Addressing Sensitivity and Non-Uniqueness in the Determination of Enzyme Kinetic Parameters

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Accurate determination of enzyme kinetic parameters is critical for model-based design and intensification of biocatalytic processes, particularly in microscale systems. While Michaelis-Menten kinetics provides a foundational framework, its extension to reversible, multi-substrate, and inhibited reactions introduces significant challenges in parameter estimation-most notably, parameter sensitivity and non-uniqueness.

This study systematically investigates these challenges across three case studies of increasing complexity: (i) mono-substrate Michaelis-Menten kinetics, (ii) reversible enzymatic reactions with four parameters, and (iii) a six-parameter reversible mono-substrate kinetic model with substrate and product inhibition. In the first two cases, we show that vastly different parameter sets can yield nearly indistinguishable model fits to experimental data, exposing the limitations of classical graphical and nonlinear regression methods. In the mono-substrate case based on real experimental data, two parameter sets differing by nearly two orders of magnitude produce virtually identical model outputs, demonstrating practical non-uniqueness even for simple kinetic models.

For the six-parameter inhibited system, a theoretical and numerical analysis reveals intrinsic non-uniqueness of the parameter estimation problem, characterized by an infinite family of parameter vectors yielding identical solutions. These results demonstrate that parameter non-uniqueness is not merely a consequence of experimental noise, but a structural property of complex kinetic models, emphasizing the need for more robust and structurally informed modeling approaches in biocatalysis.

Keywords

enzyme kinetics, kinetic parameter estimation, parameter non-uniqueness

Introduction

Accurate determination of kinetic constants for enzyme-catalyzed reactions is essential for the design and optimization of biocatalytic processes, especially in microreactor systems, where transport phenomena are often minimized, and the reaction rate becomes the dominant performance factor. In most cases, enzymatic reactions follow Michaelis-Menten kinetics, which provides a simplified yet powerful model to describe substrate saturation effects and to extract meaningful kinetic parameters.

However, as the complexity of biocatalytic systems increases—with reversible reactions, multiple substrates, and product or substrate inhibition—the classical Michaelis-Menten framework is no longer sufficient. It must be extended to account for bisubstrate mechanisms), inhibition effects, and equilibrium constraints. Although such extensions are necessary for accurate modeling, they introduce additional parameters and significantly complicate parameter estimation[1,2].

Two principal approaches are traditionally used for kinetic parameter estimation: graphical plotting (GP) and nonlinear regression (NLR). Graphical methods rely on linearization of the rate equations under varying initial conditions (e.g., Lineweaver-Burk, Hanes, Dixon plots), whereas NLR techniques use numerical optimization to minimize the deviation between experimental and simulated data. While GP methods are simple and intuitive, they often fail for complex models. NLR methods, although more powerful, are prone to parameter sensitivity and non-uniqueness, particularly in systems with four or more fitting parameters[2,3].

Recent studies have demonstrated that in multi-parameter models – such as reversible reactions with inhibition or bisubstrate formulations – many distinct t parameter combinations can yield nearly indistinguishable model outputs[2]. This be-

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havior indicates that the parameter estimation problem may be ill-posed: the objective function may lack a well-defined global minimum, or the Jacobian matrix may exhibit near-zero eigenvalues, corresponding to flat directions in parameter space[3,4].

As emphasized by Woodley and co-workers[1,2], the development of robust biocatalytic processes requires not only accurate experimental data but also structurally informed modeling and parameter estimation strategies that reduce ambiguity. Approaches such as initial rate analysis, perturbation methods, and Jacobian-based identifiability analysis are therefore becoming increasingly important in modern enzymology.

This paper addresses these challenges through three illustrative case studies of increasing complexity. The first case examines a mono-substrate Michaelis-Menten system, focusing on parameter sensitivity under noise-free and noisy experimental conditions. The second case considers a reversible mono-substrate biotransformation described by a four-parameter kinetic model. The third case investigates a six-parameter kinetic model describing a reversible mono-substrate biotransformation with substrate and product inhibition and demonstrates intrinsic parameter non-uniqueness through null-space analysis of the Jacobian matrix. Mechanistically related enzyme kinetic models, such as ping-pong bi-bi formulations, share a similar rational structure and therefore exhibit the same non-uniqueness behavior.

In micro(bio)reactor systems, reliable estimation of kinetic parameters is particularly important because the selected kinetic model directly defines the reaction characteristic time, which—together with the residence-time and diffusion characteristic times—forms the basis for time-scale analysis and model-based design of microscale bioreactors. Our findings therefore emphasize the need for more robust and structurally informed modeling approaches in biocatalysis.

Sensitivity and non-uniqueness challenges in determining enzyme kinetic parameters

To systematically investigate how parameter sensitivity and non-uniqueness emerge in kinetic modeling, three representative enzymatic systems of increasing complexity were analyzed. Each case study builds upon the previous one—starting from the classical mono-substrate Michaelis–Menten model, extending to a reversible four-parameter mechanism, and culminating in a six-parameter reversible model with competitive inhibition. This stepwise approach allows direct comparison of theoretical and practical identifiability, illustrating how small increases in model complexity and experimental noise can turn a well-posed estimation problem into an intrinsically ill-posed one.

Case 1: Sensitivity analysis of mono-substrate Michaelis-Menten kinetics

In this section, we perform a sensitivity analysis on the classical Michaelis-Menten kinetic model, describing an irreversible, mono-substrate enzymatic biotransformation. The rate equation is given by:

$$V = \frac{V_{max} \cdot S}{K_m + S} \tag{1}$$

where V represents the reaction rate (mol m^{-3} s^{-1}), V_{max} denotes the maximum reaction rate (mol m^{-3} s^{-1}), S is the substrate concentration (mol m^{-3}), and K_m signifies the Michaelis constant (mol m^{-3}).

To investigate the practical challenges of estimating the two fundamental Michaelis-Menten parameters (V_{max} and K_m), a set of experiments was conducted in a stirred batch reactor using a model mono-substrate enzymatic biotransformation. The reaction was performed at a selected initial substrate concentration under optimized conditions with respect to pH, temperature, buffer composition, and free enzyme concentration, ensuring that the process was kinetically controlled. The experimental data analyzed in this first case study originate from a real laboratory investigation of the enzymatic reduction of acetophenone to 1-phenylethanol catalyzed by alcohol dehydrogenases (ADHs), previously conducted in our laboratory. All data points shown in Fig. 1 correspond to experimentally measured substrate concentrations, where each point represents the average of three independent measurements with satisfactory experimental reproducibility. For the purpose of this work, only substrate depletion is considered, while product formation is not shown.

As a traditional method, we applied the Lineweaver–Burk linearization to estimate kinetic parameters. This approach is applicable exclusively to the basic form of the Michaelis–Menten equation (Equation 1), which describes mono-substrate biotransformations under initial rate conditions. The parameters can be estimated from a single time-course experiment, where a set of data points—preferably selected from the steeper part of the exponential decay curve, before the plateau region—is transformed and fitted. Alternatively, and more commonly, the method is applied by determining initial rates at several different starting substrate concentrations, enabling a reliable construction of the double-reciprocal plot. This approach trans-

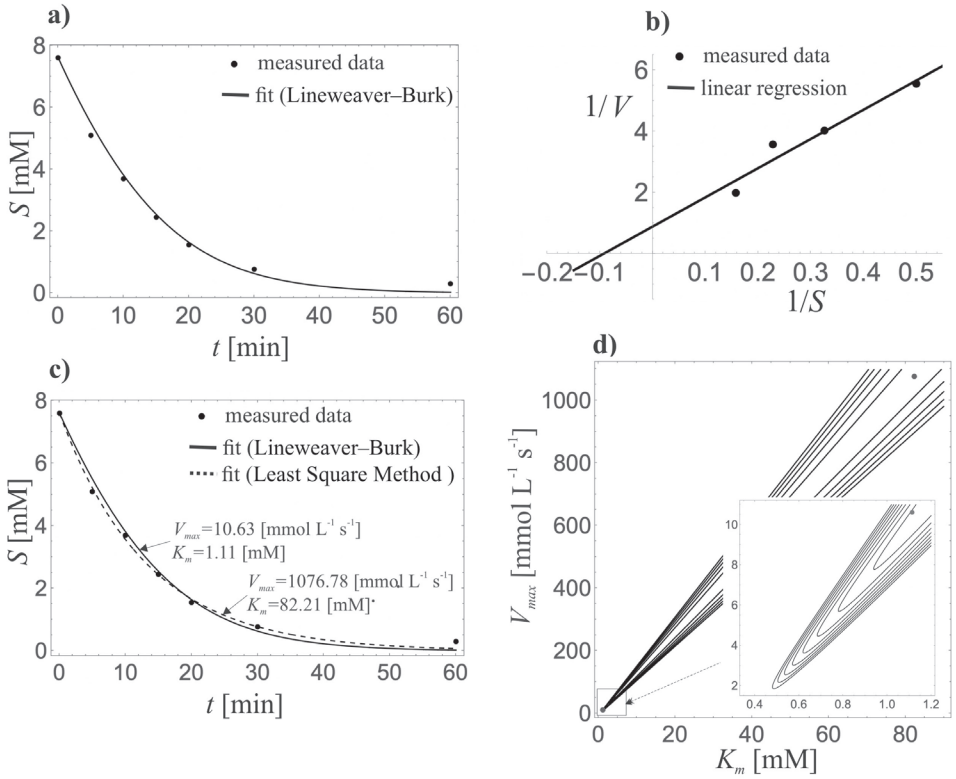

Fig. 1 – *Sensitivity analysis of mono-substrate Michaelis-Menten kinetics with two estimated parameters. 1a) Simulated time-concentration profile using kinetic parameters obtained from Lineweaver–Burk linearization. Experimental data points (black dots) lie close to the fitted curve. 1b) Lineweaver–Burk linearization of Michaelis-Menten kinetics. Black dots represent transformed measurement data (first five points), and the line is the linear regression used for parameter estimation. 1c) Comparison of model fits using two different sets of kinetic parameters. Both curves closely follow the data, illustrating practical non-uniqueness despite significantly different parameter values. 1d) Contour plot of the error function in the V_{max} – K_m parameter space. The narrow valley indicates a region of low error, confirming parameter insensitivity and the existence of infinite near-optimal solutions.*

forms the Michaelis-Menten equation into a linear form for variables $\frac{1}{V}$ and $\frac{1}{S}$:

$$\frac{1}{V}=\frac{K_m}{V_{max}}\frac{1}{S}+\frac{1}{V_{max}} \tag{2}$$

For the given time-course measurement (in minutes) of substrate concentration at a selected initial substrate concentration, S_0=8.0 [mM], we successfully applied the Lineweaver–Burk linearization using the first five data points. This allowed for sufficiently accurate estimation of both kinetic parameters, with the slope and intercept of the resulting double-reciprocal plot providing the values of V_{max} and K_m, respectively. It should be noted that the Lineweaver–Burk method is based on reciprocal reaction rates, which are taken as the inverse of the initial rate of product formation; thus, all $1/V$ values plotted are positive, as shown in Fig. 1b. The resulting parameters, when used in the integrated form of the rate equation, adequately reproduce the experimental trend (solid curve in Fig. 1a), demonstrating good agreement between the model and the measured substrate depletion (Fig. 1a).

To further refine the parameter estimates, we employed a nonlinear least-squares optimization method, fitting the model directly to the full time-course data. For a given parameter pair (V_{max} and K_m), the solution $S(t)$ of the initial value problem:

$$S'(t)=\frac{V_{max}\cdot S(t)}{K_m+S(t)},\ S(0)=S_0 \tag{3}$$

yielding the sum of squared deviations from the measured data (t_i,S_i), $i=1,\ldots,n$,

$$f(V_{max},K_m)=\sum_{i=1}^{n}\left(S_i-S(t_i)\right)^2 \tag{4}$$

where $f(V_{max},K_m)$ represents the error function used to quantify the deviation between the experimental data and model predictions.

The resulting best-fit parameters reduced the overall value of the error function slightly, and the corresponding model curve (dashed line in Fig. 1c) closely matched the experimental data. However, a surprising result emerged: the parameter set obtained via nonlinear regression differed by nearly a factor of one-hundred from that estimated using the Lineweaver–Burk method, even though both model predictions were nearly indistinguishable throughout the entire reaction time course. This example of practical non-uniqueness highlights the insensitivity of the model output to large variations in parameter values.

To visualize this effect more clearly, the error function $f(V_{max}, K_m)$ – defined as the sum of squared deviations between the measured and simulated substrate concentrations – was evaluated over a wide range of parameter combinations. The resulting contour plot (Fig. 1d) shows a narrow, elongated, and apparently open valley of low error values, indicating that multiple parameter pairs yield similarly good fits. This behavior reflects a strong correlation between the parameters and the absence of a clearly defined global minimum – characteristic of structurally ill-posed estimation problems.

Such situations are particularly problematic when only a single experimental condition (i.e., one initial substrate concentration) is used. They underscore the need for global parameter fitting across multiple conditions, as well as complementary tools such as sensitivity analysis and structural identifiability assessment.

Case 2: Sensitivity analysis of a four-parameter kinetic model of a reversible enzymatic reaction

As a representative case illustrating near parameter non-uniqueness in more complex enzymatic systems, we consider the reversible hydration of fumaric acid to L-malic acid catalyzed by fumarase in permeabilized *S. cerevisiae* cells. The reaction proceeds without observable enzyme deactivation over the experimental time window, and at the tested substrate concentrations (≤ 10 mM), product inhibition was negligible. The kinetic model is given by the reversible Michaelis–Menten mechanism [5]:

$$r_1 = -\frac{V_{max_1} C_F}{K_{M,f} + C_F};\ V_{max_1} = k_1 \cdot \gamma$$
$$r_2 = -\frac{V_{max_2} \cdot C_M}{K_{M,m} + C_M};\ V_{max_2} = k_2 \cdot \gamma \quad (5)$$

where r_1 and r_2 (mol m^{-3} s^{-1}) are the rates of fumaric acid hydration and L-malic acid dehydration, respectively, C_F (mol m^{-3}) is the concentration of fumaric acid, and C_M (mol m^{-3}) L-malic acid in batch experiments with free permeabilized *S. cerevisiae* cells. Parameters V_{max1} (mol m^{-3} s^{-1}), k_1 (mol kg^{-1} s^{-1}) and $K_{M,f}$ (mol m^{-3}) are maximal reaction rate, specific rate constant defined per biocatalyst mass, and Michaelis constant for fumaric acid hydration, respectively, and V_{max2} (mol m^{-3} s^{-1}), k_2 (mol kg^{-1} s^{-1}) and $K_{M,m}$ (mol m^{-3}) are the corresponding parameters for L-malic acid dehydration, while γ (kg m^{-3}) is the biocatalyst (yeast cells) concentration.

The system thus involves four kinetic parameters: V_{max1}, $K_{M,f}$, V_{max2}, $K_{M,m}$. These were estimated by simultaneous fitting of batch biotransformation time-courses for five different initial fumaric acid concentrations. Parameter estimation was performed using least squares minimization over all five batch time courses, where the parameters were first approximately located through interactive graphical exploration (using the Manipulate function in Wolfram Mathematica) and then optimized[5].

Two different parameter sets provided nearly indistinguishable fits to all experimental data:

- Set I (solid line in Fig. 2a):
  V_{max_1} = 0.0083 [mM s^{-1}],
  $K_{M,f}$ = 0.986 [mM],
  V_{max_2} = 0.0078 [mM s^{-1}],
  $K_{M,m}$ = 3.304 [mM]
- Set II (dashed line in Fig. 2a):
  V_{max_1} = 0.0072 [mM s^{-1}],
  $K_{M,f}$ = 1.634 [mM],
  V_{max_2} = 0.0263 [mM s^{-1}],
  $K_{M,m}$ = 39.534 [mM].

Both sets resulted in good fits: Set I: absolute error = 0.07448; Set II: absolute error = 0.1081. Here, the absolute error is defined as the sum of squared deviations between the experimentally measured and simulated concentration profiles over the entire time course. Despite significant parameter divergence – particularly in V_{max2} and $K_{M,m}$ – both models predicted nearly identical concentration trajectories (Fig. 2a).

To better understand the cause of this practical non-uniqueness, an error function landscape was analyzed by varying the two Michaelis constants, $K_{M,f}$ and $K_{M,m}$, while keeping other parameters fixed (Fig. 2b). The resulting least-squares error contours revealed a broad, flat valley with minimal error across a wide range of $K_{M,m}$ values. This is a clear manifestation of the structural ill-posedness of the parameter estimation problem in multi-parameter enzymatic models.

This example reinforces the insights from Case I. Even with multiple data sets and four parameters, practically indistinguishable model outputs may arise from widely different parameter sets. Such insensitivity in model outputs can severely limit the utility of fitted parameters for mechanistic interpretation or for predictive use outside the training conditions.

However, in the case of two or four kinetic parameters, there theoretically exists a unique solution to the minimization problem, i.e., a global minimum of the error function.

Unfortunately, this holds true in practice only under "ideal" or synthetic experimental conditions, as demonstrated in the following example.

First, we select a previously determined parameter set obtained from real experimental data (e.g., Set I). Based on the kinetic model for the given biotransformation (Eq. 5), we then generate synthetic data for a wide range of experimental conditions.

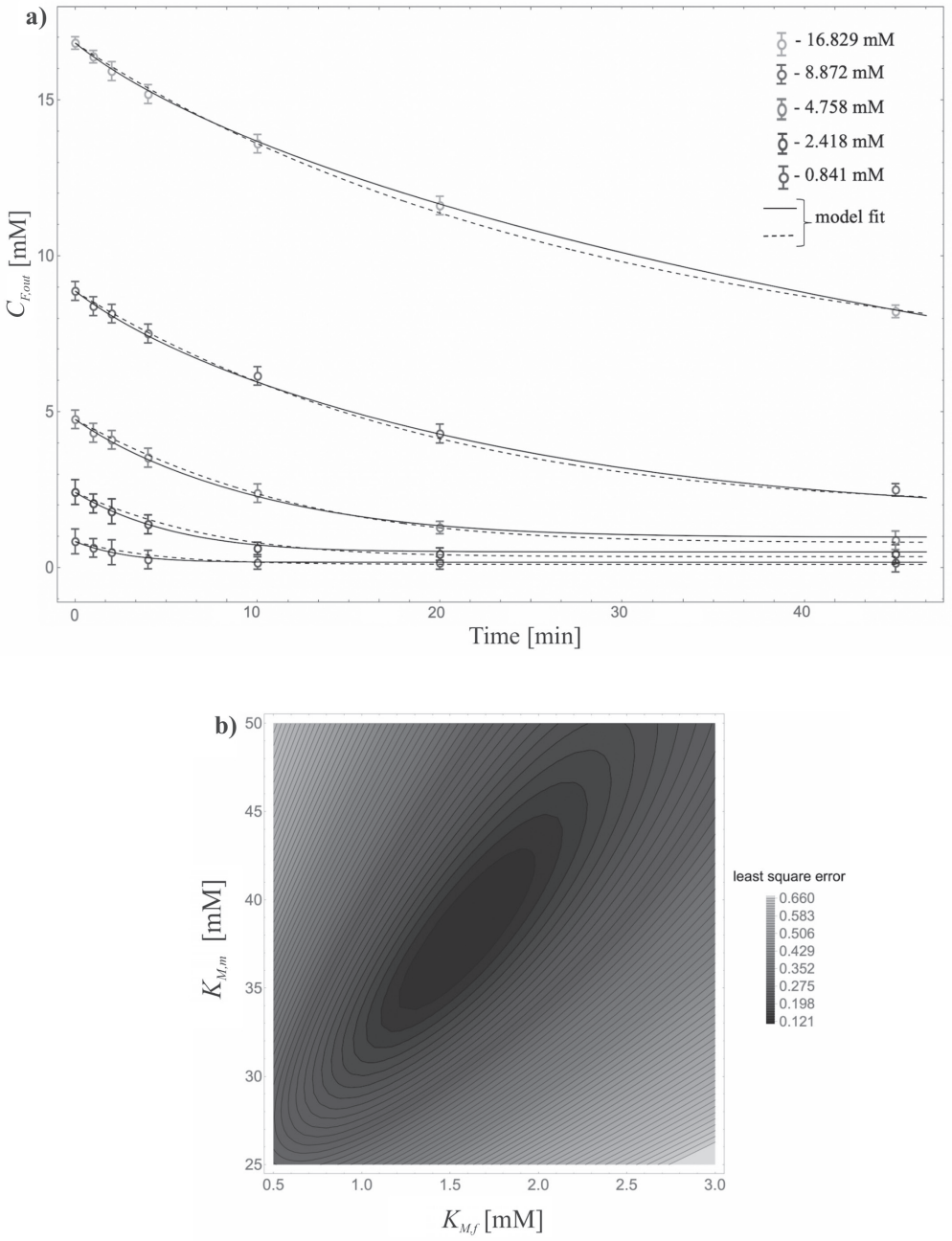

Fig. 2 – *a) Experimental data (mean of two measurements with indicated standard deviations) on the time course of the fumaric acid concentration in a batch process at various initial concentration indicated in the legend and at a given concentration of free cells together with mathematical model simulations comprising the reaction kinetics (Eq. 5) at two sets of kinetic parameters resulting in two model fits: [–] Set I; […] Set II. b) Graphical presentation of the parametric sensitivity of the two parameters out of four for second set […], namely $K_{M,f}$ and $K_{M,m}$, shown as least square error isolines. [Reproduced with permission from[5]: © 2024 Elsevier B.V. All rights reserved. Licensed for reuse under permission from Elsevier (License Number: 1665601-1).*

These may include various initial substrate concentrations, and, in the case of a reversible reaction, different initial product concentrations. The key feature of such ideal or synthetic data is that they perfectly follow the proposed kinetic model equations – in other words, the expected absolute error between the generated data and the model predictions is exactly zero. Naturally, it is unrealistic to expect real measurements to ever achieve this level of agreement, regardless of the precision of analytical methods or experimental execution.

As shown in Fig. 3a, the optimization successfully reconstructs the exact same parameter set that was originally used to create the synthetic data, with an absolute error of zero (Fig. 3b). This confirms the theoretical existence of a global minimum in the four-parameter kinetic model.

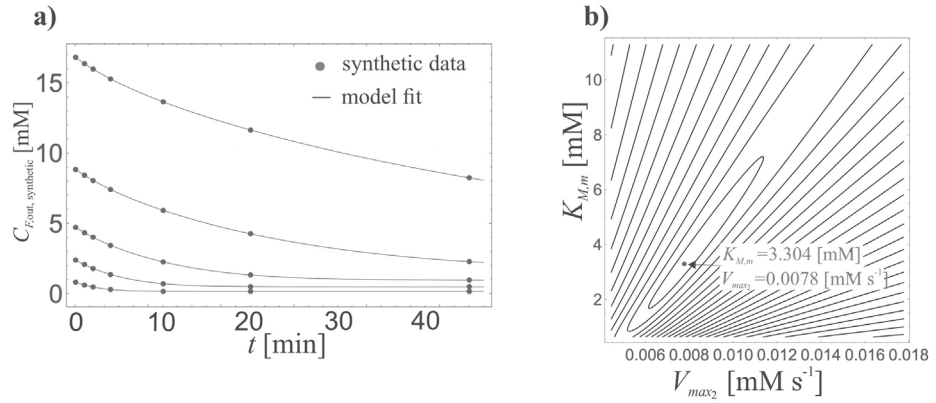

Fig. 3 – *a) Comparison of synthetic data (red dots) with model simulations (solid lines) obtained after re-fitting the model by the conventional least-squares method. The fitted curves exactly overlap with the synthetic data generated from the predefined parameter set (Set I: V_{max_1} = 0.0083 [mM s^{-1}], $K_{M,f}$ = 0.986 [mM], V_{max_2} = 0.0078 [mM s^{-1}], $K_{M,m}$ = 3.304 [mM]). b) Contour plot of the least-squares error function in the parameter space of V_{max_2} and $K_{M,m}$. The global minimum (red dot) coincides with the true parameter values used for generating the data, confirming that the optimization procedure reconstructs the exact parameter set with zero absolute error. This result demonstrates the theoretical existence of a unique global minimum in the four-parameter kinetic model.*

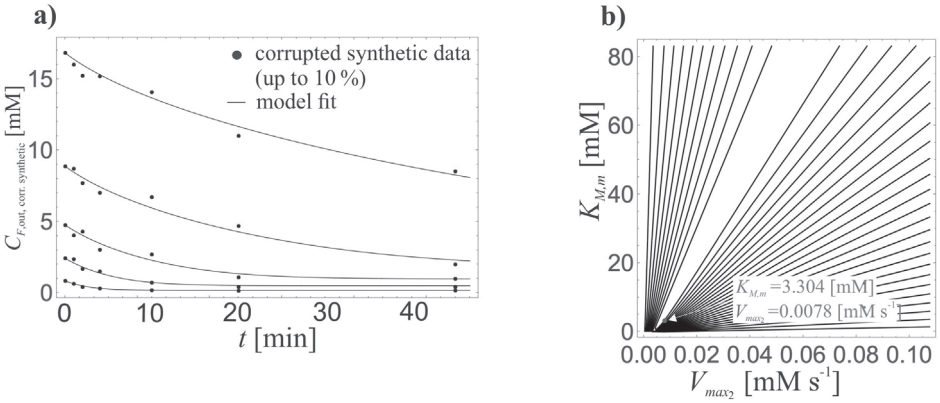

Fig. 4 – *a) Synthetic data perturbed by ±5 % random noise (blue dots) compared with model simulations obtained by least-squares fitting (solid lines). The fitted curves no longer coincide perfectly with the data, mimicking realistic experimental variability and analytical uncertainty. b) Corresponding contour plot of the least-squares error function in the parameter space of $K_{M,m}$ and V_{max_2}. The previously sharp global minimum (see Fig. 3b) broadens into an extended flat valley of nearly constant error, revealing the disappearance of a unique solution and demonstrating practical non-uniqueness under noisy conditions.*

Once the synthetic dataset is generated (Fig. 3a), we intentionally disregard the original parameter values used to generate them. We then apply the standard minimization procedure to refit the model and determine the parameters solely based on these synthetic data.

However, this behavior changes dramatically if we randomly or systematically perturb the synthetic data, shifting the data points away from the model curve by just a few percent (Fig. 4a). With such deviations – mimicking real experimental noise – the theoretical minimum disappears from the accessible parameter space, and we transition into a domain of "infinite valleys" or flat canyons in the error surface. In this region, many very different parameter sets yield similarly low errors, but with significantly different numerical values (Fig. 4b). This again clearly demonstrates the practical non-uniqueness and poor identifiability in multi-parameter enzymatic models – especially in reversible systems and when experimental data are limited or noisy.

Case 3: Addressing non-uniqueness in the six-parameter reversible Michaelis–Menten model with competitive product inhibition

This section investigates the non-uniqueness issue inherent in more intricate enzymatic reaction models. Specifically, we focus on the reversible

Michaelis–Menten model extended with competitive product inhibition, which involves six kinetic parameters:

$$V = \frac{V_{max_f} \cdot S}{K_{M,S}\left(1+\frac{P}{K_{i,P}}\right)+S} - \frac{V_{max_r} \cdot P}{K_{M,P}\left(1+\frac{S}{K_{i,S}}\right)+P} \quad (6)$$

where V is the overall reaction rate (mol m^{-3} s^{-1}); S and P denote the concentrations of substrate and product, respectively (mol m^{-3}); V_{maxf} and V_{max_r} are the maximum reaction rates in the forward and reverse directions (mol m^{-3} s^{-1}); $K_{M,S}$ and $K_{M,P}$ are the corresponding Michaelis constants (mol m^{-3}); and $K_{i,P}$ and $K_{i,S}$ represent the inhibition constants for the product and substrate in the competitive inhibition terms (mol m^{-3}).

The kinetic equations for this model are more complex than in the previous cases and account for inhibition effects in both directions of the reversible reaction. We analyze the challenges associated with determining unique parameter values in such systems, considering the implications for biocatalytic reaction optimization and process design. It is worth noting that a similar problem of non-uniqueness would also arise if one considers the kinetic rate equations describing the Ping–Pong Bi–Bi mechanism, which also typically involves six parameters.

To illustrate the problem of non-uniqueness in the six-parameter reversible Michaelis–Menten model with competitive inhibition (Eq. 6), we refer to a real laboratory case of a reversible mono-substrate biotransformation exhibiting both substrate and product inhibition. From experimental data, six kinetic parameters corresponding to Eq. (6) were determined using a conventional least-squares optimization. Using these parameters, synthetic (ideal) data were generated for an initial substrate concentration of S_0 = 5 [mM]. The time courses of substrate and product concentrations are shown in Fig. 5a,

Table 1 – *Six parameter sextuples obtained by independent optimization of Eq. (6) against the same synthetic data set. All sets yield identical model fits (absolute error = 0).*

V_{maxf}	$K_{M,S}$	$K_{i,S}$	V_{max_r}	$K_{M,P}$	$K_{i,P}$
12.40	71.48	59.72	3.633	21.64	13.60
17.96	105.8	219.6	4.897	30.91	14.76
36.75	221.6	219.6	4.897	30.91	16.28
36.75	221.6	475.0	5.294	33.82	16.28
92.02	562.3	489.3	2.906	16.31	31.93
93.49	571.5	603.4	5.374	34.41	17.27
…	…	…	…	…	…

where the synthetic points are completely covered by the model fit.

Next, we intentionally "forgot" the original parameter set and re-estimated the six kinetic parameters by applying the same least-squares procedure to the synthetic data. As expected for ideal data, the absolute sum-of-squares error equals zero. Remarkably, the optimization procedure yielded not a single but several distinct parameter sextuples, all providing virtually identical fits to the data (Fig. 5a) and resulting in a relative numerical error indistinguishable from zero (Fig. 5b). The six parameter sets summarized in Table 1 represent a specific example of distinct parameter sextuples that reproduce identical model fits. In the following section, we provide a mathematical proof demonstrating that, in fact, an infinite number of such sextuples can satisfy the same model equations and yield indistinguishable data fits.

The mathematical proof presented further herein demonstrates that the observed non-uniqueness is not accidental but inherent to the model structure. An infinite continuum of parameter sextuples can reproduce identical outputs for the reversible Michaelis–Menten kinetics with inhibition (Eq. 6).

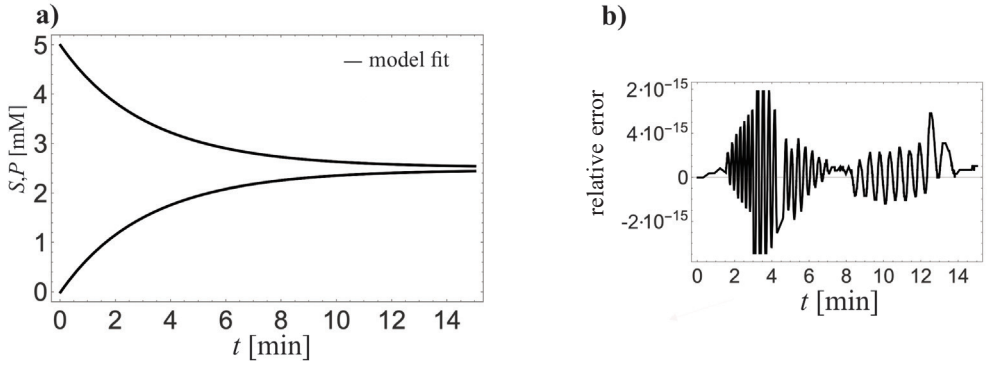

Fig. 5 – *a) Simulated time-courses of substrate (S) and product (P) concentrations generated from the kinetic model (Eq. 6) using ideal synthetic data with S_0 = 5 [mM]. The synthetic points are completely overlapped by the model fit, as multiple parameter sextuples reproduce the same trajectories. b) Relative error profiles corresponding to six independently optimized parameter sextuples (listed in Table 1), showing only negligible numerical noise, thereby confirming perfect agreement with the synthetic data and demonstrating non-uniqueness.*

The same principle extends to mechanistically related systems, including the Ping–Pong Bi–Bi mechanism, which also typically involves six kinetic parameters and exhibits the same intrinsic ambiguity.

It is important to note that classical statistical uncertainty quantification tools, such as bootstrap resampling or confidence interval estimation, are not suitable for addressing intrinsic non-uniqueness. These methods presuppose the existence of a unique optimum, whereas in structurally non-identifiable models the solution space forms a continuous manifold of equivalent parameter sets.

Mathematical proof of non-uniqueness for the six-parameter Michaelis–Menten-based kinetic models

While searching for parameters, it was shown that a system with six parameters (without experimental data and physical background) is non-unique – with different sets of parameters, the same (identical) solution to the given initial problem can be obtained. This non-uniqueness of the six parameters can also be proven.

We are solving the initial value problem:

$$S'(t) = R_{2,p}\left(S(t)\right) - R_{1,p}\left(S(t)\right) \tag{7}$$

$$S(0) = S_0 \tag{8}$$

where we have rational functions:

$$R_{1,p}(x) = \frac{p_1 \cdot x}{p_2\left(1 + \frac{S_0 - x}{p_6}\right) + x} \tag{9}$$

$$R_{2,p}(x) = \frac{p_4 \cdot \left(S_0 - x\right)}{p_5\left(1 + \frac{x}{p_3}\right) + \left(S_0 - x\right)} \tag{10}$$

and p is a parameter of ordered sextuple of numbers:

$$p = \left(p_1, p_2, p_3, p_4, p_5, p_6\right) \tag{11}$$

If we obtain two parameters $(p_1,p_2,p_3,p_4,p_5,p_6)$ and $(q_1,q_2,q_3,q_4,q_5,q_6)$ for which the rational function $R_{2,p}(x) - R_{1,p}(x)$ is the same for all real x to the rational function $R_{2,q}(x) - R_{1,q}(x)$, then the initial problems:

$$S'(t) = R_{2,p}\left(S(t)\right) - R_{1,p}\left(S(t)\right), \quad S(0) = S_0 \tag{12}$$

and

$$S'(t) = R_{2,q}\left(S(t)\right) - R_{1,q}\left(S(t)\right), \quad S(0) = S_0 \tag{13}$$

will have the same solution.

Alternatively, expressed differently:

$$R_{2,p}(x) - R_{1,p}(x) = R_{2,q}(x) - R_{1,q}(x) \tag{14}$$

for all $x \in \mathbb{R}$

or more concisely:

$$Q_p(x) = Q_q(x) \quad \text{for all } x \in \mathbb{R} \tag{15}$$

where:

$$Q_p(x) = R_{2,p}(x) - R_{1,p}(x) \tag{16}$$

How can parameters p and q (two 6-tuples) be chosen so that equation (15) is satisfied? Consider any two parameters p and q and bring the difference $Q_p(x) - Q_q(x)$ to the common denominator, placing them on the same side of the equation. This results in a fourth-degree polynomial in the numerator, which must be equal to 0 for all real x (developed by Wolfram Mathematica):

$$c_0 + c_1 x + c_2 x^2 + c_3 x^3 + c_4 x^4 = 0 \tag{17}$$

Coefficients c_0, c_1, c_2, c_3, c_4 are polynomials in variables $p_1, p_2, p_3, p_4, p_5, p_6, q_1, q_2, q_3, q_4, q_5$ and q_6. Equation (17) is satisfied for all real x only if all coefficients are equal to 0:

$$c_0 = 0,\ c_1 = 0,\ c_2 = 0,\ c_3 = 0,\ c_4 = 0 \tag{18}$$

Fix an arbitrary parameter q and find all parameters p (to prove the existence) close to q for which $Q_p(x) = Q_q(x)$ for all real x, or equivalently, the equation (18) holds. Since we are looking for p close to q, we write:

$$p = q + u \tag{19}$$

where $u = \left(u_1, u_2, u_3, u_4, u_5, u_6\right)$ is small.

We now have a function: $f : \mathbb{R}^6 \to \mathbb{R}^5$ defined by:

$$f\left(u_1, u_2, u_3, u_4, u_5, u_6\right) = \left(c_0, c_1, c_2, c_3, c_4\right) \tag{20}$$

In each c_i, the fixed $q_1, q_2, q_3, q_4, q_5, q_6$ appear, and instead of p's, we write $p_i = q_i + u_i$ for indexes $i = 1,2,\ldots,6$. It can be readily seen that:

$$f(0) = 0, \text{ since } Q_{q+0}(x) = Q_q(x) \tag{21}$$

We use the following theorem[6]:

If f: $\mathbb{R}^m \to \mathbb{R}^n$ is a smooth function with constant rank of the Jacobian matrix in the neighborhood of 0 and $f(0) = 0$, then the null space of the Jacobian matrix evaluated at the origin, J, i.e., the solution space of the corresponding homogeneous system Jh=0, is tangent to the zero set of the function f:

$$N = \left\{u \in \mathbb{R}^m, f(u) = 0\right\} \tag{22}$$

In our original problem, it turns out (with the aid of Wofram Mathematica) that the dimension of the null space of the matrix J is 2; therefore, there exists a two-dimensional surface in $\mathbb{R}^6$ that has this two-dimensional plane as its tangent space.

Discussion and perspectives

The results presented in this study provide a consistent picture of how parameter sensitivity and non-uniqueness emerge in enzyme kinetic modeling. As the kinetic description becomes more complex—from simple mono-substrate reactions to reversible and inhibited systems—the confidence in estimated parameters decreases and the limitations of empirical rate equations become evident. This trend highlights the need for structurally informed modeling and experimental strategies that ensure both reliable parameter estimation and meaningful physical interpretation of kinetic constants.

A well-documented issue in multi-parameter kinetic modeling is the lack of uniqueness in parameter estimation. Even when the underlying model is structurally identifiable, the parameter space often contains elongated valleys of nearly constant error, where many parameter combinations fit the data equally well. This phenomenon, referred to as sloppiness, has been extensively described for biological and enzymatic systems[7,8]. In practice, models with six or more parameters – such as reversible Michaelis–Menten kinetics with inhibition or the Ping–Pong Bi–Bi mechanism – rarely yield a single, well-defined global minimum, but rather sets of multiple parameters whose differences fall within numerical noise, with relative errors on the order of 10^{-15} in terms of data fitting. These results highlight the importance of experimental design and multi-condition global fitting to mitigate non-uniqueness[9,10].

Another important aspect is that kinetic parameters such as V_{maxf}, K_M, and K_i do not always retain a clear physical meaning under practical experimental conditions. While these parameters are theoretically well defined, their experimental estimates can lose interpretability when the assumptions underlying the Michaelis–Menten model (e.g., quasi-steady-state, substrate excess, negligible product inhibition) are not strictly fulfilled. As shown by Schnell and Maini[11], even small deviations from these assumptions can lead to unreliable estimates of V_{maxf}, K_M. Similarly, Choi *et al.*[12] emphasized that K_M and k_{cat} often cannot be independently determined due to limited data quality, and Verheijen and Heijnen[13] noted that *in vivo* parameter estimation is strongly affected by multicollinearity. Thus, while parameters such as V_{maxf}, K_M are widely used as apparent measures of catalytic efficiency and substrate affinity, their limited physical robustness in real systems further aggravates the non-uniqueness problem.

It is worth noting that enzymatic biotransformation mechanisms have, for almost a century, been described predominantly by empirical Michaelis–Menten-type models. Because of their empirical origin, these equations—formulated as rational functions—tend to exacerbate parameter non-uniqueness. As a result, they often lack clear physical grounding for their kinetic constants. None of the parameters explicitly capture fundamental physicochemical properties such as the electrostatic potential, the dynamic surface charge distribution of an enzyme, or the charge characteristics of its substrate. This conceptual gap underscores the empirical nature of conventional kinetic constants and explains why their estimation is so sensitive to experimental design and data variability.

Recent advances in computational chemistry and biophysics are paving the way toward a new generation of enzyme kinetic models that move beyond empirical Michaelis–Menten descriptions. Methods such as transition-state theory combined with Eyring's equation provide kinetic parameters directly linked to activation enthalpies and entropies, offering a clear physicochemical interpretation. Molecular dynamics (MD) simulations and hybrid quantum-mechanics/molecular-mechanics (QM/MM) approaches enable the analysis of substrate binding, conformational flexibility, and reaction-energy profiles at atomic resolution. In particular, the electrostatic preorganization model developed by Warshel and co-workers highlights the crucial role of internal electric fields in enzyme catalysis, directly connecting surface charge distributions and electrostatic potentials with catalytic efficiency. Brownian dynamics and Poisson–Boltzmann formalisms further allow the inclusion of long-range electrostatic interactions in binding kinetics. Together, these developments suggest that

Table 2 – *Challenges in enzyme kinetics*

Problem	Consequence	Key references
Non-uniqueness and "sloppiness" in multi-parameter models	Multiple parameter sets give indistinguishable fits; global fitting across conditions required	7–10
Loss of physical interpretability of kinetic parameters	V_{max}, K_m, K_i not robust under real experimental conditions; unreliable estimates	11–13
Empirical nature of Michaelis–Menten formalism	Rational functions intensify non-uniqueness; lack direct link to physicochemical properties	11
Toward mechanistic, physics-based models	Transition state theory, QM/MM, MD, electrostatic preorganization, Brownian dynamics	14–16

future enzyme kinetics will increasingly rely on mechanistic, physics-based models that capture molecular-level properties, thereby complementing or even replacing classical empirical formalisms[14–16]. The key conceptual and methodological challenges discussed are summarized in Table 2, which highlights how parameter non-uniqueness, empirical model limitations, and the search for mechanistic formulations collectively shape the future of enzyme kinetics research.

Conclusions

This study systematically examined the sensitivity and non-uniqueness challenges encountered in determining enzyme kinetic parameters, covering systems of increasing complexity – from the classical two-parameter Michaelis–Menten model to reversible and inhibited multi-parameter mechanisms. The results clearly demonstrate that, even when models are structurally identifiable, parameter estimates may remain practically non-unique, with multiple parameter combinations providing equally good fits to experimental data. In particular, parameter sets differing by up to two orders of magnitude were shown to yield nearly indistinguishable model outputs in experimentally based case studies.

A mathematical proof further confirmed that in six-parameter kinetic systems, an infinite family of parameter sextuples can yield identical model predictions, revealing that such non-uniqueness is intrinsic to the model structure itself and cannot be resolved by additional data or statistical refinement.

These findings emphasize the importance of combining multi-condition global fitting with sensitivity and identifiability analysis to ensure meaningful parameter estimation. They also highlight the need to move beyond empirical rate equations toward mechanistically grounded, physics-based kinetic models that establish direct links between measurable molecular properties and catalytic function. Such approaches are essential for reliable model-based design and intensification of biocatalytic processes, particularly in microscale and microreactor systems.

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References

1. *Al-Haque*, *N.*, *Santacoloma*, *P. A.*, *Neto*, *W.*, *Tufvesson*, *P.*, *Gani*, *R.*, *Woodley*, *J. M.*, A robust methodology for kinetic model parameter estimation for biocatalytic reactions, Biotechnol. Prog. **28** (2012) 1186. doi: https://doi.org/10.1002/btpr.1588
2. *Ringborg*, *R. H.*, *Woodley*, *J. M.*, The application of reaction engineering to biocatalysis, React. Chem. Eng. **1** (2016) 10. doi: https://doi.org/10.1039/c5re00045a
3. *Villadsen*, *J.*, *Nielsen*, *J.*, *Lidén*, *G.*, Bioreaction Engineering Principles, Springer, New York, 2011, pp 1–591. doi: https://doi.org/10.1007/978-1-4419-9688-6
4. *Banga*, *J. R.*, Optimization in computational systems biology, BMC Syst. Biol. **2** (2008) 47. doi: https://doi.org/10.1186/1752-0509-2-47
5. *Menegatti*, *T.*, *Plazl*, *I.*, *Žnidaršič-Plazl*, *P.*, Model-based design of continuous biotransformation in a microscale bioreactor with yeast cells immobilized in a hydrogel film, Chem. Eng. J. **483** (2024) 149317. doi: https://doi.org/10.1016/j.cej.2024.149317
6. *Rudin*, *W.*, Principles of Mathematical Analysis, McGraw-Hill, New York, 1976, pp 1–342.
7. *Chis*, *O. T.*, *Banga*, *J. R.*, *Balsa-Canto*, *E.*, Structural identifiability of systems biology models: A critical comparison of methods, PLoS One **6** (2011) e27755. doi: https://doi.org/10.1371/journal.pone.0027755
8. *Gutenkunst*, *R. N.*, *Waterfall*, *J. J.*, *Casey*, *F. P.*, *Brown*, *K. S.*, *Myers*, *C. R.*, *Sethna*, *J. P.*, Universally sloppy parameter sensitivities in systems biology models, PLoS Comput. Biol. **3** (2007) e189. doi: https://doi.org/10.1371/journal.pcbi.0030189
9. *Raue*, *A.*, *Kreutz*, *C.*, *Maiwald*, *T.*, *Bachmann*, *J.*, *Schilling*, *M.*, *Klingmüller*, *U.*, *Timmer*, *J.*, Structural and practical identifiability analysis of partially observed dynamical models by exploiting the profile likelihood, Bioinformatics **25** (2009) 1923. doi: https://doi.org/10.1093/bioinformatics/btp358
10. *Villaverde*, *A. F.*, *Banga*, *J. R.*, Structural properties of dynamic systems biology models: Identifiability, reachability, and initial conditions, Processes **5** (2017) 29. doi: https://doi.org/10.3390/pr5020029
11. *Schnell*, *S.*, *Maini*, *P. K.*, A century of Michaelis–Menten kinetics: Reliability of the K_M and v_{max} estimates, Comments Theor. Biol. **8** (2003) 169. doi: https://doi.org/10.1080/08948550302453
12. *Choi*, *B.*, *Rempala*, *G. A.*, *Kim*, *J. K.*, Beyond the Michaelis–Menten equation: Accurate and efficient estimation of enzyme kinetic parameters, Sci. Rep. **7** (2017) 17018. doi: https://doi.org/10.1038/s41598-017-17072-z
13. *Heijnen*, *J. J.*, *Verheijen*, *P. J. T.*, Parameter identification of in vivo kinetic models: Limitations and challenges, Biotechnol. J. **8** (2013) 768. doi: https://doi.org/10.1002/biot.201300105
14. *Warshel*, *A.*, *Sharma*, *P. K.*, *Kato*, *M.*, *Xiang*, *Y.*, *Liu*, *H.*, *Olsson*, *M. H. M.*, Electrostatic basis for enzyme catalysis, Chem. Rev. **106** (2006) 3210. doi: https://doi.org/10.1021/cr0503106
15. *Garcia-Viloca*, *M.*, *Gao*, *J.*, *Karplus*, *M.*, *Truhlar*, *D. G.*, How enzymes work: Analysis by modern rate theory and computer simulations, Science **303** (2004) 186. doi: https://doi.org/10.1126/science.1088172
16. *Kamerlin*, *S. C. L.*, *Warshel*, *A.*, At the dawn of the 21st century: Is dynamics the missing link for understanding enzyme catalysis?, Proteins **78** (2010) 1339. doi: https://doi.org/10.1002/prot.22654