cEST: a flexible tool for calorimetric data analysis

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Abstract

A new MS-Excel utility based on the EST speciation tool (cEST) specifically designed to be applied to the analysis of calorimetric data is presented in this work. The cEST utility is able to fit calorimetric data with species of arbitrary stoichiometry and also automatically provides a complex statistical analysis of data fitting. This latter aspect is often very useful to discriminate the goodness of fit for different models. As cEST runs under MS-Excel, it is flexible in its implementation and allows a straightforward data import and graphing. Furthermore, it is open source and can be used within both Windows and MacOS operating systems.

The applicability of cEST is tested towards data of different origin: experimental data, where the complex formation between Ag^{+}, Co^{2+} and Cd^{2+} ions and terpyridine in anhydrous DMSO is studied, and simulated ITC points for biomolecular interactions with either two or three-binding sites.

In the case of metal complex formation, the combination with regression statistics allows the choice of the best model among those for which convergence is achieved. In this case, the Akaike Information Criterion (AICc) is employed for selecting the model for the metal-terpyridine speciation. Our analysis, based on independent calorimetric data, provides models and thermodynamic parameters which are in good agreement with those of the original works obtained by combining different complementary techniques. Also, in all the examined cases, the results obtained for the biomolecular interactions provide thermodynamic parameters which are strictly in line with the published results.

1. Introduction

The equilibrium constant (K) for a chemical reaction in solution and the associated standard Gibbs free energy of reaction (ΔG^0) is a key parameter in the study of a vast number of chemical and biochemical phenomena, but often it is not sufficient for a complete understanding of the origin of the stability of a formed species. The determination of the associated standard reaction enthalpy (ΔH^0) and entropy (ΔS^0) give access to assessing the strength of the interactions between a ligand and a substrate, the de-solvation and solvation processes of reactants and products and solvent reorganization. While the methods for the determination of K are numerous and essentially related to the measurement of the equilibrium concentration of one or more species (e.g. spectrophotometry, potentiometry, fluorimetry…) the ΔH^0 can be obtained in two ways: by van't Hoff equation and by

titration calorimetry. The first method needs the measurement of K at two temperatures (at least) and is known to provide ΔH^0 with large errors which can be slightly reduced by the interpolation of data at several temperatures. Furthermore, this method relies on the assumption that C_p is constant, which is not always valid in a wide range of temperatures [1,2].

Titration calorimetry is a powerful tool in the study of chemical[3] and biochemical systems[4] in order to obtain the ΔH^0 for a given equilibrium reaction since it measures directly the heat exchanged, due to the processes occurring in the measurement cell when the reagents are mixed. As the heat is the quantity measured, also heterogeneous systems can be studied, such as the interaction of a solute with a solid dispersed in a solution[5].

In all these applications the stability constant K (and therefore ΔG^0) can be obtained by independent experiments, such as potentiometry or spectrophotometry[6–14], or be a parameter which is obtained simultaneously to the ΔH^0 in the calorimetric data analysis[6,14–16].

Nowadays, the improvements in performance of modern calorimeters and the availability of fast computers and data analysis programs allow to obtain K and ΔH^0 values for many chemical systems. Many programs able to analyze and produce thermodynamic parameters from calorimetric data are available directly from calorimeters manufacturers (for example Origin or TA Assistant), but do not allow flexibility in the reaction models since 1:1 or 2:1 (ligand to metal ratio) species are considered. This limitation makes these tools quite easy to use, but useful for simple equilibria or to determine apparent constants and enthalpies. Other software [17,18] developed by experts in solution equilibria are more powerful as they are fast, intuitive in their use and, above all, they do not limit the complexity of the chemical equilibria to be studied.

Often there is the need to apply statistical tests to the obtained results. Indeed, when dealing with calorimetric data for simultaneous equilibria, several models can fit the data, and in absence of independent experiments which provide information about the number and/or stoichiometry of the species present in solution, a statistical analysis is of great utility.

Some years ago we developed the chemical speciation tool EST in the form of a MS-Excel plugin[19] which, given the total concentrations and the stability constants, was used to provide the equilibrium concentrations for a system of any complexity.

In this work we developed a new version, now called cEST, which is able to provide dynamically multiple equilibria simulations and relative thermodynamic parameters which is particularly suitable to treat calorimetric data. For data obtained from calorimetric titrations, the least squares method used is based on the search of the parameters (K and ΔH^0) which minimize the sum of squared differences between the experimental (q_{exp}) and calculated (q_{calc}) heat at each titrant addition:

$$U = \sum_{i,1}^{n} \left(q_{calc,i} - q_{exp,i}\right)^2 \quad (1)$$

The minimum search algorithm is managed by MS-Excel Solver which acts on the parameters present in defined cells of the active spreadsheet.

In this release, new functionalities are presented: the plugin in the original version has been modified to *i)* automatically update the total concentrations and q_{calc} values and *ii)* iteratively restart the search by recalling Solver. However, Solver does not provide errors on the final parameters nor statistical analysis for the model employed in the fitting procedure. To this purpose, the SolverStat utility[20] is directly recalled in this new release to obtain both errors on the K and ΔH^0 values and a series of statistical data for the fitting. This improved version of EST, operating in combination with Solverstat, has several advantages with respect to commercial software: flexible, since it can be easily customized by users; open source, the codes can be downloaded and modified; portable, any computer supporting MS-Excel can run it with either Windows or MacOS operating systems. Last, data import and graphing are very easy as they are done with the usual MS Excel procedures

The reliability of cEST is here checked by applying it to the analysis of published data concerning chemical equilibria in solution, such as Ag^+, Co^{2+} and Cd^{2+} complex formation with terpyridine (terpy) in anhydrous dimethylsulfoxide (DMSO)[8,21] and simulated thermodynamic parameters for multiple overlapping binding equilibria associated to two- or three-binding-site models occurring in biological interactions[22]. Furthermore, new simulated titrations where the formation of polynuclear metal complexes occurs are analyzed to evidence the capability of detecting such type of species by calorimetric data alone.

2. Methods and experimental data

To use cEST it is necessary to copy the .xla file in the working directory or in the MS-Excel add-ins folder. The add-in must be enabled and allowed by the security check of MS-Excel. The preparation of a MS-Excel file to run cEST is described in the Electronic Supplementary Material.

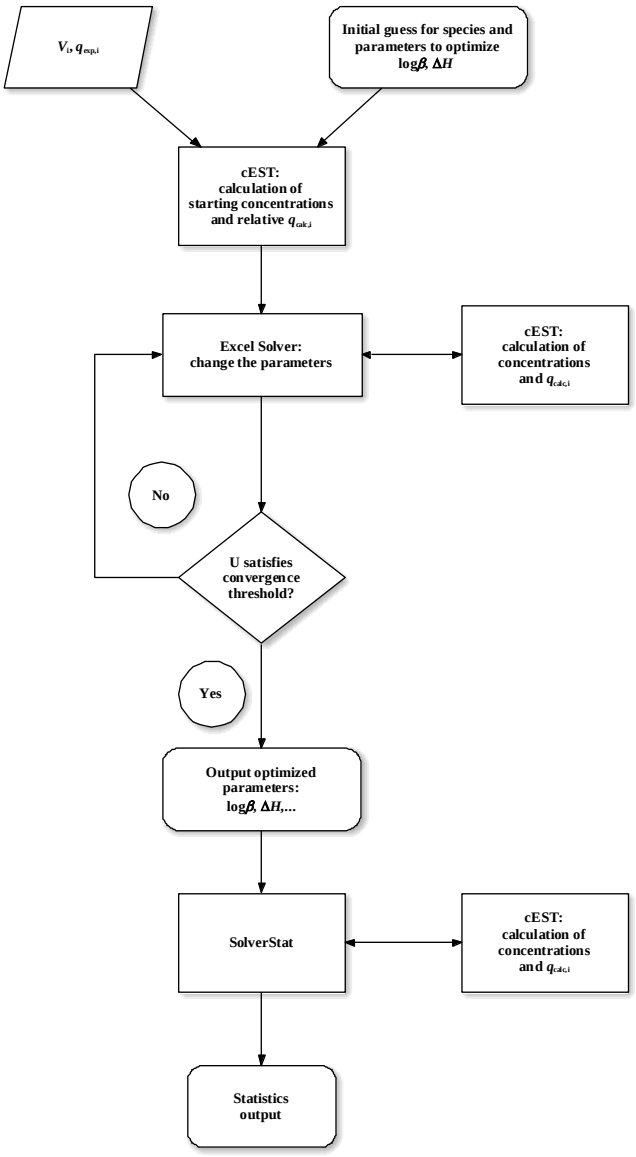
The speciation calculations carried out in cEST are described in detail in the previous paper[19]. As far as the calorimetric implementation is concerned, q_{calc} can be defined as:

$$q_{calc} = \sum_{j,1}^{m} \left(\delta n_j \cdot \Delta H_j\right) + \sum_{k,1}^{l} (Q_k) \quad (2)$$

where δn_j is the number of moles variation of a j product calculated from a set of m equilibrium constants and ΔH_j is its formation enthalpy. In the second term of the equation (2) Q_k represents the contribution to the heat due to other l not compensated processes (e.g. precipitations, side reactions).

This term can be calculated in a way defined by the user or considered negligible. In principle, no limitations on the complexity of the systems investigated exist, i.e. many simultaneous equilibria, and, if required, the initial concentrations of the species can be refined.

In cEST the δn_j values are calculated by using the K_j values for the m species formed (entered in the spreadsheet as overall formation constants, $\log\beta_{j,}$), and the total concentrations of reagents in the calorimetric cell (Scheme 1). After multiplying the δn_j values by the associated test values of ΔH_j^0 the objective function value U is calculated. At this stage, Solver is recalled to by the macro to update the log K_j and ΔH_j^0 and starts the next cycle until convergence is met (Scheme 1). The convergence criterion and other minimization parameters can be set in the Solver dialog window.



Scheme 1. Calorimetric data analysis workflow

It should be noted that in the old release, the dynamic system that allowed to reach the best fit of experimental data needed a manual modification of the associated Visual Basic for Applications (VBA) macro, which is now automatically applied in cEST. Three templates are provided: a) one sheet with a generic dynamic model; b) one sheet able to process more model sheets (i.e. titrations with different experimental conditions); c) one specific sheet to operate in combination with Solverstat.

The typical worksheet presents the stoichiometry and stability constants of the species formed and the standard enthalpies in the top left of the worksheet. Any parameter to be optimized can be selected in the Solver parameter list. Note that Solver allows also to put limits to the parameters (e.g. non-negative logK_j): this can be useful if used carefully.

Three sets of experimental and simulated data, representative of different operating conditions, have been used for testing cEST, which are discussed below.

2.1 Complex formation in non-aqueous solution

Calorimetric titrations have been carried out using a Tronac Isoperibol calorimeter with the reactants concentrations and other experimental details specified in ref. [8,21] and related data for the complex formation of Ag^+, Cd^{2+} and Co^{2+} metal ions with terpy in DMSO are analyzed by cEST in this work. In the previous papers the formation constants of the AgL_j and CdL_j complexes (j = 1, 2) were determined by potentiometric technique, using ion selective electrodes, while those of CoL_j complexes by means of UV-Vis spectrophotometric titrations using Cd^{2+} as a competitive ion[7,23–26]. In all cases the data analysis were performed by Hyperquad program[27]. The enthalpy changes were then analyzed by running the program Letagrop Kalle[28] to the calorimetric data, imposing the formation constants determined independently by potentiometry or spectrophotometry[8,21].

In these experiments, the calorimeter was equipped with a 25mL cell which was initially filled with 20mL of metal solution. Therefore, the solution volume inside the cell is increased by addition of the titrant. The concentration of a generic component at any *i* addition has been calculated as in equation (3):

$$TA_i = \frac{[TA_0 \cdot Vc_0 + CAb \cdot Vb_i]}{Vc_0 + Vb_i} \quad (3)$$

Vc_0 is the initial volume in the cell, Vb_i is the added volume of titrant at step (or time) *i*.

Although cEST is applied to treat calorimetric data for metal complex formation in non-aqueous solution, the extension to the study of reactions in water is quite easy. In the latter case, an additional reagent (H^+) is present and water formation and the (eventual) ligand protonation equilibria (with the relative enthalpies) need to be taken into account in the fitting.

2.2Multinuclear complexes

Calorimetric data where the metal in the cell (C_{Mc}) was titrated with the ligand in the burette (C_{Lb}) were simulated in the following conditions: V_{C0} = 20.0 mL; titration #1 C_{Mc} = 2 mmol dm^{-3}, C_{Lb} = 50 mmol dm^{-3}; titration #2 C_{Mc} = 20 mmol dm^{-3}, C_{Lb} = 500 mmol dm^{-3}. Realistic noise was applied to the simulated data by adding random samples from a normal distribution with mean 0 and standard deviation of 0.01 J. The "true" values of the formation constants and $\Delta H°$ were: for ML $\log\beta_{1,1}$ = 2 and $\Delta H°_{\beta_{1,1}}$ = -20 kJ mol^{-1}, for M_2L_2 $\log\beta_{2,2}$ = 5. The two sets of titration data were fitted simultaneously by cEST. Data were fitted with a model including the ML, ML and M_2L_2 or the M_2L_2 species. Different "true" values of $\Delta H°_{\beta 2,2}$ were tested to evidence the conditions in which the two species can be differentiated on the basis of calorimetric data only.

2.3 Biomolecular interactions

Recently [22], an algorithm based on a nonlinear regression analysis of ITC data has been developed in MATLAB to model multiple-binding-site equilibria in biomolecular interactions. In the specific case the algorithm has been applied for the analysis of quadruplex DNA binding a small ligand or a protein. The algorithm was meant to improve ITC data analysis to unravel the complicated binding equilibria often occurring in biomolecular interactions[29–32].

In order to check the reliability of cEST even in this application and with the aim to compare our results with those previously obtained and validated, ITC titration points were generated with the program developed in ref. [22], with the same conditions (i.e. simulated random noise, concentrations) and parameters (binding constants, molar enthalpy changes) used in the original paper and related to two-competitive- (Table 1, case 2, p.235 in in ref. [22]) and three-competitive processes models (Table 2, p.236 in ref. [22]). This was done by using MATLAB-developed software downloaded by the authors website [22]. Then, the generated ITC points were processed by using cEST.

In this application the calorimetric data were simulated for a titration in which a microcalorimeter equipped with completely filled cell is used: therefore the loss/addition of volume at each titration point must be taken into account.

For a fixed volume calorimeter, the concentration of a generic component at addition i is given by equation (4):

$$TA_i = \frac{[TA_{i-1} \cdot (Vc - \Delta Vb_i) + CAb \cdot \Delta Vb_i]}{Vc} \quad (4)$$

Where TA_i is the total concentration of component A at step i (corrected for dilution and for displacement from the calorimeter cell as titrant is added), V_c is the cell volume (constant), ΔVb_i is the volume injected at step i (assumed to be equal to that diplaced from the cell) and CAb is the concentration of A in the burette. In this type of calorimeter, the burette displaces a volume from the sample cell to a non-active reservoir: the displacement is considered to occur instantaneously.
To fit the ITC data, a sequential (stoichiometric) binding model was used [33]: in this example two or three ligand molecules L (typically a small ligand molecule or a protein) bind to the receptor molecule M (typically a protein or a macromolecule) in a sequential mode $M + jL \rightleftarrows ML_j$.

However, it should be underlined that the design of cEST does not limit the use of multiple independent binding sites.

3. Data analysis

3.1 *Complexes in non-aqueous solution*

The stability constants and enthalpy changes obtained by running cEST on Ag^+ / terpy data sets are reported in Table 1 together with the statistical analysis obtained by Solverstat. The experimental data sets together with the layout of MS-Excel are reported in the Electronic Supplementary Material.
The *Coefficient of Multiple Determination* R^2 and *Adjusted Coefficient of Multiple Determination* R^2_{adj} in Table 1 indicate the fraction of total variability in the data shown by the regression model[34]. The R^2_{adj} is superior to the common R^2 value as it is sensitive to addition of irrelevant variables (R^2_{adj} decreases whereas R^2 increases or at least stays constant). But R^2 and R^2_{adj} are not always appropriate tools in nonlinear regression [35] and the most simple and informative measure of goodness-of-fit for regression models, both linear and nonlinear, is the root mean square error (RMSE), defined as the square root of the residual mean square. The RMSE may be viewed as the “average” discrepancy between the observed data and their predicted values. Hence, its magnitude, especially when one also considers the precision in the original data, is useful in assessing whether a given model truly fits the data well[35].

Another subset selection tool available is the Akaike Information Criterion (AIC)[36] which is available in Solverstat also in the small-sample-size corrected version (AICc). This parameter it is based on measuring the fit given by SSE (Sum of Squared Errors) and correcting it for the number of regressors: the best model will show lowest value of AICc [36]. Some simple rules of thumb are often useful in assessing the relative merits of models in the set: models having a difference of less than 2 have substantial support (evidence), when the difference is between 4 and 7 they have considerably less support. Models having a difference over 10 have essentially no support [37]. Finally the Residual Sum of Squares (RSS) is the sum of squared residuals between predicted and observed values[34].

For the Ag^+/terpy system seven different models have been checked to fit the experimental data, taking into account different combination of Ag_iL_j mononuclear and polynuclear species (i = 1, 2; j =1, 2, 3), as this ions is known to form also polynuclear species in non-aqueous solutions [8,38–40]. In the last run (Model 7) the stability constants were not minimized, being imposed the values reported in ref.[21].

The Models 4-6, in which polynuclear species were considered, did not reach convergence, excluding formation of these species. The concentration range of Ag^+ ion in the calorimetric titrations was chosen in such a way (total metal concentration, $C°_{Ag^+}$ = 7 - 29 mmoldm^{-3}) that eventual polynuclear complexes should be detected, if present. The goodness of Model 3 is not comparable to Model 1 and 2, just if only R^2 and R^2_{adj} terms are considered, whereas to discriminate between Model 1 or 2 it is necessary to examine the value of AICc term: the lower is its value, the better is the adequacy of the model. Therefore, the combined analysis of calorimetric data with cEST and Solverstat shows that Model 2 is the best choice to describe Ag-terpy system, in agreement with the existence of the mononuclear AgL_j (j = 1, 2) species found also by potentiometry. Also the values of stability constants and enthalpy values are strictly in line with the values previously reported (see Table 1, bottom and ref.[21].

For Cd^{2+} and Co^{2+} complex formation with terpy, four different models were analyzed taking into account different combinations of only 1:1 and 1:2 metal-to-ligand stoichiometry, as commonly only mononuclear species have been detected for complex formation of Cd^{2+} and Co^{2+} with nitrogen donor ligands in solution[21,23,25], apart when macrocyclic multidentate ligands were considered[39–41]. The simultaneous minimization of stability constants and enthalpies were run with cEST for the experimental data reported in the spreadsheet in ESI (Sheet “data” in Ag-terpy.xls) and the results are entered in Table 2, Models 1-3, together with their errors and regression analysis as found with Solverstat combination. Model 4 in Table 2, both for Co^{2+} and Cd^{2+} systems, represents the fit obtained when the stability constants reported in ref. [8] are kept constant in cEST worksheet.

From the analysis of data and following a screening of the regression data as above reported for the Ag^{+}/terpy system, it clearly emerges that the best models are Model 2 and 1 for Co^{2+} and Cd^{2+} respectively, again in line with what found with independent potentiometric, spectrophotometric and calorimetric techniques. Also, the stability constants and enthalpy changes are in line with published data[8].

3.1. Multinuclear complexes

As described above, some metal ions have the ability to form multinuclear complexes of M_iL_j in solution [21,40,42,43]. Such species can be of difficult identification from calorimetric data alone and often require the simultaneous fitting of several titrations with different concentrations of the metal ion[21,38]. To check the performance of the analysis made by cEST, calorimetric data where the metal is titrated with the ligand were simulated on the basis of a model including either the ML species alone or in co-presence with the M_2L_2. This test is quite challenging since the two species form in the same ligand/metal ratio range. From the analysis of the results in Table 3, three points are worth to be noted: *i)* the R^2 and R^2_{adj} are not useful to discriminate among the models in any case; *ii)* on the contrary, the RMSE, AICc, e RSS parameters are able to show that the best model is when the M_2L_2 is included; *iii)* Results reported in Table 3 show that non-significant (NS) parameter is obtained (the value is outside of the 95% confidence interval) when fitting simulated data with $\Delta H°_{\beta 2,2} < -10$ kJ mol^{-1}. Therefore, it is possible to predict if the presence of M_2L_2 species can be detected by calorimetry in the experimental conditions considered.

3.2 Biomolecular interactions

The resulting best fit parameters, binding constants and molar enthalpy changes, obtained with cEST by simultaneous fit of generated ITC data for two-competitive and three-competitive processes are reported in Tables 4 and 5 respectively, together with the original simulated parameters reported by the authors[22]. The initial supposed parameters are reported in the same Tables as “true” values. The statistical evaluation obtained with cEST/Solvestat are also reported in the same tables (see the above paragraph for their definitions). It should be underlined that in ref. [22] also the number of binding sites per mole of titrated substrate (*n*) is adjusted by fitting.

The two cases reported in Table 4 and 5 have been chosen among those reported in ref. [22] as the most representative because, for these cases, the authors provided also the standard deviation

estimated from 95% confidence interval and therefore data are more suitable for a comparison with those obtained by cEST [22]. The best-fit parameters obtained with both programs are in excellent agreement and returned parameters very close to the "true" values used to create the simulated ITC data sets. The binding constants are slightly overestimated by both MATLAB and cEST while the third-process molar enthalpy changes exhibit the larger uncertainties by using both models. The regression statistics used by cEST demonstrates that all the two- or three- competitive processes models are very well defined and have acceptable errors.

4. Conclusions

An EST-based[19,20] tool, now called cEST, with new functionalities added to analyze calorimetric data is presented in this work. This new cEST utility presents several advantages with respect to commercial software, especially flexibility, since it is based on a MS Excel plugin which also allows an easy data import and graphing. Furthermore, it is open source and can be used within both Windows and MacOS operating systems. The cEST utility can be directly used in conjunction with Solverstat to provide statistical analysis of data fitting, which is often useful to discriminate the goodness of fit for different models.

Different sets of data have been considered: the experimental ones for the complex formation between Ag^{+}, Co^{2+} and Cd^{2+} ions and terpy in DMSO (calorimeter with variable sample volume), simulated data with formation constants involving a multinuclear species (ML and M_2L_2) and simulated data for an ITC titration using a calorimeter with fixed cell volume. The results obtained by cEST successfully converged towards values of thermodynamic parameters which are strictly in line with the published results in all the examined cases. In addition, the combination with regression statistics present in Solverstat results to be an important add-in for the choice of the best model among those for which convergence is achieved.

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TABLES

Table 1. Regression analysis, thermodynamic parameters (standard errors in parentheses) found by cEST-Solverstat for the analysis of calorimetric data obtained for the Ag^+/terpy systems in DMSO [21]. The $\log\beta_{i,j}$ and $\Delta H°_{\beta i,j}$ values (kJ mol^{-1}) refer to the reactions $iAg + jL \leftrightarrows Ag_iL_j$ (i =1 , 2; j =1, 2, 3). Charges omitted for clarity.

Model		AgL	AgL_2	AgL_3	Ag_2L	Ag_2L_2	R^2	R^2_{adj}	RMSE	AICc	RSS
1	$\log\beta_{i,j}$	2.04 (0.04)					0.9944	0.9943	0.758	-106.614	111.435
	$-\Delta H°_{\beta i,j}$	47.1 (0.9)									
2	$\log\beta_{i,j}$	3.15 (0.06)	4.95 (0.06)				0.9998	0.9998	0.155	-726.123	4.625
	$-\Delta H°_{\beta i,j}$	26.2 (0.3)	48.5 (0.6)								
3	$\log\beta_{i,j}$		5.89 (0.18)				0.9931	0.9931	0.836	-68.097	135.634
	$-\Delta H°_{\beta i,j}$		40.2 (0.4)								
4	$\log\beta_{i,j}$	(+)	(+)	(+)			No convergence				
	$-\Delta H°_{\beta i,j}$										
5	$\log\beta_{i,j}$	(+)	(+)			(+)	No convergence				
	$-\Delta H°_{\beta i,j}$										
6	$\log\beta_{i,j}$	(+)	(+)		(+)		No convergence				
	$-\Delta H°_{\beta i,j}$										
7	$\log\beta_{i,j}$	fixed	fixed				0.9997	0.9997	0.171	-690.841	5.656
	$-\Delta H°_{\beta i,j}$	27.0 (0.1)	51.3 (0.2)								
Ref. [21]	$\log\beta_{i,j}$	3.03(0.01)	4.68(0.1)								
	$-\Delta H°_{\beta i,j}$	26.6 (1)	50 (2)								

Table 2. Regression analysis, thermodynamic parameters (standard errors in parentheses) found by cEST-Solverstat for the analysis of calorimetric data obtained for the $Co^{2+}Cd^{2+}$/terpy systems in DMSO[8]. The β_j and $\Delta H°_{\beta j}$ values (kJ mol^{-1}) refer to the overall reactions $M + jL \leftrightarrows ML_j$ (M= Co, Cd; j =1, 2,). Charges omitted for clarity.

Model		CoL	CoL_2	R^2	R^2_{adj}	RMSE	AICc	RSS
1	$\log\beta_j$		10.3 (1.0)	1.0000	1.0000	0.0641	-516.591	0.369
	$-\Delta H°_{\beta j}$		63.2 (0.1)					
2	$\log\beta_j$	4.98 (0.15)	9.83 (0.15)	1.0000	1.0000	0.0141	-801.431	0.0175
	$-\Delta H°_{\beta j}$	29.9 (0.2)	63.36 (0.02)					
3	$\log\beta_j$	Fixed	Fixed	1.0000	1.0000	0.0157	-783.327	0.022281
	$-\Delta H°_{\beta j}$	30.44 (0.03)	63.33 (0.02)					
Ref. [8]	$\log\beta_j$	5.56 (0.15)	10.52 (0.15)					
	$-\Delta H°_{\beta j}$	33 (1)	63 (1)					
		CdL	CdL_2	R^2	R^2_{adj}	RMSE	AICc	RSS
1	$\log\beta_j$	2.46 (0.06)	4.62 (0.03)	0.9999	0.9999	0.0426	-927.500	0.258
	$-\Delta H°_{\beta j}$	10.7 (0.1)	28.4 (0.5)					
2	$\log\beta_j$	1.03 (0.05)		0.9971	0.9971	0.301	-351.12	13.1
	$-\Delta H°_{\beta j}$	71.0 (5.6)						
3	$\log\beta_j$		4.90 (0.03)	0.9998	0.9998	0.0719	-775.021	0.744
	$-\Delta H°_{\beta j}$		23.9 (0.1)					
4	$\log\beta_j$	fixed	fixed	0.9999	0.9999	0.276	-921.803	0.0438
	$-\Delta H°_{\beta j}$	11.2 (0.1)	27.40 (0.04)					
Ref. [8]	$\log\beta_j$	2.27 (0.02)	4.57 (0.05)					
	$-\Delta H°_{\beta j}$	10.6 (0.3)	27.8 (0.3)					

Table 3. Regression analysis, thermodynamic parameters (standard errors in parentheses) simulated data for the reaction iM + jL ⇆ M_iL_j (i =1 , j =1 and i = 2 j = 2). The "true" values of the formation constants and $\Delta H°$ were: for ML $\log\beta_{1,1}$ = 2 and $\Delta H°_{\beta_{1,1}}$ = -20 kJ mol^{-1}, for M_2L_2 $\log\beta_{2,2}$ = 5. Data are reported for several "true" values for $-\Delta H°_{\beta_{2,2}}$. NS = non-significant.

$-\Delta H°_{\beta_{2,2}}$ (kJ mol^{-1})		ML	M_2L_2	R^2	R^2_{adj}	RMSE	AICc	RSS
ML Only	$\log\beta_{i,j}$	2.00 (0.01)		1.0000	1.0000	0.011	-955.955	0.0124
	$-\Delta H°_{\beta_{i,j}}$	20.01 (0.01)						
	$\log\beta_{i,j}$	2.00 (0.01)	-6 (10^6) NS	1.0000	1.0000	0.011	-951.676	0.0124
	$-\Delta H°_{\beta_{i,j}}$	20.01 (0.02)	0.0 (10^3) NS					
	$\log\beta_{i,j}$		5.9 (10^6) NS	0.9984	0.9984	0.165	-379.444	2.8432
	$-\Delta H°_{\beta_{i,j}}$		37.8 (0.1)					
3	$\log\beta_{i,j}$	2.16(0.01)		0.9999	0.9999	0.026	-774.431	0.0685
	$-\Delta H°_{\beta_{i,j}}$	16.12 (0.03)						
	$\log\beta_{i,j}$	2.01 (0.02)	4.95 (0.08)	1.0000	1.0000	0.011	-952.110	0.0123
	$-\Delta H°_{\beta_{i,j}}$	19.8 (0.4)	0.7 (4.2) NS					
5	$\log\beta_{i,j}$	2.15 (0.01)		1.0000	1.0000	0.024	-791.167	0.0585
	$-\Delta H°_{\beta_{i,j}}$	16.34 (0.03)						
	$\log\beta_{i,j}$	2.01 (0.02)	4.94 (0.08)	1.0000	1.0000	0.011	-952.216	0.0123
	$-\Delta H°_{\beta_{i,j}}$	19.8 (0.4)	2.7 (3.7) NS					
10	$\log\beta_{i,j}$	2.14 (0.01)		1.0000	1.0000	0.019	-836.567	0.0381
	$-\Delta H°_{\beta_{i,j}}$	16.89 (0.02)						
	$\log\beta_{i,j}$	2.01 (0.02)	4.93 (0.08)	1.0000	1.0000	0.011	-952.490	0.0123
	$-\Delta H°_{\beta_{i,j}}$	19.7 (0.3)	8.0 (2.7) NS					
15	$\log\beta_{i,j}$	2.12 (0.01)		1.0000	1.0000	0.015	-883.550	0.0245
	$-\Delta H°_{\beta_{i,j}}$	17.45 (0.02)						
	$\log\beta_{i,j}$	2.01 (0.02)	4.93 (0.07)	1.0000	1.0000	0.011	-952.765	0.0122
	$-\Delta H°_{\beta_{i,j}}$	19.7 (0.3)	13.7 (1.8)					
20	$\log\beta_{i,j}$	2.11 (0.01)		1.0000	1.0000	0.013	-917.969	0.0177
	$-\Delta H°_{\beta_{i,j}}$	18.00 (0.02)						
	$\log\beta_{i,j}$	2.01 (0.01)	4.93 (0.07)	1.0000	1.0000	0.011	-953.024	0.0122
	$-\Delta H°_{\beta_{i,j}}$	19.7 (0.3)	19.5 (1.1)					

Table 4. Two-competitive-site model in which a two B molecules are binding to the receptor molecule A. The ITC data have been generated by "true values" with random noise as in ref. [22]. The $\log\beta_j$ and $\Delta H_{\beta j}$ (kcal mol^{-1}) values obtained by MATLAB algorithm [22] and cEST with their standard deviations are reported.

		AB	AB_2	R^2	R^2_{adj}	RMSE	AICc	RSS
This work	$\log\beta_j$	8.31 (0.01)	14.31 (0.01)	1.0000	1.0000	0.00200	-629.256	$1.877 \cdot 10^{-4}$
	$-\Delta H_{\beta j}$	-11.937 (0.002)	-16.964 (0.001)					
Ref.[22]	$\log\beta_j$	8.29 (0.01)	14.28 (0.02)					
	$-\Delta H_{\beta j}$	-11.950 (0.001)	-16.900(0.002)					
"true values"	$\log\beta_j$	8.30	14.30					
	$-\Delta H_{\beta j}$	-12.00	-17.00					

Table 5. Three-competitive-site model in which three ligand molecules B are binding to the receptor molecule A: ITC data have been generated by "true" values reported in the Table with random noise and other details as in ref. [22] log β_j and $\Delta H_{\beta j}$ (kcal mol^{-1}) obtained in ref. [22] and by simultaneous best fit with cEST.

		AB	AB_2	AB_3	R^2	R^2_{adj}	RMSE	AICc	RSS
This work	$\log\beta_j$	8.017 (0.004)	14.02 (0.01)	18.01 (0.01)	1.0000	1.0000	0.001074	-689.737	$5.195 \cdot 10^{-5}$
	$-\Delta H_{\beta j}$	-11.932 (0.001)	-19.921 (0.003)	-23.95 (0.01)					
Ref.[22]		8.03 (0.01)[$]	14.05 (0.01)[$]	18.10 (0.01)[$]					
		-12.000 (0.001)[$]	-19.990 (0.002)[$]	-24.23 (0.05)[$]					
"true values"	$\log\beta_j$	8.00	14.00	18.00					
	$-\Delta H_{\beta j}$	-12	-20	-24					

[$] Standard error estimated from 95% confidence interval