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Analytical and numerical approaches to the analysis of progress curves: A methodological comparison

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ABSTRACT

Accurate models of the reaction kinetics of enzymatic reactions are essential for the design of biocatalytic processes. While many experimental studies still build on initial slope analysis, progress curve analysis offers the potential for modelling enzymatic reactions with a significantly lower experimental effort in terms of time and costs, but requires the solution of a dynamic nonlinear optimization problem. There are many different approaches for solving this problem for parameter regression, building on the experimental progress curve data. In order to provide some guidance for selecting an appropriate approach, this study presents a detailed comparison of two analytical and two numerical approaches analysing their strengths and weaknesses on the basis of three case studies. The analytical approaches build on the implicit and explicit integrals of the respective reaction rate equations, while the numerical approaches consider the direct numerical integration of the differential mass balance equations as well as the transformation of the dynamic problem to an algebraic problem by means of spline interpolation of the reaction data. In particular, the dependence of the results on the initial parameter estimates is evaluated, showcasing that the numerical solution with spline interpolation shows a lower dependence on the initial values providing parameter estimates comparable to the analytical approaches, which are however limited in applicability.

1. Introduction

Enzymes, frequently referred to as nature's catalysts, have been a fundamental component in many industries for a considerable time [1–3]: The food industry, for instance, utilizes these biocatalysts extensively to boost the production of beverages, bread, cheese, and other products. Enzymes are also critical to analytical laboratories, playing a crucial role in precise assays to maintain the accuracy of diagnostic tests and quality control for various products. The pharmaceutical industry employs enzymes to produce intricate molecules, hastening the discovery and development of drugs. Enzymes exhibit unmatched proficiency in hastening chemical reactions under mild conditions, which has the potential to revolutionize the effectiveness of commercial chemical processes and lead to environmentally sustainable practices [1–5].

Nonlinear differential equations are essential for realistically modelling the behaviour of enzymatic reactions, as they can describe complex interactions and time-dependent processes. They make it possible to describe substrate and product concentrations over time, taking into account effects such as substrate inhibition, feedback or allosteric regulation. This is particularly useful in systems with multiple reaction steps or dynamic environments such as bioreactors. The use of such equations makes it possible to analyse enzyme behaviour in order to gain deeper insights into reaction process and make more precise predictions for biotechnological applications. Understanding the mechanism of the individual reactions is crucial to unlock the full potential of enzymes, which is of particular important for commercial applications. This understanding is expressed mathematically through the concept of reaction kinetic models, which describe the rate at which the reactions occur depending on the individual composition and operating conditions such as pH and temperature, as well as intrinsic parameters of the enzyme itself. These intrinsic parameters, often in the form of affinity constants for substrates and products, play a critical role in shaping the enzyme's activity [6–9].

The most basic of all kinetic models is the Michaelis-Menten equation for the reaction rate

$$-\frac{dc_S}{dt} = v = v_{\max}\frac{c_{S(t)}}{K_M + c_{S(t)}} \tag{1}$$

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which describes an irreversible uni-uni reaction with the maximal reaction rate v_{max}, the Michaelis constant K_M and substrate concentration c_S. Starting from this basic form, the equation can be extended in terms of other reaction mechanisms, including more substrates and/or products, different types of inhibitions, reversible or irreversible reaction steps, etc. [8]. Most enzymatic reactions exhibit complex and nonlinear behaviour that mandate complex nonlinear kinetic models to capture the true behaviour of these processes. The formulation of such models often involves numerous parameters that must be estimated from experimental data. Two commonly used methods for parameter estimation of enzyme kinetic models are the initial slope analysis and progress curve analysis (PCA). The former was already used by Michaelis and Menten themselves more than a century ago to determine the kinetic reaction parameters of invertase [10,11]. The initial slope method is simple to apply and the mathematical solution can be performed by means of a spreadsheet, which is one of the reasons why it is still used in many studies today [8,12–14]. A major strength of the initial slope approach is that for a simple reaction according Eq. (1) the results for v_{max} and K_M can be extracted very well from a graphical analysis. However, it is experimentally demanding to generate enough and dedicated data for parameter regression, as the initial slope method generates a single datapoint in an individual experiment. The approach in PCA seeks to track the concentration of substrate and/or product with time, providing a much larger set of data for an individual experiment compared to traditional initial slope experiments. The experiment is therefore not cancelled after leaving a linear range, but continued until equilibrium or full conversion is reached. Classically, this method also involves an integration of the dynamic mass balances with the reaction kinetic model, which describes the current change in concentrations at a single point in time, based on the local conditions, i.e. concentrations and temperature. Although the possibilities and advantages of PCA over the initial slope method have been pointed out since the 1970s [15–17], many studies of enzyme kinetics still avoid its use and build on the mathematically simpler initial slope method [14,18–20].

The parameter estimation problem for PCA constitutes a dynamic nonlinear regression problem, the solution of which can be a challenging [6]. While nonlinear regression problems are frequently solved to determine accurate parameter estimates, by minimizing the difference between model predictions and experimental data, it is important to note, that a sufficiently rich and reliable set of data is a prerequisite to identify the correct parameter values [21]. Furthermore, correlation of the individual model parameters can significantly limit the identifiability of the individual parameters and prevent the reliable solution for any parameter regression problem. Yet, parameter correlation is a common challenge for enzyme kinetic models [22]. However, even if the parameters of the individual model are structurally identifiable and the kinetic data is sufficiently rich and reliable the resulting optimization problem for parameter regression is usually nonconvex for enzyme kinetic models, making it difficult to find the best solution that corresponds to the best-fit parameters, as multiple local minima may exist. Consequently, good initial values for the parameters are critical to the success of nonlinear regression, when using local solvers. Depending on the specific solver, poor initial values can lead to convergence problems [23], instability, or parameter estimates that do not reflect the underlying biology [24]. As a consequence the solution to the nonlinear regression problem for enzyme kinetic models is a complex task, as the model may have multiple interconnected parameters [23–26].

To overcome these challenges several strategies have been proposed. Data preparation and smoothing can significantly improve the results of the subsequent parameter estimation. Furthermore, differentiation of the dynamic data allows for the transformation of the original dynamic nonlinear regression problem to an algebraic problem, avoiding the need for an additional integration and significantly reducing the computational load for the solution of the optimization problem. However, small errors in noisy data can be significantly amplified when numerical differentiation is used on the raw data. The use of smoothing splines that are first fitted to the experimental data as a surrogate model do not only provide a regularization of the raw data, but also allow for a direct symbolic computation of the respective derivatives to subsequently solve an algebraic nonlinear regression problem [27,28]. These techniques are quite popular in chemical reaction modelling, but can also be applied for the identification of enzyme kinetic models [29,30]. In addition, alternative methods can be applied to improve the data analysis implementing e.g. reconvolution [12,13] to improve the identifiability for a limited resolution of the available experimental data.

The dependence of the initial values for nonlinear regression can be overcome in various ways, using global deterministic optimization or global search methods [25]. E.g., multi-start grid search is a simple, yet oftentimes effective global search strategy, which systematically explores a range of initial values to identify a set of candidates that yield reasonable fits [31,32]. Sensitivity analysis further helps to assess the impact of parameter variations on model predictions, guiding the selection of initial values that are less sensitive [33,34]. The significant effect that different solvers and solution methods can have on the results of a parameter estimation on the basis of PCA has been nicely illustrated by Zavrel *et al.* [22] for an irreversible hydrolysis reaction catalysed by enzyme penicillin amidase using in-silico data as well as experimental data.

The current work provides an extended analysis of the different approaches for modelling enzyme kinetics, focussing on the PCA approach. Three different methods are compared, considering either a direct integration of the differential mass balances based on an analytical or numerical integration approach, as well as the transformation of the dynamic problem to an algebraic problem, making use of smoothing splines. The different methods for parameter estimation are described in detail in Section 2. The analysis of the different methods is performed on the basis of three case studies, including irreversible and reversible uni-uni and uni-bi reactions, as well as the evaluation of in-silico data and real noisy experimental data. The fundamental models and the specific case studies are described in detail in Section 3, while the specific results are described and analysed in Section 4. Finally, Section 5 provides a summary and conclusions.

2. Methods for parameter regression of enzyme kinetics

Fig. 1 provides an overview of the different methods for parameter estimation of enzyme kinetic models, applied in literature, as well as those in specific considered in the current study. The initial slope method seeks the identification of individual parameters through dedicated experiments, using only the linear segment at the very beginning of the experimental progress curve for a nonlinear regression on the basis of the rate equation or a linear regression to a linearized form of the rate equation [35,36]. This approach is well-established and straightforward, yet it requires a substantial number of experiments to capture each of the initial rates [37,38]. It is also based on the assumption that the concentrations during the linear phase do not change considerably, which requires quick and accurate process analytics and may not always be valid. Recently, Frère *et al.* [39] gave a good overview of why the initial slope method should be applied with great caution.

The focus of the current work is on PCA, for which such assumptions are not necessary. In PCA, the individual concentrations of substrates and/or products are tracked over time, providing a sampling of the dynamic data representing the reaction progress. This dynamic data is further used for nonlinear regression, building either on the integration of the reaction rate equations, or fitting the kinetic model to further processed data derived from a differentiation of the PCA data. For the former, either an analytical or a numerical integration approach can be used. The mentioned methods have already been used by different authors for the analysis of progress curves and have been investigated but have rarely compared with each other regarding their strengths and weaknesses. Deogratias *et al.* [40] have studied *in-silico* experiments for a

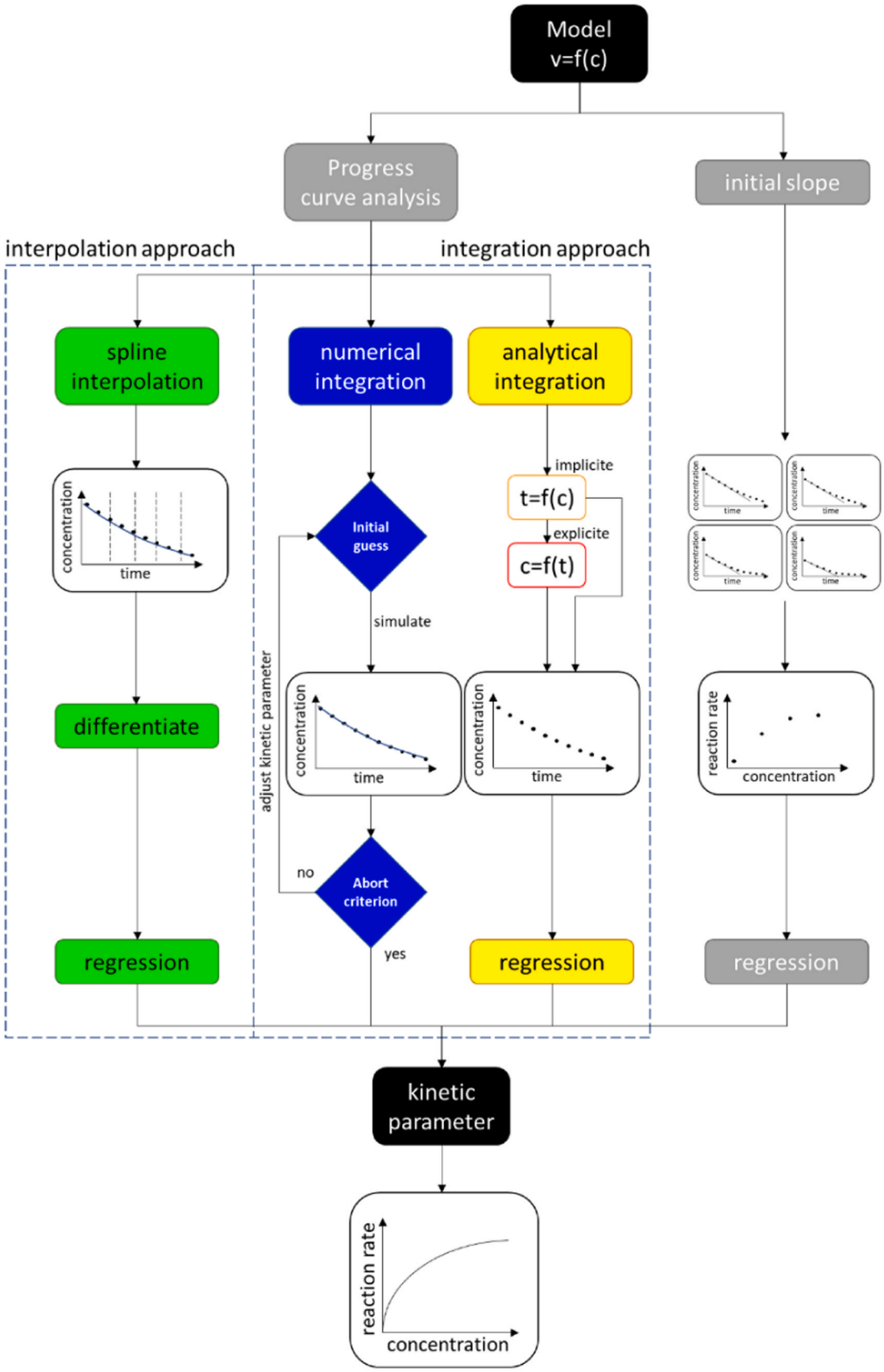

Fig. 1. Overview of methods and approaches for parameter estimation of enzymatic reaction models.

uni-uni reaction without and with different inhibitions. Artificial noise with up to 4 % was added to the data and it was found that the parameters could still be identified with high accuracy based on the least squares estimation for the analytical explicit form of the kinetics. A comparison with other methods or higher noise levels was not performed. Goličnik [41] compared the results of a nonlinear regression based on *in-silico* initial slope experimental data and *in-silico* PCA data performing nonlinear regression of the explicit integral of the kinetics without adding additional noise and with 2 % and 4 % added noise for a uni-uni reaction without inhibition. As expected, the calculated uncertainties with the explicit integral were significantly smaller than with the initial slope method. Paar *et al.* [42] used a PCA dataset with constant error for the evaluation of the explicit integral compared to the application of an implicit integral as well as a numerical integration based on initial slope experiments as well as different linearizations for an uni-uni reaction without inhibition. As expected, the calculated standard errors of the explicit integral were smaller than for the numerical integration as well as the linearizations. Unfortunately, no standard errors were calculated for the implicit integral. Zavrel *et al.* [22] compared five different computer programs with different solvers for the analysis of an uni-uni reaction with competitive inhibition with a normally distributed standard deviation of 0.002 mM at starting concentrations of 0.075 mM - 0.1 mM. The analysis was performed using initial slope experiments and PCA. For the former, it was found that smoothing splines improved parameter estimation results considerably. For the second, it was found that as the number of data points increased, the deviation decreased - although the computer programs studied performed differently depending on the number of data points. The reported advantages and disadvantages of the mentioned methods for progress curve analysis are summarized in Table 1.

Since previous studies have only focussed on a partial comparison of the individual methods [22,40–42], the current study seeks a comprehensive comparison of the analytical and numerical approaches. The overall study builds on a common PCA dataset for all methods, facilitating a direct comparison of resulting parameter estimates. Through this analysis, we aim to shed light on the strengths and limitations of each approach, providing researchers with valuable insights into choosing an appropriate method for enzymatic reaction kinetic parameter determination.

2.1. Nonlinear regression for an analytical integration

If the differential mass balance that is defined by the reaction rates can be integrated analytically, an algebraic optimization problem can be solved for parameter regression, by minimizing the least squares function for the concentration data. Depending on the type of reaction kinetic model, analytical integration can either yield an implicit relationship between time t and concentration c in the form of $t = f(c)$ [8,43], or an explicit form by transforming the implicit form into $c=f(t)$, by a series expansion using the Lambert W function [44]. Fernandes *et al.* [45] demonstrated the superiority of the implicit analytical approach over the initial slope method for *in-silico* data for simple reactions with inhibition. However, the implicit form is not intuitive to use and the objective function for nonlinear regression must be chosen with care as at standard regression usually the deviation in y-error is minimized: For the implicit approach this ends up in a minimization the deviation in time, which usually is quite accurate. However, it is generally advisable to minimize the deviation in concentration, as this can usually be measured less accurately. These may be parts of the reason why this method has not been adopted to a larger extend, even though it has been known for over 50 years [46,47]. The explicit form has the advantage of a simpler fit, however there seems a lack of usable software. It was also successfully applied for reversible inhibition kinetics [40], but cannot be effectively used if only data from initial slopes are available [18]. Yet, the complexity of the implicit equation and the lack of analytic integrals for general rate equations limit the broad applicability of analytical integration.

2.2. Numerical integration

As an alternative to the analytical integration, the differential mass balance can be integrated by numerical integration, for which various integration methods, such as the forward or backward Euler method, or different Runge-Kutta methods are available, as e.g., Matlab does provide a range of *ode* solvers combining Runge-Kutta methods of different order to automatically adjust the step size. The parameter regression problem for the numerical integration approach is mostly solved as a single (or multiple) shooting problem, for which the objective function is evaluated in each iteration, by performing the numerical integration of the differential mass balance on the basis of the kinetic model with the current parameter estimates and calculating the least squares functional for the measured composition data [48]. For such nonlinear regression problems, the objective is typically formulated as minimisation of the least squares function for the difference between the measured data and the model predictions. The challenge of parameter identifiability arises when different parameter sets provide similar model predictions, which is particularly problematic with so-called ‘sloppy models’. These models either have parameters which show low sensitivity in the objective, i.e. some parameters hardly influence the model and can therefore not be determined uniquely from the data, or parameters that are strongly correlated. To handle these problems, regularisation [22,49] or Bayesian approaches [50] are often used to introduce additional information or prior knowledge. Alternatively, sensitivity analyses can help to identify critical parameters and reduce the model to a manageable number of relevant parameters [51] [Ref]. Also model-based design of experiments provides a systematic tool for the discrimination between competing model candidates with minimum experimental effort [52]. This method of least squares minimization is e.g. implemented in the software tool Encora. It offers the advantage of general applicability to all kinds of kinetic models, but optimization of the parameters is more complex as the parameter sensitivities are not easily derived. Using Encora and *in-silico* data Straathof [48] reports that for progress curve data with an added 2 % noise, the deviation of the reaction parameter was 5–25 %. Similar results were reported for other software tools employing numerical integration where a Gaussian noise of 0.002 was considered for a fluorescence signal in the range of 0–0.15 [53].

For the current investigations the numerical integration approach implemented in the software Encora and published by Straathof [48], is

Table 1
Advantages and disadvantages of analytical and numerical approaches for progress curve analysis.

		Advantages	Disadvantages
Analytical approaches	Implicit form	Accurate results due to the analytical integration.	Limited number of kinetics equations described in literature. Non-intuitive form for the kinetic equation. Objective function must be chosen with care.
	Explicit form	Accurate results due to the analytical integration. Intuitive fit and more flexibility for objective function.	Lowest number of kinetics equations described in the literature. Available equations are based on one component, e.g. no bi-bi reactions.
Numerical approaches	Numerical integration	High flexibility, each kinetic equation can be applied. Free and commercial computer programs available.	Results depends on initial values. Less accurate than analytical approach.
	Spline interpolation	High flexibility, each kinetic equation can be applied. Comparatively robust with poor initial values.	Not yet established as a standard approach in the biotech community. Less accurate than analytical approach.

used, which can also be seen as a benchmark for an established tool in this study. Encora provides access to 204 enzyme kinetic models, including those considered for the case studies in Section 3. The fitting procedure starts with the definition of initial parameter estimates and uses a fourth-order Runge-Kutta method for numerical integration [54]. The optimization is performed with the derivative-free Nelder-Mead simplex method, minimizing the sum of squared residuals, also providing estimates of the variance [48]. For all calculations a maximum of 2000 iterations were specified, so that the stop criterion, which was a lack of adjustment in the parameter estimates beyond 1 % was generally reached first [54]. For case study 2 the maximum step size was adapted to 1, and the maximum number of iterations to 1000. Other options were taken as default.

2.3. Spline interpolation

Another viable option to derive an algebraic optimization problem for parameter regression builds on the differentiation of the measured concentration data. However, doing this with numerical differentiation directly on the basis of noisy experimental data is an ill-posed problem [27]. Tikhonov regularization and smoothing splines have been used for the estimation of reaction rates from concentration data in chemical reactions for more than 50 years [55]. Cubic spline interpolation is used to interpolate the course of the experimental data. The considered time horizon is discretized by n knots into individual intervals in each of which polynomials are defined [56–58], according to Eqs. (2) and (3).

$$c_{S,Spline}(t) = \begin{Bmatrix} c_{S,Spline,1} \\ c_{S,Spline,2} \\ \cdots \\ c_{S,Spline,n} \end{Bmatrix}, \tag{2}$$

$$c_{S,Spline,i}(t) = a_i \bullet t^3 + b_i \bullet t^2 + c_i \bullet t + d_i, \tag{3}$$

The substrate concentration c_S as function as well as its first two derivates need to be continuous over the defined time t, here a, b and c are factors that describe the polynomial. The spline polynomials are twice continuously differentiable and continuity at the knots is assured by means of additional boundary conditions in the fitting process. When characterizing the substrate concentration c_S two additional constraints are considered in the fitting process, demanding a monotonous decrease of c_S over time (Eq. (4)) and a concave form of the curve (Eq. (5)):

$$\frac{dc_{S,Spline}}{dt} \leq 0 \tag{4}$$

$$\frac{d^2c_{S,Spline}}{dt^2} \leq 0 \tag{5}$$

The spline interpolation is used not only to smooth the data, but also to enable differentiation of the smoothed data to obtain the reaction rates. The resulting spline interpolation can subsequently by differentiated to derive an explicit function for the reaction rate which can further be evaluated for discrete time points within the considered time span. The parameter regression problem is thus posed as algebraic optimization problem for the reaction rates as described by Eq. (6) [59].

$$\frac{dc_{S,Spline}}{dt} = v_{S,Spline}(t), \tag{6}$$

Previous studies have shown that the splines approach leads to a higher possibility of convergence than the implicit approach, when using poor initial guesses [60]. The spline approach is advantageous in its versatile application for different reaction kinetics. While the spline interpolation may distort the experimental data in case of perfect experimental data, real experimental data is almost never perfect and smoothing splines have demonstrated the capability for an effective regularization of noisy data. Noisy data can further complicate parameter estimation and lead to overfitting if the data is not smoothed sufficiently, while sparse data can make interpolation inaccurate [22, 61]. In addition, the choice of node density and degree of smoothing requires careful manual tuning, which is often difficult with limited data. These manual adjustments makes it in general less attractive to use at present.

2.4. Further information on applying the methods in Matlab

Based on the specific function derived by analytical integration an algebraic nonlinear least squares regression problem is posed for the concentration data and solved with the help of the *lsqcurvefit* function in Matlab. All kinetic parameters are considered as positive variables without upper bound and different initial values are tested for each case study. The specific options used for *lsqcurvefit* can be found in the Gitlab repository. For the current study total least square (TLS) regression was performed with the Matlab toolbox by Petras and Bednarova [62], where the errors in x- and y-direction are equally weighted. For the spline interpolation the shape modeling language toolbox by D'Errico [63] was used. The subsequent regression analysis was conducted using the *lsqcurvefit* function, again. For calculation of the error bars the *nlparci* function was used.

3. Case studies

Three case studies with increasing complexity are considered to evaluate the different PCA-based parameter estimation methods. First, the analytical approaches are compared with the numerical integration and spline interpolation for an irreversible uni-uni reaction without and with different inhibitions. The performance of the different methods is compared on the basis of *in-silico* experimental data in the presence of different noise levels. The second case study evaluates the performance of the different methods for the original experimental data of Michaelis and Menten [10], who have investigated an irreversible uni-bi reaction. The third case study considers a full reversible uni-uni reaction for which the different methods are compared on the basis of own experimental data.

3.1. Irreversible uni-uni reaction with and without inhibition

In the irreversible uni-uni reaction an enzyme (E) and a substrate (S) react to form an enzyme-substrate complex (ES), which then dissociates into enzyme (E) and product (P). The underlying reaction mechanism is given in Scheme (1), where the corresponding reaction rate for a non-inhibited reaction is given by Eq. (1).

$$E + S \rightleftharpoons \mathrm{ES} \rightarrow \mathrm{E} + \mathrm{P} \qquad \text{(Scheme 1)}$$

This reaction mechanism provides the basic framework for the study of enzyme reactions. In addition, different types of inhibition, as described by Cornish-Bowden [8], such as uncompetitive, non-competitive, and competitive inhibition can be considered to obtain a comprehensive picture of different enzymatic reactions. A key assumption is that the inhibitor is not converted and the inhibition is fully reversible. Also partial inhibition is neglected.

In competitive inhibition (cf. Eq. (7a)), an inhibitor molecule competes with the substrate for binding to the active site of the enzyme. This means that the inhibitor and substrate cannot bind to the enzyme at the same time. The addition of a competitive inhibitor results in a decrease in reaction rate because less substrate can bind to the enzyme. However, the maximum reaction rate can be restored by adding more substrate. Non-competitive inhibition (cf. Eq. (7b)) is a special case of mixed inhibition, were the inhibitor binds to both the enzyme and the enzyme-substrate complex. This results in less efficient conversion to a product. For non-competitive inhibition the affinity constants of the inhibitor for the enzyme (K_I) and enzyme-substrate complex (K_{IU}) are the same. As they are the same here, both are referred as K_I. In uncompetitive

inhibition (cf. Eq. (7c)), the inhibitor binds only to the enzyme-substrate complex, defined by the constant K_U. This prevents the release of products from the enzyme-substrate complex, effectively inhibiting enzyme activity. Unlike competitive inhibition, the addition of more substrate further enhances inhibition by promoting the formation of more enzyme-substrate complexes to which the inhibitor can bind.

$$v = v_{\max}\frac{c_{S(t)}}{K_M(1+\frac{c_I}{K_I})+c_{S(t)}} \tag{7a}$$

$$v = v_{\max}\frac{c_{S(t)}}{(1+\frac{c_I}{K_I})(K_M+c_{S(t)})} \tag{7b}$$

$$v = v_{\max}\frac{c_{S(t)}}{K_M+c_{S(t)}(1+\frac{c_I}{K_U})} \tag{7c}$$

The analytical integration of the original Michaelis-Menten equation (Eq. (1)) with respect to c_S results in the implicit expression in Eq. (8a) [8]. Assuming that the terms for inhibition represent constants, since the inhibitors are not converted to by-products by the enzyme, Eq. (8b-d) are obtained as analytical integrals for competitive inhibition, non-competitive inhibition as well as uncompetitive inhibition, with $c_{S,0}$ as initial substrate concentration. Accordingly, these equations do not apply, for example, to product inhibition, since in this case the product concentration does not represent a constant.

$$t = -\frac{1}{v_{\max}}\left(K_M\ln\frac{c_{S(t)}}{c_{S,0}}+(c_{S(t)}-c_{S,0})\right) \tag{8a}$$

$$t = -\frac{1}{v_{\max}}\left(K_M(1+\frac{c_I}{K_I})\ln\frac{c_{S(t)}}{c_{S,0}}+(c_{S(t)}-c_{S,0})\right) \tag{8b}$$

$$t = -\frac{1+\frac{c_I}{K_I}}{v_{\max}}\left(K_M(1+\frac{c_I}{K_I})\ln\frac{c_{S(t)}}{c_{S,0}}+(c_{S(t)}-c_{S,0})\right) \tag{8c}$$

$$t = -\frac{1+\frac{c_I}{K_U}}{v_{\max}}\left(K_M\ln\frac{c_{S(t)}}{c_{S,0}}+(c_{S(t)}-c_{S,0})\right) \tag{8d}$$

The implicit form can be transformed to an explicit form as was first described in 1997 by Schnell and Mendoza [64] by the use of the Lambert W function. Further extensions to different kinds of inhibitions and applications have been presented by various authors [38,40,65]. Yet, these analytical integrals of enzyme kinetics are only used by few specialist [66,67]. For further details on the use of the Lambert W function and its application in enzyme catalysis the interested reader is referred to relevant works [41,43,44,64,65,67]. The resulting explicit forms for the reaction rate without inhibition, as well as competitive inhibition, non-competitive inhibition and uncompetitive inhibition are listed in Eq. (9a-d), with W representing the Lambert W function.

$$c_{S(t)} = K_M W\left(\frac{c_{S,0}}{K_M}\exp(\frac{-v_{\max}t+c_{S,0}}{K_M})\right) \tag{9a}$$

$$c_{S(t)} = K_M\left(1+\frac{c_I}{K_I}\right)W\left(\frac{c_{S,0}}{K_M(1+\frac{c_I}{K_I})}\exp(\frac{-v_{\max}t+c_{S,0}}{K_M(1+\frac{c_I}{K_I})})\right) \tag{9b}$$

$$c_{S(t)} = K_M W\left(\frac{c_{S,0}}{K_M}\exp(\frac{-\frac{v_{\max}}{(1+\frac{c_I}{K_I})}t+c_{S,0}}{K_M})\right) \tag{9c}$$

$$c_{S(t)} = \frac{K_M}{(1+\frac{c_I}{K_U})}W\left(\frac{c_{S,0}}{\frac{K_M}{(1+\frac{c_I}{K_U})}}\exp(\frac{-\frac{v_{\max}}{(1+\frac{c_I}{K_U})}t+c_{S,0}}{\frac{K_M}{(1+\frac{c_I}{K_U})}})\right) \tag{9d}$$

3.2. Irreversible uni-bi reaction with product inhibition

As irreversible uni-bi reaction the original Michaelis-Menten kinetic with data by Michaelis and Menten [11] is selected. The reaction mechanism is given by Scheme (2).

$$\begin{aligned} &E+S \rightleftharpoons \mathrm{ES} \rightarrow \mathrm{E}+\mathrm{P}_1+\mathrm{P}_2\\ &E+P_1 \rightleftharpoons \mathrm{EP}_1\\ &E+P_2 \rightleftharpoons \mathrm{EP}_2 \end{aligned} \qquad \text{(Scheme 2)}$$

According to Goličnik [43] the reaction rate v for the reaction according to Scheme (2) is given by Eq. (10a), while the integrated explicit form is given by Eq. (5b). As the experimental data from Michaelis and Menten were given as ratio of product and initial substrate concentration, Eq. (10b) is reformulated to Eq. (10c). The model has four kinetic parameters, with $v_{\max}$ and K_S, K_{P1} and K_{P2} representing the affinity constant of the substrate, product 1 and product 2, respectively. K_P represents the general product inhibition constant and can be calculated from the individual product inhibition constants.

$$v = \frac{v_{\max}c_S}{K_S(1+c_{P1}/K_{P1}+c_{P2}/K_{P2}+c_S)} = \frac{v_{\max}c_S}{K_S(1+c_P/K_P)+c_S} \tag{10a}$$

$$c_{S(t)} = K_S^* W\left(\frac{c_{S,0}}{K_S^*}\exp(\frac{-v_{\max}^* t+c_{S,0}}{K_S^*})\right) \tag{10b}$$

$$\frac{c_{P(t)}}{c_{S,0}} = 1-\frac{K_S^*}{c_{S,0}}W\left(\frac{c_{S,0}}{K_S^*}\exp(\frac{-v_{\max}^* t+c_{S,0}}{K_S^*})\right) \tag{10c}$$

with

$$v_{\max}^* = \frac{v/K_S}{1/K_S-1/K_P}$$

$$K_S^* = \frac{1+c_{S,0}/K_P}{1/K_S-1/K_P}$$

$$K_P = \frac{K_{P1}K_{P2}}{K_{P1}+K_{P2}}$$

3.3. Reversible uni-uni reaction

A reversible uni-uni reaction can be described as a two-step reaction following Scheme (3a), or as a three-step reaction, assuming either a rapid equilibrium with a rate-limiting second step (Scheme 3b), or a steady state where the change in concentration of ES and EP over time is assumed to be zero (Scheme 3c) [8].

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E}+\mathrm{P} \qquad \text{(Scheme 3a)}$$

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E}+\mathrm{P}, \text{rapid equil.}, \mathrm{k}_2 << (\mathrm{k}_{-2}+\mathrm{k}_3),\ \mathrm{k}_{-2} << (\mathrm{k}_{-1}+\mathrm{k}_2) \qquad \text{(Scheme 3b)}$$

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E}+\mathrm{P}, \text{steady state} \qquad \text{(Scheme 3c)}$$

All three mechanisms result in the same kinetic model, stated in Eq. (6a). The analytical integral in explicit form is given in Eq. (6b) [44]. However, it should be noted that the definitions of the parameters K_f, K_r, v_f, and v_r as composite factors of different rate constants (k values) are different for each reaction mechanism [8]. Index f represents the forward reaction, index r represents the backward reaction. While for Schemes (3a) and (3c) the K values are represented as different affinity

constants, for Scheme (3b) the K values are dissociation constants.

$$v = \frac{\frac{v_f}{K_f}c_S - \frac{v_r}{K_r}c_P}{1 + \frac{c_S}{K_f} + \frac{c_P}{K_r}} \tag{6a}$$

$$c_{S(t)} = c_{S,0} - \Delta c_{S,\infty} + K_M^* W\left(\frac{\Delta c_{S,\infty}}{K_M^*}\exp\left(\frac{-v_{\max}^* t + \Delta c_{S,\infty}}{K_M^*}\right)\right) \tag{6b}$$

with

$$\Delta c_{S,\infty} = c_{S,0} - c_{S,\infty} = \frac{v_f c_{S,0}/K_f}{v_f/K_f + v_r/K_r}$$

$$v_{\max}^* = \frac{v_f/K_f + v_r/K_r}{1/K_f - 1/K_r}$$

$$K_M^* = \frac{1 + c_{S,0}/K_f}{1/K_f - 1/K_r} - \Delta c_{S,\infty}$$

4. Results

The following subsections discuss the results obtained by the different methods for the three case studies. For all case studies the confidence intervals are calculated by the use of *nlparci* function in Matlab.

4.1. Case study 1: irreversible uni-uni reaction

Despite its simplicity, this reaction mechanism is frequently described in the literature with or without inhibition [68–73]. In order to avoid a bias the *in-silico* data for parameter estimation, data are not generated with the analytical integrals described in (Scheme 3a). (4a)-(4d). Instead, a numerical integration with the *ode45* solver in Matlab is used to generate 1.000 data points over a time horizon of 30-minutes, using $K_M = 1$ mM, $v_{max} = 1$ mM min^{-1}, and $K_i = 5$ mM. Random noise is further added to the concentration data, considering a normal distribution 0–10 % by the use of Matlab's *randn* function. For parameter estimation 50 evenly spaced data points are extracted for testing the different approaches. An initial substrate concentration of 10 mM, and three different inhibitor concentrations of 1 mM, 10 mM and 20 mM are considered. Data are listed in the Gitlab repository.

As the experimental data were noisy up to 10 % and an irreversible reaction was considered, it occurs that some substrate concentrations are negative. Respectively, the product concentrations occur higher than the initial concentration of the substrate. The authors are aware that this fact is physically impossible. Usually this can be avoided by accurate analysis of the concentrations. However, for the implicit approach, an ln-term is applied, cf. (Scheme 3a). If a concertation is zero or smaller, these data cannot be analysed. Therefore, we have set the substrate concentration to 10^{-10} mM if a value was below zero.

To test the capability of the different methods to cope with the noisy data, the "true" parameter values used for data generation are provided as initial guess. Thus, each method should in principle be able to maintain the true values if the data represents the model accurately enough. Fig. 2 illustrates the normalized results for the individual parameter estimation with the different methods for perfect data without noise, as well as 5 % and 10 % noise, without inhibition (left) and with competitive inhibition (right). Data with other noise levels and other inhibition mechanisms show similar behaviour, are omitted from further discussion here, but are provided in the Gitlab repository. The error bars represent the 95 % confidence intervals for the estimated parameters.

All methods effectively maintain the true parameter values when provided perfect data without noise, furthermore showing high precision in the estimates, for both models. In case of added noise, the parameter estimates of the different methods deviate to different degrees, with the implicit form showing the highest deviation and the spline interpolation showing in average the best results. The latter highlights the benefits of the regularization that comes with the spline interpolation, effectively attenuating the random noise. While the implicit form of the analytical integration results in larger distortion of the parameter estimates, the explicit approach maintains values quite close to the true parameter.

The difference can in this case be explained by the effect of the noise on the objective function. As the noise was introduced to the concentration, the error was introduced on the x-axis for the implicit form (t = f (c)). As such the objective function in the ordinary least square (OLS) minimization aims at a modulation of the measurement times. This problem is e.g. overcome when considering the total least square (TLS) minimization instead, which considers the residuals in *t* and *c* equally.

Fig. 3 shows the results of the parameter estimation with all methods for the reaction without inhibition and 10 % noise. Comparing the exact course of the curve (cf. zoom-in at the right Fig. 3), all methods underestimate the true kinetics. This is to be expected, as the scattering in the late course of the reaction only considers positive deviations due to

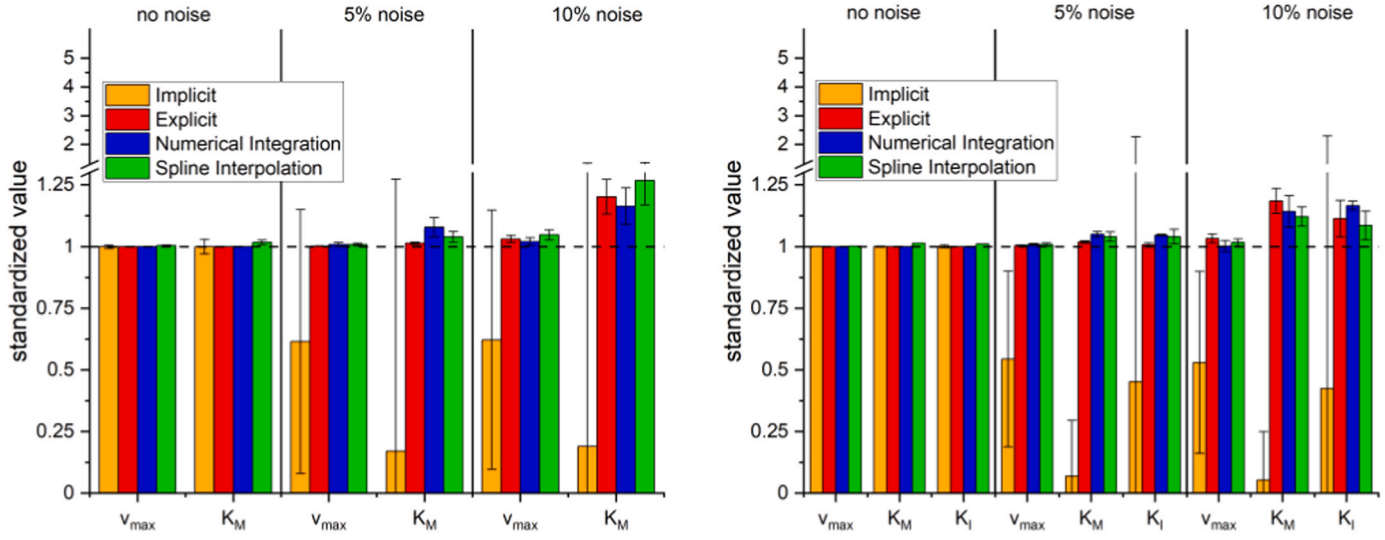

Fig. 2. Normalized values for estimates with different approaches for uni-uni reaction without inhibition (left) and competitive inhibition (right) at different noise levels.

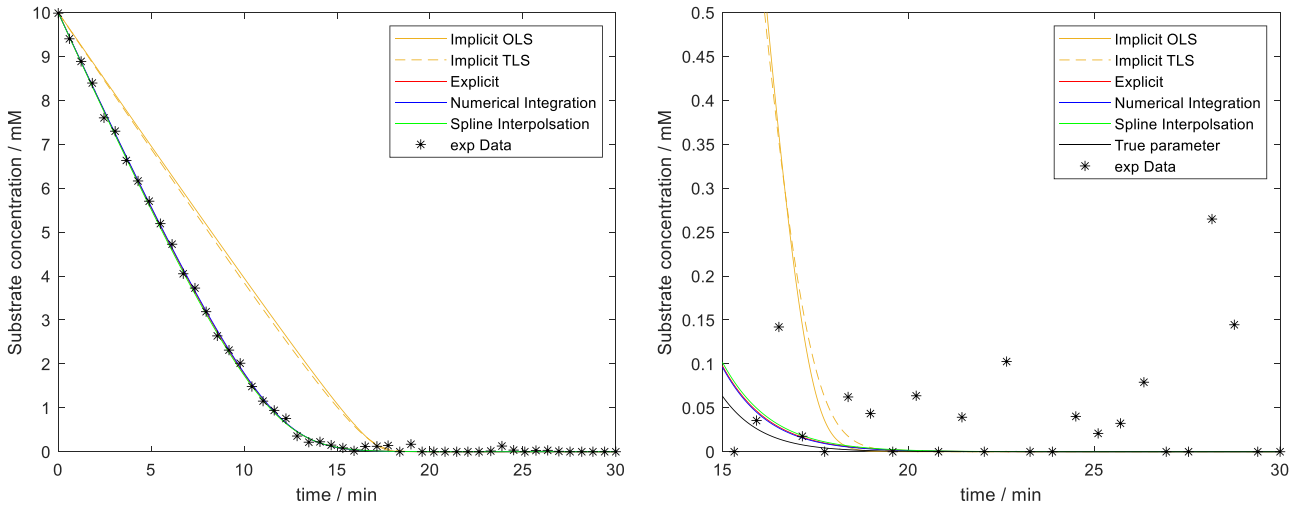

Fig. 3. Experimental data for an irreversible uni-uni reaction without inhibition and 10 % noise. Lines represent different approaches for parameter estimation. Left: total view of all approaches and experimental data as c_t. Right: Zoom in to 15 – 30 minutes.

the noise, whereas the negative deviations were set to 10^{-10} mM. The implicit approaches both show very poor agreement. This is due to the fact that the deviation in the x-direction is minimized and therefore the values in the late course of the reaction in particular significantly influence the result in the wrong direction. If only values from the first 20 minutes are considered for the regression, both implicit models show better agreement.

This can also be conducted from Fig. 4, which shows the simulated progress curve with all methods for the reaction with competitive inhibition ($c_I = 20$ mM) and 10 % noise. Due to the inhibition the overall reaction rate is decreased and therefore the reaction time expands. While for the reaction without inhibition a nearly full conversion is reached after 15 minutes, it takes up to 25 minutes for the inhibited reaction. Furthermore, the numerical methods and the explicit integral in particular are able to describe the true kinetics very well. It is noticeable that the TLS method of the implicit function performs particularly well, because even in the late course several data points are still clearly above zero and thus the deviation in x- and y- direction can be minimized well. In contrast, the implicit OLS method continues to perform very poorly. As the algorithm minimizes the deviation in the x-direction, the present data points lead to an uneven distribution along the y-axis and thus to a weighting of the values at low y-values. This leads to a distortion of the estimated parameters.

While the previous analysis evaluated the capability of the different methods to maintain the true parameters during the regression to noisy data, it is also important to evaluate the capability to identify the true parameters when using different initial values. Therefore, a grid search was performed to identify the ranges of initial estimates for which the individual methods perform well. Fig. 5 provides an illustration of these ranges, given that the initial guess for the v_{max} and K_M parameter are changed simultaneously.

Interestingly, only the numerical integration is able to calculate the true parameter from a low initial value for the parameters, while the spline interpolation performs well for the largest range of initial values, covering rather higher initial values for the parameters. The analytical approaches are more sensitive to poor initial guesses. It needs to be noted that the local optimization methods applied in the current work did actually fail to converge for initial values below 0.1.

Overall, the analytical integration with the implicit equation shows several weaknesses. A remedy could be to reformulate the regression problem as t-f(c)= 0. However, this increases the already unintuitive nature of the implicit function. Also, when applying the implicit approach, a boundary condition would be that the concentrations must be evenly distributed over the measuring points. However, this

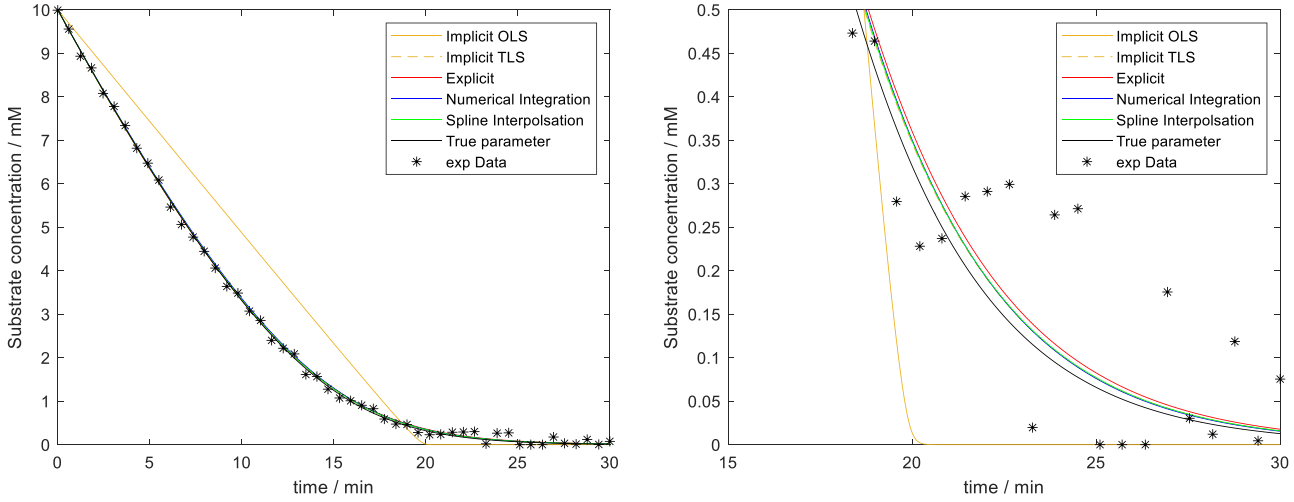

Fig. 4. Experimental data for an irreversible uni-uni reaction with competitive inhibition and 10 % noise. Lines represent different approaches for parameter estimation. Left: total view. Right: Zoom in to 10 – 15 minutes.

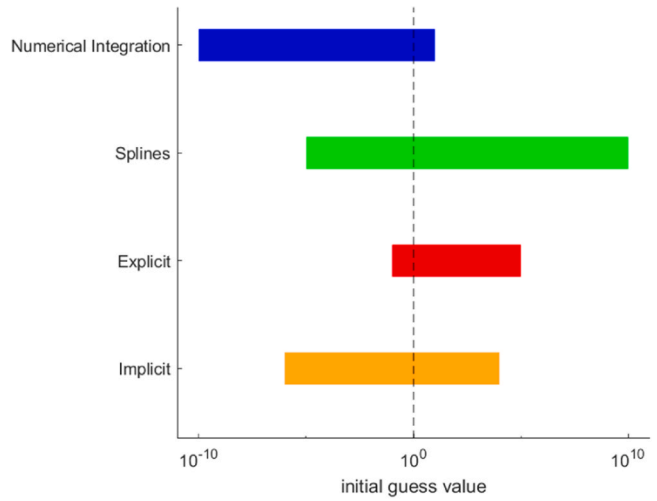

Fig. 5. Range of initial values from which the true parameter values are identified within one order of magnitude.

condition cannot be met as well during real experiments as a uniform distribution of the sampling intervals. Consequently, the implicit equation will not be considered in the following discussion. So far, the spline interpolation shows the best performance.

4.2. Case study 2: irreversible bi-uni reaction

Michaelis and Menten investigated the hydrolysis of sucrose to fructose and glucose as an example of an irreversible bi-uni reation. Of course, hydrolysis is a bi-bi mechanism as water molecules are a substrate. However, water is present in excess in biological systems, so the water concentration is considered constant [10,11,74–76].

The original work of Michaelis and Menten has been translated by Johnson and Goody [10]. Beside the fact, that they used the initial slope method, the data cover the full range of a progress curve. A total of seven experiments were performed, but in agreement with Johnson and Goody [10], we only use five experiments with 5–7 data points each. Four of the five experiments lasted until equilibrium was reached.

Based on their data, Michaelis and Menten determined the kinetic parameters by the use of initial slopes, with $v_{max} = 0.76$ mM min^{-1}, $K_S = 16.7$ mM and $K_P = 35.1$ mM. These are again used as initial guess for parameter estimation with the different methods. Goličnik [43] used analytical integration with the explicit equation for parameter regression and found a very good agreement with data by Michaelis and Menten (cf. Table 2). The same results are obtained in the current work for the analytical integration approach, with only minor variation for the K_P value. This small difference may be caused by the different algorithms for nonlinear regression, since Goličnik used Mathematica with automatic selection of the optimization algorithm and the current work used Matlab with the Trust-Region-Reflective-algorithm.

Since parameter estimates with the same accuracy are also determined with the spline interpolation approach, the precision of the parameter estimates is evaluated on the basis of the confidence intervals. However, both methods do also show comparable parameter precision for this case. Only the parameter estimates of the numerical integration approach show a lower accuracy and precision. It also shows a high sensitivity for settings of integration steps used in Encora. In this study the number of integration steps was increased to 20. Beside the numerical integration, the K_P value is the most unsecure one, which may be due to the fact, that this value is a lump value of K_G and K_F but was individually addressed by Encora.

As mentioned in the introduction section the values for v_{max} and K_S can be roughly extracted from the graphical analysis of Michaelis and Menten's diagram. If the results of Michaelis and Menten for the initial rates are presented in a classical Michaelis and Menten's diagram, we can assume for further analysis that especially $v_{max} = 0.76$ and $K_S = 16.5$ mM represents the true parameters quite well, reflecting the correct biochemical mechanism. For complex reactions, however, a graphical analysis is much more complex. In order to evaluate the dependence of the different PCA methods for parameter estimation the results are again evaluated for different initial values, resulting in the data listed in Table 3. While the spline interpolation approach calculates the same values for the kinetic parameters with high accuracy and precision, independent from the set of initial values, the results of both, the analytic and numerical integration approach fluctuate largely with the initial values. Even with initial values quite close to the true values, the results of these methods may already deviate from the "true" values.

Based on 35 data points and three parameters to estimate, the critical t value for a level of 95 % significance is 2.036. For the explicit method, the t-values are very high, especially for v_{max}, which indicates a consistent significance of this parameter across most initial sets. This indicates that v_{max} has a strong influence in this model. The t-values for K_S and K_P are also high, but not as pronounced as for v_{max}, suggesting somewhat more variable influence of these parameters on the model. With the numerical integration method, the significance of v_{max} is also striking, especially with higher values assumed at the initial guess, which indicates possible numerical instabilities. The high t-value in certain initial sets shows that the effect may not be captured linearly or is amplified by numerical artefacts. The low t-values of K_S and K_P in certain initial sets show that these are not considered significant in these cases, which could indicate an insufficient fit in the initial values or model limits. The spline interpolation method provides consistent t-values between the parameters, although v_{max} also shows quite consistently high t-values, which confirms its significant role. From a methodological point of view, the spline method indicates a stronger stability of the parameter evaluation. Overall, the analysis shows that different methods and initial values can significantly influence the statistical significance of the model parameters, with v_{max} consistently appearing as the most significant parameter, while K_S and especially K_P vary more depending on the initial setup.

Already for this slightly more complex second case study, the analytical integration with the explicit equation and the numerical integration do not show a robust performance for varying initial values in the nonlinear regression. Yet, the spline interpolation shows a good agreement with the true parameters, for a wide range of initial values. While good initial values can be evaluated on the basis of initial slope experiments, these cause additional experimental effort and cost. Remarkable is the size of the confidence intervals, which suggest a good agreement with the model in all cases, even it is assumed not to be true.

Table 2
Kinetic parameters according to Eq. 10a-c for a simultaneous multiple progress curve fitting with original data by Michaelis and Menten [11].

	Original work by Michaelis Menten (also used as initial values in this work)	Reanalyzed results using the explicit approach by Goličnik [43]	Reanalyzed results using the explicit approach (this work)	Reanalyzed results using the numerical integration approach (this work)	Reanalyzed results using the spline interpolation approach (this work)
v_{max} [mM min^{-1}]	0.76	0.76 ± 0.03	0.76 ± 0.01	0.76 ± 0.01	0.78 ± 0.02
K_S [mM]	16.7	16.5 ± 1.4	16.5 ± 0.8	19.7 ± 1.0	16.9 ± 1.3
K_P [mM]	35.1	36.7 ± 6.5	36.5 ± 4.3	51.8 ± 7.2	31.2 ± 5.4

Table 3
Kinetic parameters for the hydrolysis of sucrose calculated with varying initial values and different methods.

Initial values sets	Explicit	t-value	Numerical integration	t-value	Spline interpolation	t-value
$v_{max} = 0.76$ [mM min^{-1}]	0.76 ± 0.01	29.35	0.76 ± 0.01	29.26	0.78 ± 0.02	30.03
$K_S = 16.7$ [mM]	16.5 ± 0.8	12.44	19.7 ± 0.96	14.84	16.9 ± 1.3	12.74
$K_P = 35.1$ [mM]	36.5 ± 4.3	5.98	51.8 ± 7.17	8.49	31.2 ± 5.4	5.11
$v_{max} = 1$ [mM min^{-1}]	0.76 ± 0.01	29.26	0.72 ± 1.48	27.72	0.78 ± 0.02	30.03
$K_S = 1$ [mM]	16.5 ± 0.8	12.43	23.55 ± 131.31	17.75	16.9 ± 1.3	12.74
$K_P = 1$ [mM]	36.7 ± 4.3	6.01	0.35 ± 2.54	0.06	31.2 ± 5.4	5.11
$v_{max} = 10$ [mM min^{-1}]	0.74 ± 0.02	29.49	13.25 ± 0.69	510.14	0.78 ± 0.02	30.03
$K_S = 1$ [mM]	14.7 ± 1.7	11.08	1.61 ± 0.52	1.2136	16.9 ± 1.3	12.74
$K_P = 0.01$* [mM]	26.55 ± 6.3	4.35	0.01 ± 0.00	0.2638	31.2 ± 5.4	5.11
$v_{max} = 10$ [mM min^{-1}]	0.67 ± 0.09	25.60		NA	0.78 ± 0.02	30.03
$K_S = 1$ [mM]	1.04 ± 0.6	0.78	Approach does not converge with these initial values	NA	16.9 ± 1.3	12.74
$K_P = 0.001$* [mM]	0.94 ± 0.6	0.15		NA	31.2 ± 5.4	5.11
$v_{max} = 1000$ [mM min^{-1}]	1000 ± 0.3	38501		NA	0.78 ± 0.02	30.03
$K_S = 1$ [mM]	1 ± 0.0	0.75	Approach does not converge with these initial values	NA	16.9 ± 1.3	12.74
$K_P = 1000$* [mM]	1000 ± 36.1	163.88			31.2 ± 5.4	5.11
$v_{max} = 100$ [mM min^{-1}]	14.6 ± 0.68	562.12	117.17 ± 0.33	4511	0.78 ± 0.02	30.03
$K_S = 1$ [mM]	255.3 ± 16.7	192.43	1.93 ± 0.03	1.45	16.9 ± 1.3	12.74
$K_P = 10$* [mM]	4.98 ± 0.6	0.8161	6.6 ± 0.012	1.08	31.2 ± 5.4	5.11

* In Encora, the values $K_{M,FRU}$ and $K_{M,Glc}$ are given, from which K_P can be calculated. Here too, the results are strongly dependent on the initial values and settings. In this study, K_P was calculated as follows 0.01 = 1000 • 0.01/(1000 +0.01); 0.001 = 1000 • 0.001/(1000 +0.001); 1000 = 1111 • 10000/(1111 +10000); 10 = 10000 • 10/(10000 +10). Settings were used as described above.

4.3. Case study 3: reversible uni-uni reaction

In the last case study, a reversible reaction is investigated, represented by the isomerization of glucose to fructose by an isomerase. Since the results for the true parameters have been known in case studies 1 and 2, we now assume no information about the kinetic parameters to see, how the different approaches perform. The reaction is catalysed by an isomerase, which is well known [12,77–80] and kinetic parameters for isomerase have been widely described in literature, as it is used on an industrial tons per year scale [81], e.g. for the production of sweeting syrup [20,82]. Here, we focus on the performance of the different approaches for poorly planned experiments for the example of Sweetzyme IT, a commercial isomerase from *Streptomyces murinus*.

For data of an isomerase, we performed experiments as follows: the reaction took place in a 150 mL glass reactor at 60°C in 0.01 M $MgCl_2$ (by Bernd Kraft) and 0.05 M TRIS (>99.3 % by Carl Roth) HCl buffer (pH 7.5) as ideal stirred tank reactor. Glucose (>99.5 % by Carl Roth) was added for the desired concentration (75 mM or 15 mM). 1.3 g l^{-1} Sweetzyme IT extra (from *Streptomyces murinus* by Novozymes) was used. Samples were collected and measured at defined sampling time. The samples were centrifuged (1 min, 700 rpm) before analysis. The product concentration was analyzed based on Seliwanoff's test, as described before [12]. Error bars for experimental data points represents the accuracy of analysis, in terms of manual errors during dilution, error of balance and calibration line of Seliwanoff's test. As the $K_{M,GLC}$ value for glucose as substrate for Sweetzyme IT extra for similar reaction conditions (60°C, additional Mg^{2+}) is in the range of 408 mM to 700 mM [12,79,80] we performed experiments below these values, to see which approach is able to calculate parameters according to literature values. In the literature, it is quite common to estimate the parameters of the reverse reaction only from the progress curve of the forward reaction, although the information for the parameters of the reverse reaction is not directly available experimentally. As initial values we defined four sets: the first one is the poorest, as it uses randomly 1 as initial value. Please note, that the initial values of $K_{M,GLC}$ and $K_{M,FRU}$ must differ according to the definition of v^*_{max} and K^*_M (cf. equation (11)) otherwise the denominator would be 0. Therefore, we use 1.1. The second set bases on the initial rate of the 75 mM experiment of the forward reaction and 10 % of the value for the backward reaction as well as on the used concentrations for the K_M values. The third and the fourth sets base on literature data. Please note, that for set three [80] $v_{GLC} < v_{FRU}$ and $K_{M,GLC} < K_{M,FRU}$, while for set four [79] $v_{FRU} < c_{GLC}$ and $K_{M,FRU} < K_{M,GLC}$. The results for the calculated kinetic parameters with different approaches are shown in Table 4.

As in case studies 1 and 2, the splines approach again shows no dependency of initial values. For all sets $v_{GLC} = 0.27$ mM min^{-1} g^{-1}, $v_{FRU} = 0.32$ mM min^{-1} g^{-1} where $K_{M,GLC} = 219$ mM and $K_{M,FRU} = 86$ mM. The explicit approach also shows the same qualitative results, where $v_{GLC} < v_{FRU}$ and $K_{M,GLC} > K_{M,FRU}$, independent of the initial values. As the confidence intervals of the explicit approach are smaller, these results seem more precisely. A comparison of the two approaches shows that the calculated parameters partly coincide within the limits of accuracy or are at least not far apart. However, as was shown in case study 2 we cannot really state, which approach is better based on the confidence intervals. Just slightly higher confidence intervals than the explicit approach shows the numerical integration. However, as in both other case studies, the calculated values highly depend on the initial parameters. Especially for the first set the results are poor, compared to the other approaches. For the initial values sets 2–4 the parameter for glucose are quite similar, but for fructose the results differ more and are not within the different confidence intervals. The numerical integration approach does show a good agreement for the forward reaction with the explicit approach but no quantitative overlap with the other approaches for the backward reaction. Please note that for the explicit and spline approaches calculate $v_{GLC} < v_{FRU}$ and $K_{M,GLC} > K_{M,FRU}$, while for the numerical integration $v_{GLC} < v_{FRU}$ but $K_{M,GLC} < K_{M,FRU}$ are calculated. However, the proportion of these values reported in literature [79,80,83,84] also show that there is disagreement on this point. In all cases the relatively high K_M values for glucose and fructose were not estimated by any approach.

Fig. 6 shows the experimental data as well as simulation and confidence intervals with calculated parameters according to initial value set 2 for all approaches. As can be seen, for all approaches experimental data are covered in the range of accuracy. It can also be seen, that the deviation of the simulation of all approaches is quite small even the calculated parameters differ from each other.

Neither based on a graphical analysis of the progress curve nor on the basis of the confidence intervals can a statement be made as to which approach reflects the true parameters. However, the qualitative statements from case studies 1 and 2 can also be found here.

Based on 22 data points and 4 parameters to estimate, the critical t value for a level of significance 95 % is 2.101. In the explicit approach, the t-values are consistently low, particularly for the parameters v_{GLC}, v_{FRU}, and $K_{M,FRU}$. This suggests that these parameters may not have a

Table 4
Kinetic parameters and well as absolute and relative 95 % confidence intervals for sweetzyme IT at 60°C, calculated with different approaches and varying initial values.

Initial values sets	Explicit	t-value	Numerical integration	t-value	Spline interpolation	t-value
v_{GLC} = 1 mmol min^{-1} g^{-1}	0.23 ± 0.02 (6.5 %)	0.0781	1.00 ± 0.1 (10.2 %)	0.3372	0.27 ± 0.02 (7.2 %)	0.0911
$K_{M,GLC}$ = 1 mM	168 ± 12 (6.9 %)	1.7932	1002 ± 103 (10.3 %)	10.7124	219 ± 17 (7.6 %)	2.3413
v_{FRU} = 1 mmol min^{-1} g^{-1}	0.47 ± 0.04 (9.0 %)	0.0609	0.01 ± 0.01 (64.2 %)	0.0013	0.32 ± 0.04 (11.3 %)	0.0411
$K_{M,FRU}$ = 1.1 mM	103 ± 9 (9.2 %)	0.9197	9 ± 4 (42.9 %)	0.0807	86 ± 10 (11.7 %)	0.7713
v_{GLC} = 0.5 mmol min^{-1} g^{-1}	0.23 ± 0.02 (6.5 %)	0.0781	0.22 ± 0.02 (6.7 %)	0.0741	0.27 ± 0.02 (7.2 %)	0.0911
$K_{M,GLC}$ = 15 mM	168 ± 12 (6.9 %)	1.7932	165 ± 12 (7.0 %)	1.7540	219 ± 17 (7.6 %)	2.3413
v_{FRU} = 0.05 mmol min^{-1} g^{-1}	0.47 ± 0.04 (9.0 %)	0.0609	1.45 ± 0.13 (9.1 %)	0.1861	0.32 ± 0.04 (11.3 %)	0.0411
$K_{M,FRU}$ = 75 mM	103 ± 9 (9.2 %)	0.9197	324 ± 30 (9.2 %)	2.9058	86 ± 10 (11.7 %)	0.7713
v_{GLC} = 0.486 mmol min^{-1} g^{-1}	0.23 ± 0.02 (6.5 %)	0.0781	0.23 ± 0.02 (6.7 %)	0.0776	0.27 ± 0.02 (7.2 %)	0.0911
$K_{M,GLC}$ = 497 mM	168 ± 12 (6.9 %)	1.7932	167 ± 12 (7.0 %)	1.7854	219 ± 17 (7.6 %)	2.3413
v_{FRU} = 0.804 mmol min^{-1} g^{-1}	0.47 ± 0.04 (9.0 %)	0.0609	1.06 ± 0.10 (9.1 %)	0.1360	0.32 ± 0.04 (11.3 %)	0.0411
$K_{M,FRU}$ = 822 mM	103 ± 9 (9.2 %)	0.9197	236 ± 23 (9.2 %)	2.1166	86 ± 10 (11.7 %)	0.7713
v_{GLC} = 0.292 mmol min^{-1} g^{-1}	0.23 ± 0.02 (6.5 %)	0.0781	0.22 ± 0.02 (6.7 %)	0.0742	0.27 ± 0.02 (7.2 %)	0.0911
$K_{M,GLC}$ = 700 mM	168 ± 12 (6.9 %)	1.7932	165 ± 12 (7.0 %)	1.7640	219 ± 17 (7.6 %)	2.3413
v_{FRU} = 0.187 mmol min^{-1} g^{-1}	0.47 ± 0.04 (9.0 %)	0.0609	1.51 ± 0.14 (9.1 %)	0.1938	0.32 ± 0.04 (11.3 %)	0.0411
$K_{M,FRU}$ = 450 mM	103 ± 9 (9.2 %)	0.9197	335 ± 31 (9.2 %)	3.0044	86 ± 10 (11.7 %)	0.7713

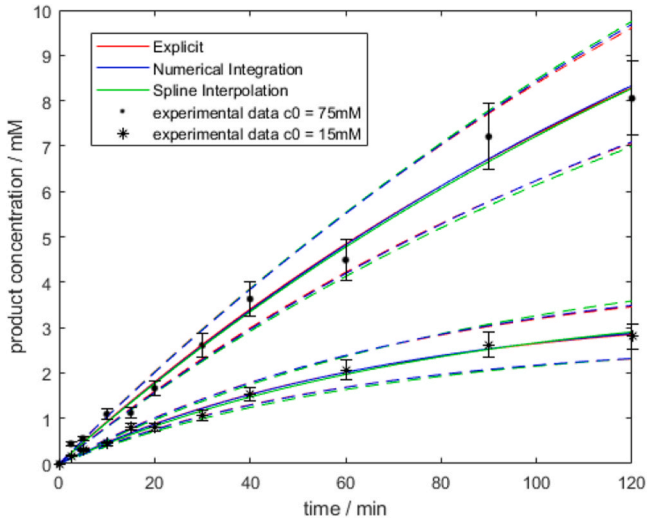

Fig. 6. Experimental data of sweetzyme IT at 60°C in 0.01 M $MgCl_2$, 0.05 M TRIS, pH 7.5 for c_0 = 75 mM and 15 mM. Solid lines represent regression curves with different approaches, dashed lines represent 95 % confidence intervals. Error bars represent experimental error.

strong statistical influence in this model, which could indicate that the model or the data do not effectively capture the effects of these parameters. The parameter $K_{M,GLC}$ shows slightly more significance. For the numerical integration, the t-values vary somewhat. $K_{M,GLC}$ occasionally shows higher t-values, but none of the parameters consistently achieve high values. This also indicates limited significance for the parameters with this method, although the values seem to be more sensitive to the initial guess. The spline interpolation consistently shows lower t-values for most parameters. Again, $K_{M,GLC}$ exhibits slightly higher t-values, but the overall significance remains questionable. The relatively high uncertainties in the estimated parameters, especially for extreme initial guesses, highlight the challenges associated with the estimation process.

The mathematical analysis of the progress curve with unknown true parameter shows an unclear result. As in case studies 1 and 2 the splines approach shows one of the highest reproducibility of calculated parameters in respect to different initial values. Also, the explicit approach shows highest reproducibility. Again, numerical integration has a high dependency of calculated parameters. However, none of the results shows an agreement with literature values but is has to be mentioned, that also literature values show high differences [12,79,80]. This could be explained e.g. by different batches of enzyme from supplier or small changes in reaction conditions, which can influence the results immensely [21].

5. Conclusions

In this study four different approaches for the progress curve analysis were investigated. Despite all of them are already known, till now there was no comprehensive comparison for the application of these approaches. The analytical implicit approach is known for half a century. However, this approach has not really established in the community. The main weakness has been stated as fast enzyme decay is not considered [17]. However, with modern computer tools, the implementation of a simultaneous enzyme decay is not a big challenge. Based on the results in this study the implicit approach is not well compatible with a standard regression, as the objective function minimizing the y-error does not fit to an experimental error in concentration measurement. The analytical explicit approach shows a good performance within this study. But currently it seems “unnoticed outside of the specialist community” [66]. This may be due to the fact, that only a limited number of integrals are available and especially integrals for industrial relevant two-substrate kinetics are not known to our best knowledge. Therefore, numerical approaches represent a good and flexible to all kind of kinetics alternative as they are able to be used directly on the differentiated form of the reaction rate. The numerical integration is widely used and available in diverse software tools. However, results based on this approach shows a high dependency on initial values in this study. Therefore, this approach needs some fundamental knowledge about the underlying biochemistry. The spline approach, also known for decades, seems to leads an unknown existence for the use of enzyme kinetic modelling. This approach is only found in a very small number of publications. However, based on our findings, it could be a promising alternative, when it comes to parameter estimation based on progress curve analysis. Unlike the numerical integration, spline integration also bears the potential to apply gradient-based global optimization solvers for the nonlinear regression, such as BARON [85] or Antigone [86], which can in principle extend the range for convergence to the whole design space for the parameters. Yet, these algorithms are computationally more demanding and the computational effort increases with problem size, which scales with the number of data points.

As this study has shown, the estimated kinetic parameters of a progress curve analysis highly depend on the procedure used. However, in most of the current studies detailed information about exact procedure are unmentioned, namely: used regression function, used algorithm, initial values, abort criterion, etc. In future, this information needs to have the same importance as it is stated for experimental

procedures, were (mostly) all information is given in respect to used buffer composition, pH, temperature, reactants, etc. For reproducibility and transparency this information is mandatory.

All details about the used modelling procedures can be found in our Gitlab repository.

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CRediT authorship contribution statement

von Ziegner Francesca: Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Skiborowski Mirko:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Waluga Thomas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors report there are no competing interests to declare.

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The authors report there are no competing interests to declare.

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