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Calibration of nanowatt isothermal titration calorimeters with overflow reaction vessels

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ABSTRACT

Obtaining accurate results with nanowatt titration calorimeters with overflow cells requires mass calibration of the buret injection volume, chemical calibration of the reaction vessel effective volume, and chemical calibration of the calorimetric factor used to convert the measured electrical signal to heat rate. Potential errors in electrical calibration of power compensation calorimeters require validation of the calorimetric factor with chemical reactions with accurately known stoichiometries and enthalpy changes. The effective volume of the reaction vessel can be determined from the endpoint of a quantitative reaction with known stoichiometries. Methods for calibration and potential calibration errors to be avoided are described. Publication of results obtained must include data on calibrations and sufficient raw data to assess precision and accuracy of the results.

Nanowatt isothermal titration calorimetry (ITC)[1] can provide accurate measurements of the enthalpy changes of reactions, and for favorable cases can be used to obtain accurate values of equilibrium constants, but only if the calorimeter is properly calibrated. The absence of systematic errors in procedures, injection volume, reaction vessel effective volume, and the calorimetric factor used to convert the measured electrical signal to heat rate all must be demonstrated with standard reactions. Demonstration that accurate results can be obtained for a standard reaction has historically been a requirement for acceptance of any calorimetric measurement, and the very small volumes and heat effects currently used in most ITC measurements require extraordinary care and attention to detail.

Four different nanowatt isothermal titration calorimeters that operate with overflow cells are currently commercially available: the VP-ITC and ITC_{200} from GE Healthcare (MicroCal) and the Nano ITC Standard Volume (SV) and Nano ITC Low Volume (LV) from TA Instruments. All four calorimeters are differential power compensation calorimeters, and the primary measurement is heat rate, which is derived from the power supplied by a control heater that is used to maintain a steady-state, approximately zero temperature difference between the sample and reference cells, but cooling for power compensation is done differently in the GE Healthcare calorimeters than in the TA Instruments calorimeters. The power from the control heater is compensated by a constant cooling mechanism that is passive in the GE Healthcare calorimeters and actively controlled in the TA Instruments calorimeters. The Nano ITC-SV and VP-ITC have approximately 1-ml reaction vessels, and the Nano ITC-LV and ITC_{200} have approximately 0.2-ml reaction vessels. The Nano ITC-SV is available with either gold or hastelloy reaction vessels. The Nano ITC-LV has a gold reaction vessel. Both GE Healthcare calorimeters have hastelloy reaction vessels. The different materials used to construct the reaction vessels have significantly different thermal conductivities that lead to differences in time constants and control software. Titrations are typically incremental, with 20–30 injections of 1–10 μl from a motor-driven syringe with a stainless steel needle that also serves as a stirrer. Reaction vessels in the GE Healthcare calorimeters are flat horizontal cylinders with rounded edges. The TA Instruments calorimeters are upright cylinders with conical ends. This difference in vessel shape creates differences in mixing properties.

An ideal power compensation calorimeter would always maintain the system in an exact isothermal condition; therefore, changes in the control power to maintain that isothermal condition would accurately measure heat from reactions. This is impossible in real systems for two reasons. The first reason involves sudden changes in heat input such as those that occur during an injection of titrant that cannot be exactly compensated by the control circuit. Furthermore, these transient nonisothermal conditions cause transfer of heat between the sample cell and surroundings, including the reference cell, and this may alter the supposedly constant cooling power. The second problem arises from inevitable differences in heat distribution in the system from heat effects from reaction and from the control heater. These differences cause

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[1] *Abbreviations used:* ITC, isothermal titration calorimetry; CBS, *p*-carboxy benzene sulfonamide; CAII, carbonic anhydrase II.

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heat exchange with the surroundings and reference cell to differ between the heat effects from a chemical reaction and the heat effects from the control heater. In addition to the control heater with which measurements are made, an electrical calibration heater is included in these calorimeters. Electrical calibration of calorimeters, however, can be subject to significant error because of failure to transfer all heat to the calorimeter vessel, because of heat generation in lead wires, and because of a different distribution of heat from the calibration heater than from a chemical process [1]. Because there is potential for systematic errors in calibration with the electrical calibration heater, a multiplicative calorimetric factor determined with a standard chemical reaction should be used to make measured changes in control power equal to the heat effects from a reaction. Even if the calorimetric factor is believed to be unity, this must be demonstrated with a standard reaction.

The effective volume of the reaction vessel in overflow vessels is not identical to the physical volume of the reaction vessel as supplied by the manufacturer and, therefore, must also be calibrated chemically [2]. The effective volume depends on how much volume is displaced by the stirrer and how much of the overflow tube is accessed by the reactant in the vessel during a reaction.

Although suppliers typically warranty the syringes used to manufacture burets to 1% linearity, the entire buret with drive system requires calibration to ensure accuracy of delivered volume. Buret calibration for total delivery volume can be conveniently done by mass, but because of their small size, individual injection volumes are difficult to measure and are best tested by chemical reaction.

A test to determine the variability of the calorimetric factor, injection volume, and effective reaction vessel volume among calorimeters that were not chemically calibrated was done with common reagents in seven Nano ITC-LVs in five laboratories and six Nano ITC-SVs with gold cells in four laboratories. Results of the mean of 39 experiments with the Nano ITC-LVs gave a relative standard deviation of 3.6% in the endpoint and 7.6% in the enthalpy change. The results from the 41 experiments done in Nano ITC-SVs gave a relative standard deviation of 2.1% in the endpoint and 4.1% in the enthalpy change. These errors indicate the magnitude of errors from lack of chemical calibration and not the precision obtained from a given calorimeter.

Literature surveys [3,4] show that nanowatt titration calorimeters with overflow cells have not been accurately calibrated; thus, previously published data are open to question. Results presented in the literature often suggest that solution concentrations, injection volumes, and/or cell volumes were inaccurate; time intervals between injections were too short or incomplete mixing occurred; and poor conditions were chosen for equilibrium constant determinations. Publications typically include only a reference to the calorimeter model and manufacturer, a sample of raw data, a titration curve with data points, and a fitted curve. Rarely is any information on calibration given. For example, Baranuskienė and coworkers [5] attempted to apply known reactions for comparison of the accuracy of electrically calibrated calorimeters but did not describe how electrical calibrations were done, did not calibrate the effective volume of the reaction vessel, and did not test the accuracy of assumed titrant injection volumes. The stoichiometries reported are inaccurate, and this could be caused by inaccurate solution concentrations, a wrong reaction vessel effective volume, inaccurate titrant injection volumes, and/or incorrect analysis of the data. Thus, their conclusions about the accuracy of different calorimeters are not supported by their data.

Currently, the most common use of calorimeters is for simultaneous determination of equilibrium constants and enthalpy changes. The only reactions previously recommended [6,7] as test reactions for this method are the reaction of aqueous $BaCl_2$ with 18-crown-6 with log $K_f = 3.771 \pm 0.015$ and $\Delta_R H = -31.4 \pm 0.2$ kJ/mol and the reaction of *p*-carboxy benzene sulfonamide (CBS) with carbonic anhydrase II (CAII) with $K_f = 1 \times 10^6\ M^{-1}$ and $\Delta_R H = -44$ kJ/mol. The usefulness of the $BaCl_2$/18-crown-6 reaction depends on the dynamic range of the calorimeter. The equilibrium constant is relatively small; so, to obtain $50 < C_R K_f < 500$ (C_R is the reactant concentration) and thus obtain a titration curve with the optimal shape [8], the 18-crown-6 concentration must be between 8.5 and 85 mM and the $BaCl_2$ concentration must be greater than 150 mM. At these concentrations, a 5-μl injection produces more than 2 mJ, which is outside the linear dynamic range of most calorimeters. In addition, the reagents are not readily available as directly weighable materials. As shown by the large errors (i.e., standard deviations of ±15% in stoichiometry, ±22% in K_f, and ±25% in $\Delta_R H$) from a round-robin test in 14 laboratories [7], the CBS/CAII reaction is not a suitable standard. The reactions of Ag^+ with halides as proposed by Baranuskienė and coworkers [5] are not recommended because of possible precipitation and plugging of the buret needle, difficulties with removal of the precipitates from the reaction vessel, and potential for reactions of the ions with the materials of construction. Others have suggested the use of a protein inhibitor reaction such as reaction of RNase A with 2′-CMP. This possibility is now moot because 2′-CMP is no longer readily available. Furthermore, no protein is likely to meet the challenge of being readily available in high purity, stable, easy to use, and not sensitive to small errors in preparations. We note the 0.9 ± 0.1 stoichiometry of carbonic anhydrase reactions in Fig. 4 of Baranuskienė and coworkers' [5] article, which they (along with others [9]) consider to be "good" for proteins. In the round-robin test involving 14 laboratories, the enthalpy of binding of CBS to bovine CAII varied by ±25% [5,7], and although it is not clear whether this was due to variability of materials, procedures, or calorimeters, it is clear that this reaction is not a suitable standard. Even though the reaction of Ca^{2+} with ethylenediaminetetraacetic acid (EDTA) was recently introduced by GE Healthcare as a test kit, irreproducibility by other preparations as a result of sensitivity to ionic strength and pH makes the reaction unsuitable as a calibration reaction.

To determine the likely systematic errors from lack of chemical calibration in simultaneous determinations of equilibrium constants, enthalpy changes, and stoichiometry, a round-robin test was run with the reaction of bicarbonate and HCl ($pK_a = 6.35$, $\Delta_R H = -9.15$ kJ/mol) with common reagents supplied to the participants. Results from calorimeters that were not chemically calibrated after manufacture gave $pK_a = 6.01 \pm 0.12$ (5.4% low), $\Delta_R H = -9.26 \pm 0.70$ kJ/mol (1.2% high), and $n = 0.993 \pm 0.036$ (0.7% low) for 39 observations in a Nano ITC-LV calorimeter; $pK_a = 6.22 \pm 0.06$ (2.0% low), $\Delta_R H = -8.53 \pm 0.35$ kJ/mol (6.7% low), and $n = 1.026 \pm 0.022$ (2.6% high) for 41 observations in a gold cell Nano ITC-SV calorimeter; and $pK_a = 6.22 \pm 0.09$ (2.0% low), $\Delta_R H = -8.54 \pm 0.37$ kJ/mol (6.7% low), and $n = 1.042 \pm 0.027$ (4.2% high) for 24 observations in a hastelloy cell Nano ITC-SV calorimeter. These results are given only to indicate the clear need for chemical calibration to obtain accurate values and not as a comparison among different calorimeters.

The purposes of this article are, first, to propose methods for testing calorimeter performance; second, to propose methods and reactions for calibration of injection volume, reaction vessel effective volume, and calorimetric factor; third, to propose reactions for testing overall performance of experiments and procedures for determination and analysis of data; and lastly, to reiterate requirements to ensure the accuracy of published data. These issues have not been adequately addressed previously.

Calibration procedures

Table 1 is a list of symbols, the calibration parameters to be determined, and the methods for doing so. Note that determination

Table 1
Parameters to be calibrated, procedures, and interdependence of parameters.

Parameter	Procedure	Dependence	Equation
Detection limit (D_B)	Titrate water into water	Depends on precision of injection volume and temperature control	D_B = 2(standard deviation of mean heat per injection)
Blank heat (Q_B)	Titrate water into water or titrant into blank solvent	Depends on ΔT between titrant and titrate	$Q_B = \Delta T(V_{inj})$(heat capacity of water) or $Q_B = \Delta T(V_{inj})$(heat capacity of titrant) + (heat of dilution of titrant)
Injection volume (V_{inj})	Determine mass of water delivered	Independent of other parameters	Mass/density of water
	Determine heat per injection (Q_R) with quantitative reaction	Depends on titrant concentration (C_T) and calorimetric factor (f)	$V_{inj} = (Q_R - Q_B)/(C_T)(-\Delta_r H)$ Eq. (1)
	Determine reaction endpoint	Depends on titrant concentration (C_T), reactant concentration (C_R), and reaction vessel volume (V_{RV})	$V_{ep} = (V_{RV})(C_R)/(C_T + C_R)$ Eq. (2)
Calorimetric factor (f)	Determine heat per injection (Q_R) with quantitative reaction	Depends on injection volume (V_{inj}), titrant concentration (C_T), and blank heat (Q_B)	$f = (V_{inj})(C_T)(-\Delta_R H)/(Q_R - Q_B)$ Eq. (3)
	Determine total heat (Q_R) from catalyzed reaction	Depends on reactant concentration (C_R), reaction vessel volume (V_{RV}), injection volume (V_{inj}), and blank heat (Q_B)	$f = (C_R)(V_{RV} - V_{inj})(-\Delta_R H)/(Q_R - Q_B)$ Eq. (5)
Effective volume of reaction vessel (V_{RV})	Determine reaction endpoint	Depends on titrant concentration (C_T), reactant concentration (C_R), and injection volume (V_{inj})	$V_{RV} = (V_{ep})(C_T + C_R)/(C_R)$ Eq. (7)
	Determine total heat (Q_R) from catalyzed reaction	Depends on blank heat (Q_B), injection volume (V_{inj}), and reactant concentration (C_R)	$V_{RV} = [(Q_R - Q_B)/(C_R)(-\Delta_R H)] + (V_{inj})$ Eq. (8)

of the injection volume by mass is the only calibration that can be done independent of the other calibrations.

Performance tests: detection limit and titrant equilibration

The detection limit of a calorimeter is best determined by titration of water into water (or solvent into solvent). The slope of a plot of these data (i.e., heat per injection versus injection number, as illustrated in Fig. 1) will be near zero if the buret is operating properly. The detection limit (D_B) is equal to twice the standard deviation of the average heat per injection in such a blank titration. Excessive variation (i.e., $D_B > 2$ μJ/injection) may indicate a buret or temperature control problem. A test of this at 25 °C in seven Nano ITC-LVs in five different laboratories gave 2.9 ± 0.7 μJ/injection for the average of 18 titrations with 2.5-μl injections. A test in six Nano ITCs with gold cells in four laboratories gave 3.4 ± 1.7 μJ/injection for the average of 18 titrations with 5-μl injections. Uncertainties are given as twice the standard deviation of the mean (D_B).

The syringe buret in these calorimeters is not thermostatted at the calorimeter temperature; instead, thermal equilibration of the titrant is done during passage of titrant through the syringe needle/stirrer. Two effects – (i) a temperature difference between the titrant and titrate and (ii) viscous flow – cause the heat per injection to differ from zero. The heat per injection from viscous flow can be calculated from the dimensions of the injection needle, the injection rate, and the titrant viscosity [10] and is usually negligible compared with the effect from the temperature difference. The blank heat is typically exothermic when the calorimeter is operated at temperatures near room temperature and below and endothermic when the calorimeter is operated above room temperature. Near ambient temperature, a blank heat effect larger than approximately 1 μJ/μl usually indicates an inadequately cleaned buret or reaction vessel or that the stirrer shaft is rubbing on the entry tube. The collars on the stirrer shafts are commonly misinterpreted as bearings, but the purpose of these is to limit mixing between the liquid in the tube above the collar with liquid in the reaction vessel. If these collars touch the walls of the fill tube because of misalignment of the buret with the fill tube or because the stirrer shaft is bent, rubbing of these collars on the fill tube will cause frictional heating of the titrant.

Buret calibration

The best way to calibrate the buret is by weighing the amount of water delivered by a known number of steps of the stepper motor. If the syringe can be removed from the drive mechanism, this can be done by overfilling the syringe, attaching the syringe to the fully open drive mechanism to remove the overfill, removing and weighing the filled syringe, reattaching the syringe to the drive, delivering a known number of steps, and weighing the syringe and remaining water. The difference in mass and the density of water at the delivery temperature can then be used to determine the volume delivered. If the syringe cannot be removed from the drive mechanism, a similar procedure can be used, but the entire drive mechanism must be weighed.

The buret injection volume (V_{inj}) can also be calculated from the heat per injection with a quantitative reaction with a known enthalpy change, $\Delta_R H$, but the result depends on prior knowledge of the calorimetric factor:

$$V_{inj} = (Q_R - Q_B)/(C_T)(-\Delta_R H). \quad (1)$$

In Eq. (1), C_T is the titrant concentration, $\Delta_R H$ is the enthalpy change for the reaction, Q_R is the measured heat per injection that depends on the calorimetric factor (f), and Q_B is the blank correction. $\Delta_R H$ must be corrected to experimental conditions. The consistency of individual injection volumes is calculated as twice the standard deviation of the mean heat per injection.

The total volume delivered by the buret, and thus V_{inj}, can also be calculated from the endpoint of a titration with a quantitative reaction, but the result depends on prior knowledge of the effective reaction vessel volume, as shown in Eq. (2):

$$V_{ep} = (V_{RV})(C_R)/(C_T + C_R), \quad (2)$$

where V_{ep} is the titrant volume at the endpoint, V_{RV} is the effective volume of the reaction vessel, C_R is the reactant concentration, and C_T is the titrant concentration. Eq. (2) is derived assuming a single injection, but it correctly identifies the dependence of V_{ep} on other variables. The equation for multiple injections is not simply written and adds unnecessary complexity to this discussion. The endpoint is typically identified as the inflection point in a curve fitted to a sigmoidal plot of the heat per injection versus injection number, but the endpoint volume may be more accurately located as the intersection of the two straight lines in a plot of total heat versus

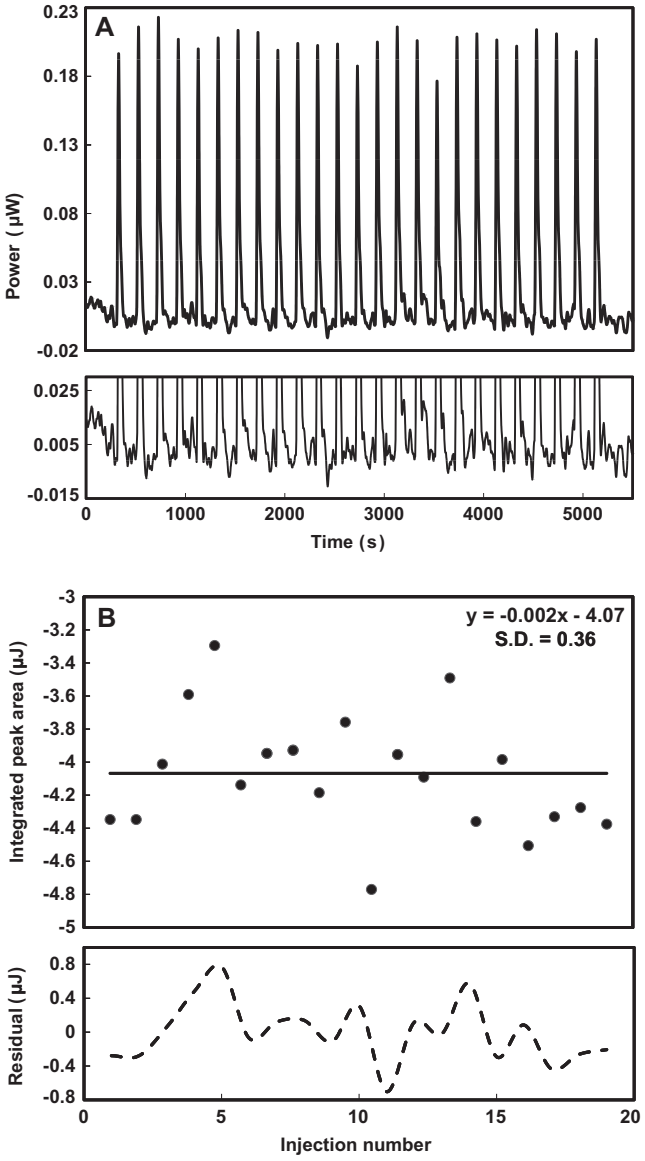

Fig.1. Determination of detection limit and titrant equilibration. (A) Raw data for 5-μl injections of water into water in a Nano ITC-SV at 25 °C. An expanded view of a portion of the data is shown in the lower part of the plot to show the return to baseline between injections. The titration consisted of 20 injections at 200-s intervals with a stirring speed of 350 rpm. Prior to titration, the instrument baseline was auto-equilibrated to a slope less than 0.1 μW/h and a peak-to-peak standard deviation less than 10 nW prior to the first injection. (B) Integrated peak area plotted against the injection number. The average heat per injection (4.07 ± 0.36 μJ) was calculated by fitting the integrated data points to a first-order linear equation ($y = -0.002x - 4.07$). The instrument detection limit ($D_B = \pm 0.72$ μJ) is equal to twice the standard deviation (0.36 μJ/injection) of the average heat per injection. The residual plot in the lower portion of the panel shows the deviations of individual points from the fitted model. S.D., standard deviation.

injection number. Any reaction from diffusion from the buret needle prior to the first injection affects only the vertical axis on such a plot and, thus, has no effect on the determined endpoint volume. The sigmoidal plot is less accurate for reactions that are quantitatively complete at the endpoint because only one or two data points occur near the endpoint, and a fitted sigmoidal curve often overestimates the titrant volume at the endpoint.

Determination of calorimetric factor

The calorimetric factor (f) can be determined from the heat per injection with a quantitative reaction, but the measured value for f depends on prior knowledge of the injection volume. The calorimetric factor is calculated as

$$f = (V_{inj})(C_T)(= \Delta_R H)/(Q_R - Q_B), \tag{3}$$

where V_{inj} is the volume of titrant injected, C_T is the concentration of titrant, $\Delta_R H$ is the enthalpy change for the reaction, Q_R is the heat per injection during the reaction, and Q_B is the heat per injection during a blank titration. The injection volume should be chosen to optimize the total heat per injection. The results should be tested for consistency by plotting the corrected heat per injection versus injection number. The slope of this plot should be near zero. Results may be averaged over several injections, and individual injections may be deleted from consideration because of mixing in the buret needle or injection of a bubble. Twice the standard deviation of the mean value for several injections is equal to the detection limit (D_R) for determination of a heat of reaction. The relative uncertainty in f ($\delta f/f$) includes the uncertainty in all of the variables and is calculated by Eq. (4):

$$\delta f/f = [(\delta V_T/V_T)^2 + (\delta C_T/C_T)^2 + (\delta \Delta_R H/\Delta_R H)^2 + (\delta Q_R/Q_R)^2 + (\delta Q_B/Q_B)^2]^{0.5}, \tag{4}$$

where δ indicates the uncertainty in each of the variables.

The calorimetric factor can also be determined from the total heat produced on injection of a catalyst into a known amount of substrate for a reaction with a known enthalpy change. The measured value of f in this case depends on prior knowledge of the effective volume of the reaction vessel and the injection volume as shown in Eq. (5):

$$f = (C_R)(V_{RV} - V_{inj})(-\Delta_R H)/(Q_R - Q_B). \tag{5}$$

The enthalpy change must be corrected to the experimental conditions. The relative uncertainty in f in this case is given by

$$\delta f/f = [(\delta C_R/C_R)^2 + (\delta V_{RV}/V_{RV})^2 + (\delta \Delta_R H/\Delta_R H)^2 + (\delta Q_R/Q_R)^2 + (\delta Q_B/Q_B)^2]^{0.5}. \tag{6}$$

Determination of effective volume of reaction vessel

The only way to determine the effective cell volume in overflow cells is by determining the amount of a reaction that actually occurs in the cell when it is overfilled with a reactant of a known concentration. This can be done by determining the endpoint of a titration with a quantitative reaction or by determination of the heat produced when a catalyst is injected into the reaction vessel with a known concentration of substrate. The effective volume, V_{RV}, in the first case is given by

$$V_{RV} = V_{ep}(C_T + C_R)/C_R \tag{7}$$

and in the second case is given by

$$V_{RV} = (Q_R - Q_B - V_{inj} C_R \Delta_R H)/(C_R)(-\Delta_R H), \tag{8}$$

where Q_R is the total heat measured. Eq. (7) is derived assuming a single injection but correctly identifies the dependence of V_{RV} on other variables. The equation for multiple injections is not simply written and adds unnecessary complexity to this discussion.

Interdependence of calibrated parameters

The discussion above and the equations in Table 1 clearly show that determination of the buret injection volume by mass is the only calibration that can be done independent of the other two calibration parameters. Accurate determination of the calorimetric factor (f) and effective reaction vessel volume (V_{RV}) from heat per injection and reaction endpoint, respectively, depend on having

an accurate V_{inj}. V_{RV} and f are interdependent when determined with a catalyzed reaction, and so one of the other methods must also be used. Therefore, achieving independent calibration of all three parameters requires first calibration of V_{inj} by mass, followed by calibration of f from heat per injection and then calibration of V_{RV} from endpoint determination. Alternatively, f or V_{RV} may be determined from the total heat from a catalyzed reaction if one of the other methods determines the other parameter. The methods chosen for calibration should be those that most resemble the experiments to be done after calibration.

Quantitative reactions for buret calibration, determination of the chemical calorimetric factor, and effective volume of the reaction vessel

Reaction of strong acid with excess strong base or basic buffer for calibration of injection volume and calorimetric factor

Titration of a well-defined solution of a strong acid into a solution of a strong base, or of a basic buffer solution containing a large excess of base, is suitable for determination of the buret injection volume (V_{inj}) and chemical calibration factor (f). Solutions of sulfamic acid, HCl, $HClO_4$, and HNO_3 are suitable titrants, but HCl corrodes stainless steel at concentrations greater than 1 mM and, even in dilute solution, HCl and HNO_3 are sufficiently volatile to cause problems by eventual corrosion of electrical components. Sulfamic acid ($pK_a = 1$, $\Delta_{diss}H = +0.41$ kJ/mol [11]) may be considered as a strong acid at concentrations below 1 mM where less than 1% is undissociated, and as a solid sulfamic acid is a weighable acidimetric standard, but because of slow hydrolysis, solutions must be freshly prepared. Sulfuric acid is not suitable because the second proton ionization is incomplete ($pK_{a2} = 2$) and has a relatively large $\Delta_R H$. NaOH and 2-amino-2-hydroxymethyl-propane-1,3-diol with $pK_a = 8.06$ (also known as Tris, tris(hydroxymethyl)aminomethane, and THAM) are the best characterized bases for this purpose, with enthalpy changes of reaction with strong acid of −55.8 and −47.4 kJ/mol, respectively, at infinite dilution at 25 °C [6,11,12]. Because the base is present in large excess, the concentration does not need to be known accurately.

Obtaining accurate results with these reactions requires special care to eliminate the effects of CO_2. CO_2 cannot be entirely eliminated from water without extraordinary effort; neither boiling nor vacuum reduces the CO_2 concentration below approximately 0.01 mM [13], a concentration that is significant compared with the concentrations of reagents typically used for ITC. Carbonic acid has an effective $pK_{a2} = 10.33$ and $pK_{a1} = 6.35$, with enthalpy changes for protonation equal to −15 and −9.15 kJ/mol, respectively [11–13]. Because these enthalpy changes are less negative than those for protonation of hydroxide ion or Tris, if carbonate or bicarbonate reacts, less heat will be produced than expected. Therefore, experimental conditions must be adjusted to prevent reaction of carbonate. Hydroxide ion is more basic than CO_3^{2-}; therefore, any CO_3^{2-} in the solution will not react as long as hydroxide ion is present in large excess (at least 10× any CO_3^{2-} present). Tris is less basic than CO_3^{2-} but more basic than HCO_3^-, and any CO_2 in the solution will be converted into HCO_3^- in a Tris buffer. Therefore, if Tris is present in large excess (at least 10× any HCO_3^- present) in a Tris/HTris+ buffer, the bicarbonate will not react with injected strong acid. Suggested conditions are titration of 40 mM base with 1 mM acid. In the case of Tris, early injections should be deleted because of possible reaction with CO_3^{2-}.

Fig. 2 shows sample data for titration of 1 mM HCl into 40 mM Tris. Data can be analyzed by fitting the heat per injection to a first-order linear equation. The slope should be negligibly small. The heat per injection can be calculated from the average or from the intercept, which gives the least squares value for the heat per injection. The uncertainty, as twice the standard deviation of the mean for 20 5-µl injections, is typically ±14 µJ/injection ($n = 5$ replicate titrations) as determined in a Nano ITC-SV with a gold reaction vessel.

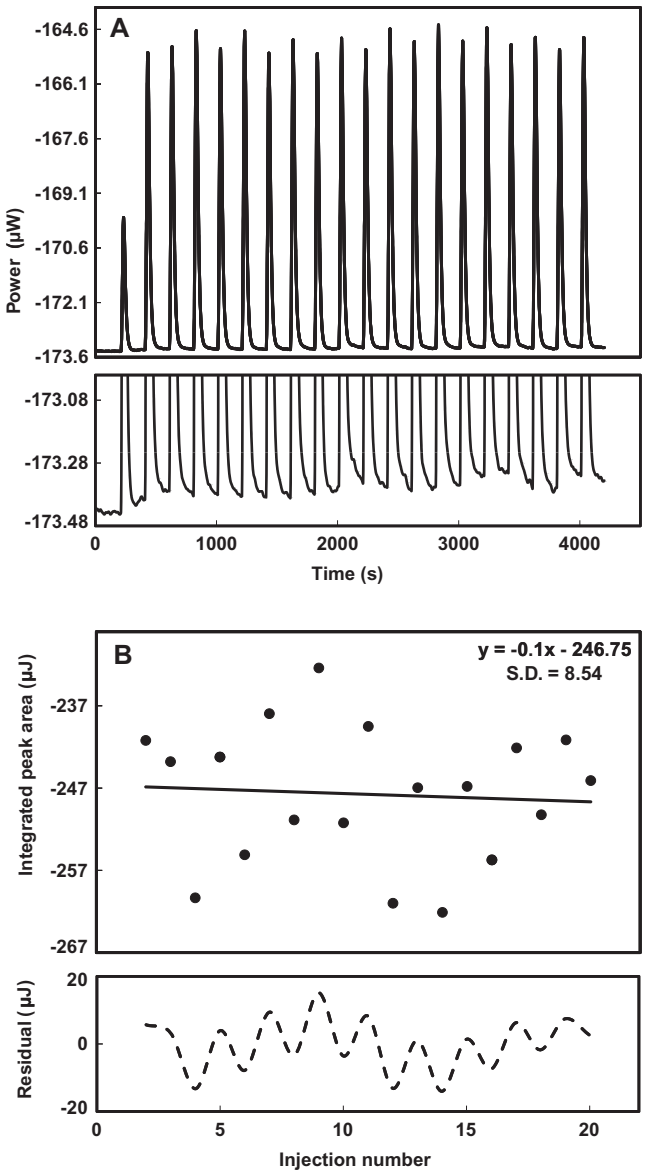

Fig.2. Determination of the chemical calorimetric factor. (A) Raw data for 5-µl injections of 1.036 mM HCl into 40 mM Tris in a Nano ITC-SV at 25 °C. The titration consisted of 20 injections at 200-s intervals with a stirring speed of 350 rpm. Prior to titration, the instrument baseline was auto-equilibrated to a slope less than 0.1 µW/h and a peak-to-peak standard deviation less than 10 nW. The electrical calibration factor was 1.000, buret calibration was done by a mass of water, and solutions were prepared from deionized boiled water, Fluka standard HCl solution, and Sigma ACS reagent-grade Tris. An expanded view of a portion of the data is shown in the lower part of the plot to show the return to baseline between injections. The expanded figure shows that the peaks are not fully baseline resolved; injections should have been done at longer time intervals. (B) Integrated peak area plotted against injection number. The first point was masked from data fitting. The heat per injection (247.75 µJ) was calculated by fitting to a first-order linear equation ($y = -0.1x - 246.75$) and taking the midpoint. The slope is negligible given that it causes a maximum error of ±1 µJ. The standard deviation for the fit of the first-order equation is ±8.5 µJ/injection; the residual plot in the lower portion of the panel shows the deviations of individual points from the fitted model. Note the nonrandom structure caused by too short injection intervals. The chemical calibration factor calculated from this titration is 1.016 = (5 µl/inj)(1.036 nmol/µl)(47.4 µJ/nmol)/(247.75–4.07 µJ/inj). See Eq. (3). S.D., standard deviation.

As much as possible, CO_2 should be removed from water used to prepare both the titrant and titrate by boiling and cooling in a closed container with a CO_2 sorbent. Vacuum degassing, as is commonly done with nanowatt ITC, is not sufficient for CO_2 removal because dehydration of H_2CO_3 is slow at lower temperatures. If CO_2 is present in the acid titrant, it will react with the basic titrate and reduce the heat produced. Solutions in equilibrium with the normal concentration of CO_2 in laboratory air are approximately 1 mM CO_2, and injection of 10 µl of such a solution into 1 ml of base produces only −42 kJ/mol hydroxide, whereas reaction of strong acid with hydroxide ion produces −55 kJ/mol. Reversing the titration by using the base as a titrant [5] obscures, but does not solve, the problem of CO_2 because CO_3^{2-} and/or HCO_3^- in the titrant will react with the acid in the reaction vessel to reduce the heat effect but will not be detected because the carbonate reactions do not affect the shape or apparent stoichiometry of the titration curve.

Measured enthalpy changes should be corrected for heat of dilution of the titrant [7] to the ionic strength in the reaction vessel. Data for these corrections can be found in Ref. [14], or a blank titration can be done to determine the correction. Measured blanks, corrected for the blank injection heat from a water-into-water titration, should agree closely with heats of dilution calculated from literature. If not, the dilution reaction must be different from that supposed. Two different acids and two different bases can be run to verify that adsorption of ions on surfaces is not a significant source of heat during the titration.

Acid–base titration for calibration of buret delivery and effective volume of the reaction vessel

Because of interference by CO_2, reaction of acids with strong bases or bases with $pK_b > 7$ other than HCO_3^- is not recommended for this purpose. The endpoint of a titration of strong acid into $KHCO_3$ solution ($\Delta_R H = -9.15$ at 25 °C [13]) is not affected by CO_2 sorption and has a sufficiently sharp endpoint as long as the concentration of HCO_3^- is greater than 0.1 mM. $KHCO_3$ is not hygroscopic and is readily available with sufficient purity; thus, it is a weighable standard material for this purpose. Fig. 3 shows a sample set of data for titration of $KHCO_3$ with HCl. With any given calorimeter, results at the 95% confidence level from repeated experiments should be within ±1% with three replicates for the endpoint stoichiometry.

Catalyzed reaction for determination of effective volume of the reaction vessel and calorimetric factor

The total heat produced by enzyme-catalyzed hydrolysis of sucrose to (fructose + glucose) with $\Delta_R H = -14.95$ kJ/mol at 25 °C [6] can be used to determine the effective volume or calorimetric factor. This reaction does not have a strong dependence on buret injection volume or suffer any effects of CO_2 contamination. The volume of enzyme solution injected should be kept to a minimum to minimize displacement and dilution of the sucrose solution. Care must also be taken to ensure that no enzyme reaches the sucrose solution prior to the injection. Reaction of 5 mM sucrose with 0.5 units of invertase (β-D-fructofuranosidase) in a 0.2-ml calorimeter and 3 mM sucrose with 3.3 units of invertase in a 1-ml calorimeter goes to completion in approximately 60 min. Fig. 4 shows a sample data set for this reaction. This method is appropriate for calibration of instruments used for determination of reaction kinetics. The 95% confidence interval for replicate experiments in a given calorimeter should be ±0.6% (three replicates).

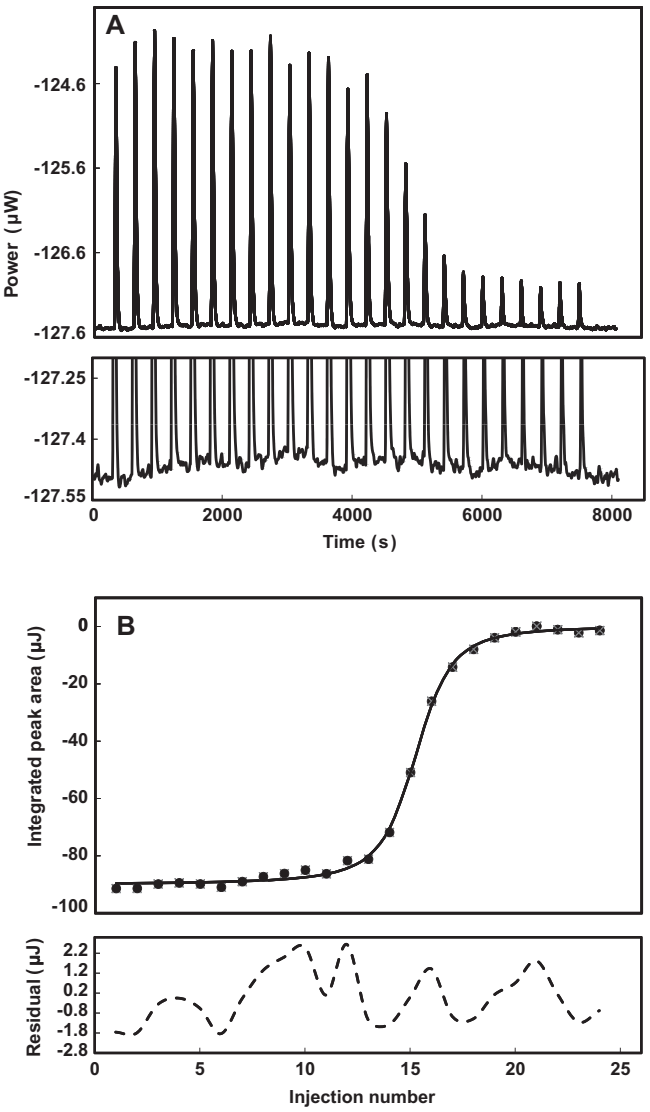

Fig.3. Acid–base titration for calibration of buret delivery or effective volume of the reaction vessel. (A) Raw data for 10-µl injections of 1.036 mM HCl into 0.1798 mM $KHCO_3$ in a Nano ITC-SV at 25 °C. Solutions were prepared from deionized boiled water, Fluka standard HCl solution, and Mallinckrodt analytical reagent-grade $KHCO_3$. The titration experiment consisted of 25 injections at 300-s intervals with a stirring speed of 350 rpm. An expanded view of a portion of the data is shown in the lower part of the plot to show the return to baseline between injections. Prior to titration, the instrument baseline was auto-equilibrated to a slope less than 0.1 µW/h and a peak-to-peak standard deviation less than 10 nW. The buret was calibrated by a mass of water. (B) Integrated peak area plotted against injection number. The stoichiometry (0.987) was calculated from the fit of a 1:1 reaction model (continuous line) assuming V_{RV} = 950 µl. The effective reaction vessel volume calculated from this titration is 938 µl = (950 µl)(0.987).

Combined tests of calorimeter performance

Heats of dilution

Obtaining correct results for heats of dilution depends on having accurate values for f, V_{inj}, and V_{RV} as well as the concentration of the titrant. Dilution of 2% (w/w) 1-propanol in water is a convenient and appropriate titrant [6]. Under these conditions a plot of the heat per injection versus injection number or total volume injected will be near linear with a significant slope. To prepare the titrant, the 1-propanol must be dry but can be purchased as a dry reagent or dried with molecular sieves. The heat per injection at 25 °C can be calculated by

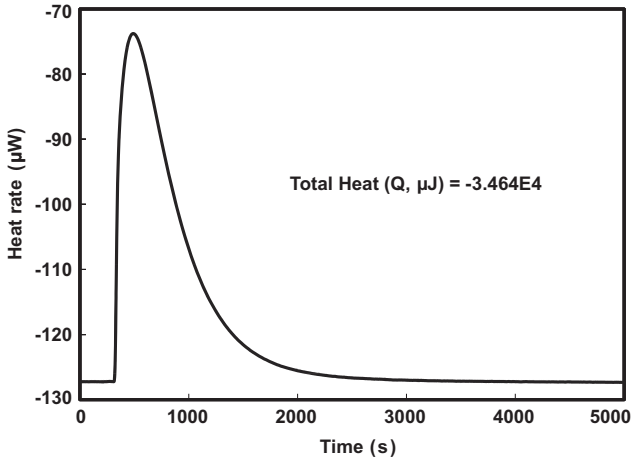

Fig.4. Catalyzed reaction for determination of effective volume of the reaction vessel or calorimetric factor. Raw data for a single 20-μl injection of invertase (grade VII from baker's yeast, Sigma) into 2.499 mM sucrose (EMD Chemicals) in 0.1 M acetate buffer (pH 5.65) in a Nano ITC-SV at 25 °C are shown. The buret was loaded with 10 μl of invertase (3 units) in 0.1 M acetate buffer (pH 5.65) and then with 10 μl of buffer to prevent diffusion of enzyme prior to titration. The chemical calibration factor was 0.995, the stirring speed was 350 rpm, and the calorimeter was auto-equilibrated to a slope less than 0.1 μW/h and a peak-to-peak standard deviation less than 10 nW. The total heat (3.464×10^4 μJ) was calculated by integrating the peak area above the baseline. The effective reaction vessel volume calculated from this experiment is 947 μl = 20 μl + {(34,640 − 12 μJ)/[(2.499 nmol/μl)(14.95 μJ/nmol)]}. See Eq. (8).

$$Q_{\mathrm{inj}} = n_{\mathrm{b}}[c_1(m_{\mathrm{f}} - m_{\mathrm{b}}) + c_2(m_{\mathrm{f}}^2 - m_{\mathrm{b}}^2)], \tag{9}$$

where n_{b} is moles of propanol per injection, m_{f} is the molality of propanol in the reaction vessel after an injection, m_{b} is the molality of propanol in the titrant, $c_1 = 558 \pm 9$ J kg mol^{-2}, and $c_2 = 158 \pm 8$ J kg^2 mol^{-3} [6]. Fig. 5 shows a sample set of data. The intercept should be within ±12 μJ/inj, and the slope should be within ±0.6 μJ/inj at the 95% confidence level for three replicates with a given calorimeter.

Dilution of aqueous sucrose and urea solutions into water can be used similarly [6], but because of the large difference in density between the titrant and water, mixing in the reaction vessel might be incomplete. NaCl solutions have also been proposed for this purpose [2], but the heat of dilution is quite small [14] compared with that for *n*-propanol, sucrose, or urea.

Simultaneous determination of enthalpy changes and equilibrium constants

In addition to accurate values for the calorimetric factor, effective cell volume, injection volume, and concentrations of the titrant and titrate solutions, accurate simultaneous determination of enthalpy changes and equilibrium constants requires properly chosen conditions. The value of $C_{\mathrm{R}}K_{\mathrm{f}}$, where C_{R} is the concentration of reactant and K_{f} is the formation constant for the reaction, must be near 100 [8]. The optimal $C_{\mathrm{R}}K_{\mathrm{f}}$ value is a compromise between conditions that produce near complete reaction at the beginning of the titration and conditions that produce incomplete reaction at the equivalence point. At $C_{\mathrm{R}}K_{\mathrm{f}}$ values greater than approximately 500, the number of data points in the curved portion around the equivalence point is usually not sufficient to obtain an accurate K_{f} value. Because of the paucity of data points around the equivalence point, errors in K_{f} increase rapidly at $C_{\mathrm{R}}K_{\mathrm{f}}$ values above 500. At $C_{\mathrm{R}}K_{\mathrm{f}}$ values less than approximately 50, K_{f} and $\Delta_{\mathrm{R}}H$ are interdependent, and although it is theoretically possible to obtain separate values, large systematic errors may occur because the fitted values of K_{f} and $\Delta_{\mathrm{R}}H$ are very sensitive to small errors in concentrations, baseline, and so forth. The effects of such errors on K_{f} and $\Delta_{\mathrm{R}}H$ increase rapidly as $C_{\mathrm{R}}K_{\mathrm{f}}$ decreases below 50. Thus, the $C_{\mathrm{R}}K_{\mathrm{f}}$ value necessary to obtain accurate values for both K_{f} and $\Delta_{\mathrm{R}}H$ is between 50 and 500 [8].

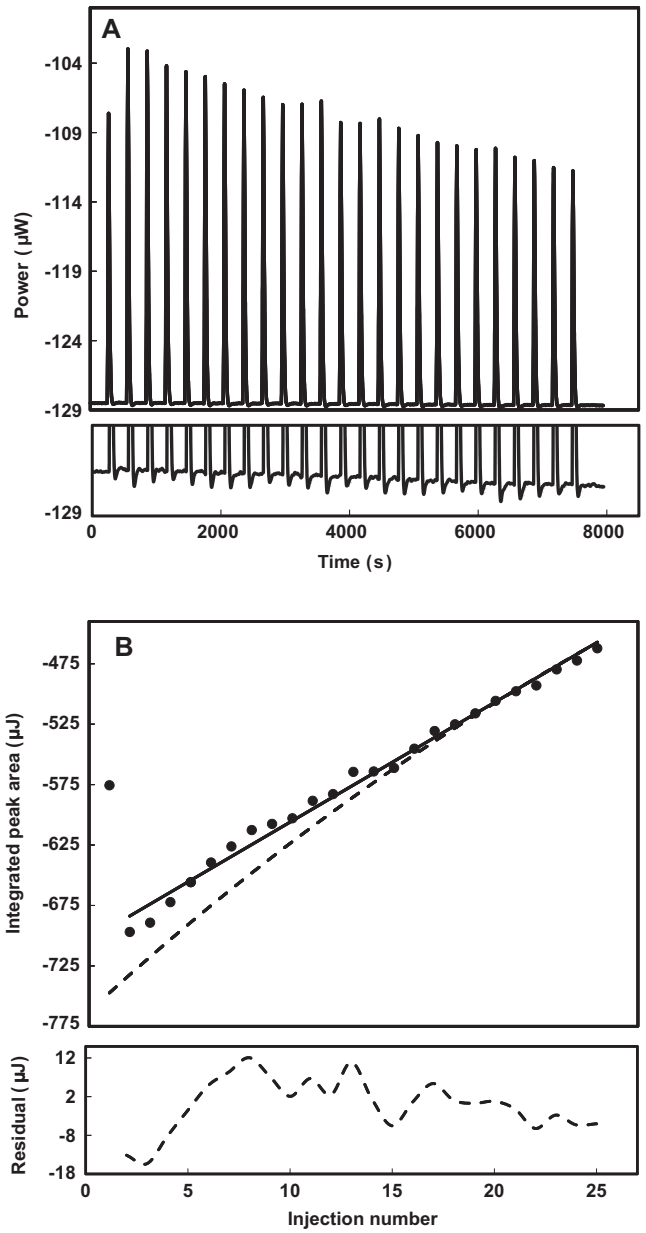

Fig.5. Determination of heats of dilution. (A) Raw data for 10-μl injections of 2.004% (w/w) 1-propanol in water (Sigma HPLC-grade) into water in a Nano ITC-SV at 25 °C are shown. The titration consisted of 25 injections at 300-s intervals with a stirring speed of 350 rpm. Prior to titration, the instrument baseline was auto-equilibrated to a slope less than 0.1 μW/h and a peak-to-peak standard deviation less than 10 nW prior to the first injection. The buret was calibrated by mass, $V_{\mathrm{RV}} = 950$ μl, and the chemical calibration factor was 1.000. (B) Integrated peak area plotted against injection number. The equation obtained by fitting the data to a first-order linear equation is $y = 9.851x - 693.65$ with a standard deviation of ±7.10. Data calculated with Eq. (9) as shown by the dotted line are slightly curved. The difference between the measured and calculated curves is likely caused by an incorrect V_{RV}.

The stoichiometric equivalence between the titrant and titrand is another parameter often determined simultaneously with K_{f} and $\Delta_{\mathrm{R}}H$. This parameter is extremely sensitive to small errors in input data when $C_{\mathrm{R}}K_{\mathrm{f}}$ is less than approximately 50, and accuracy improves with increasing $C_{\mathrm{R}}K_{\mathrm{f}}$ values above 50. Values for K_{f} and $\Delta_{\mathrm{R}}H$ determined for a test reaction should not be accepted if n does not equal 1 ± 0.01.

Titration of $KHCO_3$ with strong acid is a suitable test system for this purpose. The literature gives $pK_a = 6.35$ and $\Delta_R H = -9.15$ kJ/mol [12,13] (see Fig. 3). Solutions of both reactants can be prepared by weight if sulfamic acid is used as titrant. The determined values of K_f and $\Delta_R H$, however, do depend on the concentration of CO_2 in the solvent water, as shown in Fig. 6. The concentration of $KHCO_3$ should be approximately 0.2 mM, which makes $C_R K_f = 450$. Higher concentrations are not in the optimal range for determination of K_f and may produce bubbles of CO_2 on acidification. At this concentration, 10-μl injections of 1 mM acid give heats of injection of approximately 90 μJ at the beginning of the titration. Results of replicate experiments in a given calorimeter should be within ±0.15 kJ/mol for $\Delta_R H$ and within 1.5% for the stoichiometry at the 95% confidence interval with three replicates. Such conditions are typical of the conditions under which many protein–ligand binding reactions are studied.

Standards for publication

Because ITC is used to obtain thermodynamic data that are of worth in the long term only if the values are accurate, papers submitted for publication must contain sufficient information for reviewers and editors to properly assess the accuracy of reported results. At the very least, this must include a comparison of results obtained on an accepted standard reaction with the literature values for the standard. Electrical and chemical calorimetric factors, effective cell volume, and buret calibration together with experimental conditions should be reported. Preparation of solutions for chemical calibration must be reported in sufficient detail to ensure that the experimenter is aware of and has taken precautions to avoid or minimize potential errors. If calibration is done with the calibration heater, electrical heater power and duration of heating should be reported, especially if these are significantly different from heat effects from a reaction. Statements such as "according to the manufacturer's instructions" are not sufficient because such instructions change over time. The method used to correct for secondary reactions, blank effects, and baseline must be reported.

Customary practice for reporting ITC data has been to provide plots of heat rate data and integrated data (i.e., heat per injection) versus time and injection number, respectively. If properly presented, such plots can be very useful for assessing data quality. Plots of raw heat rate data should be presented as obtained, before any baseline correction has been done. The lower portion of the plot should be expanded to show the actual shape of the trailing edge of the injection peaks to determine whether mixing and the time between injections is sufficient for the reaction to reach equilibrium and for the calorimeter to achieve baseline steady-state. Plots of integrated data should be presented together with a residual plot (i.e., a plot of the deviations of individual points from a fitted model) to assess the precision of the fit. Actual data (i.e., μJ/injection), and not normalized data (i.e., kJ/mol), should be presented to make the size of the measured effect apparent for comparison with the detection limit and dynamic range of the calorimeter. For chemical standards, the actual data for heat per

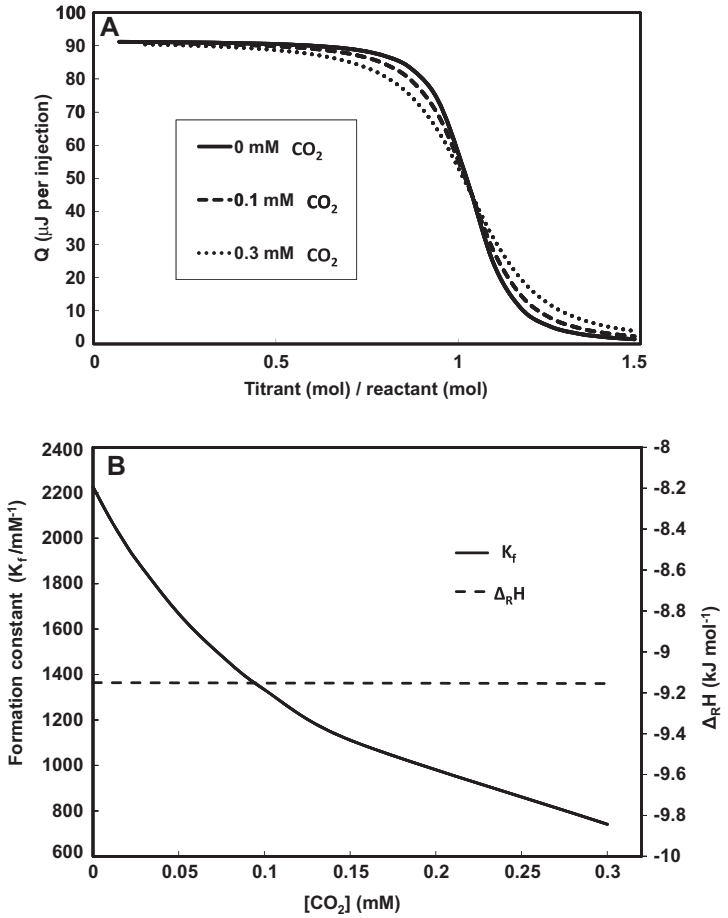

Fig.6. Effect of CO_2 on simultaneous determination of enthalpy changes and equilibrium constants in titrations of $KHCO_3$ with strong acid. (A) Simulated data for titration of 2 mM acid into 0.15 mM $KHCO_3$ with varying concentrations of CO_2 in the solvent water. Integrated heat per injection plotted against the ratio of moles of titrant to moles of reactant is shown. (B) Formation constant (K_f) and $\Delta_R H$ calculated, assuming no CO_2 from simulated data with CO_2.

injection should be presented with an overlain plot of data calculated with standard values. Such a comparison makes contamination by a reactive impurity immediately apparent.

Discussion

Although Baranuskienė and coworkers [5] were more thorough than most authors, their results illustrate some of the deficiencies of current practices in nanowatt ITC. Plots of raw heat rate data have been baseline corrected and are not presented with a fine scale so that the tail portion of the peaks can be discerned. Time between injections is only stated to be "3–4 min intervals" and "stirring was 150–400 rpm," so it is difficult to judge the quality of the raw data. The data for 0.5 mM HNO_3 titrated with 5 mM Tris base appear to be close to baseline resolved, but the raw data for AgI precipitation are clearly not resolved, indicating that chemical equilibrium and/or calorimeter steady-state was not achieved between injections in this experiment. No statement regarding data analysis for quantitative reactions was made, but plotted data indicate that $\Delta_R H$ was obtained by fitting the data to an independent sites model with n, K_f, and $\Delta_R H$ as fitting parameters. The effective volume of the reaction vessel, which is not given, is a hidden parameter in these calculations, and it is not clear how this affects the results. Only three values of n, the stoichiometric ratio of reactant to titrant, are given in Baranuskienė and coworkers' article: $n = 0.928$ for titration of HNO_3 with Tris, $n = 0.9595$ for titration of NaI with $AgNO_3$, and $n = 0.9 \pm 0.1$ for titration of recombinant human CAII with various ligands. These values indicate a systematic error in the buret delivery volume, the effective volume of the reaction vessel, the concentration of the titrant, or the data analysis. Calculating $\Delta_R H$ as the heat per injection divided by the moles injected could have been used to eliminate the effective reaction vessel volume as a source of this error. Stoichiometries should have been determined from the endpoint in plots of total heat versus total moles injected.

Baranuskienė and coworkers [5] stated that "calorimeters were electrically calibrated according to the manufacturer's instructions," so we assume that they used the manufacturer's default settings. Thus, the heat effects used in the chemical tests for the calorimetric factor, 1–2 mJ/injection, were likely larger than in the electrical calibrations and probably beyond the linear dynamic range. The results from the Nano ITC-III were in poorer agreement with the literature than those from the VP-ITC or ITC_{200}, but this is not an indictment of the calorimeter; rather, it is an indication of a failure to properly calibrate the calorimeter. Increasing the calorimetric factor or decreasing the default cell volume for the Nano ITC-III by 11% would bring those results into the same agreement with the literature values as the other calorimeters. The stoichiometries found were not reported, and errors in solution concentrations could account for the different results from different calorimeters.

Chemical standards should be run across a range of concentrations for two reasons: first, to demonstrate linearity of the calorimetric factor over the dynamic range and, second, to check for the presence of reactive impurities in the standard solutions. If a reactive impurity is present from solvents, its effect increases with dilution of the reactants. If the impurity is in the reagent used as a standard, the relative error is independent of the concentration. Effects from contaminants that are negligible when working with reagents that are more concentrated and the associated larger heat effects might not be negligible when working with more dilute solutions and smaller heat effects. A case in point is the calorimetric acid–base reactions found in super-dilute NaCl solutions and published in support of the homeopathic theory that water has a "memory" [15]. In fact, the results are fully explained by assuming that the water was in equilibrium with atmospheric CO_2. The stoichiometry found in titrations of standards should always be reported as a check on the accuracy of concentrations and buret delivery.

A common practice in ITC is to use data points after the main reaction is complete as a correction for the heat of dilution of the titrant. Mizoue and Tellinghuisen [16] demonstrated the potential for error in this approach. To avoid such errors, dilution data should always be tested for consistency with known heats of dilution [14].

Summary

Calorimetric measurements, especially at the very small volumes, low concentrations, and heat effects currently used in most ITC measurements, require extraordinary care and attention to detail to obtain accurate results. Three parameters must be accurately calibrated: the calorimetric factor used to convert the electrical signal to heat rate, the injection volume, and the effective cell volume. Chemical standards for calibration and testing of nanowatt titration calorimeters with overflow reaction vessels have been proposed. Despite the shortcomings in their presentation, Baranuskienė and coworkers [5] did a considerable favor to the field by pointing out the necessity of chemical standards for obtaining accurate data. We totally agree with their statement that "validation should be reported in the experimental section of every ITC manuscript," and we hope that experimenters, reviewers, editors, and standards organizations will come together to make this a reality.

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