Affirmation of the Separate Rate-law Method for Determining $v_{\text{max}}$ and $K_M$ of the Michaelis Reaction

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Abstract. The maximum-velocity, $v_{\text{max}}$, and the Michaelis-constant, $K_M$, are the essence of the Michaelis-Menten equation. The value of $v_{\text{max}}$ is determined when the reaction is in zeroth-order and $K_M$ is in first-order. With a slight change in the notion of $v_{\text{max}}$ concept, both were used as the basis for a new determination method called Separate Rate-law Method (SRlM). As yet, the Lineweaver - Burk and Eadie - methods were the most commonly used. Both combine zeroth-order and first-order data. The purpose of this study was to validate the quality of all methods. The research was carried out theoretically. All were tested by applying them to the same literature data, and the results were compared. The assessment is based on the $v_{\text{max}}$ and $K_M$ values obtained; doubling $[E]$ will double $v_{\text{max}}$ but the same $K_M$. The results show that the two present methods are inaccurate. Both give $v_{\text{max}}$ and $K_M$ values even if the reaction is always first-order. Based on the integral method, two variants of the new method, $SRlM_{IM}$ and initial rate, $SRlM_{IRM}$, were also introduced. All new methods give more accuracy, which results in the method accuracy of $SRlM \approx SRlM_{IM} > SRlM_{IRM}$.

1. Introduction

According to Michaelis-Menten, enzyme-catalyzed reactions proceed by following the reaction mechanism in Eq. (1-2).

\[
\begin{align*}
E + S & \rightleftharpoons ES \\
k_1 & \\
ES & \rightarrow E + P \\
k_2 & 
\end{align*}
\]

Using the Steady-State Approximation and after some steps, the rate law is written in Eq. (3).

\[
v = \frac{k_2[E][S]}{K_M+[S]} \tag{3}
\]

Where $v$, $k_2$, $[E]$, $[S]$, and $K_M$ are rate, enzyme rate constant, enzyme concentration, substrate concentration, and Michaelis constant. $K_M$ is a measure of the enzyme's affinity for its substrate, and it has value in the range of $10^{-6}$ to $10^{-2}$ M. In experiments, the initial concentration of the enzyme is usually made very small compared to substrate concentration. In data analysis, the initial rate method ($IRM$) is the most commonly used.
In Eq. (3), the order with respect to $[E]$ is first-order, but the order with respect to $[S]$ is uncertain. If $[S] >> K_M$, Eq. (3) becomes Eq. (4), in which the reaction is zeroth-order to $[S]$ and first-order to $[E]$.

$$v = k_2 [E]$$

(4)

In this condition, all enzymes react with the substrate, and the reaction is in the maximum velocity or $v$ will approach $v_{\text{max}}$. Then, Eq. (4) becomes Eq. (5).

$$v = v_{\text{max}} = k_2 [E]$$

(5)

However, if $[S] << K_M$, Eq. (3) becomes Eq. (6), in which the reaction is of the first-order to $[E]$ and to $[S]$.

$$v = \frac{k_2 [E][S]}{K_M}$$

(6)

Inserting Eq. (5) into Eq. (6) gives Eq. (7).

$$v = \frac{v_{\text{max}}[S]}{K_M}$$

(7)

Inserting Eq. (5) into Eq. (3) gives Eq. (8).

$$v = \frac{v_{\text{max}}[S]}{K_M+[S]}$$

(8)

Three problems arise. First, there is a need for a new method that followed the basic assumptions of Michaelis reaction. The method must be based on Eq. (5) and Eq. (7) and can be used as a standard method. Second, during the reaction process, the enzyme/catalyst concentration will be constant, but the substrate decreases. Depending on the ratio of the initial concentration of substrate and enzyme, the reaction can be zeroth-order at the beginning reaction; however, it then becomes first order at near-end reaction or always being the first-order reaction. There is no check for the real rate law at these two conditions in all of the present equations for Michaelis-Menten or catalyzed reaction fitting-constants determination. When both data are combined, this can cause problems. For example, if the first happens, it cannot give the correct values for $v_{\text{max}}$ and $K_M$ because the reaction changes from zeroth-order to first-order at the near-end. If the latter happens, it will contradict the essence of Eq. (5) and Eq. (7).

Third, $v_{\text{max}}$. will be achieved if all enzymes/catalyst reacts with the substrate. However, the Michaelis reaction is first-order to enzyme/catalyst concentration. Thus, doubling enzyme/catalyst concentration will double the value of $v_{\text{max}}$ as well. That is to say, $v_{\text{max}}$ is not a constant. In the present equations, $v_{\text{max}}$ is used to determine $K_M$ without considering this fact. In addition, save for first-order reaction, $IRM$ or the differential method, will give different rate constant values at a constant time of reaction.

The present methods for determining $v_{\text{max}}$ and $K_M$ are based on Eq. (8). However, it is difficult to determine both fitting-constants in this equation because the plot of $v$ against $[S]$ is hyperbolic. To cope with, the linear form is usually used, such as that introduced by Lineweaver-Burk ($L-B$) [1] and by Eadie [2]-Hofstee ($E-H$) [3], Hanes [4], and Espenson. The most commonly used is $L-B$ and $E-H$, as presented in Eq. (9-10), respectively.

$$\frac{1}{v_o} = \frac{1}{v_{\text{max}}} + \frac{K_M}{v_{\text{max}}[S]_o}$$

(9)
The linear plot of \( \frac{1}{v_o} \) against \( \frac{1}{[S]_0} \) gives \( v_{\text{max}} = \frac{1}{\text{intercept}} \) and \( K_M = \frac{v_{\text{max}}}{\text{slope}} \).

The linear plot of \( (v_o) \) against \( (v_o/[S]_0) \) gives \( v_{\text{max}} = \text{intercept} \) and \( K_M = -\text{slope} \). Both equations use and share the outset and near-end data. Based on the above theoretical description, this study aims to introduce new methods based on Eq. (5) and Eq. (7) to prove that the present methods for \( v_{\text{max}} \) and \( K_M \) determination in Michaelis reactions are ambiguous and to introduce a better notion of \( v_{\text{max}} \) and the real constant in Michaelis reactions.

2. Method

2.1. Reaction model

This study theoretically used the enzymatic reaction data taken from the hydrolysis of phenylacetate catalyzed by enzyme acetylcholinesterase [6] and the hydrolysis of methyl-hydro cinnamate catalyzed by enzyme chymotrypsin [7]. These two hydrolysis data are presented in Table 1 and Table 2, respectively.

| Table 1. Concentration data for fitting-constants determination in the hydrolysis of phenylacetate catalyzed by enzyme acetylcholinesterase [6]. |
|---|
| [PhOAc], 10^{-4} M | 22.5 | 16.3 | 12.6 | 9.73 | 8.45 | 7.18 | 6.21 | 5.55 |
| \( v_i \), 10^{-7} M s^{-1} | 7.24 | 6.35 | 5.32 | 4.61 | 4.53 | 4.02 | 3.38 | 3.26 |

| Table 2. Concentration data for fitting-constants determination in the hydrolysis of methyl hydro-cinnamate catalyzed by enzyme chymotrypsin [7]. |
|---|
| [Methyl hydro-cinnamate], 10^{-3} M | 30.8 | 14.6 | 8.57 | 4.60 | 2.24 | 1.28 | 0.32 |
| \( v_i \), 10^{-8} M s^{-1} | 20 | 17.5 | 15.0 | 11.5 | 7.5 | 5.0 | 1.5 |

The data can be divided into two parts and be assumed as the outset and near-end data. However, there is no data for studying the effects of changing enzyme or catalyst concentration. To cope with, the other model used is the reaction of acetone with bromine, catalyzed by \( H \). Because there are two substrates, then the reaction occurred in conditions of excessive [Acetone] to [Bromine]. It is believed that in these conditions, the reaction has the rate law as presented in Eq. (11).

\[ \nu = \frac{k_{\text{obs}}[H^+][Br_2]}{\kappa[Br_2]} \]

Where \( k_{\text{obs}} = k \) [Acetone] and \( \kappa \) are the observed rates constant and catalytic constant, respectively. When Eq. (12-13) was assumed, Eq. (11) is similar to Eq. (8). The absorbance unit data (measured at 400 nm) is presented in Table 3 [8, 9].

\[ k_{\text{obs}} [H^+] = v_{\text{max}} \]

\[ \kappa = K_M \]

2.2. Framework and Data Interpretation Technique.

2.2.1. Separate Rate-law Method (SRIM)
All data were analyzed using Eq. (9) and Eq. (10) and using the new equation. With slight changes in the notion of the maximum velocity concept, a new method, called Separate Rate-law Method or SRM, for $v_{\text{max}}$ determination is derived from Eq. (5).

Table 3. Absorbance data for fitting-constants determination in the reaction of acetone (0.8 $m$) with bromine 0.01 $m$ catalyzed by hcl (hcl 0.050 $m$, hcl 0.100 $m$, and hcl 0.200 $m$)

| $t/s$ | $[H^+]$  | 0.050 $M$ | Outset | Near-end | $[H^+]$  | 0.100 $M$ | Outset | Near-end | $[H^+]$  | 0.200 $M$ | Outset | Near-end |
|-------|-----------|-----------|--------|----------|-----------|-----------|--------|----------|-----------|-----------|--------|----------|
| 10    | 0.468     | 0.463     | 0.059  | 0.055    | 0.483     | 0.473     | 0.073  | 0.066    | 0.455     | 0.437     | 0.109  | 0.096    |
| 20    | 0.463     | 0.458     | 0.055  | 0.052    | 0.473     | 0.463     | 0.066  | 0.059    | 0.437     | 0.420     | 0.096  | 0.084    |
| 30    | 0.458     | 0.454     | 0.052  | 0.050    | 0.463     | 0.454     | 0.059  | 0.053    | 0.420     | 0.403     | 0.084  | 0.074    |
| 40    | 0.454     | 0.449     | 0.050  | 0.047    | 0.454     | 0.444     | 0.053  | 0.048    | 0.403     | 0.387     | 0.074  | 0.065    |
| 50    | 0.449     | 0.444     | 0.047  | 0.044    | 0.444     | 0.434     | 0.048  | 0.043    | 0.387     | 0.372     | 0.065  | 0.057    |
| 60    | 0.444     | 0.044     | 0.434  | 0.043    | 0.372     | 0.057     |        |          |           |           |        |

Note: For IRM, the end of the one-time interval is considered as the initial value for a new (and the same) time interval.

\[ v = -d[S]/dt = v_{\text{max}} \] (14)

\[ -[S]_o d\frac{[S]/[S]_o}{dt} = v_{\text{max}} \] (15)

Introducing $\beta = \text{ faction of substrate in the solution} = [S]/[S]_o$ into integral form of Eq. (15) gives Eq. (16).

\[ -\int \frac{d[S]}{dt} d\beta = v_{\text{max}} \int_0^t dt \] (16a)

\[ [S]_o (1 - \beta) = v_{\text{max}} t_{\beta} \] (16b)

\[ v_{\text{max}} = \left( [S]_o (1 - \beta_{zeroth}) \right) / t_{\beta} \] (16)

The equation for $K_M$ determination is derived from Eq. (7).

\[ v = -d[S]/dt = \left( \frac{v_{\text{max}}}{K_M} \right) [S] \] (17a)

\[ -d([S]/[S]) = \left( \frac{v_{\text{max}}}{K_M} \right) dt \] (17)

Introducing $\beta = [S]/[S]_o$ into integral form of Eq. (17) gives Eq. (19).

\[ -\int_0^t \frac{d(\beta)}{\beta} = \left( \frac{v_{\text{max}}}{K_M} \right) \int_0^t dt \] (18)

\[ -\ln \beta = \left( \frac{v_{\text{max}}}{K_M} \right) t_{\beta} \] (19a)

\[ K_M = \frac{v_{\text{max}} t_{\beta}}{\ln \beta_{first}} \] (19)
Eq. (16) can only be used if the rate is zeroth-order. For example, in Table 4, for \([H^+] = 0.050 \, M\), \([S]_0 = 0.468, \beta = (0.463/0.468 = 0.9893)\), and for \([H^+] = 0.100 \, M\), \([S]_0 = 0.483, \beta = (0.473/0.483)= 0.9793\).

Eq. (19) can only be used if the reaction is first-order. Differ with zeroth-order reaction, here \(\beta\) is constant. The value used is not \(\beta = (0.055/0.059)\), but the variance of the 5 values of \(\beta\) (for \([H^+]\) 0.050 \(M = 0.9431\) and for \([H^+]\) 0.1000 \(M = 0.8996\)).

2.2.2. Data analysis

All methods are then used to determine the \(v_{\text{max}}\) and \(K_M\) of the five reaction models. The results are presented in Table 4 through Table 8.

2.2.3. Data interpretations

A method is valid if \(v_{\text{max}}\) is determined when the reaction is zeroth-order and \(K_M\) when the reaction is first-order. The \(v_{\text{max}}\) value is relative to the enzyme (catalyst) concentrations. However, \(K_M\) value must be the same. A method will be ambiguously notified if it combines the outset and near-end data, regardless of the same or different reaction order.

3. Results and discussion

3.1. First case

SRIM is an integral method; therefore, it is interesting to compare SRIM and SRIM_{IM} together with SRIM_{IRM}. The advantage of IRM is that it directly gives the correct reaction order. However, the rate constant, \(k\), value is uncertain. Save for zeroth-order reactions, \(k\) values are always different from IM. The correct value should be consulted with the Integral Method (IM) [10]. However, it is difficult to differentiate between integers and half-integers and even between sequences of integer order with IM. The actual value obtained from this method can be equated using the correction factor in Eq. (20-21).

\[
C_f = 1 \quad \text{for} \quad n = 0 \quad (20)
\]
\[
C_f = \frac{1-\beta}{\beta \ln \beta} \quad \text{for} \quad n = 1 \quad (21)
\]

With Eq. (20), for zeroth-order, \(k_{\text{IRM}} = k_{\text{IM}}\), and so on, \(v_{\text{max}}_{\text{IRM}} = v_{\text{max}}_{\text{IM}}\). However, in Table 8, for \([H^+] = 0.05 \, M\), \(v_{\text{max}}_{\text{IRM}} \neq v_{\text{max}}_{\text{IM}}\) the percentage difference is 14.53%. The percentage difference of \(v_{\text{max}}\) values between SRIM and \(\text{SRIM}_{\text{IM}}\) relative to \(\text{SRIM}_{\text{IM}}\) is 7.67%; this is slightly higher than the maximum allowable of 7%. The reason is that although \(\text{SRIM}_{\text{IRM}}\) gives the correct order, the regression coefficient is 0.0265, which means there almost no correlation. For the same case, \(\text{SRIM}_{\text{IM}}\) gives a regression coefficient of 0.9996 (Table 6.).

In this study, SRIM is taken as the standard method. It is only because the SRIM is directly derived from Eq. (5) and Eq. (7). Actually, SRIM is impractical to use. The order must determine in advance by IM. However, from these steps, \(v_{\text{max}}\) and \(K_M\) can be directly determined by \(\text{SRIM}_{\text{IM}}\). As shown in Table 8, the percentage difference between \(\text{SRIM}_{\text{IM}}\) and \(\text{SRIM}_{\text{RM}}\) relative to SRIM is for \(v_{\text{max}}\) was 3.99% and for \(K_M\) was 2.49%. Therefore, it is better to use SRIM_{IM} as the standard. The only drawback is to obtain a definite order of the reactions, and two different integral equations must be tested; zeroth-order and first-order. However, this can be overcome by using Eq. (22).

\[
t_1 = \beta^{(i-1)(1-n)} t_{\beta} \quad (22)
\]
Where $\beta$, $t_b$, $t_i$, and $n$ are the remaining reactant fractions (must be kept constant), first time observation, following observations, observation, and reaction order, respectively [12, 13]. For example if $n = 0$ and $\beta = 0.5$ then $t_1 = t_b$, $t_2 = \frac{1}{2} t_b$, and $t_3 = \frac{1}{4} t_b$. Then, if observations are made simultaneously, the rate will not be the same; it is faster. Thus, for $n = 1$, $t_i$ will always be the same with $t_b$.

3.2 Second case
There are three misconceptions in the L-B and E-H methods. First, both equations are based on IRM. IRM is best for determining reaction order but not for rate constants. There are two different laws.

Second, they do not consistently adhere to the basic concept of determining $v_{\text{max}}$ and $K_M$. The value of $v_{\text{max}}$ must be determined when the reaction is zeroth-order (at the outset) and $K_M$ when the reaction is first-order (at near-end). However, neither method requires an examination of the real rate law. Instead, they use the combination of the outset and near-end data. There are values for $v_{\text{max}}$ and $K_M$ even if the reaction is half-order at the outset and first-order at the end (Table 4) or always first-order (Table 7). In Table 8, the $v_{\text{max}}$ value obtained by all methods is almost the same. Theoretically, the value obtained by L-B and E-H should be lower compared to the new method. With the new method, $v_{\text{max}}$ is determined when the reaction is zeroth-order, the reaction at its maximum velocity. This is because both methods are basically based on IRM (or differential method). There may be problems in using IRM for constant time interval observation data. In Table 5, the $v_{\text{max}}$ value by the SRIRM is higher. As expected, the $K_M$ values obtained by the new methods are relatively higher than those of L-B and E-H. The $K_M$ value depends on $v_{\text{max}}$.

| Method | A        | B        | $r$   | Order | $V_{\text{max}}$ | $v_{\text{max}} / K_M$ | $K_M$          |
|--------|----------|----------|-------|-------|------------------|----------------------|----------------|
|        |          |          |       |       |                  |                      |                |
| Outset | -4.6786  | 0.55     | 0.994 | 0.5   |                  |                      |                |
| Near-end| -3.7877  | 0.83     | 0.986 |       |                  |                      |                |
| L - B  | All data | 824855.5 | 1254.81 | 0.9927 | -1.21x10^{-6} | 1254.81               | 1.52x10^{-3}  |
| E - H  | All data | 1.17x10^{-6} | -1.44x10^{-3} | -0.9654 | 1.17x10^{-6} | 1.44x10^{-3}  |                |

Third, however, the main misconception of the L-B and E-H methods concerns their equations. If $v = v_{\text{max}}$ then both Eq. (15) and Eq. (16) must give:

$$K_M = 0 \quad (23)$$

Eq. (20) states that $v_{\text{max}}$ and $K_M$ cannot be determined using a combination of outset and near-end data [11]. In actual, the used combined data is against Eq. (8). If $V_0 = v_{\text{max}}$, Eq. (8) also gives Eq. (20). These means that the value of $v_{\text{max}}$ and $K_M$ must be determined separately; $v_{\text{max}}$ when $[S] \gg K_M$ (zeroth -order to $[S]$) and $K_M$ when $[S] \ll K_M$ (first-order to $[S]$).

3.3. Third case.
Unusually, in Table 4, the order of $[S]$ is half-order. Such cases occur when the enzyme or substrate is a weak acid or base. According to Eq. 7, $[S]$ is first-order; however, the estimation using the modified method in the present study results in half-order. Therefore, the Michaelis reaction rate-law presented in Eq. 7 should be re-considered. In Table 8, $v_{\text{max}}$ changes with the change in $[H^+]$. However, dividing $v_{\text{max}}$ for $[H^+]$ 0.100 $M$ by that at $[H^+]$ 0.100 $M$ gives a value of about 2. Since the order must be integer or half-integer, the order to $[H^+]$ is $n = 1$ which is the most general order for a catalyst or enzyme. The division results also give relatively the same value for $k_{\text{obs}}$ (the observed catalyst constant), where $k_{\text{obs}} = k_0 [H^+][\text{Acetone}]$. These facts mean that the term maximum velocity is not a suitable notion for $v_{\text{max}}$; the better is the maximum relative velocity. The real constant is the observed catalyst constant $k_{\text{obs}}$ (or generally $k_c$ for a single substrate).
### Table 5. Results of data analysis of hydrolysis of methyl hydro-cinnamate catalyzed by chymotrypsin

| Method     | A       | B     | r   | n   | $v_{\text{max}}$ | $v_{\text{max}} / K_M$ | $K_M / v_{\text{max}}$ | $K_M$  |
|------------|---------|-------|-----|-----|------------------|--------------------------|--------------------------|--------|
| Outset     | -6.359  | 0.22  | 0.9906 | 0   |                  |                          |                          |        |
| Near-end   | -4.901  | 0.84  | 0.9993 | 1   |                  |                          |                          |        |
| $S_{RI}M_{IR}$ |         |       |       |     | 4.38x10^{-7}     |                          |                          | 3.49x10^{-2}  |
| $L - B$    | 4373009.1 | 19939.679 | 1.0000 | -   | 2.29x10^{-7}     | 19939.679                | 4.56x10^{-3}              |        |
| $E - H$    | -0.230x10^{-7} | -4.60x10^{-3} | -1.0000 | 2.30x10^{-7} | 4.60x10^{-3}   |                          |                          |        |

### Table 6. Results of statistic analysis data of reaction of acetone with bromine catalyzed by $[H^+]$

| Method     | $[H^+]$ (M) | Outset                        | Near - end                        |
|------------|--------------|-------------------------------|-----------------------------------|
|            | A     | B     | r   | n   | A     | B     | r   | n   |
| $IRM$      | 0.050 | -3.2653 | 0.16 ≈ 0 | 0.0265 | 0   | -1.9664 | 1.22 ≈ 1 | 0.4323 | 1   |
|            | 0.100 | -3.0057 | 0.01 ≈ 0 | 0.0072 | 0   | -2.0485 | 0.96 ≈ 1 | 0.9957 | 1   |
|            | 0.200 | -2.3841 | 1.05 = 1 | 0.9692 | 1   | -1.9439 | 0.97 ≈ 1 | 0.9950 | 1   |
| $IM$       | 0.050 | -0.749 | -1.04x10^{-3} | -0.9995 | l   | -2.780 | -5.65x10^{-3} | -0.9974 |         |
|            | 0.100 | -0.706 | -2.13x10^{-3} | -0.9998 | 0   | -2.510 | -1.06x10^{-2} | -0.9999 |         |
|            | 0.200 | -0.747 | -1.66x10^{-3} | -0.9995 | 0   | 0.117  | -1.04x10^{-3} | -0.9957 |         |
| $L - B$    | 0.050 | 1904.1 | 84.084 | 0.7818 | 0.050 | 4.91x10^{-4} | -7.54x10^{-2} | -0.7008 |       |
|            | 0.100 | 897.151 | 48.4317 | 0.9601 | 0.100 | -1.08x10^{-3} | -4.73x10^{-2} | -0.9999 |       |
|            | 0.200 | 481.77 | 43.8227 | 0.9610 | 0.200 | -1.96x10^{-3} | -7.54x10^{-2} | -0.9012 |       |
| $E - H$    |         |       |       |     |       |       |       |       |
Table 7. Effects of catalyst concentrations changes on the reaction of acetone with bromine by SRIM, SRIMRM, SRIMIM, L-B, and L-H

| $[H^+]$ (M) | Methods      | $v_{\text{max}}$ M s$^{-1}$ | $v_{\text{max}}/K_M$ | $K_M$ Abs | $1/v_{\text{max}}$ | $v_{\text{max}}$ Abs | $K_M/ v_{\text{max}}$ | $K_M$ Abs | $v_{\text{max}}$ M | $K_M$ M |
|-------------|--------------|-----------------------------|-----------------------|-----------|-------------------|----------------------|------------------------|-----------|-----------------|--------|
| 0.050       | SRIM         | 5.01x10$^{-4}$              | 8.55x10$^{-2}$        |           |                   |                      |                        |            |                 |        |
|             | SRIMRM       | 5.43x10$^{-4}$              | 1.08x10$^{-2}$        | 5.03x10$^{-2}$ |                   |                      |                        |            |                 |        |
|             | SRIMIM       | 4.74x10$^{-4}$              | 5.65x10$^{-3}$        | 8.39x10$^{-2}$ |                   |                      |                        |            |                 |        |
|             | L-B          | 1904.1                     | 84.084                | 1904.1    | 5.25x10$^{-4}$    | 84.084               | 4.41x10$^{-2}$         | 3.28x10$^{-6}$ | 2.76x10$^{-4}$ |        |
|             | E - H        |                            |                       |           | 4.91x10$^{-4}$    |                      |                        |            |                 |        |
| 0.100       | SRIM         | 1.00x10$^{-3}$              | 9.45x10$^{-2}$        |           |                   |                      |                        |            |                 |        |
|             | SRIMRM       | 9.87x10$^{-4}$              | 8.94x10$^{-3}$        | 0.1104    |                   |                      |                        |            |                 |        |
|             | SRIMIM       | 9.74x10$^{-4}$              | 1.06x10$^{-2}$        | 9.19x10$^{-2}$ |                   |                      |                        |            |                 |        |
|             | L-B          | 897.151                    | 1.12x10$^{-3}$        | 48.4317   | 5.39x10$^{-2}$    | 7.00x10$^{-6}$        | 3.37x10$^{-4}$         | 2.96x10$^{-4}$ | 2.96x10$^{-4}$ |        |
|             | E - H        |                            |                       |           | 1.08x10$^{-3}$    |                      |                        |            |                 |        |
| 0.200       | L - B        | 481.77                     | 2.08x10$^{-3}$        | 43.8227   | 9.10x10$^{-2}$    | 1.30x10$^{-5}$        | 5.69x10$^{-4}$         | 2.96x10$^{-4}$ | 2.96x10$^{-4}$ |        |
|             | E - H        |                            |                       |           | 1.96x10$^{-3}$    |                      |                        |            |                 |        |

Table 8. The effect of changing the $[H^+]$ on the reaction order and the values of $v_{\text{max}}$ and $\kappa$ in the reaction of acetone with bromine.

| Methods | $[H^+]$ M | $v_{\text{max}} M s^{-1}$ | Ratio | $n$ | $k_{obs} M s^{-1}$ | $\chi^{10^{6}}$ | $\sigma_{\chi}$ | $\sigma_{PD}$ % | $K_M x 10^4 M$ | Ratio | $\chi x 10^{4}$ | $\sigma_{\chi}$ | PD % |
|---------|-----------|--------------------------|------|----|-------------------|-----------------|-----------------|-----------------|----------------|-------|-----------------|----------------|-------|
| SRIM    | 0.100     | 6.25x10$^{-6}$           | 2.00 $\approx$ 2     | 1   | 6.25x10$^{-5}$    | 6.26            | 5.00x10$^{-8}$  | 1               | 5.91           | 1.11  | 5.63           | 2.85x10$^{-1}$ | 5.91  |
|         | 0.050     | 3.13x10$^{-6}$           |                  |    | 6.26x10$^{-5}$    |                |                 |                 | 5.35           |       |                 |                 |       |
| SRIMRM  | 0.100     | 6.17x10$^{-6}$           | 1.82 $\approx$ 2     | 1   | 6.17x10$^{-5}$    | 6.48            | 3.05x10$^{-7}$  | 3.51           | 6.90           | 2.20  | 5.02           | 1.88x10$^{-1}$ | 10.83 |
|         | 0.050     | 3.39x10$^{-6}$           |                  |    | 6.78x10$^{-5}$    |                |                 |                 | 3.14           |       |                 |                 |       |
| SRIMIM  | 0.100     | 6.09x10$^{-6}$           | 2.06 $\approx$ 2     | 1   | 6.09x10$^{-5}$    | 6.01            | 8.50x10$^{-8}$  | 3.99           | 5.74           | 1.10  | 5.49           | 2.50x10$^{-1}$ | 2.49  |
|         | 0.050     | 2.96x10$^{-6}$           |                  |    | 5.92x10$^{-5}$    |                |                 |                 | 5.24           |       |                 |                 |       |
| L - B   | 0.100     | 7.00x10$^{-6}$           | 2.13 $\approx$ 2     | 1   | 7.00x10$^{-5}$    | 6.78            | 2.20x10$^{-6}$  | 8.48           | 3.37           | 1.22  | 3.07           | 3.05x10$^{-1}$ | 45.47 |
|         | 0.050     | 3.28x10$^{-6}$           |                  |    | 6.56x10$^{-5}$    |                |                 |                 | 2.76           |       |                 |                 |       |
| E - H   | 0.100     | 6.75x10$^{-6}$           | 2.20 $\approx$ 2     | 1   | 6.75x10$^{-5}$    | 6.45            | 3.05x10$^{-6}$  | 8.31           | 2.96           | 1.60  | 2.42           | 5.40x10$^{-1}$ | 57.02 |
|         | 0.050     | 3.07x10$^{-6}$           |                  |    | 6.14x10$^{-5}$    |                |                 |                 | 1.88           |       |                 |                 |       |
| L - B   | 0.200     | 1.30x10$^{-5}$           |                  |    |                   |                |                 |                 | 5.69           |       |                 |                 |       |
| E - H   | 0.200     | 1.23x10$^{-5}$           |                  |    |                   |                |                 |                 | 4.71           |       |                 |                 |       |
4. Conclusions

Three new methods for determining the fitting constant of the Michaelis-reaction have been introduced. The \( SRIM \), \( SRIM_{RM} \), and \( SRIM_{FM} \) methods are reliable for determining the fitting-constants of enzyme-catalyzed reaction, in which the order of the accuracy method for these three methods is \( SRIM \approx SRIM_{RM} > SRIM_{FM} \). The Lineweaver-Burk and Eadie-Hofstee methods are not recommended for the determination of the fitting-constants of an enzyme-catalyzed reaction. The better notion of the concept of \( v_{max} \) is the relative maximum rate, not the maximum rate. The true constant is \( k_{obs} \) (or \( k_c \) if the substrate is single).

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