Supporting Information for

Thermal Activity in Affinity Separation Techniques such as Liquid-Liquid Extraction analyzed by Isothermal Titration Calorimetry (ITC) and Accuracy Analysis of the Technique in the Molar Concentration Domain

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Abstract

In this supporting information document, experimental methods to minimize the error in isothermal titration calorimetry (ITC) are discussed. These are essential to maximize experimental accuracy. Furthermore, a more extensive discussion on theoretical ITC-curves is included that supports the ITC-curve fitting discussion in the main article.
Minimizing the error in experimental ITC results

To obtain the most accurate results with ITC, the experimental parameters that can be varied are the number of injections \( n_{\text{inj}} \), the injection volume \( V_{\text{inj}} \), the concentration of samples or extractant in the measuring cell \([B]\), the titrant concentration in the syringe \([A]_{\text{syr}}\) and the final ratio of titrant concentration over sample concentration in the sample cell after the experiment \( R_m\) (\( = \frac{([A]_{\text{tot}})}{([B]_{\text{tot}})}_{\text{final}} \)).

Increasing the number of injections does not always reduce the error, since volume errors are important for volumes under 7 µL and injection energies may become too small.\(^1\) In contrast to earlier published values of \( n_{\text{inj}} = 30 \), now values around \( n_{\text{inj}} = 10 - 15 \) are recommended,\(^2,4\) taking into account that the integrated heat of injection should be above 10 µcal, or 42 µJ.\(^5\) The error in titrant concentration can also be very significant.\(^2\) The number of injections is limited by the syringe volume, sample cell volume, titrant concentration \([A]_{\text{syr}}\) and initial concentration in the measuring cell \([B]_0\), where in general \( \frac{[A]_{\text{syr}}}{[B]_0} \) should be between 20-50.\(^5\) These concentrations can be related to the equilibrium constant. Wiseman introduced a \( c \)-value to describe the shape of the binding isotherms of ITC and optimize experimental conditions, such as the concentration in the sample cell \([B]\). The definition of the Wiseman \( c \)-value is shown in eq (S1), where \( K \) is the equilibrium constant.\(^6\)

\[
c = [B]Kn \tag{S1}
\]

Several guidelines can be found for the value of \( c \), where \( c \) should in general be between 1 and 1000,\(^2\) and more ideally between 10-100.\(^5\) For too low \( c \)-values the calculation of \( n \) and \( \Delta H \) becomes problematic, whereas for too high \( c \)-values the calculation of \( K \) is problematic. When the stoichiometry is known, experiments with \( c < 10 \) can also be very accurate.\(^7\) Although multiple values for \( c \) are reported, successful analysis can be performed even at \( c < 1 \) when the titration range is increased from the normal \( R_m = 2 \), to \( R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c} \) with \( R_m \geq 1.1 \).\(^2,4\) According to Tellinghuisen\(^4\) successful analysis is less determined by the \( c \)-value itself than by the total detectable heat and the maximal conversion of
the complexation reaction. Another advice is to remove the first (small) injection from the data points, to account for diffusive titrant loss.\textsuperscript{2,4} In the fitting of the data a correction by a constant value can be applied to account for drift.\textsuperscript{3}

For the experiments with 0.12 M TOA a higher value for $R_m$ was chosen because the slope of the S-curve in the last part of the curve was steeper than for the other two types of experiments. Because of the higher sample concentration, for the experiments with 0.48 M TOA a smaller sample vial was used to allow for sufficient $R_m$. The disadvantage of a smaller total volume is that the number of injections could not further be increased significantly without making concessions that could lead to a too small injection volume.

Furthermore, important assumptions that are used in ITC analysis are that binding is reversible and equilibrium is reached between injections. It is therefore important to ensure that the signal is back to the baseline before the next injection. Also the heat of dilution of the ligand and (macro)molecule influences the measurements, for this blanks should be performed of titrating ligand into the buffer solution and of the buffer solution in the macromolecule.\textsuperscript{8} It should be taken into account that performing blanks uses the assumption that the heat of dilution is equal for dilution into the pure diluent and into the solvent mixture.\textsuperscript{3} Lastly, $\Delta H$ is not (necessarily) constant with temperature, so experiments at different temperatures might be required.\textsuperscript{9}

**Diluent effects**

The parameter fitting of the experimental data in Figure 3 is performed using MATLAB by a least squares minimization of the error between experimental and calculated data based on the model equations. The results are shown in Table S1. It can be seen that in the case of toluene for the sequential reaction model $\Delta H_{1,1}$ is higher than $\Delta H_{n+1,1}$, indicating that indeed the interaction with the first acid is stronger
than with the following acids in the diluent toluene. In the case of 1-octanol it is clear that the (1,1)-
complex is the main complex formed as $K_{1,1}$ is high and $K_{n+1,1}$ is very low for the sequential reaction
model. This can also be concluded from the low stoichiometry $n = 1.2$ in the fit of the single reaction
model for 1-octanol.

Table S1 Parameter fit for the $\Delta H$, $K$ and $n$-values for interaction of acetic acid with 0.24 M TOA in
toluene, MIBK, heptane and 1-octanol at 20°C, fitted with both the sequential reaction model (top) and
single reaction model (bottom), described in eq (1), (2), (7) and eq (8)-(9), respectively. Isotherms are
shown in Figure 3.

| Sequential reaction model | $\Delta H_{1,1}$ (kJ/mol) | $K_{1,1}$ | $\Delta H_{n+1,1}$ (kJ/mol) | $K_{n+1,1}$ | $n$ | Residue (%) |
|--------------------------|---------------------------|-----------|----------------------------|-------------|----|-------------|
| Toluene                  | -32.4                     | 10.7      | -13.0                      | 141         | 1.6| 3.2%        |
| MIBK                     | -22.2                     | 5.6       | -20.4                      | 47.5        | 1.5| 4.5%        |
| Heptane                  | -17.9                     | 22.5      | -20.9                      | 45.4        | 1.9| 5.7%        |
| 1-octanol                | -28.8                     | 90.7      | -27.6                      | 0.8         | 0.8| 2.2%        |

| Single reaction model    | $\Delta H_{n,1}$ (kJ/mol) | $K_{n,1}$ | $n$ | Residue (%) |
|--------------------------|----------------------------|-----------|-----|-------------|
| Toluene                  | -20.6                      | 37.7      | 2.8 | 4.8%        |
| MIBK                     | -16.7                      | 28.4      | 3.4 | 4.0%        |
| Heptane                  | -15.4                      | 25.8      | 3.8 | 4.1%        |
| 1-octanol                | -31.8                      | 28.7      | 1.2 | 8.9%        |

Theoretical ITC curves for single site model and dual site model

Figure S1a shows a theoretical isotherm for a system based on the one reaction formation of the 1:1
complex (see eq (1) and (2)). Figure S1b shows a theoretical isotherm for a reaction system based on
two reaction equations (see eq (1), (2), (5) and (6)) where the enthalpy of complexation is larger for the
interaction of the first ligand than for the second ligand. In this isotherm a double S-shape is visible.
Figure S1 Shape of theoretical isotherms for a) a single reaction model with 1:1 stoichiometry (0.83 M extractant, 17.5 M titrant, $K = 30$, $\Delta H = -35 \text{ kJ/mol}$) and b) a sequential reaction model with 1:1 and 2:1 stoichiometry of the complexes (0.59 M extractant, 17.5 M titrant, $K_{1,1} = 1.0 \cdot 10^4$, $\Delta H_{1,1} = -35 \text{ kJ/mol}$, $K_{2,1} = 200$, $\Delta H_{3,1} = -18 \text{ kJ/mol}$).

Lessons from literature for fitting of multiple site models

Brautigam\textsuperscript{10} studied the fitting of isothermal data based on a two-site and a three-site model. In general both the two-site and three-site binding model show good results. For the fitting a clear biphasic (clear S-shape) isotherm was required for the two-site model and three distinct phases were required for the three-site model.\textsuperscript{10} Monte Carlo analysis of the fitting of such a three-sites model was performed by Freyer et al.\textsuperscript{5}. Three $K$ values and three $\Delta H$ values were fitted from a virtual set of 1000 simulated experiments in which a noise of approximately 1 µJ was applied with injection heat ranging up to 170 µJ. The average error in the resulting parameters was between 0.5 - 6% for $\Delta H$ and 7 – 10% for $K$. When the fitting of the different parameters is compared, in this case $\Delta H$ and $n$ were better fitted than $K$.\textsuperscript{5}

Fitting an increased number of parameters may not always yield a single solution to the parameter fit,\textsuperscript{10} and an appropriate starting position for fitting is necessary to optimally determine the best-fit solution.\textsuperscript{11}
Care should be taken to avoid local minima when fitting multiple site models, which may be done through restricting the parameters to realistic values, but should be performed very carefully. Fitting of a monophasic isotherm with the three-site binding scenario for the reveal of two parameters was successfully performed using strongly constrained parameters. Another option to improve fitting is a global fit on the basis of multiple experimental data sets to derive one set of thermodynamic parameters. This is what Freiburger et al. did in the analysis of ITC data on folding and unfolding of allosteric enzymes based on a sequential two-site binding model. The results were fitted globally for different temperatures and combined with the van ‘t Hoff equation, to improve accuracy.

Fitting statistics of single – reaction model.

Fitting statistics were also determined for the single reaction model. For the single reaction model it was assumed that $K_{n,m} = 45$, $\Delta H = -20 \text{ kJ/mol}$, $n = 2.8$ and $m = 1$. For the single reaction model the parameter fit was independent of the initial guess values, see the results in Table S2. The standard deviation in the fitted parameter $K_{n,1}$ is 2.4%, this implies that $K_{n,1}$ is more sensitive to deviations in the data than the other parameters, with a relative standard deviation of 0.24% in $n$ and 0.39% in $\Delta H_{n,1}$. However, even in this case the deviation in the fitted parameter $K_{n,1}$ is not excessively larger than the standard deviation of 1% in the data that was fitted. The same procedure was performed for simulated datasets with a normalized error in the initial volume, in the amount of moles of extractant present and in the concentration in the syringe. This resulted in similar standard deviations in the parameter fits as those shown in Table S2, so the parameter fit is not extra sensitive to any of these factors. Compared to the parameter fit analysis based on experimental results, see Table 3, the results based on simulated data with a 1% normalized error show significantly lower relative standard deviations. This supports the hypotheses that the error in the heat measured is either larger, not normally distributed or that other factors induce significant errors.
Table S2 Parameter fit for $K_{n,1}$, $n$, $\Delta H_{n,1}$ and fit residue for fitting 1000 series of simulated data with the single reaction model of equations (8)-(10).

| Dataset       | $K_{n,1}$ | $n$ | $\Delta H_{n,1}$ (kJ/mol) | Residue of fit (%) |
|---------------|-----------|-----|---------------------------|--------------------|
| Simulated dataset average | 45.03     | 2.80 | -20.0                     | 0.8 %              |
| standard deviation | 1.1       | 0.0068 | 0.077                     | 0.1 %              |
| $\sigma = 0.01$ relative standard deviation (%) | 2.4       | 0.24 | 0.39                      |                    |

Effect of extractant concentration on data fitting

The Monte-Carlo analysis was also performed at a lower concentration of extractant than that applied in Table S2 and Table 6. For these simulated data sets the amount of extractant ($n_{base}$) was $3.3 \cdot 10^{-4}$ mol, corresponding to an initial sample concentration of 0.12 M TOA. The results are shown in Table S3 for the single reaction model and in Table S-3 for the sequential reaction model. Compared to the fitting for the 0.24 M TOA model of Table S2 and Table 6, the results are very similar for both standard deviation and residue of fit, with the only exception that for the sequential model in Table S3 the standard deviation in the residue of the fit is lower as a result of the lower extractant concentration. This may be an effect of the less strong steep part of the isotherm at lower concentration. Comparing the relative standard deviations of Table S3 and Table S4 with the experimental results of a 0.12 M TOA system (see Table 3 and Table 4), it can be seen that for this system the differences are smaller for both reaction models, implying that the experimental error is reduced in the 0.12 M TOA system with the 50% acetic acid titrant.

Table S3 Parameter fit for $K_{n,1}$, $n$, $\Delta H_{n,1}$ and fit residue for fitting 1000 series of simulated data with the single reaction model of equations (8)-(10) for an initial extractant concentration of 0.12 M.

| Dataset       | $K_{n,1}$ | $n$ | $\Delta H_{n,1}$ (kJ/mol) | Residue of fit (%) |
|---------------|-----------|-----|---------------------------|--------------------|
| Simulated dataset average | 44.99     | 2.80 | -20.0                     | 0.8 %              |
| standard deviation | 0.98     | 0.0089 | 0.098                     | 0.1 %              |
| $\sigma = 0.01$ relative standard deviation (%) | 2.2       | 0.32 | 0.49                      |                    |
Table S4 Parameter fit for $K_{1,1}$, $\Delta H_{1,1}$, $K_{n+1,1}$, $\Delta H_{n+1,1}$, $n$ and fit residue for fitting 170 series of simulated data with the sequential reaction model of eq (1), (2), (7), (11) for and initial extractant concentration of 0.12 M.

| Dataset                           | $K_{1,1}$ (kJ/mol) | $\Delta H_{1,1}$ | $K_{n+1,1}$ (kJ/mol) | $\Delta H_{n+1,1}$ | $n$ | Residue of fit (%) |
|----------------------------------|--------------------|------------------|-----------------------|--------------------|-----|--------------------|
| Simulated dataset $\sigma = 0.01$. | average            | 9.20             | -31.7                 | 229                | -11.2| 1.70              | 2.1%        |
|                                  | standard deviation | 0.53             | 0.95                  | 22                 | 0.60 | 0.02              | 0.3%        |
|                                  | relative standard deviation (%) | 5.8             | 3.0                   | 9.7                | 5.4  | 1.3               |             |
| Original values used as initial value | average            | 12.0             | -28.0                 | 118                | -15.0| 1.60              | 0.8%        |
|                                  | standard deviation | 0.14             | 0.170                 | 3.3                | 0.14 | 0.011             | 0.2%        |
|                                  | relative standard deviation (%) | 1.1             | 0.61                  | 2.8                | 0.95 | 0.66              |             |

Comparison with reported ITC accuracy in literature

Table S5 shows experimental specifications and a summary of the accuracy reported in this work in combination with results published for other applications. In most of the systems in Table S5, either Origin or the Omega software that was delivered with the Microcal machines was applied. Other (free) software for ITC data analysis are NITPIC and SEDPHAT. NITPIC is a software program to process ITC data, that can fit parameters based on multiple models and also calculates the errors in the experimental data. SEDPHAT can apply several binding models and statistical tools in combination with global fitting of parameters.

Table S5 Fitting procedures and accuracy reported for parameters obtained from ITC experiments.

| System                          | Energy [kJ/mol] | [B] | [A] | K | Error | Fitting | Machine | Ref |
|---------------------------------|-----------------|-----|-----|---|-------|---------|---------|-----|
| Acetic acid with TOA in toluene | Up to 20        | 0.12 - 0.48 M (c = 13-34) | 9 - 18 M | 25-40 | Standard deviation, single reaction: 1.3-13 % in K, 1.6-3.5 in n, 0.76-3.3 in $\Delta H$, sequential reaction: 6.0-37% in $K_{1,1}$, 2.7-5.0 in $\Delta H_{1,1}$, 23-29 in $K_{n+1,1}$, 5.1-12 in $\Delta H_{n+1,1}$ and 1.4-4.7% in n | Least squares, MATLAB | TAM III | This work |
| Ba$^{2+}$ with 18-crown-6 ether  | Up to 40        | 1.0 mM | ~ 42 mM | | | Least squares, 1:1 complexes, Origin | | |
| (Thio)phenols with phosphine oxide and phosphate | Up to 16 | 1-10 mM | 10-100 µM | $10^2$ - $10^4$ | 1-10% in n, 8-46% in K, 4-14% in $\Delta H$, 0-6% in $\Delta G$, 4-30% in $\Gamma$ | Origin software, one-set-of-sites | Microcal VP-ITC | 14 |
| Carboxybenzenesulfonamid e CBS and bovine CAII | 10 µM (c = 10) | 720 µM | | | Relative standard error of $\gamma$: 3% in K and 1% in $\Delta H$, inter-laboratory statistic error of ~20%. | Origin least squares, 1:1 complexes | GE/MicroCal VP-ITC | 2 |
| Simulated data and data of bio-molecular interaction | Up to 42 | 190 µM | 4500 µM | $10^7$ | Noise level 0.42 kJ/mol | Two-site, three site, SEDPHAT | Malvern VP-ITC | 10 |
| Peptide-lipid | Up to 80 | 20M | 200 µM | $10^3$ - $10^5$ | 2-10% in $\Delta H$ | Customized model | Microcal MC2 | 15 |
Cyclodextrin-heptanoate system; Up to 13 kJ/mol
Heptanoic acid – heptylamine system

Inclusion of cyclodextrin and nicotinic/ascorbic acid

Monte-Carlo analysis of simulated data

General

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