1∶1 B-H plots still show a linear feature, contradictory to the fact of 1∶2 binding interaction.

There is also a problem that both 1∶1 and 1∶2 B-H methods (represented in Eq.(3)) cannot give correct information of the stoichiometry of the 1∶2 binding interactions. Fig.4 is an example, showing the results in the range of $1/C_B^0$ from 0.1 to 0.5 L·mol^{-1}, or r from 5000 to 25000. The 1∶1 B-H plot is seen to be linear in Fig.4a, whereas the 1∶2 B-H plot shows a nonlinear feature in Fig.4b. However, the ratio of C_{PB_2}/C_{PB} was found to vary from 10 to 2, indicating that C_{PB_2} is greater than C_{PB} at the equilibrium state. It means that the percentage of 1∶2 binding interaction is obviously more than that of the 1∶1 binding interaction. As a result, both B-H plots give incorrect information of the stoichiometry of the 1∶2 binding interaction.

Then, we consider the case when K_1 is much greater than K_2, existing in many binding interactions, such as the ones be-

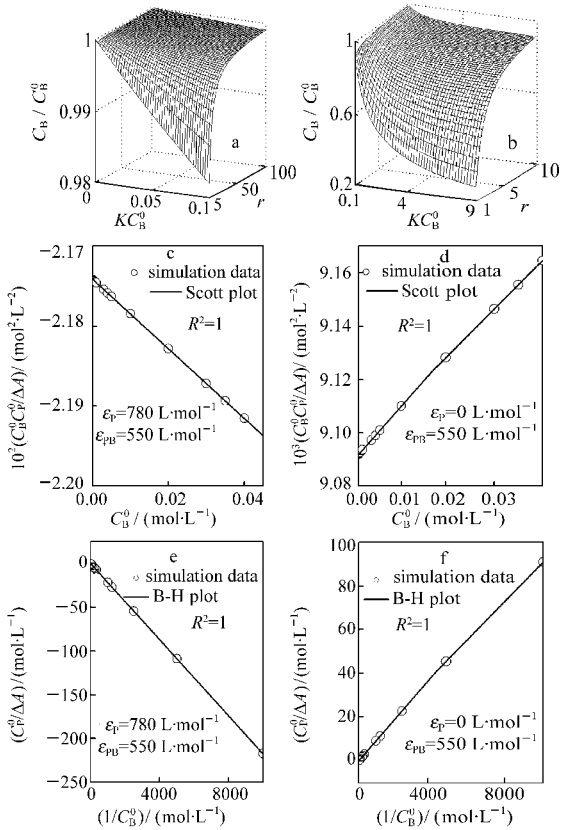

Fig.2 The relationship between C_B/C_B^0, r(=C_B^0/C_P^0) and KC_B^0 (a, b), 1∶1/1∶2 Scott plots (c, d), and B-H plots (e, f)

KC_B^0<0.1, K=0.2 L·mol^{-1}, C_P^0=4×10^{-4} mol·L^{-1}

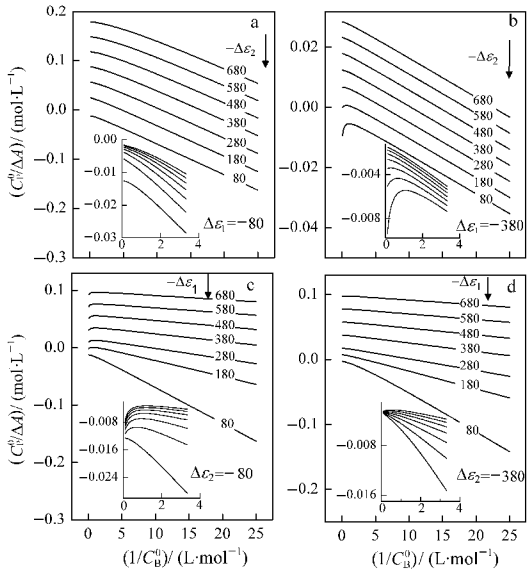

Fig.3 1∶1 B-H plots of 1∶2 interactions with K_1=2 L·mol^{-1} and K_2=1 L·mol^{-1}

Each of the curves is adjusted by an offset constant to separate the plots. The origins of the curves are shown as insertions. $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ are in L·mol^{-1}.

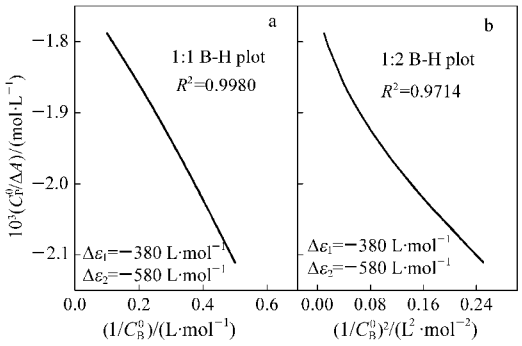

Fig.4 1:1 (a) and 1:2 (b) B-H plots of the 1:2 binding interactions with K_1=2 L·mol^{-1} and K_2=1 L·mol^{-1}

tween diazines and phenol derivatives[38]. Representative values of the equilibrium constants are K_1=20 L·mol^{-1} and K_2=0.2 L·mol^{-1}. It is found that $C_B/C_B^0 > 0.99$ can be guaranteed with these parameters, meaning that the approximation condition of the B-H method is satisfied. Using the assigned absorption coefficients (see the Simulation method section) and further taking $\Delta\varepsilon_1=\Delta\varepsilon_2$, 1:1 B-H plots have been simulated, and the results in the range of $1/C_B^0$ from 0.2 to 2 L·mol^{-1} (r from 12500 to 1250) are shown in Fig.5. Clearly, these are all very good linear plots ($R^2 \geqslant 0.9996$) and the concentration ratio of C_{PB_2}/C_{PB} was found to be in the range from 0.1 to 1, indicating that the second step of the interaction can not be ignored in these cases. This means that the 1:1 B-H method gives incorrect stoichiometric information because the reaction is a 1:2 binding interaction. Other than the situation $\Delta\varepsilon_1=\Delta\varepsilon_2$, 1:1 B-H method has been always found to show nonlinear plots for 1:2 interactions when K_1 is much greater than K_2.

2.2.2 Strong binding interactions

Nowadays, for strong binding interactions, such as the complexation of cyclodextrins, both 1:1 and 1:2 B-H methods are widely used. Our simulation work showed that both B-H plots could provide incorrect results, especially when $\Delta\varepsilon_1 > \Delta\varepsilon_2$, which is the most probable case in hypochromic binding interactions.

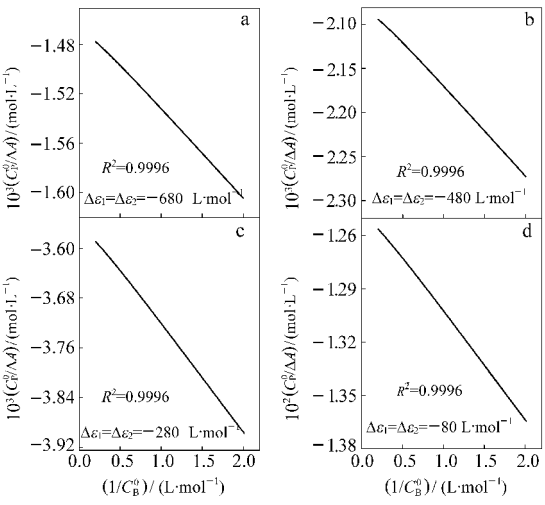

Fig.5 1:1 B-H plots of 1:2 interactions with K_1=20 L·mol^{-1} and K_2=0.2 L·mol^{-1}

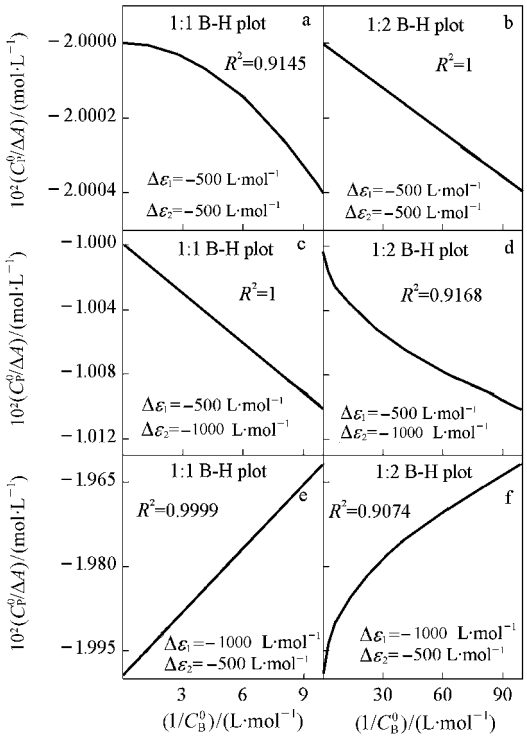

Fig.6 1:1 and 1:2 B-H plots of 1:2 interactions with K_1=1000 L·mol^{-1} and K_2=500 L·mol^{-1}

To assure the approximation of the B-H method, the r range from 250 to 25000 ($1/C_B^0$ from 10 to 0.1 L·mol^{-1}) was selected. C_{PB_2}/C_{PB} was found to be in the range of 1−5000, with the simulation values of K_1 and K_2 listed in section 1.2, suggesting that the second step of the interaction is the dominating step. By taking various values of K_1 and K_2 as testing values, our simulation work showed that the 1:1 and 1:2 B-H plots showed differences in behavior with the various relationships between $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$. Fig.6 shows the representative results with K_1=1000 L·mol^{-1} and K_2=500 L·mol^{-1}. It is thus clear that when $\Delta\varepsilon_1=\Delta\varepsilon_2$, both B-H plots showed correct stoichiometric information (Fig.6 (a, b)). However, when $\Delta\varepsilon_1 \neq \Delta\varepsilon_2$, both B-H plots generated incorrect results (Fig.6(c−f)). In the case of $\Delta\varepsilon_1 > \Delta\varepsilon_2$, linear 1:1 plots and nonlinear 1:2 plots are seen in Fig.6 (c, d). In the case of $\Delta\varepsilon_1 < \Delta\varepsilon_2$, even negative equilibrium constants can occur because of positive slopes and negative intercepts of the plots as demonstrated in Fig.6(e, f). Furthermore, our simulation indicates that it is the ratio of $\Delta\varepsilon_1/\Delta\varepsilon_2$, rather than the absolute values of $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$, that plays a more important role in governing the correctness of the B-H method.

3 Conclusions

B-H method is a popular method that is being widely used in determining the stoichiometry of either 1:1 or 1:2 binding interaction, and has wide applications. However, in this study, through computer simulation we demonstrated that the method can be incorrect in many cases and is not restricted to the condition expressed in Eq.(4)[34]. This included both weak and strong interactions, where the 1:1 B-H plots showed a linear feature

and/or the 1:2 B-H plots showed a nonlinear feature.

Due to the fact that both equilibrium constants and molar absorption coefficients can exert their influences on the validity of the method, it is very hard, if not impossible, to find out the exact conditions under which the problem arises. To solve the problem, nonlinear regression analysis using Eq. (10) is recommended[39–41]. However, attention must be paid to a previous conclusion that such nonlinear method cannot be used if Eq. (4) is satisfied[34]. Thus, other experimental and/or theoretical methods should be employed to obtain independent stoichiometric information[42]. Combination of different methods will improve the reliability of the stoichiometry determined.

B-H method can also be used to determine equilibrium constants of binding interactions. For 1:1 interactions, the initial concentration ratio between the ligand and central species (r_0) is a key parameter in the determination of the equilibrium constant (K). For the accurate determination of K, the safe value of r_0 is 100, with an error smaller than 1%. For weak binding interactions, r_0 can be as small as 1 or even without limitation. Theoretical analysis shows that the inequation, $1/(KC_P^0) \geqslant 10$, proposed before[31] for the safe application of the B-H method is actually a condition to secure $C_B/C_B^0 > 91\%$, a quite less stringent requirement for the approximation of $C_B^0 \approx C_B$. If the percentage is increased to 95% or 99%, the inequation should be $1/(KC_P^0) \geqslant 19$ or $1/(KC_P^0) \geqslant 99$. It is also demonstrated that the condition $KC_B^0 > 0.1$ is not the safe condition to validate the B-H method. These conclusions are of importance in the design of the concerned experiments.

On the basis of this study and results from the literature, a few rules in the determination of equilibrium constant can be suggested. (1) Use molarity rather than mole fraction or molality as concentration unit[26]. (2) The minimum concentration ratio C_B^0/C_P^0 should be sufficiently large. (3) At least one order of magnitude of the range of C_B^0 should be used when C_P^0 is kept as a constant[18]. (4) Sufficiently large $1/(KC_P^0)$ value should be applied. A threshold value of 10 was suggested[31], but 20 is better.

In addition to absorption spectroscopy, the B-H method has also been used in other techniques such as NMR and fluorescence spectroscopy. Regardless of whether it is absorption coefficient (ε) in absorption spectroscopy, or chemical shift (δ) in NMR, or fluorescence quantum yield (ϕ) in fluorescence spectroscopy, they can all be discussed in a similar way. The conclusions we draw in this study can be taken as general conclusions.

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