Easy Kinetics: a software that simplify enzyme's kinetics characterization

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Abstract

Easy Kinetics is an intuitive software based on an original algorithm, that can compute the main kinetic constants, the Hill coefficient of cooperativity, the speed of catalysis at any substrate concentration chosen and the corresponding kinetic graph of an enzymatic kinetics, needing only two spectrophotometric measurements conducted in duplicate. In this way Easy Kinetics simplify the modern approach for the study of enzyme's kinetics by needing less spectrophotometric measures in comparison to those required by any traditional commercial software but in any case showing with the latter a significant concordance of results.

Introduction

The '60s represented the golden age for the characterizations of enzymatic kinetics, but still today studying enzymatic kinetics continues to attract interest both in basic research, due to the continuous new enzymes discovered, and in industrial processes (e.g. design of enzymatic reactors), so it is important to know the kinetic parameters of the enzymes used to optimize the production processes of the molecules of interest [1]. In the past a researcher had to perform repeated spectrophotometric measurements (at least 8 conducted in duplicate) in samples containing the enzyme: possible cofactors if requested by the reaction and different substrate concentrations. Then from these data the most used method consists into draw the kinetic graphs and extract graphically, from that of reciprocal doubles, the values of K_m and V_{max}. Original alternative methods for k_m and V_{max} determination have been proposed, but even these require multiple spectrophotometric measurements and determine graphically the output values [2]. With the advent of computers and their diffusion among scientist, this process has been greatly optimized, minimizing human errors and obtaining graphs and kinetic parameters from precise and reliable algorithms. However, until today, these software (e.g. GraphPad Prism 8© [3]) continue to require multiple spectrophotometric measurements, on which then regression models are implemented to predict the change of the reaction speed to different substrate concentrations, generating curves on which calculate the K_m and V_{max} parameters [4], but unless the data fit the assumed relationship extremely well, considerable unconscious bias may be introduced in drawing the curves, and such graphical analysis furnishes only an approximation of the real kinetics parameters searched. Indeed if any of the experimental velocities measured with the spectrophotometer deviate very much from the corresponding values calculated from the fitted curve, the errors in the regression model used became sizeable, and the kinetic parameters thus calculated will not reflect their real values [5]. The software presented here uses an original algorithm, shown and explained below, able to directly return the main kinetic constants, the Hill coefficient of cooperativity, the speed of catalysis at any substrate concentration chosen and the corresponding kinetic graphs, from only two spectrophotometric measurements conducted in duplicate. Easy Kinetics saves not only the time of the researcher by performing a fast kinetic analysis, but also allows a considerable saving of materials and money to be used (e.g. minimum amounts of enzyme and substrate) in according to facilitate research. In addition, the user-friendly interface and the simple input information needed by this software, allow its use in this research field not only by most experienced researchers, but also by students and novice scientists.

Materials and methods

Original Algorithm used in the evaluation of K_m and V_{max}:

The original software's algorithm, used for the evaluation of K_m and V_{max} from only two spectrophotometric measures, is based on the trigonometric demonstration shown in **Fig.1**. The graph of the reciprocal doubles relates the reciprocal of the catalysis velocities (ordinates) and of the substrate concentrations (abscissa). Note two points of this graph, whose velocity of each has been calculated through the spectrum-photometer at known substrate concentrations, it can be said that for these passes one and only one straight line. This line will have an unknown inclination "a" and will intersect the cartesian axes in points $\frac{1}{V_{max}}$ and - $\frac{1}{K_m}$, also unknown. By tracing the projections of the two known points on the cartesian axes, we observe that the lines $y = y_2$ and $y = 0$ are parallel and are intersected by the studied line. Since the internal alternate angles are equal, we can therefore state that "a" = "a_1". Considering now instead the lines $y = y_2$ and $y = y_1$, also parallel and intersected by the usual straight line, we observe that "a_1" = "a_2" for the previous reason, which therefore will also be equal to "a". This implies that:

$$\tan(a) = \frac{y_2 - y_1}{x_2 - x_1}$$

But also $\frac{z}{x_2} = \tan(a)$, with $z = y_2 - \frac{1}{V_{max}}$, so we have:

$$\frac{1}{V_{max}} = y_2 - \mathrm{z} = y_2 - (\tan(a) * x_2) = y_2 - \frac{x_2 * (y_2 - y_1)}{x_2 - x_1}$$

Once calculated $\frac{1}{V_{max}}$, we can have $\frac{1}{K_m}$through the following relation:

$$\left| -\frac{1}{K_m} \right| = \frac{\frac{1}{V_{max}}}{\tan(a)}$$

Inverting the two values found are finally $K_m$and V_{max}; and from there by subsequent biochemical relations the other kinetic parameters are deduced.

Fig 1. Graphical trigonometric demonstration of the K_m and V_{max} evaluation

Because of Easy kinetics receives in input only two spectrophotometric measurements (despite being performed in duplicate), if one of these measures is anomalous, it isn't correct by other measurements as occurs when we have multiple measurements and we perform regression models for points; so the software may fall in error. To fix this problem and minimize experimental bias, measurements must be conducted in duplicate and if both the two absorbances per minute measured at the same substrate concentration fall within the range of their mean ± 10% of their mean, then the algorithm can considers the average value between the two as the measurements to be used, otherwise we have to repeat the two measurements for the substrate concentration considered, because of too much difference found in those entered.

Kinetics equations used from literature:

Following are reported the main equation used by the software [6-10]:

$$nH = log_{[S]}(K_m * \frac{V_0}{V_{max} - V_0})$$

is the equation used for the calculus of the Hill coefficient

$$V_0 = \frac{\text{Vmax} * [S]^{nH}}{Km^{nH} * [S]^{nH}}$$

is the equation used for the graph building once V_{max} and K_m were computed

$$V_0 = \frac{\text{Vmax} * [S]^{nH}}{[S]^{nH} * \left(1 + S_i * \left(\frac{[S]}{K_i}\right)^{nH}\right) + Km^{nH}}$$

is the equation used for the calculus of the V_0 at a set chosen substrate concentration once we found V_{max} and K_m

$$V_{moli} = \frac{V_0 * L_f}{O * \varepsilon}$$

is the equation used for the conversion of the V_0 previously found and expressed in ΔAbs/min in a new V_0 expressed in μmoli of reporter product generated per minute

$$\text{U} = \frac{Abs_{high}}{\left(\frac{L_f}{L_i}\right) * O * \varepsilon}$$

is the equation used for the calculus of the enzyme's units in the sample

$$C_p = \frac{\text{Abs}_{protein} - \text{Abs}_{blank}}{0.064 * \text{O}}$$

is the equation used for the calculus of the protein's concentration in the Bradford assay

$$S_{activity} = \frac{\text{U}}{C_p}$$

is the equation used for the calculus of the enzyme's specific activity

$$K_{cat} = \frac{\text{P.M} * V_{max}}{\varepsilon * O * C_p}$$

is the equation used for the calculus of the enzyme's K_{cat}

$$C_{efficiency} = log_{10}\frac{K_{cat}}{K_m}$$ is the equation used for the calculus of the enzyme's catalytic efficiency

Where [S] is the substrate concentration; S_i can worth 1 if substrate inhibition is present or 0 if substrate inhibition is absent; K_i is the inhibition constant calculated at very high substrate's concentration as:

$$K_i = \frac{(100 * K_m)^2}{\frac{(100 * K_m) * V_{max}}{Abs_{(100 * K_m)} - K_m - (100 * K_m)}}$$ when substrate inhibition is present

$$K_i = 1$$ when substrate inhibition is absent

L_f is the final volume of the sample; L_i is the initial volume of the sample; ε is the extinction molar coefficient of the product; O is the optical path of the spectrophotometer; Abs_{high} is the measured absorbance at very high substrate concentration; $Abs_{protein}$ is the measured absorbance for the protein's solution; Abs_{blank} is the measured absorbance for the solution without protein and P.M. is the molecular weight of the reporter product.

Software design and implementation:

Easy Kinetics was developed in C# language with a GPL-3.0 license, both for the versatility of C# and the design object-oriented, in Windows 10 environment (with October update installed), this is for the diffusion of Windows 10 and the consequent ease distribution of the software. The software installation package can be downloaded freely as windows application on Microsoft Store at the URL:https://www.microsoft.com/it-it/p/easy-kinetics/9nx1f4q5fpg5?activetab=pivot:overviewtab) or alternatively on the repository GitHub (DOI: 10.5281/zenodo.3242785) at the URL:https://github.com/ekin96/EasyKinetics. Easy Kinetics allows the user to operate in different masks 5 different kinetic analyzes: Simple Enzyme Kinetics, Inhibition Kinetics, Enzymatic Units Assay, Calculation of Abs / min, Bradford Assay. By performing the two main analyzes, "Simple Enzyme Kinetics" and "Inhibition Kinetics", the application allows to process the experimental data and graphically extrapolates the impact on the Reaction Rate as the concentration of the enzymatic substrate varies. Furthermore Easy Kinetics has been optimized to self-detect possible substrate-inhibition kinetics.

Results

Easy Kinetics user-friendly interface:

Once the program is launched, from the side menu it's possible to choose the type of function wanted. For example "SIMPLE ENZYME KINETICS" allows to perform an enzymatic characterization on a single enzyme, determining basic kinetic parameters such as V_{max}, K_m, the Hill coefficient and the speed of catalysis expressed both, in ΔAbs per minute and in μmoli of reporter product generated per minute, starting from only two double spectrophotometric measurements of ΔAbs per minute on two samples containing a different substrate concentration. However it is possible to extend this analysis by executing in the same window a Bradford assay and the evaluation of the enzymatic units present in the sample, obtaining kinetic parameters such as K_{cat}, the catalytic efficiency and the specific activity of the enzyme in the analyzed starting sample. Instead "INHIBITION KINETICS" allows to verify the presence of an inhibition on the enzyme by a certain molecule, and in the case of quantifying this inhibition. Two values of ΔAbs per minute are given to the software for two samples with the same quantity of enzyme but different substrate concentration and not containing the inhibitor, and other two values of ΔAbs per minute are given for another two samples with the same quantity of enzyme but different substrate concentration (also can be different from the previous ones) but containing the inhibitor at a chosen

concentration. Finally in output comes the values of K_m and V_{max} with and without the inhibitor, the type of inhibition, the percentage of still active enzyme and the speed of catalysis expressed both, in ΔAbs per minute and in μmoli of reporter product generated per minute, for a chosen substrate concentration with and without the inhibitor at the previous concentration. Both "SIMPLE ENZYME KINETICS" and "INHIBITION KINETICS" given the opportunity to generate the graph of enzyme's speed of catalysis in function of the substrate concentration, and in addition are able to self-detect inhibition of the catalytic activity operated by the substrate. The last three functions concern the execution of a simple Bradford assay, the evaluation of the enzymatic units presents in the sample and the calculation of the absorbance variation per minute given the single absorbance values over time (this last function is particularly useful if old spectrophotometers are used to conduct this type of study).

Fig 2. Simple Enzyme Kinetics starting environment: input and output fields are characterized respectively by a withe and colored background. Below the title there is a brief descriptive guide that explains the general guidelines to follow during the experiment

Evaluation of the K_m and V_{max} accuracy:

The applicability and accuracy of Easy Kinetics have been statistically proved on six kinetic characterization scenarios, that are resumed below:

Enzyme	Substrate	K_{m_trad}[mM]	V_{max_trad}(dA/min)	K_{m_Easy}[mM]	V_{max_Easy}(dA/min)
Bovine ALDH	Glyceraldeide	0.2946	0.0227	0.3270	0.02395
Bovine ALDH	Acetaldeide	0.37035	0.0409	0.4951	0.04485
Bovine ALDH	NAD+	0.02465	0.0259	0.02625	0.0272
Bovine ALDH	Para-Nitrophenyl-Acetate	0.0213	0.0537	0.0215	0.05296
Bovine X.O.	Xanthine	0.0058	0.0646	0.0049	0.0629
Bovine X.O.	Hypoxanthine	0.0119	0.0531	0.01231	0.0546

Tab 1. Values of K_m and V_{max} for bovine xanthine oxidase and for bovine aldehyde dehydrogenase for different substrates calculated both: traditionally, through the classic methods based on multiple spectrophotometric measurements at different substrate concentrations (K_{m_trad} and V_{max_trad}), and with Easy Kinetics, using for every substrate of the enzymes only two of the previous ones spectrophotometric measurements in duplicate (K_{m_Easy} and V_{max_Easy}).

Below are presented the graphs of the reciprocal doubles, obtained by traditional commercial software through multiple measurements of the absorbance change per minute produced by bovine's xanthine oxidase and aldehyde dehydrogenases for different substrate at different concentrations. By these graphs were obtained the previous values K_{m_trad} and V_{max_trad}:

Fig 3. graphs of reciprocal doubles obtained with traditional method through multiple measurements of the absorbance change per minute produced by bovine's xanthine oxidase and aldehyde dehydrogenases for different substrates at different concentrations.

Finally the results were analyzed using the software *R* [11], at first two new variables named K_{m_diff} and V_{max_diff} are created, that represent for every record the difference between the traditionally obtained parameter and the Easy kinetics computed one: $K_{m_diff} = K_{m_trad} - K_{m_Easy}$ and $V_{max_diff} = V_{max_trad} - V_{max_Easy}$. Then was checked the normality distributions of this new variables with a Shapiro Wilks normality tests, obtaining that only V_{max_diff} (with W= 0.92535 and a p-value = 0.5447) follow a normal distribution. For this reason K_{m_diff} was transformed in $K'_{m_diff} = 1/ K_{m_diff}$), that this time show a normal distribution (with W= 0.89055 and a p-value = 0.3211). Subsequently a two sided t-test for paired data with a confidence range of 0.95 was done to check if the difference between the calculated and computed values for V_{max} and K_m could be significantly assumed as 0: K'_{m_diff} (t = -1.2914, df = 5, p-value 0.253) and V_{max_diff} (t = = -1.1526, df = 5, p-value = 0.3012).

Discussion

The results obtained in the previous section shows a significant compatibility between the results obtained by Easy Kinetics for V_{max} and K_m and those obtained using the traditional methods. In particular the probability that K_{m_trad} and K_{m_Easy} are equal and their discrepancy is attributable to sampling errors or distortion is 0.25, while the probability that V_{max_trad} and V_{max_Easy} are equal and their discrepancy is attributable to sampling errors or distortion is 0.30. Then Easy kinetics shows a great concordance of the results with the traditional obtained

ones. So the discrepancy between the experimental and computed results is likely to be explained by the distortion and by the sampling errors made during spectrophotometric measures collection (e.g. measures approximations, instruments sensibility, sample differences, etc.) that propagate and have an ever greater weight so many more measurements are taken.

Conclusion

In conclusion Easy Kinetics has allowed to evaluate the K_m and V_{max} for different substrates in bovine xanthine oxidase and aldehyde dehydrogenase, in a simplest way, in less time and using less material if compared to the classic experimental investigations done with other software. Furthermore, the results obtained with only two spectrophotometric measurements, by Easy Kinetics original algorithm proposed here, have also proved to be significantly concordant with the results obtained by traditional experimental investigations using multiple spectrophotometric measurements in other commercial software environment. The greatest strength of Easy Kinetics, however, remains its ease of use and its intuitive user-friendly interface that allow anyone, from the expert researcher to the young student to be able to quickly and accurately conduct experiments in the field of kinetic enzymes analysis.

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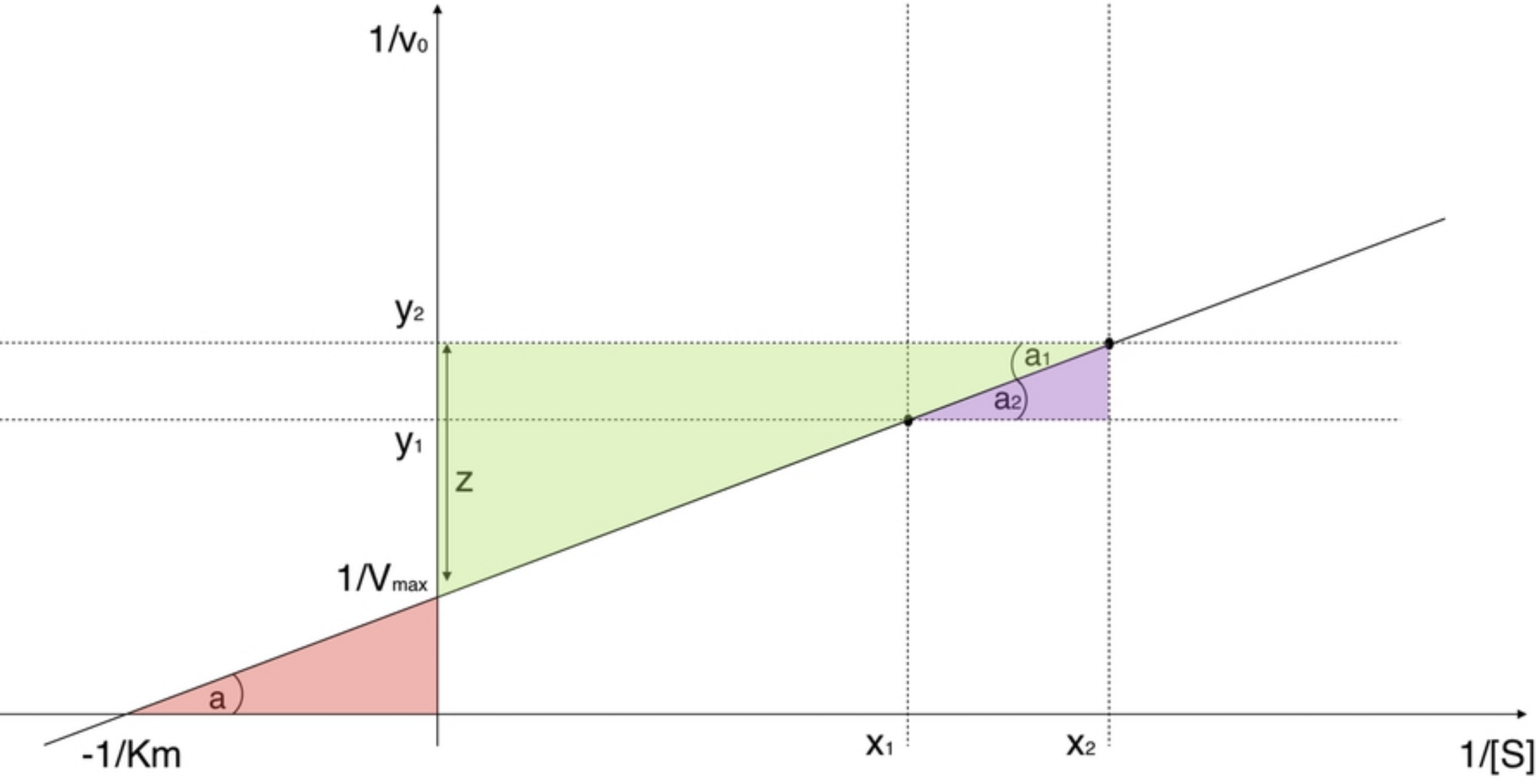

Fig1.tiff

EasyKinetics

Main | Chart

EasyKinetics

Simple Enzyme Kinetics

Inhibition Kinetics

Enzymatic Units Assay

Calculation of Δ Abs/min

Bradford Assay

SIMPLE ENZYME KINETICS (enzymes with one limiting substrate)

Prepare 4 samples containing an equal (and appropriate) volume of the starting enzyme solution, then put in 2 samples a chosen substrate concentration of [X1] μ M and in the last 2 samples a higher substrate concentration of [X2] μ M. Finally report the Δ Abs/min of the samples exposed to the specific wavelength of the reporter product.

Product Molecular Weight 0 g/mol

Set lower substrate concentration of samples 0 μ M

1st sample 0 Δ Abs/min

2nd sample 0 Δ Abs/min

Set higher substrate concentration of samples 0 μ M

1st sample 0 Δ Abs/min

2nd sample 0 Δ Abs/min

Set a sample with substrate concentration of 0 μ M

Absorbance change per minute 0 Δ Abs/min

REACTION PARAMETERS

V_{max} 0 Δ Abs/min K_m 0 μ M

n_H 0 K_{cat} 0 sec^{-1}

Catalytic efficiency : 10^ 0

BRADFORD ASSAY

Prepare 4 samples: the first 2 containing 10 μl of the protein's solution and 790 μl of ion free H2O, the other 2 containing only 800 μl of ion free H2O; then add in each one 200 μl of Bradford stain. Vortex all the samples and wait 15 min; finally report their absorbance.

Insert Abs_(595nm) of the solution with proteins:

1st measurement 0

2nd measurement 0

Insert Abs_(595nm) of the blank solution:

1st measurement 0

2nd measurement 0

Proteins conc. 0 μ g/ml

Specific activity 0

ENZYMATIC UNITS ASSAY

Molar extinction coefficient 0

Optical path 0 cm

Volume of initial solution from which the enzyme is taken 0 μ l

Total volume of final solution 0 μ l

Units/ml 0

REACTION RATE ASSESSMENT

Set substrate concentration to get Reaction Rate:

0 μ M

At this substrate concentration:

v_0 = { Δ Abs/min ; μ moli/min }

Fig2.tiff

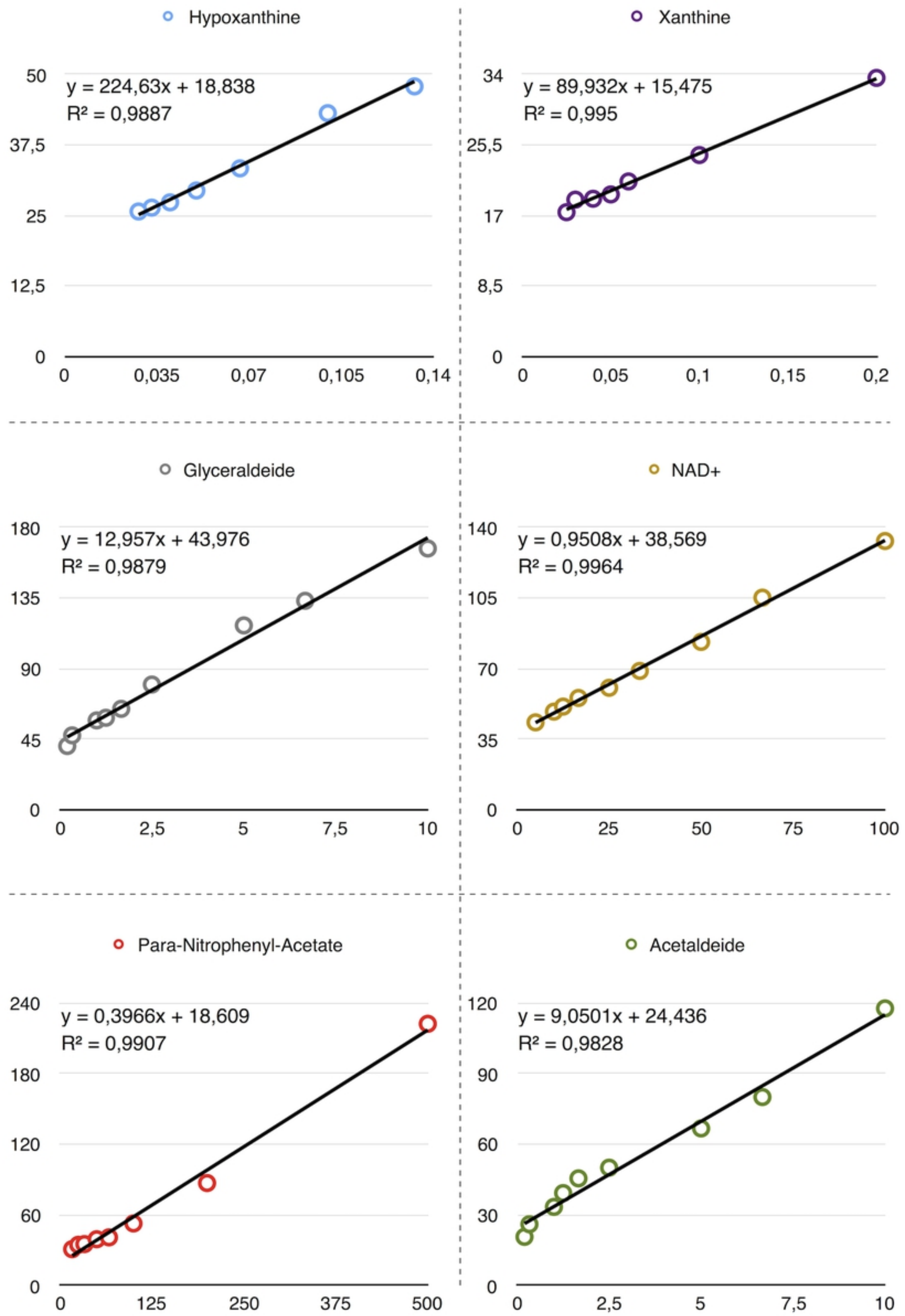
Hypoxanthine
y = 224,63x + 18,838
R² = 0,9887
50
37,5
25
12,5
0
0
0,035
0,07
0,105
0,14
Xanthine
y = 89,932x + 15,475
R² = 0,995
34
25,5
17
8,5
0
0
0,05
0,1
0,15
0,2
Glyceraldeide
y = 12,957x + 43,976
R² = 0,9879
180
135
90
45
0
0
2,5
5
7,5
10
NAD+
y = 0,9508x + 38,569
R² = 0,9964
140
105
70
35
0
0
25
50
75
100
Para-Nitrophenyl-Acetate
y = 0,3966x + 18,609
R² = 0,9907
240
180
120
60
0
0
125
250
375
500
Acetaldeide
y = 9,0501x + 24,436
R² = 0,9828
120
90
60
30
0
0
2,5
5
7,5
10

Fig3.tiff