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A bilogarithmic method for the spectrophotometric evaluation of stability constants of 1:1 weak complexes from mole ratio data

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Abstract

The absorbance changes that occur when the mole ratio of the components of ligand complex equilibria is varied while the concentration of one component is kept constant (mole ratio method) allow evaluating stability constants in favourable conditions. Values of the corresponding stability (association) constants are normally assigned on the basis of spectrophotometric analysis. Determination of stability constants can be performed by a number of linear procedures, but most of these, suffer from theoretical and practical drawbacks, e.g., linear transformation of the rectangular hyperbola type of binding constants, is valid only when one of the two species is present in a large excess. A rigorous treatment of the experimental mole ratio data for 1:1 weak complexes is carried out in this paper with the aim of eliminating some of the assumptions involved in the other methods usually applied for evaluating stability constants. Orthogonal regression is required in order to take into account the error in both axes. The method has been applied to literature data for the iron(III)-thiocyanate and nickel(II)-selenocyanate systems, as well as to a number of host–guest cyclodextrin complexes.
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Keywords: Mole ratio method; Bilogarithmic method; Orthogonal regression; Spectrophotometry; Iron(III)-thiocyanate; Nickel(II)-selenocyanate; Host–guest cyclodextrin complexes

1. Introduction

The extent of complexation is an important factor, especially in therapeutics, where the pharmacological effect of a drug is directly related to its nature (meaning in free or complexed form) (Loukas, 1997). Complexation of indomethacin with β-cyclodextrin, for example, does not eliminate the side effect of the drug because 40% of it is in free form (Djedani et al., 1990). Some non-linear least squares methods in the determination of association constants have been mentioned in the literature. Nevertheless some problems may arise because of the choice of data, initial estimates, convergence or multiple local minima, all typical of non-linear regression analysis (Douglas, 1992; Nievergelt, 1994). A new interpretation of the mole ratio method for 1:1 weak complexes is carried out in this paper and applied to data found in the analytical literature. The method devised allows a rigorous treatment of the experimental data in order to avoid some of the assumptions involved in the other methods usually applied for evaluating the stability constants. Small-molecule interactions with protein molecules are of great importance in therapeutic drug discovery process. The binding of a ligand (L) with a protein (P) to form a complex (PL) in which the total ligand is varied at fixed protein concentration constitutes a characteristic example of a 1:1 weak complex model (Fuchs and Gessner, 2001; Sharma and Agarwal, 2001). Interaction of an electron donor (D) with an electron acceptor (A), often leads, to the formation of which is known as a charge-transfer complex (Borazan and Ajuna, 1988), a phenomenon described by the equilibrium $D + A \rightleftharpoons DA$. The UV–visible spectrum of a charge-transfer complex generally reveals a bathochromic shift relative to the spectra of the starting materials, frequently permitting isolated analysis of the complex itself. The equilibrium $B + C \rightleftharpoons BC$ may also serve as a useful model for investigating the interaction between an anion and an enzyme, drugs or dyes with polynucleotides, or the entrapment of an organic entity within the hydrophobic cavity of a macrocyclic host. The progress of such reactions can be monitorized by means of experimentally determined value of some spec-

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troscopic variable, e.g., fluorescence quantum yield (Junquera and Aicart, 1999; Loukas et al., 1997; Sadlej-Sosnowska et al., 2003; Tuncer and Erk, 2005), absorption coefficient (Connors, 1987; Exner, 1997; Hoenigman and Evans, 1996; Polster and Lachmann, 1989; Vives et al., 2000), chemical shift (Ikeda et al., 2004; Loukas, 1997; Oh et al., 1998; Nowick et al., 1993; Rozou et al., 2004), etc. Relationships between the changes of the measured property of the guest on the concentration of free ligand, are hyperbolic. Stability constants have been estimated either by a non-linear fitting of the experimental data to the hyperbolic equations or by linear transformation, which facilitate the extraction of the parameters from the linear plots (Sadlej-Sosnowska et al., 2003). However, a bilogarithmic method applicable in the case of the formation of a single 1:1 complex is developed in this paper and applied to literature data. Orthogonal regression is required as a variable is involved on both sides of the model equation proposed.

2. Basic equations

Let us consider the model reaction

$$S + L \rightleftharpoons SL \tag{1}$$

the difference between S and L is shown to be only a matter of form which makes the treatment attractive and useful. The stability constant is defined as:

$$\beta_{11} = \frac{[SL]}{[S][L]} \tag{2}$$

If the total analytical concentration of S (i.e., protein, acceptor, metal ion) and L (i.e., donor, ligand ...) are denoted by T_S and T_L, respectively, consideration of material balances gives:

$$\beta_{11} = \frac{A/\varepsilon}{(T_S - A/\varepsilon)(T_L - A/\varepsilon)} \tag{3}$$

where ε is the molar absorptivity of SL. If measurements are performed with light path 1 cm, A/l should be substituted for all values of absorbance A (corrected for the absorbance of the free reagents if necessary) in the following discussion. Ionic strength, temperature and medium is assumed to be constant.

Rearranging Eq. (3) we get:

$$\beta_{11}\frac{A^2}{\varepsilon^2} - [(T_S + T_L)\beta_{11} + 1]\frac{A}{\varepsilon} + \beta_{11}T_S T_L = 0 \tag{4}$$

By multiplying Eq. (4) through $\varepsilon/(A\beta_{11}T_S)$ we have:

$$\frac{T_L\varepsilon}{A} + \frac{A}{T_S\varepsilon} = 1 + \frac{T_L}{T_S} + \frac{1}{\beta_{11}T_S} \tag{5}$$

and taking into account the definitions of mole ratio:

$$z = \frac{T_L}{T_S} \tag{6}$$

where T_S is constant through measurements and limit absorbance (hypothetical absorbance corresponding to complete reaction):

$$A_{\lim} = \varepsilon T_S \tag{7}$$

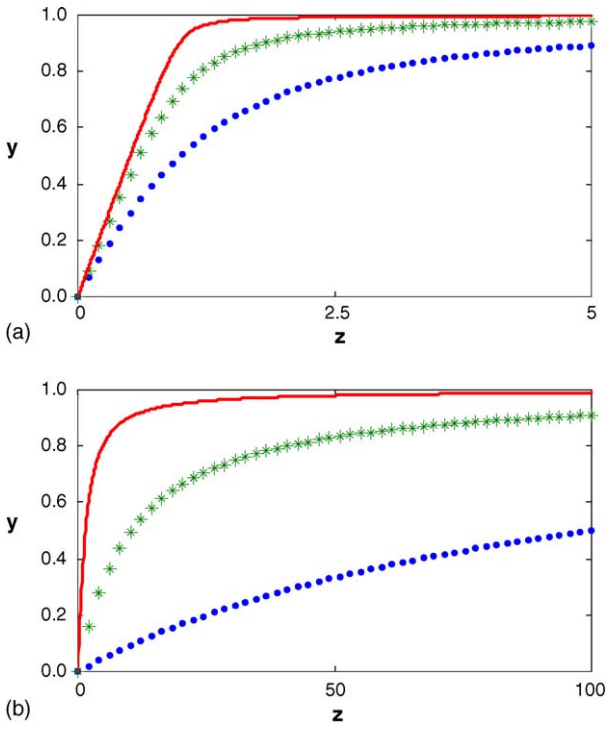

Fig. 1. Degree of complexation y vs. mole ratio z plotted for hypothetical systems: (a) at $\beta_{11}T_S = 100$, 10 and 2 (top to the bottom) and (b) at $\beta_{11}T_S = 1$, 0.1 and 0.01 (top to the bottom).

from Eq. (5) we may derive the expression:

$$y^2 - \left(1 + z + \frac{1}{\beta_{11}T_S}\right)y + z = 0 \tag{8}$$

where y denotes the degree of formation of the SL complex:

$$y = \frac{A}{A_{\lim}} \tag{9}$$

and then from Eqs. (8) and (9):

$$A = \frac{1}{2}A_{\lim}\left(1 + z + \frac{1}{\beta_{11}T_S} - \sqrt{\left(1 + z + \frac{1}{\beta_{11}T_S}\right)^2 - 4z}\right) \tag{10}$$

(the negative root of Eq. (8) is appropriate).

Eq. (10) allows drawing a family of curves, degree of complexation y versus z for given values of $\beta_{11}T_S$ (Fig. 1). The value of z required for the 99 and 99.9% degree of complexation for various $\beta_{11}T_S$ values are compiled in Table 1, and clearly indicates that in some cases, is experimentally difficult to obtain the concentration of L required to force all S in its complexed form. Linear extrapolation of the curves made where the curved plot becomes near parallel to the molar ratio axis after an excess of the variable component is added, does not lead to (Carta et al., 1981; Chriswell and Schilt, 1975; Momoki et al., 1969) reasonable results in the choice of the limiting absorbance because this limiting value is only slowly approached asymptotically if the complex is weak.

Table 1
Values of z required for 99 and 99.9% complex formation for varying $\beta_{11}T_S$

$\beta_{11}T_S$	$z_{y=0.99}$	$z_{y=0.999}$
1000	1.09	2.00
100	1.98	10.99
50	2.97	20.98
10	10.89	100.9
5	20.79	200.8
2	50.49	500.5
1	99.99	1000

On rearranging Eq. (8) we get:

$$y - 1 - \frac{1}{\beta_{11}T_S} - z\left(1 - \frac{1}{y}\right) = 0 \tag{11}$$

and then

$$z = \frac{y - 1 - (1/\beta_{11}T_S)}{1 - (1/y)} \tag{12}$$

Eq. (12) allows calculating mole ratio values, once, the degree of complexation and $\beta_{11}T_S$ values are known. Finally from Eq. (12) we may derive:

$$\frac{z}{y} + y = 1 + z + \frac{1}{\beta_{11}T_S} \tag{13}$$

and taking decadic logarithms.

$$\log\left(\frac{z}{y} - 1\right) = -\log(\beta_{11}T_S) - \log(1 - y) \tag{14}$$

Thus, the essentially non-linear nature of the related model equation was removed without simplification. A representation of the left member of Eq. (14) against $\log(1 - y)$ should give a straight line ($u = a_0 + a_1x$) of slope minus unity and intercept with the x-axis equal to $\log(\beta_{11}T_S)$. Deviations of slope from its nominal value could also indicate a problem with the model. Particular attention must been given to Eq. (14) so that the y variable is involved on both sides. Then, an error in this quantity appears in both coordinates mutually correlated in both conditions, that is, the independent variable x is not an exact quantity and the independence of errors is not fulfilled (Sayago et al., 2004). Note that when least squares are used with Eq. (14), it is biased on the assumption that the x-values are known without error. In such a situation, wrong conclusions may be drawn when conventionally weighted least squares are used for the computation of regression coefficients. The orthogonal least squares procedure (Lisý et al., 1990) must be applied, which is explained, in the following.

3. Orthogonal regression

The weighted least squares adjustment requires that β_{11} be selected such that:

$$S = \sum w_i r_i^2 = \sum w_i(y_i - \hat{y}_i)^2 = \sum w_i(y_i - a_0 - a_1x_i)^2 \tag{15}$$

should be a minimum, where i is the number of data point, and w_i is defined by (Asuero and Gonzalez, 1989):

$$w_i = \frac{1}{\mathrm{var}r_i} = \frac{1}{\sigma_{y_i}^2 + a_1^2\sigma_{x_i}^2 - 2a_1\,\mathrm{cov}(x_i, y_i)} \tag{16}$$

If S is to be a minimum, the first partial derivatives of S with respect to a_j ($j = 0, 1$) must be zero:

$$\frac{\partial S}{\partial a_j} = \sum\left[w_i\frac{\partial r_i^2}{\partial a_j} + r_i^2\frac{\partial w_i}{\partial a_j}\right] = 0 \tag{17}$$

On rearranging Eq. (17) we get:

$$\sum\left[w_i\frac{\partial r_i^2}{\partial a_j}\right] = -\sum\left[r_i^2\frac{\partial w_i}{\partial a_j}\right] \tag{18}$$

and then, taking into account that:

$$\frac{\partial r_i^2}{\partial a_0} = 2(a_0 + a_1x_i - y_i) \tag{19}$$

$$\frac{\partial r_i^2}{\partial a_1} = 2(a_0x_i + a_1x_i^2 - x_iy_i) \tag{20}$$

$$\frac{\partial w_i}{\partial a_0} = 0 \tag{21}$$

$$\frac{\partial w_i}{\partial a_1} = 2w_i^2[-a_1\sigma_{x_i}^2 + \mathrm{cov}(x_i, y_i)] \tag{22}$$

we obtain the set of normal equations:

$$a_0\sum w_i + a_1\sum w_ix_i = \sum w_iy_i \tag{23}$$

$$a_0\sum w_ix_i + a_1\sum w_ix_i^2 - \sum r_i^2w_i^2[a_1\sigma_{x_i}^2 - \mathrm{cov}(x_i, y_i)] = \sum w_ix_iy_i \tag{24}$$

As the values of a_0 and a_1 depends on weighting factors and, at the same time, weighting factors depends on a_1, an iterative algorithm for solving the system is needed (Gonzalez and Asuero, 1992).The starting guesses of parameters a_j are the values obtained from a single linear regression (setting $w_i = 1$). The new values of w_i are computed from Eq. (16) and, from these, improved values for the parameters are calculated according Eqs. (23) and (24), and so on. The convergence criterion is that k digits of each parameter are not changed in the iteration, that is:

$$|(a_{j,n+1}/a_{j,n}) - 1| < 10^{-k} \tag{25}$$

where n is the iteration number. Once the optimized values of parameters and weighting factors are known, the elements of the variance–covariance matrix $[\sigma^2(a_0), \sigma^2(a_1), \mathrm{cov}(a_0, a_1)]$ are computed by using well established relationships (Connors, 1987).

4. Weights

Assuming zero covariance and taking into account the random error propagation law (Asuero et al., 1988), we get:

$$w_i = \frac{1}{\sigma_u^2 + a_1^2\sigma_x^2} = \left[\frac{A_{\lim}\ln 10}{\sigma_A}\right]^2 \frac{1}{[z/(z-y)y]^2 + a_1^2[1/(1-y)]^2} \quad (26)$$

σ_A was taken arbitrarily as 0.001 through the calculations.

The weights calculated are normalized (Asuero and Gonzalez, 1989) by transforming each old weight into a new one, w^* in such a way that in being n the number of points on the graph we have:

$$\sum w = n \quad (27)$$

and

$$w^* = \frac{nw}{\sum w} \quad (28)$$

5. Choice of the starting value of the limiting absorbance $A_{\lim}$

For weak complexes where the concentration of L is in excess, the initial concentration is approximately the actual concentration of L in the solution; A/ε being negligible with respect to T_L and then:

$$\beta_{11} = \frac{A/\varepsilon}{(T_S - (A/\varepsilon))T_L} \quad (29)$$

Solving for A and taking into account Eq. (6) we get:

$$A = \frac{A_{\lim}\beta_{11}z}{1/T_S + \beta_{11}z} \quad (30)$$

for which

$$\frac{A}{T_S} - A_{\lim}\beta_{11}z + Az\beta_{11} = 0 \quad (31)$$

By using the method of least squares we may obtain the set of normal equations for the above case. Thus, both $\partial/\partial(A_{\lim}\beta_{11})$ and $\partial/\partial(\beta_{11})$ should vanish, leading to the conditions:

$$\left(\sum z_i^2\right) A_{\lim}\beta_{11} - \left(\sum A_i z_i^2\right)\beta_{11} = \frac{1}{T_S}\left(\sum A_i z_i\right) \quad (32)$$

$$\left(\sum A_i z_i^2\right) A_{\lim}\beta_{11} - \left(\sum A_i^2 z_i^2\right)\beta_{11} = \frac{1}{T_S}\left(\sum A_i^2 z_i\right) \quad (33)$$

where the summation is taken over all experimental points. In practice, however, the flat portion of a molar ratio curve is discarded. Solving by Cramer's rule we may estimate:

$$\beta_{11} = \frac{\left(\sum z_i^2\right)\left(\sum A_i^2 z_i\right) - \left(\sum A_i z_i^2\right)\left(\sum A_i z_i\right)}{\left(\sum A_i z_i^2\right)\left(\sum A_i z_i\right) - \left(\sum z_i^2\right)\left(\sum A_i^2 z_i^2\right)} \quad (34)$$

$$A_{\lim} = \frac{\left(\sum A_i^2 z_i\right)\left(\sum A_i z_i^2\right) - \left(\sum A_i z_i\right)\left(\sum A_i^2 z_i^2\right)}{\left(\sum z_i^2\right)\left(\sum A_i^2 z_i\right) - \left(\sum A_i z_i^2\right)\left(\sum A_i z_i\right)} \quad (35)$$

The $A_{\lim}$ value given by Eq. (35) was taken as starting value for the minimization process.

6. Minimization process

As $A_{\lim}$ is not accessible to direct measurement, a procedure for overcoming this difficulty must be followed before Eq. (14) can be applied ($y = A/A_{\lim}$). Vary $A_{\lim}$ systematically around the first estimate value obtained by applying Eq. (35) and apply the entire procedure in each case. Take the best log β_{11} value as that which minimize:

$$\text{S.D.}_A = \sqrt{\frac{\sum (A - A_{\text{calc}})^2}{N}} \quad (36)$$

where N is the number of A–z data pairs and A_{calc} is calculated from Eq. (10). A MATLAB program was written to perform this task with the process of minimization being carried out either by trial and error or by univariate search; e.g., the Fibonacci search, the golden section search or inverse parabolic interpolation by Brent's method (Press et al., 1999). Alternatively, the largest absorbance value may be taken as starting value for the minimization process.

7. Applications

The methods applied for the evaluation of stability constants of weak complexes are mostly based on graphical solutions using linear least squares regression analysis applied to known mathematical models. Most of these models, however, employ assumptions. The method of Benesi and Hildebrand has been the most popular for more than half a century (Exner, 1997). This analysis requires that the concentration of one of the associating species be kept much lower than the other, and the most important objection was that they can yield completely erroneous results when only low concentrations of the complex have been attained (Douglas, 1992; Exner, 1997; Hoenigman and Evans, 1996; Martin, 1997). On the other hand, the non-linear regression method sometimes fails to converge under non-ideal conditions. In consequence, the method devised has been applied to data found in the literature for the iron(III)-thiocyanate (Fig. 2) and nickel(II)-selenocyanate complexes, which were measured at multiple wavelengths. Absorbance diagrams of A_{λ_1} versus A_{λ_2} lead in both cases to a straight line passing through the origin (e.g., Fig. 2b), indicating that only one absorbing species in solution is found (Asuero et al., 1986; Herrador et al., 1987; Jimenez et al., 1987). Typical experimental details and absorbance versus T_S and T_L data employed are given in that follows:

Iron(III)-Thiocyanate system (Frank and Ostwalt, 1947): $[SCN^-] = 3E{-}04$ M. $\lambda = 400$ nm; $\{T_L, A\} = \{1E{-}03, 0.0998; 2E{-}03, 0.1953; 2E{-}03, 0.1807; 3E{-}03, 0.2505; 3E{-}03, 0.2450; 5E{-}03, 0.3550; 8E{-}03, 0.4600; 8E{-}03, 0.4510\}$.

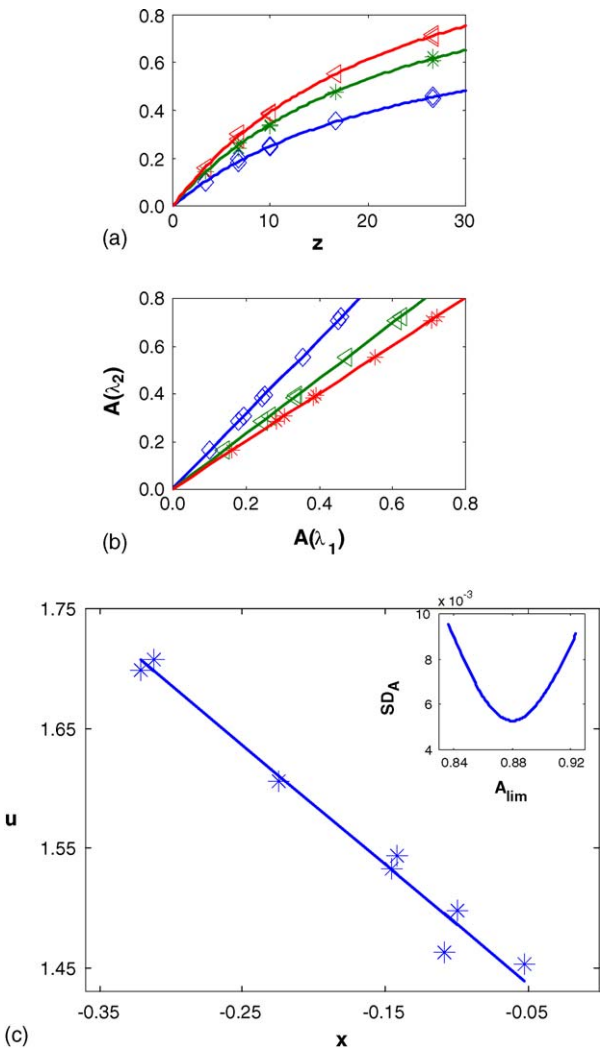

Fig. 2. (a) Absorbance data for the Fe(III)-thiocyanate system at (△) 450 nm, (✳) 440 nm and (◊) 420 nm. The curves are calculated with the β_{11} and A_{lim} values given in the text. (b) Absorbance diagram constructed for two wavelengths: (◊) 450/440 nm, (△) 540/420 nm and (✳) 450/440 nm. (c) Bilogarithmic plot for the Fe(III)-thiocyanate system at 400 nm. *Inset*: mean quadratic deviation of absorbances as a function of A_{lim} assumed.

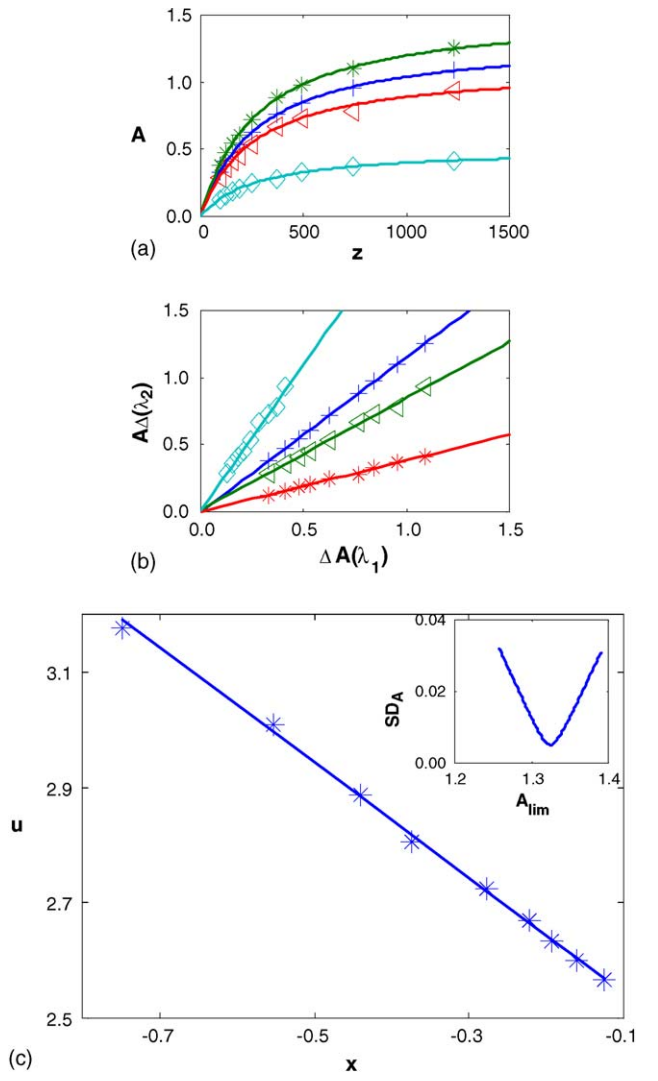

Fig. 3. (a) Absorbance data for the Nitracine Yellow-α-cyclodextrin system at (✳) 650 nm, (+) 635.4 nm, (△) 615 nm and (◊) 550 nm. (b) Absorbance difference diagram constructed for two wavelengths combinations: (◊) 615/550 nm, (+) 635.4/650 nm, (△) 615/650 nm and (✳) 550/650 nm. (c) Bilogarithmic plot for the Nitracine Yellow-α-cyclodextrin system at 650 nm. *Inset*: mean quadratic deviation of absorbances as a function of A_{lim} assumed.

Nickel(II)-selenocyanate system (Kulberg, 1974): $[Se^{2+}] = 5E{-}03$ M. $\lambda = 300$ nm; $\{T_L, A\} = \{20E{-}03, 0.0265; 40E{-}03, 0.0455; 60E{-}03, 0.0615; 80E{-}03, 0.0730; 100E{-}03, 0.0830\}$.

Frank and Ostwalt (1947) hold the thiocyanate concentration as constant, varying the iron concentration, in order to avoid the stepwise formation of higher complexes. The values of stability constants obtained for the $FeSCN^{2+}$, and $NiSeCN^{+}$ complexes at varying wavelength are compiled in Table 2. Values are reported with the same number of digits in all cases, even if they are not significant. The orthogonal weighted bilogarithmic method, proposed in this paper, leads to slope values close to the minus unity, which clearly constitutes an argument in favour of the application of the proposed method. On the other hand the agreement between the values obtained by the orthogonal weighted bilogarithmic method and the non-linear regression method of Carta and Crisponi (1982) is excellent in both cases.

Cyclodextrins are cyclic polysaccharides that form host–guest complexes with different compounds (Ikeda et al., 2004). Complexation reactions involving cyclodextrins are highly important in several fields such as pharmaceutical, chemical, environmental and food analysis, among others. They found use, e.g., for the improvement of physicochemical properties of drugs such as solubility, stability and bioavailability (Loukas et al., 1998; Oh et al., 1998; Rozou et al., 2004; Saetern et al., 2004). The stoicheiometry of this kind of host–guest complexes is dependent on the size of guest molecules though the most frequent host:guest ratio found is 1:1. The bilogarithmic method devised in this paper has also been applied to several host–guests α-cyclodextrin systems (Figs. 3 and 4). The Nitrazine Yellow-α-cyclodextrin system

Table 2
Stability constants of iron(III)-thiocyanate and nickel(II)-selenocyanate complexes

System	λ (nm)	Slope	log β_{11}	A_{lim}	S.D.$_A$
Iron(III)-thiocyanate	400	0.9982 ± 0.0580	2.136 ± 0.014 2.136 ± 0.027*	0.880 0.880*	5.281E−03
	420	0.9989 ± 0.0603	2.137 ± 0.017	1.188	7.430E−03
	440	0.9995 ± 0.0539	2.136 ± 0.015	1.376	7.649E−03
	450	0.9984 ± 0.0563	2.138 ± 0.014	1.379	8.010E−03
	460	0.9978 ± 0.0534	2.138 ± 0.014	1.341	7.496E−03
	480	0.9955 ± 0.0663	2.126 ± 0.016	1.166	7.592E−03
	500	1.0010 ± 0.0430	2.147 ± 0.010	0.862	3.892E−03
Nickel(II)-selenocyanate	305	0.9992 ± 0.0285	0.984 ± 0.006	0.104	1.682E−04
	300	1.0002 ± 0.0318	0.958 ± 0.007 0.959 ± 0.015*	0.176 0.176*	2.866E−04
	295	0.9991 ± 0.0182	0.955 ± 0.004	0.271	2.595E−04
	290	1.0011 ± 0.0278	0.971 ± 0.006	0.387	6.191E−04
	285	1.0025 ± 0.0451	0.984 ± 0.010	0.518	1.365E−04

* NLR method of Carta and Crisponi (1982).

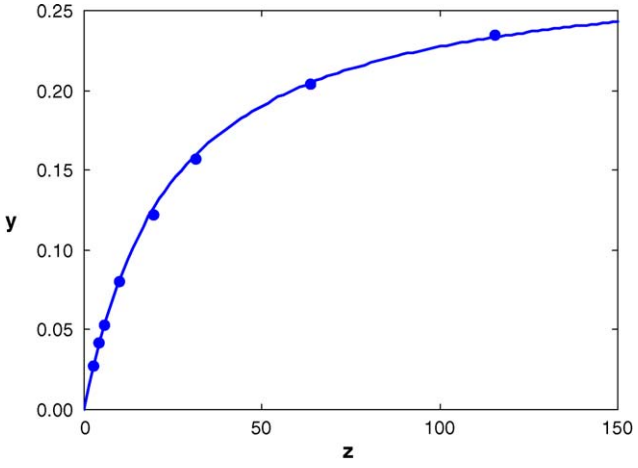

Fig. 4. Absorbance data for the *p*-nitrophenol-α-cyclodextrin system at 317 nm. The solid line in the figure is calculated with the β_{11} and A_{lim} values given in the text.

(Fig. 3) was measured at multiple wavelengths and only one complex species in solution is found from the absorbance difference diagram constructed at two wavelengths combinations (Polster and Lachmann, 1989). Typical experimental details and absorbance A (corrected for absorbance of indicator with no cyclodextrin present) versus T_S and T_L data employed are given in that follows:

Nitrazine Yellow-α-cyclodextrin system (Pendergast, 1983). 0.05 M pH 9.2 Tris buffer. [*Yellow Nitrazine*] = 4.847E−05 M. λ = 650 nm; {T_L, A} = {0.4497E−02, 0.3323; 0.5996E−02, 0.4106; 0.7495E−02, 0.4746; 0.9102E−02, 0.5296; 1.214E−02, 0.6245; 1.794E−02, 0.7646; 2.390E−02, 0.8438; 3.576E−02, 0.9539; 5.960E−02, 1.0874}
Methylorange-α-cyclodextrin system (Lin, 1981). 0.08N in HCl. [*Methyl Orange*] = 1.67E−05 M; λ = 508 nm; {T_L, A} = {0.478E−03, 0.190; 0.637E−03, 0.236; 0.972E−03, 0.321; 1.944E−03, 0.447; 3.999E−02, 0.573; 20E−03, 0.730}
p-Nitrophenol-α-cyclodextrin (Lin, 1981); [*p-Nitrophenol*] = 1.749E−04 M; λ = 317 nm; {T_L, A} = {0.443E−03, 0.027; 0.707E−02, 0.42; 1.039E−02, 0.53; 1.768E−02, 0.08;

Table 3
Stability (association) constants of some host–guests α-cyclodextrin inclusion complexes

System	λ (nm)	Slope	log β_{11}	A_{lim}	S.D.$_A$	log β_{11}*
Nitrazine Yellow-α-cyclodextrin, pH 9.2, I = 0.1	650	1.0006 ± 0.0019	1.871 ± 0.006	1.324	5.107E−03	1.870 ± 0.005
	653.4	1.0004 ± 0.0205	1.872 ± 0.007	1.524	6.385E−03	1.871 ± 0.005
	615	1.0030 ± 0.0594	1.896 ± 0.021	1.107	1.385E−02	1.881 ± 0.008
	550	0.9980 ± 0.0419	1.877 ± 0.015	0.506	4.213E−03	1.880 ± 0.011
Methyl Orange-α-cyclodextrin, 25 °C in HCl 0.08N	508	0.9974 ± 0.0071	2.833 ± 0.002	0.784	8.477E−04	2.828 ± 0.003
Methyl Orange-α-cyclodextrin, 25 °C in HCl 1.0N	508	0.9981 ± 0.011	2.818 ± 0.004	0.990	2.020E−03	2.822 ± 0.030
p-Nitrobenzoate-α-cyclodextrin in pH 9.2 Tris buffer	265	0.9895 ± 0.1102	1.822 ± 0.043	0.187	1.877E−03	1.846 ± 0.037
	315	0.9852 ± 0.0901	1.894 ± 0.034	0.106	1.905E−03	1.905 ± 0.029
p-Nitrophenol-α-cyclodextrin, 25 °C	317	1.0050 ± 0.0252	2.360 ± 0.005	0.285	1.485E−03	2.390 ± 0.018
4-Acetylbenzoate-α-cyclodextrin, 25 °C, pH 9.18 Tris buffer	253	1.0037 ± 0.0470	1.700 ± 0.015	0.123	8.172E−04	–

* Lin (1981) and Pendergast (1983).

3.409E−02, 0.122; 5.482E−02, 0.157; 11.16E−02, 0.204; 20.22E−02, 0.235}

p-Nitrobenzoate-α-cyclodextrin (Pendergast, 1983). 0.05 M pH 9.2 Tris buffer; [*p-Nitrobenzoate*] = 9.14E−05 M; λ = 265 nm; $\{T_L, A\}$ = {8.117E−02, 0.158; 5.798E−02, 0.143; 3.479E−02, 0.137; 2.319E−02, 0.110; 1.160E−02, 0.089; 0.8117E−02, 0.063; 0.5798E−02, 0.051; 0.4638E−02, 0.041}.

The stability constants determined are shown in Table 3, together with the previous values obtained by applying a weighted version of the Benesi and Hildebrand method (Lin, 1981; Pendergast, 1983). The difference in $\log \beta_{11}$ and $A_{\lim}$ values obtained, with respect to the proposed method, may be caused by the assumptions inherent in the Benesi and Hildebrand method (Exner, 1997; Hoenigman and Evans, 1996; Martin, 1997).

In spite of the modern possibility of calculating stability constants using black-box computer programs, the determination of these constants by linearized plots seems to be more prevalent (Fuchs and Gessner, 2001), probably owing to the transparency of the method used. The proposed method gives a clear indication of the existence of only a single complex in solution (a_1 slope equals to −1), it is flexible enough to allow the weighting of measurements and the computation procedure yield the best value of $\log \beta_{11}$ and its limit of error.

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