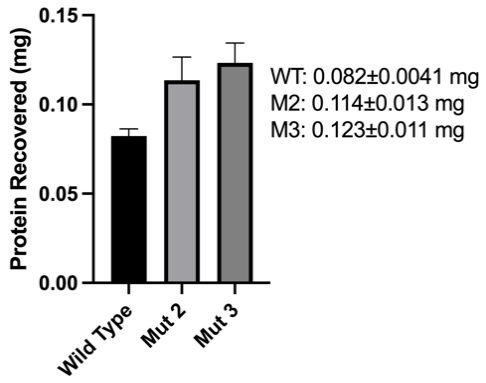
Investigation in Correlation between Structural Variation in MDH2 and pH

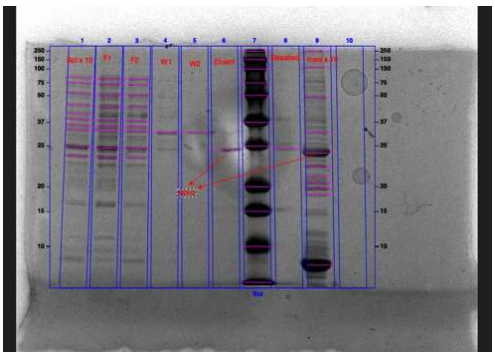
Results

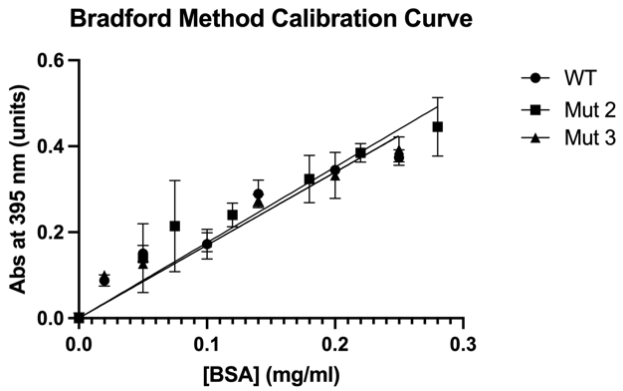
Analysis of protein purification

	Bradford	SDS-PAGE	
Variant	Mass recovered (mg)	MW	Band
WT	0.082 ± 0.004	41.28 ± 14%	MDH2
M2	0.114 ± 0.013	30.98 ± 14%	MDH2
M3	0.123 ± 0.011	40.47 ± 8.3 %	MDH2

Table 1: Summary of the isolates (Desalted Fraction) for each mutant following total protein assay and SDS-PAGE for mass confirmation and assessment of impurity.







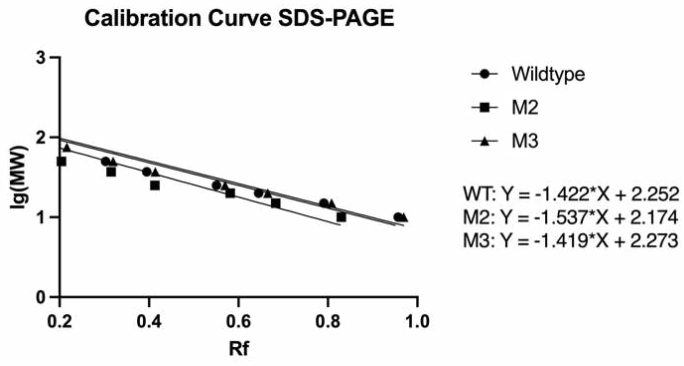


Figure1: A (top left) is the total recovery for each fraction. B (top right) SDS-PAGE of each fraction through each purification step. C (bottom left) Calibration curve for Bradford Method. D (bottom right) Calibration curve for SDS-PAGE.

In the analysis of protein purification, three protein variants were assessed: wild type (WT), M2, and M3. The mass of each isolated protein was determined using the Bradford assay, with the WT variant yielding 0.082 ± 0.004 mg, the M2 variant yielding 0.114 ± 0.013 mg, and the M3 variant showing close to M2 recovery at 0.123 ± 0.011 mg.

Mass confirmation was done through SDS-PAGE. The WT protein was estimated to have a molecular weight at 41.28 ± 14%, the M2 variant showed a lower molecular weight of 30.98 ± 14%, while the M3 variant was closer to the wild type, with a molecular weight of 40.47 ± 8.3%. Each variant was identified on the gel as the MDH2 band. We also note significant reduction in intensity and number of bands when compared to the first fraction (lane 9) at the beginning of the purification process.

Characterization of Isolate

Variant	Specific Activity	Vmax (umol/s)		Km (umol)	
	(umol/mgmin)	NADH	OAA	NADH	OAA
WT	41736 ± 29208	0.00578	0.00463	33.54	79.5
M2	1316 ± 1423	0.00193	0.0016	6.525	25.99
M3	14994 ± 17896	0.08015	0.0670	272.1	143.7

Table 2: Summary of experimental evaluation of kinetic parameters of each variant. Vmax and Km were approximated through a L-B plot.

The characterization of isolated protein variants was conducted to evaluate their enzymatic kinetic parameters. The specific activities of the wild type (WT) and mutants M2 and M3 were measured, revealing the highest specific activity in the WT of 41,736 ± 29,208 µmol/mgmin of all variants. Mutant M2 exhibited the lowest specific activity of 1,316 ± 1,423 µmol/mgmin, and mutant M3 showed a specific activity of 14,994 ± 17,896 µmol/mgmin.

When examining the maximum velocity (Vmax) of the enzymatic reaction with NADH as a substrate, the M3 variant exhibited the highest Vmax at 0.08015 µmol/s, compared to the WT's 0.00578 µmol/s, and M2's 0.00193 µmol/s. With oxaloacetate (OAA) as a substrate, the trend was similar, with M3 showing a higher Vmax (0.0670 µmol/s) relative to WT (0.00463 µmol/s) and M2 (0.0016 µmol/s).

The Michaelis-Menten constant (Km) was also determined for both substrates. For NADH, the WT exhibited a Km of 33.54 µmol, which was substantially lower than that of the M3 variant (272.1 µmol), indicating that the WT had a higher affinity for NADH. The M2 variant had a Km of 6.525 µmol for NADH, indicating the highest affinity among the three. Regarding OAA, the WT had a Km of 79.5 µmol, M2 had 25.99 µmol, and M3 had 143.7 µmol, showing M2 variant's higher affinity for OAA too.

Kinetic graphs plotted the relationship between enzyme concentration and specific activity, as well as the velocity of the enzymatic reaction with varying concentrations of NADH and OAA. These graphs confirmed the non-linear behavior typical of enzymatic reactions. The determination of Vmax and Km for each variant through a Lineweaver-Burk plot was done. A non-linear regression using GraphPad Prism for mutant M2 was also performed in addition for comparison.

Protein immunoblots gave clear bands for WT and M3 and a faint band for M2. For M2 and M3, we noted presence of bands at higher and lower MW relative to the signal of MDH2.

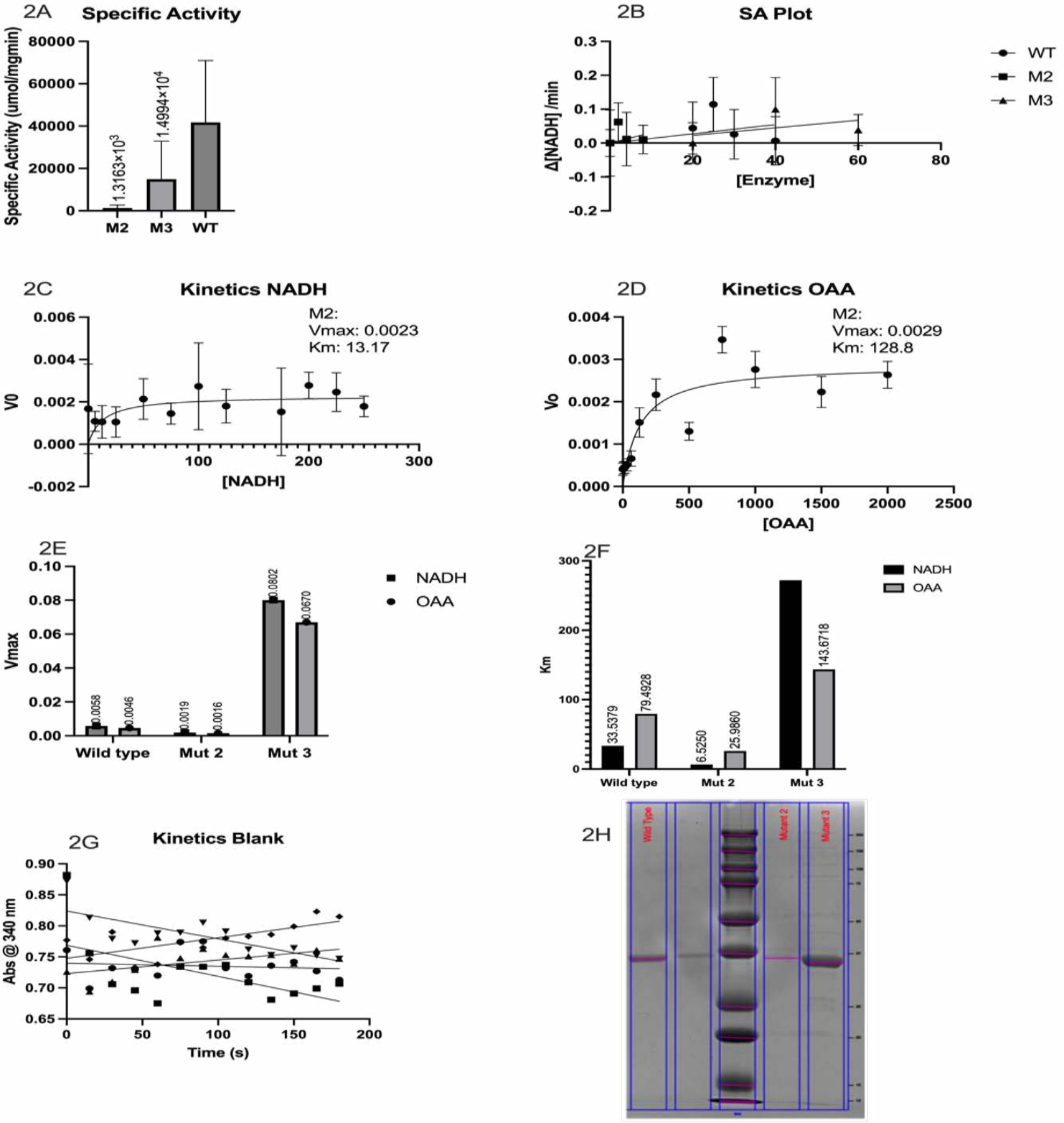


Figure 2: 2A Specific activity of the 3 variants obtained as gradient of figure 2B. Non-linear regression using GraphPad Prism was used to evaluate the kinetic parameters in figure 2C and 2D for mutant 2. L-B plot was used to estimate Vmax and Km for all 3 variants and summarized in figure 2E and 2F. For all kinetic methods, differential spectrophotometry was used with 2G as reference. Figure 2H is the protein immunoblot of all variants.

pH Variation Experimentation

pH	Specific Activity (umol/mgmin)		
	WT	M2	M3
3.2	1663.197 ± 892.247	55.774 ± 60.076	4429.83 ± 690.72
6	45808.064 ± 2452.984	1286.296 ± 150.907	12198.082 ± 1328.619
7	8716.758 ± 663.06	568.201 ± 68.075	17847.721 ± 1672.196
8	49595.345 ± 8626.8	1354.852 ± 179.15	52858.357 ± 8597.685

Table 3: Specific activity evaluated across pH range for each variant by monitoring NADH extinction at 340 nm.

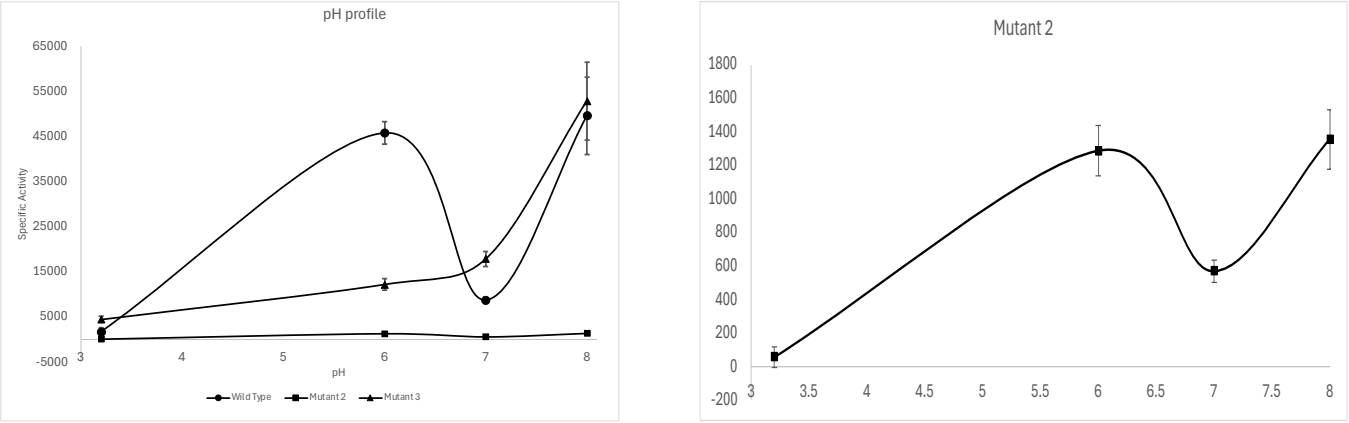


Figure 3: A (left) Plot of pH profile obtained for all variants in relation to specific activity. Figure 3B (right) is mutant 2 profile enlarged for better reference.

An investigation into the effect of pH on the enzymatic activity of protein variants wild type (WT), mutant 2 (M2), and mutant 3 (M3) demonstrated significant variability across different pH levels. The specific activity, measured in µmol/mgmin and monitored by the NADH extinction at 340 nm, was influenced by pH changes for all variants.

At a pH of 3.2, the WT displayed a specific activity of 1663.197 ± 892.247 µmol/mgmin. This activity was substantially lower than that observed at pH 6, which was the optimal pH for the WT, showing a peak specific activity of 45808.064 ± 2452.984 µmol/mgmin. The activity declined at pH 7 and further increased at pH 8although slightly lower than at pH 6.

For M2, the activity at pH 3.2 was minimal, at 55.774 ± 60.076 µmol/mgmin, but increased dramatically at pH 6, showing 1286.296 ± 150.907 µmol/mgmin, which was the peak activity for this mutant. There was a decrease in activity at pH 7 and it again picked up at pH 8 although lower than at pH 6. The bimodality of pH optima in the profile was similar to WT protein.

The M3 variant showed a consistent increase in specific activity with increasing pH values, starting at its lowest at 4429.83 ± 690.72 µmol/mgmin at pH 3.2 and reaching unexpectedly lower than WT at pH 6 with 12198.082 ± 1328.619 µmol/mgmin. Unlike WT and M2, the M3 variant maintained higher activity at pH 7 and highest of all variant's pH 8, with activities of 17847.721 ± 1672.196 µmol/mgmin and 52585.357 ± 8597.685 µmol/mgmin, respectively. It did not have the bimodal pH optima. None displayed the bell-shaped curve typical of pH profile of enzymes.

Discussion

Nickel affinity chromatography was employed to pull our protein with a His-tag from the lysate. The latter was prepared using Bug Buster reagent with a serine protease inhibitor to prevent degradation of target protein. Buffer salts were exchanged with dH_2O via size exclusion chromatography. Total protein assay was carried out for intermediate fractions and a decrease in total protein mass was noted during progress of isolation indicating purification working as intended (See Excel Workbook-Bio-Rad Assay as Supplementary Info).

Literature values provided a mass of 36 kDa for MDH2. SDS-PAGE indicated a MW of 41.28, 30.98 and 40.47 kDa for WT, M2 and M3. For the first 2, the error was of 14% and for M3, 8.3%. These small deviations form theoretical values were deemed to be random errors which could be smoothed out by repetition. These bands were characterized as signal of MDH2. Furthermore, one-sample t-tests could be carried out for each variant following replication to provide more confidence with the characterization of MDH2 by mass.

Addressing the faint signal for M2 in the purified fraction in lane 8 of SDS-PAGE, we utilized a low amount of protein. M2 had decreased activity and a greater amount was needed to generate a stable signal during kinetic assays. It is suggested for future replications to prepare more M2 compared to other fractions.

Some faint bands still persisted in the purified fraction for M2. These bands corresponded to the other proteins in the lysate as seen in the first lane which was a fraction at the beginning of the separation procedure. These bands were faint too. We deemed the isolation procedure as successful with little protein degradation as signified by lack of lower MW bands in the purified fraction. To address the remaining higher MW impurities, other methods like HPLC are suggested to remove these.

Protein Immunoblot had dark bands for WT and M3 with faint band for M2 due to the low protein amount employed as explained above. These 3 coincided, confirming identities of the MDH2 signals. Lighter bands were also noted above and below MDH2 bands in all lanes. The lower bands were attributed to His-tags separated from proteins; a degradation inherent to all separation procedures. The higher bands were of unknown origin. Replication is suggested for future references. Dimers could be suggested as the source of these, but this does not agree with M3 being unable to well form dimers and absence of these bands in WT. Attributing these to dimers would be a contradiction.

The specific activity for each variant was evaluated for all variants. As expected, we confirmed the qualitative prediction that M2 would have much lower activity compared to WT and M3. We however expected M3 to have slightly higher activity than WT but we noticed WT was significantly higher than M3. Replication is suggested instead of further seeking an explanation from the physicochemical structure of M3 and WT. One-Way ANOVA was conducted and reported no significant difference amongst the mean specific activities of the variants, suggesting that the readings obtained were attributed to randomness.

This can be seen due to the large error bars obtained in the specific activity readings. These can be attributed to several factors in

the design of the experiment and post-hoc statistical treatment of the data collected. Difference spectroscopy method was employed for background correction using a blank as reference for the assessment of specific activities and all following kinetic assays. We noted significant noise in the blank signals across 5 replicates. Ordinary Least Square (OLS) was used to assess the linear regression coefficients. Low r values were noted. This might be the source of the large error bars in the estimate of specific activity. We suggest for future replication either more replicates to smooth out the noise in the blank signal for the difference spectroscopy method or the use of a geometric method to evaluate a constant blank signal with more certainty.

Kinetic parameters were evaluated using the spectroscopic methods and statistical treatment described above. GraphPad Prism was employed for non-linear regression in addition to L-B plot estimate using Microsoft Excel. Significant difference between the Vmax for NADH and OAA for the 2 models were noted but Prism provided a higher estimate of the Km.
From the analysis of the data without considering the statistics, we could explain the higher Km of M2 by its mutation causing the binding pocket to be more open so allowing the substrate to better interact with the enzyme while also suppressing catalytic activity due to solvent exposure; effectively the substrate acting as a sort of inhibitor due to the enzyme's mutation.

Another study on enzyme kinetics discouraged the use of Linear Least square methods for the regression of coefficients due to increasing standard error with increase in enzymatic velocity. The assumption of homoscedasticity is violated due to non-homogenous variance of residuals. Similarly, we did not conduct ANOVA as academically ethical practice due to the results being unreliable from the violation of the mentioned assumption. This would be wasted effort to draw a respectable conclusion from the data without replication. The study employed orthogonal polynomials with a weighted least-square method to circumvent the inhomogeneity of residual variances in kinetic assays. This method is suggested for future replications.

For the pH range experiment, we noted similar profile with peaks at pH and 8 for WT and M2. M3 showed peak activity only at pH 8. The bimodality of peak activity would suggest another factor involved in the kinetics of MDH2. It could be attributed to the inability of M3 to form dimers and low pH promoting the formation of dimers for increased activity. A valid conclusion cannot be drawn without replication, but this provides an interesting lead to investigate the different forms MDH2 under which MDH2 is active under pH ranges.

In conclusion, our experiment highlighted the efficiency of our simple separation procedure. It provides a respectable amount of purification compared to the cost of employing HPLC to remove the remaining impurities. We faced and highlighted the significant hurdles in assessing the kinetic parameters due to the noise involved in the traditional benchtop methods used followed by simple statistical analyses. For replication, more powerful statistical methods are recommended to counteract the noise. G*Power statistical power analysis tool is suggested to finetune the experiment for more confidence in the results.

References

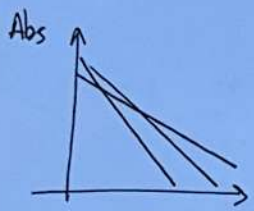
Gelpí JL, Dordal A, Montserrat J, Mazo A, Cortés A. Kinetic studies of the regulation of mitochondrial malate dehydrogenase by citrate. Biochem J. 1992 Apr 1;283 (Pt 1)(Pt 1):289-97. doi: 10.1042/bj2830289. PMID: 1567375; PMCID: PMC1131027.

Storer AC, Darlison MG, Cornish-Bowden A. The nature of experimental error in enzyme kinetic measurments. Biochem J. 1975 Nov;151(2):361-7. doi: 10.1042/bj1510361. PMID: 1218083; PMCID: PMC1172366.

Gbur, Edward; Landers, Patti; and Sharp, Roy (1990). "STATISTICAL ANALYSIS OF SPECTROPHOTOMETRIC ASSAYS IN THE PRESENCE OF INTERFERENCE," Conference on Applied Statistics in Agriculture. https://doi.org/10.4148/2475-7772.1435

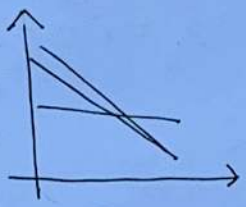
Elmore,D.T.,Kingston,A.E.& Shields,D.B.(1963) J.Chem.Soc.2070-2078

Propagation of Error in Specific Activity using Initial Rate Method.



X 2

5 replicates



Blank

5 replicates

Let error slope x2

$= \sigma_{\beta, x2}$

Let error blank

$= \sigma_{\beta, Blank}$ → These are already very large

We have 5 replicates ⇒ 5 Slopes

We evaluate the average (mean) of each slope

$$\sigma^2_{Avg\,x2} = \left(\sigma^1_{x2}\right)^2 + \left(\sigma^2_{x2}\right)^2 + \left(\sigma^3_{x2}\right)^2 + \underbrace{\left(\sigma^4_{x2}\right)^2 + \left(\sigma^5_{x2}\right)^2}$$

$$\sigma_{Avg\,x2} = \sqrt{\sum_{j=1}^{5} (\sigma_{x2\,j})^2} \qquad \sigma_{Avg\,blank} = \sqrt{\sum_{k=1}^{5} (\sigma_{blank\,k})^2}$$

$$Corrected\ Avg\,x2 = Avg\,x2 - Avg\,blank$$

$$\sigma_{corrected\,x2} = \sqrt{\left(\sqrt{\sum_{j=1}^{5} (\sigma_{x2\,j})^2}\right)^2 + \left(\sqrt{\sum_{k=1}^{5} (\sigma_{blank\,k})^2}\right)^2}$$

$$= \sqrt{\sum_{j=1}^{5} (\sigma_{x2\,j})^2 + \sum_{k=1}^{5} (\sigma_{blank\,k})^2} \xrightarrow[\text{notation}]{\text{By Property of Sigma}} = \sqrt{\sum_{i=1}^{5} \left(\sigma^2_{x2} + \sigma^2_{blank}\right)_i}$$

$$\text{St } \left(\sigma^2_{x2} + \sigma^2_{blank}\right)_i = \sigma^2_{x2\,i} + \sigma^2_{blank\,i}$$

Err S_A

$A = \varepsilon l c$

S_A

$= S_A \times \sqrt{\left(4.37^2 \times \frac{\pi^2}{19}\right) + 0.237}$

$oc = A/\varepsilon l$ $= S_A \sqrt{62.744}$ Constant $= 3.4$

~ 8 times S_A

$\sigma_{\text{Specific Activity}} = 7.92\, S_A$ $\forall n$ replicates

If we increase no. replicates $\to \infty$

$\Sigma_3 = 2\sum_{i=1}^{\infty} \frac{1}{n^2}$ $\to \pi^2/6$ $= \pi^2/3$

860 % Error

Why σ was huge.

$\times 0.225$

$\frac{1}{3.4} \times \frac{9}{40} = \frac{9}{136}$

$\Rightarrow \sigma_{\bar{x}} = \frac{1}{K_{>1}} \cdot \sqrt{\sum_{i=1}^{5} (\sigma_{x2}^2 + \sigma_{\text{blank}}^2)_i}$

Error on mix $= \frac{1}{K_2}$

$= \frac{9}{136} \sqrt{\sum_{i=1}^{5} (\sigma_{x2}^2 + \sigma_{\text{blank}}^2)_i}$ $\to \Sigma$

0.0662

Analysis of Σ_3

$\Sigma_3 = \sum_{i=1}^{5} (\sigma_{x2}^2 + \sigma_b^2)_i$

If we analyse method only,

① Assume uniform variance

s.t. $\Sigma_3 = \sum_{i=1}^{5} 2\sigma^2$

$* = 2\sum_{i=1}^{5} \sigma^2$

$\frac{1}{n} \uparrow \sigma < 1$

$\Rightarrow \Sigma_3 = 2\sum_{i=1}^{5} \frac{1}{n^2}$ when $n > 1$

$\frac{1}{2} * 3$ Σ_2

err err activity $= \text{Activity} \times \sqrt{\left(\frac{0.0662\,\Sigma}{0.014}\right)^2 + \left(\frac{0.0001}{0.0075}\right)^2}$

$\frac{\sigma_{SA}}{S_A} = 0.6$

$\Sigma_3 = \sum_{i=1}^{5} 2\sigma^2$

$= \text{Act} \times \sqrt{(4.73\,\Sigma)^2 + \left(\frac{1}{75}\right)^2}$ σ_{ox} σ was huge $\Rightarrow \frac{1}{n}$ small

$= 1.87 \sqrt{4.37^2 \left(\sum_{i=1}^{5} (\sigma_{x2}^2 + \sigma_b^2)_i\right) + \left(\frac{1}{75}\right)^2}$

σ was huge <

$\sigma_{SA} = S_A \sqrt{22.4 \times \Sigma_3 + 0.327}$ $\Sigma_3' = \sum_{i=1}^{5} \sigma_i^2 \to 0.0008$

$= S_A \sqrt{44.8\,\Sigma_3' + 0.327}$ $=$

$S_A \sqrt{19(\Sigma_3) + 0.327}$

err Specific activity $= 1316 \sqrt{\left(\frac{1.87\,\Sigma_2}{1.87}\right)^2 + \left(\frac{\text{err conc}}{\text{Conc.}}\right)^2}$

$\Rightarrow \frac{\sigma_{SA}}{S_A} = \sqrt{44.8\,\Sigma_3'} = 18$ Specific Act $\times \sqrt{4.37^2 \left(\sum_{i=1}^{5} (\sigma_{x2}^2 + \sigma_b^2)_i + \frac{1}{5625} + \frac{16}{49}\right)}$