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METHODS PAPER

Isothermal titration calorimetry in the single-injection mode with imperfect mixing

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Abstract

Isothermal titration calorimetry (ITC) is now a method of choice to obtain thermodynamic information about the interaction between two molecular partners. Most often, the method in use is the so-called multiple-injection method (MIM) consisting in distinct short-time injections of the titrant separated by sufficient delay to reach equilibrium before each new injection. However, an alternative single-injection method (SIM) exists. It consists in a unique continuous injection and, despite the fact that it is quite simple and generally faster than MIM, it is very little used. The goal of this work is to reconsider its theoretical basis. A new equation taking into account the effect of dilution resulting from the continuous titration process is obtained. It allows to consider efficiently the continuum of possibilities from perfect to imperfect mixing of the cell content. It is shown that, to good approximation, imperfect mixing can be accounted for by considering the cell volume as an adjustable parameter. Most likely, this should lead to an artificial increase of it, although one cannot reject the possibility of a decrease. The processing of experimental data on the interaction of Ba^{++} with 18-crown-6 from led to an increase by 6.9%, which resulted in a much better fit of the titration curve and improved results on the association constant *Ka* and enthalpy variation ΔH. A criterion is also obtained on the maximum injection rate to be used for maintaining quasi-equilibrium during the whole titration for the association–dissociation mechanism $A + B \rightleftarrows C$.

Keywords ITC · Single-injection method · Multiple-injection method · Titration curve equations

Introduction

Isothermal titration calorimetry (ITC) is now a widely spread technique in chemistry and biology. This is due to the development of commercially available instruments with small cell volumes and high sensitivity (down to 200 µl and 0.1 µW, respectively). The range of application of ITC extends from classical chemical reactions to lipid-membrane studies, from macromolecule–ligand to macromolecule–macromolecule interactions; ITC also allows studying bacteria in solution and in biofilms by monitoring the evolved heat attached to their metabolism (for review, Chaires et al. 2015). ITC has also been used for many years to obtain enzymatic kinetic information (for review, Bianconi 2007). Beyond enzymatic reactions, the kinetic methods (now known as *kinITC*) have been 'rediscovered' more recently (Vander-Meulen and Butcher 2011; Burnouf et al. 2012), as well as made easy to use (Dumas et al. 2016; Piñeiro et al. 2019). Interestingly, the list is not closed and new developments are being made like those on the determination of surface tension of liquids (Garrido et al. 2022).

Here, we will focus on the most common application of ITC consisting in titrating one molecule (the *titrant*) against another one initially present in the measurement cell (the *titrand*) to determine the enthalpy variation and the association constant of their interaction. This covers the vast area of all possible molecular interactions of chemical or biological interest. There are two possible methods: the multiple-injection method (MIM) consisting in distinct short-time injections of the titrant and the single-injection method (SIM) consisting in a unique continuous injection. MIM requires generally more time as each new injection has to be delivered only after equilibrium has been reached. There is a controversy as to whether a rather large number of injections of small volume are a better choice than a smaller number of injections of greater volume. The common practice is to

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use as many injections as permitted by the need of a sufficient heat signal, the rationale being that a finer sampling of the titration curve is obtained. In routine work, a simple rule of thumb consisting in ten to twenty injections is used, but it does not derive from any serious analysis. However, Tellinghuisen examined the problem and, somewhat against intuition, advocates a small number of injections (at least 5 in normal conditions, but as small as 2 or 3 in particular conditions) (Tellinghuisen 2007a). With SIM, there are not such problems and the finest possible sampling is achieved.

MIM is by far the most commonly, if not exclusively, used method with modern calorimeters and the alternative and simple SIM has been let aside although it was in common use with older instruments having large cell volumes and long response times. This is still so despite the development by Markova & Dan Hallèn of a 'continuous ITC method' (cITC) for the modern instruments (Markova and Hallen 2004). The method has been further analyzed in depth for being used with enzyme kinetics (Transtrum et al. 2015).

The aim of this work is to reconsider the basis of SIM, alias cITC, and to present the development and the consequences of a new mathematical method for processing such experimental data. The novelty lies in the unexpected finding that a corrective term for the dilution due to injection of titrant was missing in the accepted primary equation linking the thermogenesis and the variation of concentrations following the reaction taking place in the cell. This result highlights the importance of correctly appreciating the extent of mixing of the cell content. Indeed, it is known from previous works that too imperfect mixing can be extremely detrimental to obtaining results from SIM experiments (Markova and Hallen 2004). Here it is shown how to correct most of the deleterious effects of moderate imperfect mixing.

Some explanations on the origin of this work are necessary. An initial work posted on bioRχiv (Dumas 2019) was on the same topics, but considered the two methods SIM and MIM. The hypothesis of perfect mixing was made, which led to incorrect conclusions when comparing the results obtained by the various programs used for processing MIM data. Although many other considerations were of interest, it was necessary to withdraw this preprint since doubts were cast unduly on well-established programs. The present work reconsiders the problem and, being limited to SIM and rejecting the perfect mixing hypothesis, avoids completely these pitfalls.

Materials and methods

Initial considerations

With MIM, each injection gives rise to an 'injection curve', which is a 'power curve' (dQ/dt), and each power curve is integrated to yield the total heat Q produced or absorbed in the cell during each injection. The experimental data, the so-called *titration curve* (TC), or *binding isotherm*, thus correspond with MIM to discrete values $Q(V_i)$ of the function $Q(V)$ where V_i is the sum of all injected volumes from the first to the ith injection. With SIM, which may be viewed as the limit reached by MIM with infinitely many injections of infinitely small volume, a continuous power curve $\mathrm{d}Q/\mathrm{d}t$ is measured. The latter derivative may be replaced equivalently by $\mathrm{d}Q/\mathrm{d}v = \mathrm{d}Q/(\varphi \mathrm{d}t)$ where $v = V/V_{\mathrm{cell}}$ is the reduced injected volume and $\varphi = dv/dt$ (considered as constant). The interest of using the reduced quantities v and φ is of obtaining results valid for all cell volumes. We are primarily interested in an equation yielding a rigorous evaluation of $\mathrm{d}Q/\mathrm{d}v$. In this work, we consider the simple and usual situation corresponding to a single step:

$$A + B \rightleftharpoons C \quad (K_{\mathrm{a}} = k_{\mathrm{on}}/k_{\mathrm{off}},\ \Delta H) \tag{1}$$

where compound A is initially alone in the measurement cell at a concentration A_0 and compound B in the syringe at a concentration B_0 (the concentration $[X]$ of a compound X is noted simply X). Note that these neutral notations A and B are used to emphasize that one does not presuppose that a macromolecule (protein, nucleic acid,…) is initially in the cell. Note also that one considers in this work that there is only one binding site and thus that the stoichiometry number n is fixed to 1. This reaction is characterized by the thermodynamic parameters K_{a} (or $K_{\mathrm{d}} = 1/K_{\mathrm{a}}$) and ΔH. $K_{\mathrm{a}} = k_{\mathrm{on}}/k_{\mathrm{off}}$ is the association constant with k_{on} (in $M^{-1}\ s^{-1}$) and k_{off} (in s^{-1}) being, respectively, the forward and backward kinetic constants. ΔH is the enthalpy variation per mole of C produced during the reaction. If A_0 and B_0 are known, the $Q(V_i)$ can be translated into $Q(s_i)$ where the s_i are the successive stoichiometric (or molar) ratios $B_{\mathrm{tot}}/A_{\mathrm{tot}}$ with $A_{\mathrm{tot}} = A + C$ and $B_{\mathrm{tot}} = B + C$.

The 'overfill mode'

Most often, any injected volume δV of compound B implies that an equivalent volume δV of the reaction mixture has to leave the measurement cell. This corresponds to the *overfill mode* and this implies that the measurement cell is already filled at the beginning of injections. Note that, with the instruments from TA, the user may or may not choose the overfill mode. In the following, we will only consider the overfill mode.

Perfect mixing and imperfect mixing

More or less explicitly, it is often considered that the stirring of the cell content is sufficient to obtain perfect mixing despite the continuous injection of new material

and the concomitant expulsion of cell material. This is of course an idealized hypothesis. The opposite limiting situation is best understood within the frame of MIM: it corresponds to assuming that each injection is almost instantaneous, which results in negligible mixing during the very short injection time. As a consequence, the material ejected from the cell during the nth injection has the equilibrium composition reached at the end of the $(n-1)$ th injection (Tellinghuisen 2007b). In the situation of SIM, this means that the concentrations X of the species X (X = A, B, or C) ejected from the cell at an instant t (resp. reduced volume v), corresponds in fact to the quasi-equilibrium concentration $\widetilde{X}$ reached at $t-\delta t$ (resp. $v-\delta v$), where δt and $\delta v = \varphi\ \delta t$ depend on many parameters and are not known.

Perfect mixing or no mixing at all? The truth is in between these two extremes at a point depending on the total injected volume V *vs.* the cell size (hence on $v = V/V_{\text{cell}}$), and on the reduced injection rate $\varphi = \mathrm{d}v/\mathrm{d}t$ *vs.* the stirring speed, the viscosity of the solution and the kinetics of the reaction. Also important are the cell shape and the geometry of the injecting needle and stirring paddle, which determine whether some parts of the cell volume are less well stirred than others (as seen in Markova and Hallen 2004).

The dilution problem with the 'overfill mode' and perfect mixing

A simplified method for obtaining $\mathrm{d}Q/\mathrm{d}V$ is of considering that the injected volumes are very small and, therefore, that there is negligible dilution of the reaction mixture during the titration (as this was supposed in the seminal paper by Wiseman et al. 1989). As a consequence, any infinitesimal variation $\mathrm{d}C$ of the concentration of C is due to the reaction, which implies:

$$\frac{\mathrm{d}Q}{\mathrm{d}V} = \Delta H\ V_{\text{cell}}\frac{\mathrm{d}C}{\mathrm{d}V} = \Delta H\frac{\mathrm{d}C}{\mathrm{d}v} \tag{2}$$

However, this is most often an oversimplified hypothesis, particularly in the overfill mode enhancing the effect of dilution due to injection of titrant. Abandoning this hypothesis of negligible dilution and assuming temporarily that the cell content is always well mixed, the infinitesimal variations of the total concentrations of A and B are thus $\mathrm{d}A_{\text{tot}} = -A_{\text{tot}}\ \mathrm{d}V/V_{\text{cell}} = -A_{\text{tot}}\ \mathrm{d}v$ and $\mathrm{d}B_{\text{tot}} = (B_0 - B_{\text{tot}})\ \mathrm{d}V/V_{\text{cell}} = (B_0 - B_{\text{tot}})\ \mathrm{d}v$. The equation for compound B takes into account both the injection of new material and the dilution effect. After integration, it is obtained:

$$A_{\text{tot}}(v) = A_0\ e^{-v} \qquad B_{\text{tot}}(v) = B_0\ (1 - e^{-v}) \tag{3}$$

The current stoichiometric ratio is thus:

$$s(v) = \frac{B_{\text{tot}}(v)}{A_{\text{tot}}(v)} = r(e^{v} - 1) \quad (r = B_0/A_0) \tag{4}$$

which implies:

$$e^{v} = 1 + \frac{s(v)}{r} \qquad \frac{\mathrm{d}s}{\mathrm{d}v} = r + s(v) \tag{5}$$

The total concentrations can thus be expressed as functions of s considered as the independent variable:

$$A_{\text{tot}}(s) = A_0\frac{r}{r+s} \qquad B_{\text{tot}}(s) = B_0\frac{s}{r+s} \tag{6}$$

Equations (3, 4) have already been derived (Sigurskjold 2000; Herrera and Winnik 2013). They are both very simple and 'exact' (provided that complete mixing is always achieved).

Experimental data used to test the theoretical results

The data were from a cITC experiment at 25 °C on the interaction of Ba^{++} with 18-crown-6. They were obtained from an accurate digitalization of Fig. 2B in (Markova and Hallen 2004). Importantly, the quality of digitalization was much higher than the discrepancy between the curve and the initial fit mentioned in Sect. 3.5. A correction of dilution was made as mentioned in (Markova and Hallen 2004). The origin of times was displaced by 0.11 min to account for a clear lag before the abrupt rise of power in the original figure.

Using of *Mathematica*

Mathematica 11.3 from Wolfram Research was of great help during this work for all symbolic calculations (Eqs. 11, 16–18), for the numerical integration of Eq. (13) with the function `NDSolve`, and for the least-squares fit of the experimental titration curve with the function `NonlinearModelFit`. The figures were made with *Mathematica* too.

Results

Accounting for the influence of dilution within the hypothesis of perfect mixing

Here, the function $Q(v)$ in Eq. (2) is evaluated and, for that, we need to consider the effect of dilution on compound C. The infinitesimal concentration variation $\mathrm{d}C$ is the sum $\mathrm{d}C_{\text{chem}} + \mathrm{d}C_{\text{dil}}$ of a term $\mathrm{d}C_{\text{chem}}$ due to the chemical

reaction consuming A and B to produce C, and of dC_{dil} due to the dilution resulting from the injection of $dV = V_{cell}\, dv$. At this stage, one has to consider the mixing problem. We first assume perfect mixing and the obtained result will be used to relax this ideal hypothesis. If perfect mixing is achieved, the concentration of each species is the same in the whole cell volume. Therefore, for the species C, one has $dC_{dil} = -C(v)\, dv$ where $C(v)$ is its uniform concentration corresponding to the reduced injected volume v. Note that this does not imply that $C(v)$ is strictly equal to the true equilibrium concentration of C. Obviously, the heat evolved or absorbed during the titration is only linked to the variation of the concentration of C due to the chemical reaction, not to C leaving the cell (it is assumed that there is no difference in temperature and heat capacity between the injected and ejected material). Therefore, taking into account $v = V/V_{cell}$ instead of V, Eq. (2) has to be replaced by:

$$\frac{dQ}{dV} = \Delta H\, V_{cell} \frac{dC_{chem}}{dv} \tag{7}$$

From the preceding, $dC_{chem} = dC - dC_{dil} = dC + C(v)\, dv$, which gives an exact expression (within the hypothesis of perfect mixing) for the heat per injected mole of B:

$$\widetilde{Q}(v) = \frac{1}{B_0\, V_{cell}} \frac{dQ}{dv} = \frac{\Delta H}{B_0}\left[\frac{dC}{dv} + C(v)\right] \tag{8}$$

where the new notation $\widetilde{Q}(v)$ for $dQ/(B_0\, V_{cell}\, dv)$ has been introduced. Considering that $ds/dv = r + s$ (Eq. 5), an alternative form with the stoichiometric ratio s as the variable is obtained as:

$$\widetilde{Q}(s) = \frac{\Delta H}{B_0}\left[(r+s)\frac{dC}{ds} + C(s)\right] \tag{9}$$

Equations 8, 9, which do not seem to have been mentioned yet, open the way for a more accurate treatment of SIM experiments. The difference with all approximate treatments is the corrective term $C(v)$ added to dC/dv in Eq. (8). Although its justification is quite simple, its occurrence is not immediately intuitive, which explains why it remained overlooked.

Exact expressions of $\widetilde{Q}(v)$ (and $\widetilde{Q}(s)$) within the hypothesis of perfect mixing

We continue with the classical situation described by Eq. (1). Only the logic is exposed as it is not useful to show the details. From mass action law ($A \times B/C = Kd$) and the conservation equations ($A + C = A_{tot}$ and $B + C = B_{tot}$), the concentration of C is obtained as a function of A_{tot} and B_{tot} at any stage of a titration:

$$C = \frac{1}{2}\left(S - \sqrt{S^2 - 4P}\right)$$
$$S = A_{tot} + B_{tot} + K_d \qquad P = A_{tot} B_{tot} \tag{10}$$

This is readily transformed into a function $C(v)$ by replacing A_{tot} and B_{tot} with Eqs. 3, which allows to obtain the heat per injected mole $\widetilde{Q}(v)$ by using Eq. (8). Then, the result is transformed by using Eq. (5) in order to express the heat per injected mole as a function of the stoichiometric ratio $s = B_{tot}/A_{tot}$, as usually in practice. The final result is:

$$\widetilde{Q}(s) = \frac{\left(\gamma^{-1}+1\right)[Y(s) - r] - \left(\gamma^{-1} + \gamma + 2\right)s + \gamma - 1}{2\, Y(s)} \Delta H$$
$$Y(s) = \sqrt{[\gamma(s-1) + r + s]^2 + 4\gamma(r+s)}$$
$$r = B_0/A_0; \quad \gamma = r\, c = B_0/K_d \tag{11}$$

This corresponds to an exact explicit solution replacing the numerical approach described in (Poon 2010). It may be verified that for $r \to \infty$, corresponding to negligible dilution of the cell content since vanishingly small volumes of titrant would be necessary, this is identical to the Wiseman isotherm (Eq. 3 in (Wiseman et al. 1989) where X_r and r stand, respectively, for s and $1/c$ in this work).

Accounting for the influence of dilution with imperfect mixing

It is easy to modify the previous result to account for imperfect mixing with good approximation. At variance with the previous situation, $C(v)$ in Eq. 8 is no more a uniform concentration within the cell volume; instead, it has to be the particular concentration in the neighborhood of the ejection aperture and, to avoid new notations, it will be understood as such in this section. Because of the imperfect mixing and possible turbulence, one may be concerned by the time fluctuation of each concentration X (r,t) at any position $\mathbf{r}$, in particular around the ejection aperture. However, the continuous injection process in SIM is slow enough to allow considering a time average of X (r,t) within a time interval $(t - \tau, t + \tau)$ short enough to consider the average as independent of τ (hence significant of t only), but long enough to erase any significant rapid fluctuations. Therefore, it is legitimate to consider that $C(v)$ in Eq. (8) results from this time-averaging and can just be replaced by $C(v - \delta v)$ equal to the equilibrium concentration at $v - \delta v$ (resp. $t - \delta t$) where $\delta v = \varphi\, \delta t$ is an unknown, but small and likely positive term accounting for the delay due to imperfect mixing. (For complete generality, one is led to let open the possibility that $\delta v = \varphi\, \delta t$ be negative, which would mean that the state near the ejection aperture would be in advance, not late, in comparison of the average value of $C(v)$ within the cell). It is clear that the major simplification to deal here with

imperfect mixing is of invoking the theoretical equilibrium concentration of C reached at $v - \delta v$. Strictly speaking, δv and δt cannot be seen as constant along the titration process. Indeed, they are null at the very beginning of it and they rapidly reach a constant, or almost constant, value determined 'internally' by the volumes and shapes of the cell and of the stirring paddle and, 'externally', by the stirring speed and the kinematic viscosity of the solution. In the following, δv and δt will be considered as constant.

We now consider the consequence of this correction. For that, we replace $C(v)$ by $C(v - \delta v) = C(v - \varphi\,\delta t)$ in Eq. (8) and, since $\delta v = \varphi\,\delta t$ is a small correction, one may use the first-order Taylor approximation $C(v - \varphi\,\delta t) \approx C(v) - \varphi\,\delta t\,\mathrm{d}C/\mathrm{d}v$, which gives:

$$\widetilde{Q}(v) = \frac{\Delta H}{B_0}\left[(1 - \varphi\,\delta t)\frac{dC}{dv} + C(v)\right] \tag{12}$$

The derivative $\mathrm{d}C/\mathrm{d}v$ is now multiplied by $\beta = (1 - \varphi\,\delta t)$, which is slightly less than one if δt is positive. In such a case, imperfect mixing leads to too small a contribution (in absolute value) of $\mathrm{d}C/\mathrm{d}v$. Since $\beta\,\mathrm{d}C/\mathrm{d}v = \beta\,V_{\mathrm{cell}}\,\mathrm{d}C/\mathrm{d}V$, imperfect mixing is thus essentially equivalent to perfect mixing, but with too small a cell volume. Therefore, one anticipates that describing the continuous titration within the hypothesis of perfect mixing should most often require to increase artificially the cell volume to counteract the decrease of the influence of $\mathrm{d}C/\mathrm{d}v$ due to imperfect mixing. The cell volume would have to be decreased in the unlikely situation of $\delta t < 0$. One should insist on the fact that it is not a matter of correcting a wrong estimate of the cell volume, but of evaluating an apparent value of it due to imperfect mixing.

Equation for the measured heat power during a continuous injection in SIM

With SIM, the chemical reaction is perturbed at all times by the continuous injection of new material. In theory, therefore, equilibrium is never reached during the titration. However, in situations where the continuous injection is sufficiently slow, one may consider that the reaction is always very close to equilibrium. It will be examined in Sect. 3.8 what is the condition on φ to fulfill this condition. Here, we suppose, and it will be verified, that it is valid. If the instrument response time τ_{ITC} may be neglected, $\widetilde{Q}(s)$ given by Eq. (11) can be used directly to represent the evolution of the heat per injected mole during the titration. In reality, however, it cannot be neglected and it is taken into account by estimating the measured heat per mole $\widetilde{Q}_{\mathrm{m}}(s)$ by:

$$\widetilde{Q}(s) = \widetilde{Q}_{\mathrm{m}}(s) + \tau_{\mathrm{ITC}}\frac{\mathrm{d}s}{\mathrm{d}t}\frac{\mathrm{d}\widetilde{Q}_{\mathrm{m}}}{\mathrm{d}s} \tag{13}$$

which is known in calorimetry as the Tian equation [for accessible references, see (Calvet 1962; Hansen et al. 2010)]. From Eq. (5), it is obtained:

$$\frac{\mathrm{d}s}{\mathrm{d}t} = r\,\varphi\,e^{v} = \varphi\,(r + s) \qquad r = B_0/A_0; \quad \varphi = \mathrm{d}v/\mathrm{d}t \tag{14}$$

One should admit that this is an approximation in the frame of imperfect mixing since Eq. (5) derived from the perfect mixing hypothesis. However, in Eq. (13), only the product $\tau_{\mathrm{ITC}}\,\mathrm{d}s/\mathrm{d}t$ is important, which means that any small systematic error on $\mathrm{d}s/\mathrm{d}t$ resulting from this approximation can be counteracted by an opposite small systematic error on the estimate of the instrument response time τ_{ITC}. Therefore, $\widetilde{Q}(s)$ being known (Eq. 11), $\widetilde{Q}_{\mathrm{m}}(s)$ can be obtained by numerical integration of Eq. (13). Finally, $\widetilde{Q}_{\mathrm{m}}(s)$ is transformed into $\varphi\,V_{\mathrm{cell}}\,B_0\,\widetilde{Q}_{\mathrm{m}}(s)$ to translate a heat per injected mole into a thermal power. Therefore, the function $\varphi\,V_{\mathrm{cell}}\,B_0\,\widetilde{Q}_{\mathrm{m}}[s(t)]$ can be used to fit the experimental power curve $P_{\mathrm{m}}(t)$.

Experimental test of the theoretical results

The previous results were tested with one experimental dataset reported in (Markova and Hallen 2004). These data obtained with a VP-ITC-like instrument (identified by the authors as what they called a 'type 2 calorimeter') are from the continuous titration of Ba^{++} with 18-crown-6. Another experiment was reported about the titration of cytidine-2' monophosphate by bovine RNase A, but could not be used here because the raw power curve was not shown. It appeared that the fit of these high-quality data with Eq. (13) was unacceptably poor when the expected values $r = B_0/A_0 = 29.95$ and $V_{\mathrm{cell}} = 1.36$ ml were used (Fig. 1, black curve).

In the initial work (see the end of the introduction), the cell volume was used empirically as a free parameter, which surprisingly led to a perfect fit (Fig. 1, red curve) after a significant increase of it by $(6.9 \pm 0.4)\%$. In view of the analysis in Sect. 3.3, this increase is no more surprising. Importantly, the procedure also yields K_a and ΔH values in better agreement with the reference values from (Briggner and Wadsö 1991) than those obtained in (Markova and Hallen 2004) (Fig. 1, inset). In addition, the obtained value $\tau_{\mathrm{ITC}} = (19.6 \pm 0.2)\,s$ for the instrument response time agrees perfectly with the expectation for a VP-ITC-like instrument.

Although in agreement with the theoretical analysis, this increase is at odd with an observation that led to a decrease of the cell volume (Tellinghuisen 2004). However, Tellinghuisen showed convincingly that his observation resulted from the fact that the volume of the paddle shaft inserted in the cell was not subtracted from the volume of the empty cell. There is no reason to

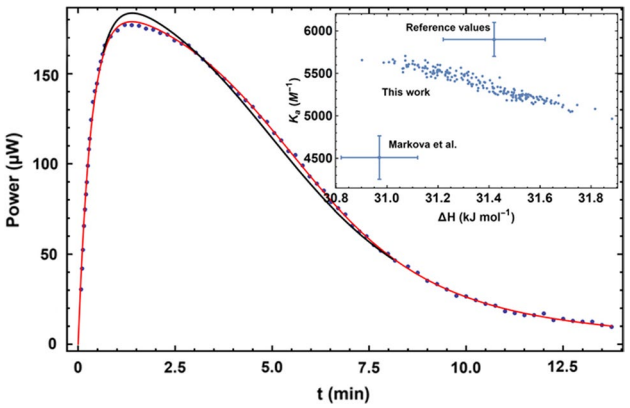

Fig. 1 Fit of a continuous SIM curve (red) with numerical integration of Eq. (13) (see text). The experimental curve was obtained as described in Sect. 2.5. A normally distributed random noise of zero mean and 0.5 μW standard deviation was added to the points to account for the small errors in the determination of their coordinates from the figure. This yielded reasonable error estimates on *Ka* and ΔH through multiple repeats of the fitting procedure each time with a new set of noise (inset: cloud of points corresponding to 200 repeats; Reference values from Briggner and Wadsö (1991)). The (black) curve fitting badly the experimental points corresponds to a fit with the nominal value of $V_{\text{cell}} = 1.36$ ml, whereas the (red) curve fitting well the experimental points was obtained with $V_{\text{cell}} = 1.069 \times 1.36 = 1.45$ ml (see text). (Colors indicated within parenthesis are for the on-line version of the article.)

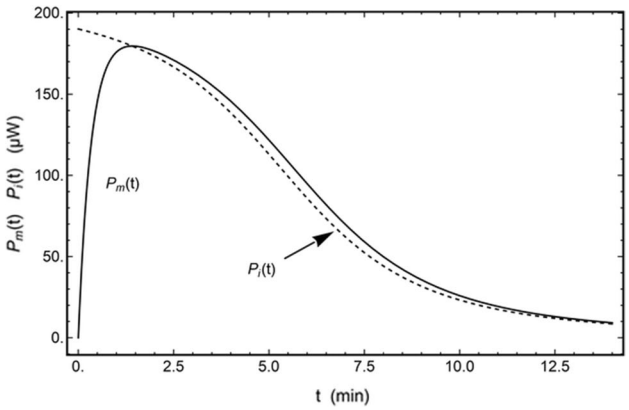

Fig. 2 Comparison of the measured and ideal power curves $P_m(t)$ and $P_i(t)$ to illustrate the apparent disappearance in $P_i(t)$ of the abrupt, but continuous rise of power in $P_m(t)$ at short times. The curves were calculated with the parameters mentioned in Fig. 1

believe that the same problem occurred with the instrument used in Markova and Hallen (2004). Finally, the following considerations strongly support the result. With $V_{\text{cell}} = 1360$ μl, the correction by + 6.9% corresponds to an artificial increase of the cell volume by $\delta V_{\text{cell}} = 94$ μl. From Eq. (12) it is obtained $\delta V_{\text{cell}} \approx \varphi\, \delta t\, V_{\text{cell}}$ and, since $\varphi = 10^{-4}$ s^{-1}, this leads to $\delta t = 669$ s ≈ 11 min. Such a time delay between the equilibrium concentration of C and its value near the ejection aperture is quite important since it almost corresponds to the duration of the titration (Fig. 1). However, Markova & Hallen noted that '*The binding constant determined in a type 2 calorimeter is about 7% lower than the corresponding value for a type 1 calorimeter*. It is striking that this percentage fits exactly with the correction by + 6.9% on the cell volume, which is rationalized by their next comment: '*A plausible explanation for this would be that the binding reaction does not occur throughout the entire volume of the type 2 calorimetric vessel due to insufficient mixing. The mixing efficiency is the most important factor for a successful cITC experiment*'. In excellent support with their conclusion is that the experiment using a stirring speed of 400 rpm was uninterpretable, whereas '*the results were significantly improved when the stirring speed was increased to 700 rpm*' (compare their Figs. 4 and 3B). There is thus good agreement with the result obtained in this work and the conclusion in Markova and Hallen (2004). One may thus consider that the proposed correction on the cell volume was successful to counteract imperfect mixing.

No other parameter can replace V_{cell} as an additional free parameter

The need of adjusting artificially the cell volume to improve the quality of fit suggests that one might equivalently adjust either the initial concentration A_0 in the cell, or the concentration B_0 in the syringe. This was tried and both methods indeed allowed to obtain exactly the same quality of fit. However, adjusting A_0 led to increase it by 6.8% and yielded the correct ΔH but underestimated *Ka* by 10%, whereas adjusting B_0 led to decrease it by 6.6% and yielded the correct *Ka* but overestimated ΔH by 6.9%. Therefore, in agreement with the analysis in Sect. 3.3, only adjusting V_{cell} is successful.

Comparison with the method used in Markova and Hallen (2004)

In (Markova and Hallen 2004), the curve to be fit was not the measured power curve $P_m(t)$ but an ideal power curve $P_i(t)$ that would be obtained with an ideal instrument having a null response time. Such an ideal power curve is represented by $\varphi V_{\text{cell}}\, B_0\, \widetilde{Q}\, [s(t)]$. According to Eq. (13), $P_i(t)$ is easily derived from $P_m(t)$ as follows:

$$P_i(t) = P_m(t) + \tau_{\text{ITC}} \frac{\mathrm{d}P_m}{\mathrm{d}t} \tag{15}$$

Equation (15) means that $P_m(t)$ is the result of the convolution of $P_i(t)$ with the instrument response function $\tau_{\text{ITC}}^{-1} \exp\left(-t/\tau_{\text{ITC}}\right)$ (Fig. 2). A convolution is an averaging

procedure, which downweighs the highest frequencies, here in $P_i(t)$. This may seem contradictory as $P_i(t)$ looks smoother than $P_m(t)$ since the abrupt raise at short times in $P_m(t)$, which is entirely determined by the value of τ_{ITC}, has disappeared in $P_i(t)$ (Fig. 2). However, this is an incorrect feeling since the abrupt rise in $P_i(t)$ is indeed present as the instantaneous jump from $P_i(t=0)=0$ to the maximum value $P_i(t\rightarrow 0^+)\approx 190\ \mu W$. Therefore, it is argued here that the two methods are not equivalent in practice because $P_i(t)$ is an ideal curve that has lost all direct marks of the instrument response time τ_{ITC}. In conclusion, fitting the measured power curve with $P_m(t)$ highlights, instead of masking, any disagreement. This appeared well with the black curve in Fig. 1 that could not be accepted as a good fit. In comparison, the fit of the ideal power curve with $P_i(t)$ did not reveal any such disagreement (Fig. 3 in Markova and Hallen 2004) and yet, led to less accurate values of the parameters (inset of Fig. 1).

Condition on the injection rate to maintain quasi-equilibrium during the titration

Here, we estimate an order of magnitude of the maximum injection rate to be used to remain close enough to equilibrium during the whole titration. These considerations are not about the mixing problem, which was examined previously, and it is assumed that mixing is perfect so that Eqs. 3,4, 5 can be used. This approximation is justified as we only seek for an order of magnitude. The continuous injection of compound B is seen as a small perturbation of the chemical equilibrium given by Eq. (1), which calls for using the relaxation methods going back to (Eigen and De Maeyer 1963). For that, all concentrations are written as $X(t)=\widetilde{X}(t)+\delta X(t)$ where $\widetilde{X}(t)$ is the equilibrium concentration corresponding to the cell content at t, and $\delta X(t)$ a small perturbation ($X=A$, B or C). This leads to kinetic equations that are linearized by neglecting the product $\delta A(t)\ \delta B(t)$. Standard calculations (not shown) of the relaxation matrix and of its eigenvalues yield the relaxation time $\tau_r(v)$ at 'any stage' of the titration [apart for very short times when the product $\delta A(t)\ \delta B(t)$ cannot be neglected]:

$$\tau_r(v)=\left\{1+c\,e^{-2v}\left[c(1-r(e^v-1))^2+2e^{2v}(1+r(e^v-1))\right]\right\}^{-1/2}k_{\mathrm{off}}^{-1} \tag{16}$$

This allows to obtain its maximum value $\tau_{r\,\max}$ reached during the titration:

$$\tau_{r\max}=\frac{1}{2}\frac{1+r}{[r(1+c+cr)]^{1/2}}\,k_{\mathrm{off}}^{-1}\sim\frac{1}{2}c^{-1/2}\,k_{\mathrm{off}}^{-1} \tag{17}$$

the approximation being for large r values. This is a true local maximum (with $\tau_r'(v)=0$) if $c>c^*=(r-1)/(r+1)$ and, otherwise, for $c<c^*$, the maximum is at $v\rightarrow 0$ (with $\tau_r'(v\rightarrow 0)<0$) and $\tau_{r\max}=(1+c)^{-1}k_{\mathrm{off}}^{-1}$. When $c>c^*$, the maximum relaxation time $\tau_{r\max}$ is reached at a stoichiometric ratio $s_{\max}$:

$$s_{\max}=\frac{r[1+c+(c-1)r]}{-1+r[1+(1+r)c]}\sim 1-c^{-1}+\frac{c^{-1}(1+c^{-1})}{r}\qquad(c>c^*) \tag{18}$$

The approximation $\tau_{r\max}\sim 1-c^{-1}$ for large r values corresponds exactly to the conclusion obtained for kinITC (Burnouf et al. 2012; Dumas 2016).

Obviously, maintaining quasi-equilibrium during the titration requires $\varphi << \tau_{r\max}^{-1}$ (with $\tau_{r\max}$ given by Eq. (17) if $c>c^*$, or by $(1+c)^{-1}\,k_{\mathrm{off}}^{-1}$ if $c<c^*$). However, considering φ alone is insufficient as, for a given φ, the higher the concentration B_0 (hence the higher $r=B_0/A_0$), the more important the perturbation due to the continuous injection of B. It is therefore necessary to consider $r\,\varphi << \tau_{r\max}^{-1}$ as the correct condition to be respected. The results (not shown) of simulations of continuous titrations, performed either without assuming equilibrium (by numerical integration of the kinetic equations), or with the approximation assuming equilibrium, add weight to the latter criterion as the maximum relative error on the concentration curves was always close to $r\,\varphi\,/\,\tau_{r\max}^{-1}$. This means that, to achieve an accuracy of order 10^{-n}, one should respect the condition:

$$\varphi<10^{-n}(r\,\tau_{r\max})^{-1}\ \Rightarrow\ \varphi<10^{-n}f(r,c)\,k_{\mathrm{off}} \tag{19}$$

where $f(r,c)$ depends on c/c^*. In the common situation corresponding to $c>1>c^*$ and $r>>1$, Eq. (17) has to be considered, which means $f(r,c)\approx 2\,c^{1/2}\,r^{-1}$. The data from (Markova and Hallen 2004) fulfill these conditions with $\varphi=10^{-4}\,s^{-1}$, $c=K_aA_0=5.5\ 10^3\times 1.66\ 10^{-3}=9.1$ and $r=B_0/A_0=29.94$, which yields from (19) the condition:

$$\varphi<10^{-n}\,2\,c^{1/2}\,r^{-1}\,k_{\mathrm{off}}\ \Rightarrow\ 10^{-4}\mathrm{s}^{-1}<10^{-n}\,0.2\times k_{\mathrm{off}}\ \Rightarrow\ k_{\mathrm{off}}>5\times 10^{n-4}\ \mathrm{s}^{-1} \tag{20}$$

This means that $k_{\mathrm{off}}>5\ \mathrm{s}^{-1}$ should hold to fulfill Eq. (20) with $n=4$. Since $K_a=5.5\times 10^3\ \mathrm{M}^{-1}$ from *cITC* (inset in Fig. 1), this is equivalent to the condition $k_{\mathrm{on}}>2.75\times 10^4\ \mathrm{M}^{-1}\mathrm{s}^{-1}$. It is fortunate that experimental values (also at 25 °C) are available as $k_{\mathrm{on}}=1.3\times 10^8\ \mathrm{M}^{-1}\mathrm{s}^{-1}$ and $k_{\mathrm{off}}=1.7\times 10^4\ \mathrm{s}^{-1}$ (Izatt et al. 1985, p.329). These values imply $K_a=7.6\times 10^3\ \mathrm{M}^{-1}$, which is in reasonable agreement with $K_a=5.5\times 10^3\ \mathrm{M}^{-1}$ from *cITC* and justifies their consideration. By far, the previous inequalities are fully

respected: the hypothesis of quasi-equilibrium at the basis of data processing was thus amply justified. Of course, in real life, neither k_{on} nor k_{off} are known and the previous criterion (Eq. 19) is more of theoretical than practical interest.

Conclusion

The results exposed in this work represent a significant improvement on methods for ITC used in the single-injection mode. The present analysis led to the prediction that fitting SIM data requires to adjust artificially the cell volume around its nominal value to counteract imperfect mixing. This was very well verified with one data set taken from the literature. The accuracy of the method used in this work is based on the validity of the hypothesis that one is always very close to equilibrium. Indeed, if the condition on the injection rate given by Eq. (19) cannot be respected, then the method in use is no more accurate. In such a case, a true kinetic analysis, as described for *kinITC* in (Burnouf et al. 2012; Dumas et al. 2016), would be necessary and become feasible if the titration curves appear to vary sufficiently with the injection rate. In favorable conditions, the *bonus* would be that, not only K_a, but *also* k_{on} and k_{off} would be obtained. This remains to be developed. Conversely, if the condition given by Eq. (19) is easily respected, it should then be kept in mind that another limitation on the injection rate comes from the need of avoiding too imperfect mixing as exemplified in (Markova and Hallen, 2004). Finally, it should be mentioned that the experimental data that were used were of good quality due to an important heat power. With less favorable conditions, it might be necessary (as this is often the case with MIM) to introduce an additional parameter to account for a non-null baseline. There is a need of using more widely SIM/*cITC* to evaluate its general efficiency and convince the users of its interest. It is hoped that the present work will participate in changing this situation.

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