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Determination of Michaelis–Menten parameters obtained from isothermal flow calorimetric data

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Abstract

Recent papers have reported [Thermochim. Acta 399 (2003) 63; Thermochim. Acta, in press] the results of a preliminary inter/intra laboratory study into the suitability of the base-catalysed hydrolysis of methyl paraben as a test and reference reaction for isothermal flow-through calorimeters. It was shown that this reaction can be used to investigate the flow characteristics of the instrument being used. It has also allowed, for the first time, the calculation of accurate values for the rate constant and for the enthalpy change, ΔH (hereafter H (enthalpy) for simplicity) of reaction directly from the calorimetric data, free from assumption. These findings have been extended to permit the direct determination of Michaelis–Menten based kinetic parameters from calorimetric data again free from assumption (except that the system conforms to Michaelis–Menten kinetic theory). This paper describes the method used for such an analysis and reports the results of a preliminary study on the urea/urease enzymatic system.

Keywords: Flow calorimetry; Enzymes; Michaelis–Menten kinetics

1. Introduction

The requirement of a chemical test and reference reaction for the validation of isothermal calorimeters has been known for a number of years [3–5]. Recently the results of an intra/inter laboratory study into the suitability of the imidazole-catalysed hydrolysis of triacetin (hereafter ICHT for simplicity) as such a test and reference reaction have been reported [6,7]. This has been followed by a number of other publications [7–11] reporting the potential applications of the triacetin reaction. The papers referenced above, however, all describe applications of the test and reference reaction for use in calorimeters operated in the static or batch mode and no consideration of solution phase reactions studied by flow-through instruments is made.

Flow-through calorimetric instruments are particularly suited to studying reactions, which have relatively short half-lives or for those in which constant stirring is required, examples include biological cell/medium interactions and enzyme/substrate reactions [12].

Data obtained from flow calorimetry can yield, through application of the appropriate equations, both thermodynamic and kinetic information such as enthalpy of reaction and rate constant for example. However, until recently, the recovery of such parameters was severely limited in its accuracy. As will be seen in the following section the calorimetric data is dependent upon the value of the effective thermal volume of the calorimetric cell (i.e. the volume of reacting solution "seen" by the calorimeter). This could not be readily measured before the introduction of a test and reference reaction for such instruments and assumptions were made as to its value. As a consequence it is likely that the majority of values reported for the thermo-kinetic parameters of interest derived from this technique (using assumptions for the thermal volume), are likely to be in error.

An identical study to that reported here was performed by Beezer et al. [17] some 30 years ago. Values were reported for the enthalpy of reaction, the Michaelis constant and the first-order rate constant. For the reasons outlined above, and detailed in previous publications, these values (and the enthalpy in particular) are likely to be in error. This paper will highlight the potential magnitude of some of these errors by a comparison of the value reported 30 years ago for the enthalpy of reaction for the urea/urease enzymatic system

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(derived using assumptions for the value of the thermal volume) with a value for the enthalpy obtained by us using an identical protocol and calorimeter but this time with a properly identified value for the thermal volume.

General equations which describe calorimetric output for static batch type calorimeters have been derived [13,14]. The principles used in the derivation of static batch type equations were also used to derive equations, which describe calorimetric output for flow-through calorimeters [15–17]. A general equation which describes the amount of material reacted, x, to any time, t, for a flowing system is described below in Eq. (1):

$$x = C - (C^{-n}(-ktC^n + kntC^n + C))^{1/(1-n)} \quad (1)$$

where x is the amount of material reacted to time t; C, concentration of the reagent in solution; n, order of reaction; k, appropriate rate constant; t, time.

The calorimetric output for a flowing system is the average signal over the time during which the reacting solution is in the calorimetric vessel, the residence time, (Eq. (1) can be written for x at any time t and for x at time $t+$) allowing equations to be written [10,16] which describe the calorimetric output for any kinetic order. Equations which describe zero-order, Eq. (2), and first-order, Eq. (3), systems are presented below:

$$\Phi = -k_0 H\tau F \quad (2)$$

$$\Phi = -FCH(1 - \mathrm{e}^{-k_1\tau})\,\mathrm{e}^{-k_1 t} \quad (3)$$

Here Φ is the calorimetric output; F, flow rate of the flowing solution; H, enthalpy of reaction; τ, residence time of the solution in the calorimetric cell.

As Eqs. (2) and (3) clearly demonstrate determination of reliable values for rate constants and enthalpy changes from experimental data (power/time data) for reacting systems studied by flow microcalorimetry requires accurate and precise values for T (which is determined from Eq. (4) through knowledge of F and V_c). The thermal volume (that is the effective operational volume of the calorimeter), V_c, can be determined (for any flow rate) through prior knowledge of accurate values for k and H and consideration of the calorimetric output from a first-order solution phase (test and reference) reaction. For a detailed discussion of these points see Ref. [2].

$$\tau = \frac{V_c}{R} \quad (4)$$

The obvious choice would be the ICHT reaction. However, O'Neill [10] reports that the ICHT reaction is unsuitable for use as a test and reference reaction for flow-through calorimeters and proposed the base-catalysed hydrolysis of methyl paraben (hereafter BCHMP for simplicity) as an alternative. The results [1,2,10] of a preliminary inter/intra laboratory trial on the BCHMP have been reported for studies using the LKB 10700-1 flow calorimeter and customized and standard thermometric flow inserts (specifically designed apparatus that allows flow-through and/or flow-mix experiments to be conducted using the thermal activity monitor (TAM)).

It was demonstrated by Kemp and Olomolayie, in our previous publications, [1,2,10], using their modified calorimetric flow insert, for TAM, that the effects of flow rate could be minimized by careful design of the insert and perhaps more importantly the arrangement of sample and reference ampoules in the calorimetric channel (more details can be found in Ref. [2]). It was found that the effects of flow rate on thermal volume were negligible, however, a significant finding was that the zero flow rate volume, V_0 (that is the effective thermal volume of the calorimetric cell when the flow rate is zero (nominally identical to the physical volume of the cell) is significantly different from the nominal (engineered) volume (approximately 30% greater: 1 and 1.29 cm^3 for the engineered and operational volumes, respectively). However, for the standard TAM flow inserts it was found that the effect of flow rate was not predictable in that Volpe and Oliveira and co-workers [1,2,10] found no significant effect with flow rate moreover they also found that the zero flow rate volume was almost identical to the nominal physical volume of the cell. In contrast Vine, using a nominally identical flow insert, reported significant variation in thermal volume with flow rate and also a significant difference between the zero flow rate volume and nominal physical volume. The results obtained by Vine are mirrored by those obtained by O'Neill using the LKB instrument [2]. The variation in thermal volume across the range experimental flow rate could be as much as 15% (0.61–0.71 cm^3). The zero flow rate volume can be as much as 60% greater than the nominal physical volume of the cell (0.73 and 0.47 cm^3, respectively). For the flow rate commonly used by us, for the LKB instrument, this equates to approximately a 25% difference (for the most common flow rate used) between effective volume and nominal physical volume. These observations clearly highlight the fact that the effect of flow rate cannot be predicted, for different instruments and/or instrumental set-up, and can introduce very large errors into values for thermo-kinetic parameters derived from flow calorimetric data. Consequently, it is necessary that such instrument characteristics are determined before any studies are performed using the calorimeter. For a more detailed discussion see Refs. [1,2,10].

The implications of these observations are clear. If the value of V_c (hence τ) used in Eqs. (2) and (3) is incorrect then the derived value for the enthalpy will also be in error. This could amount to a significant error if the nominal physical volume is used for all flow rates.

Beezer et al. [16,17] (the first to publish the flow calorimetric equations) used the equations described above to analyse data from a study of the urea/urease enzymatic system. Eqs. (2) and (3) were manipulated in order to yield Michaelis–Menten based kinetic parameters from the calorimetric data (Eqs. (5) and (6)):

$$\Phi = -k[\mathrm{E}]_{tot} V_c H \quad (5)$$

$$\Phi = -FCH(1 - \mathrm{e}^{(-k[\mathrm{E}]_{tot}/K_M)\tau})\,\mathrm{e}^{-(-k[\mathrm{E}]_{tot}/K_M)t} \quad (6)$$

Note that in this treatment $k[E]_{tot}$ is equal to k_0 and $k[E]_{tot}/K_M$ is equal to k_1.

As discussed earlier, equations such as (2) and (3) require knowledge of the residence time, τ, and thermal volume, V_c. Previously these equations could only be used if certain assumptions were made about the values of V_c (and hence τ). The procedure described by Beezer et al. [16,17] was to assume that (i) the value of V_c was the engineered volume, and (ii) from Eq. (5), that the value of $k\tau$ is small and hence Eq. (7) could be derived:

$$\Phi = -\frac{k[E]_{tot}}{K_M}[S]HV_c\, e^{(-k[E]_{tot}/K_M)t} \quad (7)$$

where $[E]_{tot}$ is the initial enzymatic concentration; K_M, Michaelis constant; [S], initial substrate concentration.

Based on these assumptions the Michaelis constant, K_M, could then be calculated using Eqs. (6) and (7). Knowledge of the Michaelis constant then permits the calculation of the remaining kinetic parameters of interest. However, it is only possible to calculate the product V_cH using this method. In order to calculate the enthalpy, H, it is necessary to know V_c. In their method V_c was again assumed to be the physical volume of the cell. As described above, for the flow rate employed by them, this is approximately 25% in error. These assumptions are clearly unacceptable if accurate values are to be found for the enthalpy of reaction.

The concept of thermal volume is discussed in Refs. [1,2,10] where it is shown that thermal volume and physical volume are not the same, and therefore, any enthalpy and rate constant values derived in this manner would be inaccurate. These publications also report the results of a secondary test and reference reaction which, for the first time, allows accurate values of thermal volume to be calculated, and therefore, permits accurate determination of values for the enthalpy change of reactions studied by flow calorimetry.

Knowledge of the thermal volume at any given flow rate allows calculation of τ and consequently allows direct determination of Michaelis–Menten parameters, free from assumption, from calorimetric data.

Through knowledge of the enthalpy for any first-order reaction (enzymatic or otherwise) can be calculated from Eq. (3). This can be done in two ways. The first method is to extrapolate the line of a $\ln \Phi$ versus t plot to $t = 0$ and define a value for Φ at $t = 0$. At this point the value of the term Eq. (3) goes to 1 and hence disappears from the equation, therefore, Eq. (3) becomes Eq. (8). If the values for F, C, and Eq. (3) (or k_1 for a non-enzymatic reaction). These values can be calculated from the slope of the $\ln \Phi$ versus t plot are known then calculation of H, from a rearrangement of Eq. (8), to give Eq. (9) is possible.

$$\Phi_{t=0} = -FCH(1 - e^{(k[E]_{tot}/K_M)\tau}) \quad (8)$$

$$H = \frac{\Phi_0}{FC(1 - e^{-(k[E]_{tot}/K_M)\tau})} \quad (9)$$

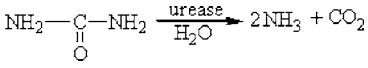
The second method is to calculate H at every time point, t, for the lifetime of the first-order reaction, and to take the average value for H across all these time points. Again this is readily achieved by a simple rearrangement of Eq. (3) to yield Eq. (10). This equation can be entered into an appropriate software package, e.g. Microsoft Excel and an algorithm written, which automatically returns the value of H at any time t hence allowing the average to be calculated.

$$H = \frac{\Phi}{FC(1 - e^{(-k[E_{tot}]/K_M)\tau})\, e^{-(k[E_{tot}]/K_M)t}} \quad (10)$$

In principle this averaging method will yield a more accurate value for the enthalpy. If the enthalpy is now known then it is trivial to calculate $k[E]_{tot}$ from the value of the calorimetric output under zero-order conditions by using Eq. (6).

The method described above shows how it is now possible to calculate directly the Michaelis–Menten parameters from isothermal microcalorimetric data free from assumption. This has been tested through a study of the urea/urease system. This enzymatic system is extremely well known and has been studied extensively over many years [18-21].

The overall reaction scheme is expressed below.



2. Experimental protocol [16]

Sodium di-hydrogen phosphate·$12H_2O$ and di-sodium hydrogen phosphate·$12H_2O$ were obtained from Sigma Aldrich (ACS reagent) and were used at a total phosphate concentration of 0.75 M for the buffer solution (pH 7.0). Urea (ACS reagent) was also obtained from Sigma Aldrich and used without further purification. Urease (ACS reagent from jack bean) was obtained from Sigma Aldrich biochemicals and stored at 5 °C. Fresh solutions of 10 units (ml buffer)$^{-1}$ were prepared for each experiment.

Different concentrations of urea were freshly prepared, in buffer, for each experiment. Concentrations of urea used were 0.02, 0.03, 0.04, 0.05, 0.1, 0.2 and 0.4 mol dm^{-3}. All experiments were performed using an LKB 10700-1 flow calorimeter operated at 298 K and a flow rate of 7.17×10^{-6} dm^3 s^{-1}. Data was collected using Picolog^{TM}.

A 50-ml aliquot of the urea solution is pre-thermostated to the operational temperature of the calorimeter and run in a continuous loop, at a known flow rate, until a stable baseline is achieved. This solution is then inoculated with 4.55 ml of a standard, fixed concentration, urease solution also buffered to pH 7.0 and the resulting calorimetric output recorded as a function of time. This is repeated for all concentrations of urea.

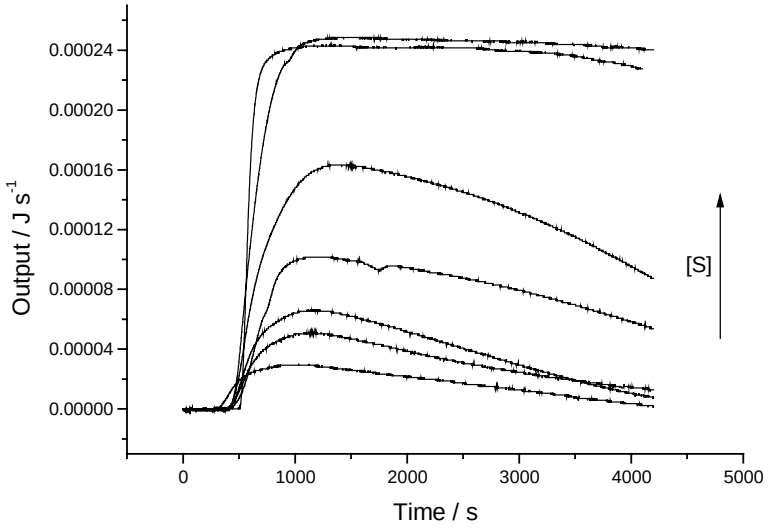


Fig. 1. Calorimetric outputs for first-order, mixed order and zero-order urea/urease enzymatic reactions.

3. Results

Experimental data was exported from Picolog to Microcal Origin and Microsoft Excel where all further data analysis was conducted.

Fig. 1 shows the observed calorimetric outputs across a range of substrate concentrations, from first-order kinetic behaviour (substrate concentration of 0.02–0.04 mol dm^{-3}) through to mixed order kinetic behaviour (substrate concentration of 0.04–0.1 mol dm^{-3}) and finally to zero-order kinetic behaviour (substrate concentration of 0.2–0.4 mol dm^{-3}).

As noted earlier, at low substrate concentration, the slope of the ln Φ versus t plot yields a straight line with slope equal to the first-order rate constant for the reaction (Fig. 2). It can be shown by rearrangement of Eq. (5) that the slope of the ln Φ versus time plot is equal to $-k[E]_{tot}/K_M$ (which is also equivalent to the first-order rate constant). Eq. (10) allows an average enthalpy across the lifetime of the first-order reaction to be calculated, this average was calculated to be -10.6 kJ mol^{-1} for all substrate concentrations within the first-order kinetic region. Table 1 shows that the two methods (described earlier) produce results that are consistent with each other. However, note that the averaging method yields a value for the enthalpy, which is more consistent over a range of substrate concentration compared with those values derived via extrapolation. It would appear, therefore, that the average value obtained, for the enthalpy, over the lifetime of the first-order reaction is indeed more precise and hence the averaging technique preferable to the extrapolation. Also

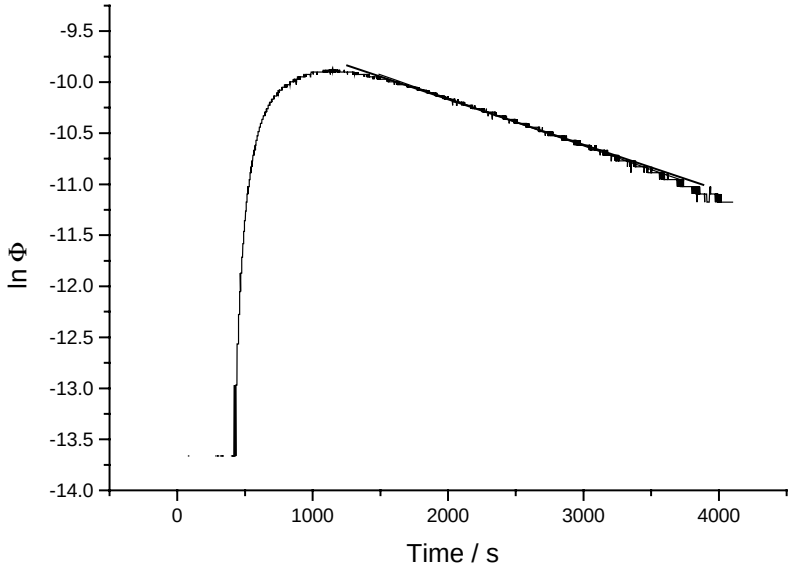


Fig. 2. A plot of ln Φ vs. t for the determination of the first-order rate constant for the urea/urease enzymatic system.

Table 1
Comparison of values obtained for H using extrapolation and average techniques

Substrate concentration (mol dm^{-3})	H from extrapolation to t = 0 (kJ mol^{-1})	H from average over lifetime (kJ mol^{-1})
0.02	−8.84	−8.83
0.03	−10.7	−10.6
0.04	−10.4	−10.6

note that at lower substrate concentrations, the derived value for the enthalpy is not consistent with those at higher concentrations. This is not unexpected and can, in part, be attributed to lack of sensitivity of the calorimeter at low concentrations of substrate. Also note at lower substrate concentrations, it is possible that the magnitude of the change in the calorimetric signal is not sufficient to derive an accurate value for the rate constant this too will impact on the accuracy of any derived value for the enthalpy. It should also be noted that the values reported for the enthalpy in this study and that of Beezer et al. [17] are representative of the overall reaction enthalpy and not just the conversion of substrate to product. This has been dealt with in some detail by Wolf et al. [21], who report values for the enthalpy for each reaction step.

It should also be noted that the calorimetric signal can be directly converted into rate by simply dividing the calorimetric signal by the enthalpy for reaction, this is demonstrated in Fig. 3.

$$\frac{\Phi\ (\mathrm{J\,s^{-1}})}{H\ (\mathrm{J\,mol^{-1}})} = \text{rate}\ (\mathrm{mol\,s^{-1}})$$

It is clear from the form of both Figs. 1 and 3 that the urea/urease system does conform to Michaelis–Menten type kinetics (i.e. saturation kinetics are observed with increasing substrate concentration). Therefore, it is appropriate to apply the equations outlined earlier in an analysis of this enzymatic system.

As described earlier, once the enthalpy is known then all other parameters become accessible. Eq. (6) describes the output for an enzymatic reaction under zero-order conditions (i.e. excess substrate). It is clear that if the enthalpy and thermal volume are known for the reaction then it is trivial to calculate $k[\mathrm{E}]_{\mathrm{tot}}$ from the zero-order output Eq. (11).

$$\frac{\Phi}{V_{\mathrm{c}}H} = k[\mathrm{E}]_{\mathrm{tot}} \tag{11}$$

where $k[\mathrm{E}]_{\mathrm{tot}} = 3.4 \times 10^{-4}$.

The slope of the $\ln \Phi$ versus time plot is linear with slope equal to the first-order rate constant which can also be shown to be equal to $-k[\mathrm{E}]_{\mathrm{tot}}/K_{\mathrm{M}}$.

Hence,

$$\text{Slope} = -\frac{k[\mathrm{E}]_{\mathrm{tot}}}{K_{\mathrm{M}}} = k_1, \qquad k_1 = 4.9 \times 10^{-4}\,\mathrm{s^{-1}}$$

$$K_{\mathrm{M}} = -\frac{k[\mathrm{E}]_{\mathrm{tot}}}{4.9 \times 10^{-4}}, \qquad K_{\mathrm{M}} = 0.07\,\mathrm{M}$$

4. Discussion

The protocol for this study was obtained from a previous paper published by Beezer et al. [17] some 30 years ago. They analysed their data as described earlier and made the assumption that the thermal volume (at their particular flow rate) was identical to the physical volume of their calorimetric cell (0.45 ml) in order to calculate a value for the enthalpy of reaction. The reported values for the rate constant, k_1, Michaelis constant, K_{M} and enthalpy, H are $4.8 \times 10^{-4} \pm 1.4 \times 10^{-5}\,\mathrm{s^{-1}}$, 0.05 M and $-33 \pm 1\,\mathrm{kJ\,mol^{-1}}$, respectively.

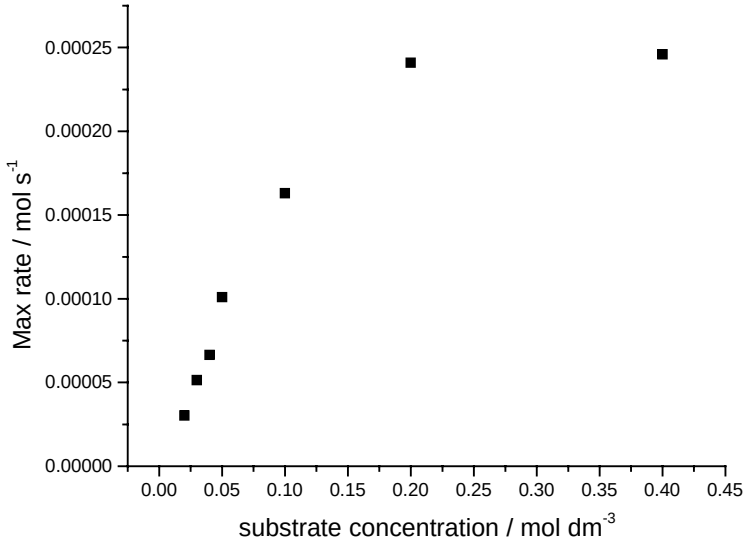


Fig. 3. Max rate vs. substrate concentration for the urea/urease enzymatic reaction.

The values calculated for the Michaelis constant, K_M, and rate constant, $k_1/(k[E]_{tot}/K_M)$ in this study correspond well with the values derived by Beezer et al. [17] in their identical study. This is to be expected since the rate constant is not governed by the thermodynamics of the system, it is merely a reflection of the rate of change in the signal. The Michaelis constant is a function of several rate constants and also is not dependent on the thermal volume of the calorimeter. However, the enthalpy is dependent on thermal volume and it was found that derived values for the enthalpy vary markedly. Beezer et al. [17] report a value of approximately $-33\,kJ\,mol^{-1}$ compared with $-10.6\,kJ\,mol^{-1}$ found in this study. This significant difference between the two can most likely be accounted for in the assumptions made by Beezer et al. for the values of the thermal volume and $k\tau$. Recall the value used for V_c in their analysis was 0.45 ml, in fact this value should have been closer to 0.65 ml, at the flow rate employed in their study (Beezer et al. performed their study with an instrument identical to that used in this study). Since the kinetic parameters (k and K_M) were found to be similar for the two studies it is likely that the difference between the two observed values for the enthalpy (-10.6 and $-33\,kJ\,mol^{-1}$) can be accounted for in the inaccurate value used for V_c in the study by Beezer et al.

5. Conclusions

This paper has described a new technique for the direct determination of Michaelis–Menten parameters, for enzymatic systems, from flow calorimetric data. The technique has been demonstrated through a study of the urea/urease enzymatic system. The results obtained from this study, using the new technique, have been compared with the results obtained from an identical study (published some 30 years ago), obtained using several (flawed) assumptions, particularly the assumption that physical volume is identical to effective thermal volume. This is not critical to the elucidation of accurate kinetic parameters, however, it has a significant impact on the derived value for the enthalpy of reaction. The results reported here have shown that the effect this variation can have is as much as 25%, at the flow rate employed in this study, and hence clearly highlight the importance of having accurate values for τ and V_c if valid thermodynamic and kinetic data are to be obtained from flow calorimetric studies. It is possible, therefore, that any values obtained from the older method of analysis (described by Beezer et al.) are inaccurate and should be treated with caution. However, it is possible to correct these data if the physical volume used to elucidate the enthalpy is known and the effective thermal volume can be calculated retrospectively.

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