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# Flow-calorimetry – a tool for investigation of reactions with incomplete conversion?[1]

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**Abstract**

In this article a simple mathematical model is presented, which can be used to describe the distribution of reactant concentrations, heat power and temperature in the column of a non-adiabatic flow-calorimeter with a one-point temperature determination. The calculations are based on the properties of a reaction catalyzed by a fixed bed of immobilized enzyme in a so-called enzyme thermistor. With this model it becomes possible to obtain kinetic parameters of enzymatic reactions from calorimetric investigations under conditions of local variation of the reaction rate along the axis of the calorimetric column. It has been demonstrated how the presented model can be used for an optimization of the experimental conditions by means of error simulation. The verification of the model using a real reaction is shown. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Flow-calorimetry; Enzyme thermistor; Immobilized enzyme; Error simulation; Kinetic parameter

## 1. Introduction

As was pointed out in an earlier publication [1] fast and reliable methods for the determination of kinetic parameters of immobilized enzymes are required. Several authors suggest the use of flow-calorimetric devices with fixed beds of immobilized enzymes for this purpose [2–4]. Gemeiner et al. suggested a mathematical model for the determination of kinetics parameters using the enzyme thermistor [5]. The model is based on the approximation of an adiabatic calorimeter and a constant reaction rate along the axis of the fixed bed. The measured temperature difference is proportional to the reaction rate and can be directly used in the kinetic equation to determine kinetic parameters. The application of the model is limited because it turned out that the enzyme thermistor is non-adiabatic and the reaction rate cannot be assumed as constant along the axis of the column.

Therefore, we simulated the conditions in such a flow-calorimeter considering non-adiabatic conditions and a change of the reaction rate along the column.

In this paper we want to explain how to use simple separate mathematical models for the reaction as well as the calorimeter to describe the distribution of reactant concentration, heat power and temperature

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along the axis of the calorimetric column and how these models can be used to determine kinetic parameters of enzyme catalyzed reactions. Further we want to show the application of the suggested models to optimize the experimental conditions. In the second part the verification of the model using a real reaction will be described.

## 2. Theory

The described models are developed based on the properties of a modified enzyme thermistor [5]. In this device the immobilized enzyme is fitted into the calorimetric column as a fixed bed. The liquid medium is flowing through this fixed bed with a constant flow rate. The models describe the local distribution of concentration, heat power and temperature along the column under steady state conditions. At the end of the column the temperature of the flowing liquid is measured by a thermistor. The temperature at the end of the column is influenced by a set of parameters including amount of enzyme, substrate concentration, heat of reaction, kinetic parameters of the investigated reaction, flow rate, heat capacity and heat resistance of the calorimeter. The fact that one group of parameters (concentrations, heat of reaction and kinetic parameters) belongs to the reaction, whereas the other group includes properties of the calorimetric device, allows to develop separate models for reaction and calorimeter.

### 2.1. The reaction model

For the description of the reaction in the column it is necessary to know the kinetic equation of the reaction. A kinetic equation for a simple enzymatic reaction

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \tag{1}$$

according to Michaelis and Menten was assumed. Possible diffusion effects of substrate into the particles of the immobilized enzyme are neglected.

The Michaelis–Menten equation is used in the following form:

$$\frac{\mathrm{d}c_\mathrm{P}}{\mathrm{d}t} = \frac{k_2 c_\mathrm{E} c_\mathrm{S}}{K_\mathrm{M} + c_\mathrm{S}}, \tag{2}$$

where $c_\mathrm{P}$, $c_\mathrm{E}$ and $c_\mathrm{S}$ are the concentration of product, enzyme and substrate, respectively, $k_2$ the rate constant of the second reaction step in (1) and $K_\mathrm{M}$ the Michaelis-constant which can be understood as a combination of the rate constants of the partial reactions in (1):

$$K_\mathrm{M} = \frac{k_{-1} + k_1}{k_2}. \tag{3}$$

If the immobilized enzyme is distributed homogeneously in the column while plug flow occurs, the fixed bed can be divided into $n$ identical segments with the volume $V_\mathrm{Seg}$,

$$V_\mathrm{Seg} = \frac{V}{n}. \tag{4}$$

If the segments are sufficiently small, every segment behaves as a perfectly stirred tank reactor, which means the fixed bed is described using tanks in series model [6]. The conversion of substrate in every segment can be calculated using the following balance:

$$(c_\mathrm{Si} - c_\mathrm{So})\dot{V} = -\frac{\mathrm{d}c_\mathrm{So}}{\mathrm{d}t} V_\mathrm{Seg}, \tag{5}$$

where $c_\mathrm{Si}$ is the input and $c_\mathrm{So}$ the output concentration of substrate in the liquid entering the segment, and $\dot{V}$ is the flow rate [6].

Combination of Eqs. (2) and (5) gives:

$$(c_\mathrm{Si} - c_\mathrm{So})\dot{V} = \frac{k_2 c_\mathrm{E} c_\mathrm{So}}{K_\mathrm{M} + c_\mathrm{So}} V_\mathrm{Seg}. \tag{6}$$

In this equation the concentration of enzyme $c_\mathrm{E}$, a quantity poorly defined for an immobilized enzyme, can be substituted by the amount of enzyme $n_\mathrm{E}$ per segment as shown by Schellenberger et al. [7]:

$$c_\mathrm{E} = \frac{n_\mathrm{E}}{V_\mathrm{Seg}}. \tag{7}$$

So the conversion for a given segment in the calorimetric column becomes:

$$(c_\mathrm{Si} - c_\mathrm{So})\dot{V} = n_\mathrm{E} k_2 \frac{c_\mathrm{So}}{K_\mathrm{M} + c_\mathrm{So}}. \tag{8}$$

Setting the output concentrations of one segment as the input concentration of the following, it is possible to calculate the concentration of substrate in every segment and so the distribution of product and substrate along the axis of the reactor under steady state conditions.

If the concentration of substrate and product in every segment is known, we can calculate the distribution of heat power $\dot{q}$. The heat power released in segment $n$ can be obtained using

$$\dot{q} = \Delta_R H c_{Pn} \dot{V}, \quad (9)$$

where $\Delta_R H$ is the heat of reaction and $c_{Pn}$ the concentration of product formed in segment $n$. The local distribution of heat power along the column forms the link to the second model, the flow-calorimeter.

### 2.2. The calorimetric model

For the development of a mathematical model of the non-adiabatic column of a flow-calorimeter a further simplification is necessary. The segments are approximated as points with uniform distribution of temperature and concentration. Eq. (10) shows the heat power balance for a segment:

$$\Delta_R H \dot{V}(c_{Pi} - c_{Pi-1}) = \frac{1}{R_{th}}(T_i - T_S) + \dot{V}\rho C_p(T_i - T_{i-1}) \quad (10)$$

In this equation $C_p$ is the heat capacity, $\rho$ the density of the flowing medium, $R_{th}$ the thermal resistance of the segment to the surroundings, $(T_i - T_S)$ is temperature difference between the segment $i$ and the surroundings and $(T_i - T_{i-1})$ is the temperature difference between segment $i$ and the previous segment.

$\Delta_R H \dot{V}(c_{Pi} - c_{Pi-1})$ is the part set free by the reaction, $1/R_{th}(T_i - T_S)$ is the heat conduction to the surroundings and the product $\dot{V}\rho C_p(T_i - T_{i-1})$ is a convective part.

For the first segment the temperature $T_{i-1}$ is the ambient temperature $T_S$.

Using both models for the reaction and the calorimeter it is possible to calculate the temperature difference $(T_i - T_S)$ for the last segment, the quantity measured with the enzyme thermistor.

### 2.3. Optimizing the experimental conditions

The described model was applied to the invertase-catalyzed hydrolysis of sucrose to glucose and fructose. The developed model was utilized to optimize the experimental strategies and conditions. The hydrolysis of sucrose by invertase was chosen as model reaction because it is known to follow over a certain range of substrate concentration a Michaelis–Menten-mechanism and because it was already used for investigation of enzyme kinetic problems [8]. Aim of the work is to determine the values for the kinetic parameters $k_2$ and $K_M$. To calculate the kinetic parameters by non-linear regression using the Matlab/Simulink platform it is possible to use the temperature difference either as a function of flow rate or of substrate concentration.

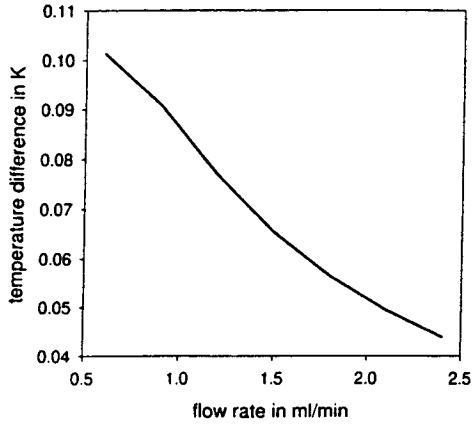


Fig. 1. Calculated temperature difference as a function of flow rate.

In Figs. 1 and 2 the calculated temperature difference as a function of both variables is shown. For flow rate realistic range was chosen, which means a range which can be used with the technical equipment

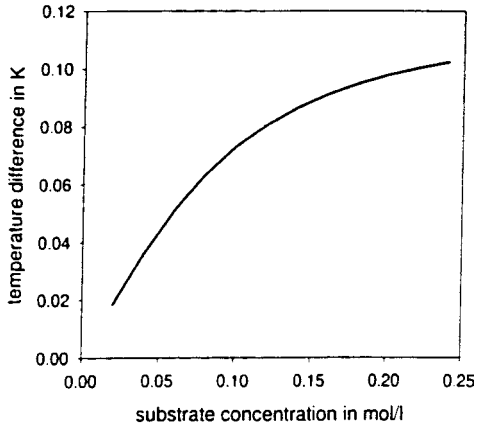


Fig. 2. Calculated temperature difference as a function of the substrate concentratioin.

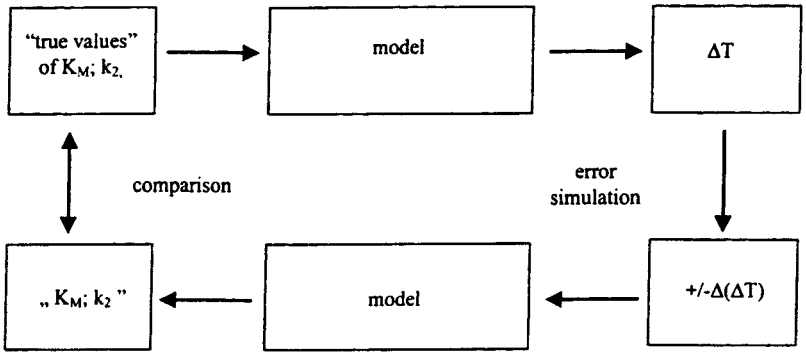


Fig. 3. Principle of the error simulation.

(0.5–2.5 ml/min). A reasonable variation of substrate concentration (0–0.25 mol/l) was applied, taking into account the values of $K_M$ (0.04 mol/l) for the free enzyme [8]. Setting the number of segments $n$ to 100 turned out to be a good compromise between expenditure for calculation and the precision of the results.

To test how reliable the mathematically "good" values are in terms of their physical meaning an error simulation was carried out. The principle of this error simulation is shown in Fig. 3.

The calculations of the functions $\Delta T = f(c_S)$ with $\dot{V} = \text{constant}$ and $\Delta T = f(\dot{V})$ with $c_S = \text{constant}$ were done with given "true" values of the kinetic parameters $K_M$ and $k_2$. These theoretical curves where varied by a random error of $\Delta T$. The error of $\Delta T$ is considered to include all other errors, e.g. of flow rate, concentration etc., because under experimental conditions all these errors will be expressed as errors of the temperature difference. In a third step the obtained "incorrect" curves were used to recalculate values for $K_M$ and $k_2$ which were compared with the given "true" values. The results of this error simulation for $K_M$ and $k_2$ are shown in Fig. 4(a) and (b). The figure shows that the Michaelis-constant is much more sensitive to errors of the measured temperature difference than $k_2$. The more reliable values for the Michaelis-constant $K_M$ as well as the rate constant $k_2$ are obtained by measuring $\Delta T$ as a function of substrate concentration than as a function of flow rate.

This can be explained because under the conditions of the studied combination of enzymatic reaction

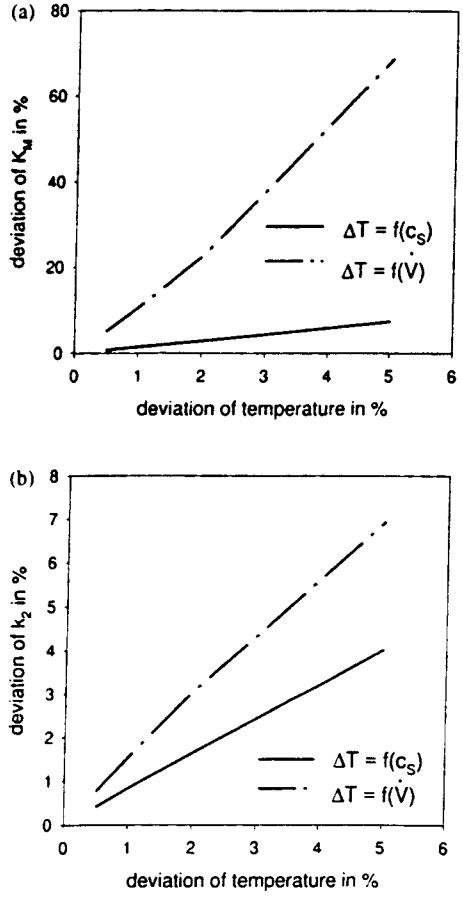


Fig. 4. (a) Relative deviation of $K_M$ recalculated from given input data. (b) Relative deviation of $k_2$ recalculated from given input data.

and flow-calorimeter, variation of $K_M$ and $k_2$ for $\Delta T = f(\dot{V})$ only lead to parallel shifts of the values of $\Delta T$, whereas the almost linear form of the function remains relatively unchanged. Thus two variables which act the same way are to calculate from an almost linear function, which leads to a great uncertainty even if the values are mathematically "good". On the other hand for $\Delta T = f(c_S)$ both kinetic parameters act in different ways. The main effect of the variation of $k_2$ is a parallel shift in the values of $\Delta T$ whereas the influence of the Michaelis-constant is mainly expressed as a change of the curvature of the function.

So the better strategy to determine the kinetic parameters of such reactions using the described flow-calorimetric device is to measure the temperature difference as a function of the substrate concentration. The optimal flow rate has to be found for the measurement of the $\Delta T = f(c_S)$ function. That's why a second error simulation was carried out, calculating the error of $K_M$ and $k_2$ as a function of the flow rate at constant error for $\Delta T$ of 2%. The result of this simulation (Fig. 5) is comparable with the first simulation. The value of the Michaelis-constant $K_M$ is much more sensitive than $k_2$ to incorrect values of the temperature difference. With a decreasing flow rate the error of $K_M$ increases exponentially, the error for $k_2$ remains relative constant.

To determine the kinetic parameter with a reasonable error, it is necessary to use a flow rate as high as possible. The conversion of the substrate and consequently the temperature difference decreases rapidly with increasing flow values and it becomes difficult to measure this temperature difference precisely. The investigation based on the developed model yields meaningful kinetic parameter in a certain range of flow rate and substrate concentrations. This "window" of experimental conditions can be shifted for different reactions. If the values for $\Delta_R H$, $K_M$ and $k_2$ are approximately known, the experimental setup can be estimated with the presented models.

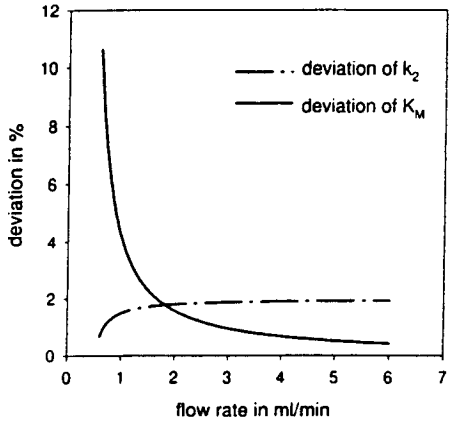


Fig. 5. Relative deviation of calculated $K_M$ and $k_2$ as a function of flow rate, using $\Delta(\Delta T) = 2\%$.

## 3. Experimental

A first verification of the calorimetric model was performed using the hydrolysis of sucrose catalyzed by invertase immobilized at Eupergit C. The reaction was used as a basis for the theoretical calculations described above. The values for $\Delta_R H$, $k_2$ and $K_M$ are known from literature for the free enzyme as well as for the enzyme immobilized at several carriers [8–12].

### 3.1. Reagents

Lyophilized invertase (β-fructofuranosidase, E.C. 3.2.1.26.) from yeast was purchased from Merck and used without further purification. As carrier for the enzyme Eupergit C®, oxirane acrylic beads (Röhm Pharma, Weiterstadt, Germany) was applied. Sucrose was purchased from Merck. All other used substances were of analytical grade.

Invertase (β-fructofuranosidase) was connected to the carrier material Eupergit C by the following procedure:

1 g of dry Eupergit C carrier was suspended in ca. 5 ml of 1 M phosphate buffer, pH = 7.5 and 100 mg of lyophilized invertase were added. After gently shaking, the mixture was allowed to react for 20 h at room temperature. The Eupergit C-invertase beads were collected at a sintered glass-filter and washed with three aliquots of 0.1 M phosphate buffer pH = 7.5. The enzyme-carrier combination was stored at 4°C in the same buffer containing 0.05%$NaN_3$ to avoid bacterial growth. The amount of enzyme bound to the carrier was determined by measuring the invertase

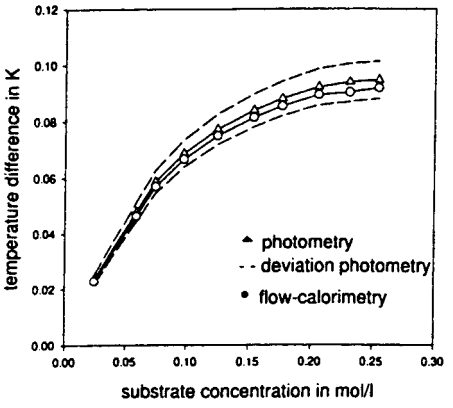


Fig. 6. Comparison of the results of photometric and calorimetric experiments for invertase.

concentration in the washing solution using the Bradford-assay [11,12].

### 3.2. Verification of the calorimetric model

The enzyme carrier complex was fitted into the column between porous plates as fixed bed. Measurements were carried out with a flow rate of 2 ml/min. The concentration of sucrose was varied in a range of 0.02–0.25 mol/l. A sodium acetate buffer (0.05 M, pH = 4.6) was used as flowing medium. To verify the calorimetric model the glucose concentration in the medium leaving the column was measured photometrically using a method given in [8]. The concentration of glucose is equal to the concentration of sucrose converted during the reaction. From the known conversion the values for $K_M$ and $k_2$ were calculated. These values were compared with the values calculated from the measured temperature differences $\Delta T$. As can be seen in Fig. 6 and Table 1 the calorimetrically determined values are in good agreement with these obtained photometrically. The measured function $\Delta T = f(c_S)$ is within the range calculated from the values determined independently. It is difficult to compare measured kinetic parameters with values given in literature, because they differ for different carrier materials, enzyme sources and experimental conditions (pH, temperature, buffer). Ranges of kinetic parameters for immobilized invertase are given in Table 1.

Table 1
Kinetic parameters for immobilized invertase

| | Flow-calorimetry | Photometry | Literature [9,10] |
|---|---|---|---|
| $K_M$ (mmol/l) | 34.2 (±4.1%) | 38 (±11%) | 29–100 |
| $k_2$ ($s^{-1}$) | 238 (±1.9%) | 250 (±5.1%) | 505–750 |

## 4. Summary

In this paper a combination of two simple mathematical models for the description of the conditions in a non-adiabatic flow-calorimeter is suggested. It could be shown how these models can be used to calculate the distribution of substrate and product concentration, heat power and temperature along the calorimetric column. The determination of kinetic parameters of an enzyme catalyzed reaction was demonstrated. The possibility to develop and test measuring strategies for flow-calorimetry was shown. In a further part a first verification of the calorimetric model was carried out.

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