Validation of a dispenser machine and enzyme kinetics for alcohol dehydrogenase

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Populärvetenskaplig sammanfattning

En måttenhet är en definierad mängd av en kvantitet, exempelvis längd eller volym, som är bestämd och anpassad antingen av överenskommelse eller av lag och det används som standard för mätningar av samma kvantitet. Mätningar är en process för att bestämma hur stort eller litet någonting är jämfört med grundläggande referenser av samma sorts kvantitet. Definitioner, överenskommelser och användningsområden av måttenheter har spelat en avgörande roll i mänsklig strävan på kunskap genom tiderna. I byteshandel spelar ofta vikt, volym med mera en stor roll för att försäkra om att det blir rättvist. Reproducerbarhet av experimentella resultat är centralt inom vetenskapliga metoder och ett standardsystem av enheter underlättar detta. Vetenskap använder ofta större och mindre måttenheter än de som används vardagligt. Det är svårt att behålla precision och noggrannhet när det kommer till att överföra väldigt små volymer, något man ofta gör i kemi. Det finns många olika redskap och tekniker som kan användas för att minska felaktigheten i dispenserade volymer, exempelvis dispensermaskiner av olika slag. Mitt arbete har gått ut på att validera en dispensermaskins precision samt noggrannhet och sedan att med hjälp av denna undersöka kinetiska parametrar hos enzymet alkohol dehydrogenas. Resultaten visade att dispensermaskinen hade god reproducerbarhet med små spädningsserier även om både noggrannheten och precisionen var bättre ju större räckvidd spädningsserien hade. En tvättvolym på 50 µl var den bästa för dispensermaskinen eftersom det var den minsta möjliga volymen som ändå medförde minst avvikelser från det väntade värdet till följd av kvarvarande rester. Genom att studera enzymets reaktionsförlopp, plottat som en så kallad Michaelis Menten kurva, kunde flera kinetiska parametrar bestämmas. Den maximala reaktionshastigheten när etanolkoncentrationen 0,2 M användes visade sig vara 6,74 nM/s och hälften av den etanolkoncentration som krävdes för att nå den maximala hastigheten var 10,5 mM. Den katalytiska effekten som enzymet hade på reaktionen beräknades vara 100,7 $M^{-1}s^{-1}$. Antalet etanolmolekyler som varje enzymsäte omvandlar till produkt per tidsenhet beräknades till 1,06 s^{-1}. En till kurva gjordes med ett mindre koncentrationsspann, etanolkoncentrationen var då 0,1 M.

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Abstract

In chemistry, it is difficult to retain the precision and accuracy when very small volumes are to be transferred. Various tools and techniques can be used but there is still some error. In an attempt to optimize the precision and accuracy of small dispensed volumes, a dispenser machine named Gradis was built. In order to examine the dispenser machine's accuracy and precision, two tests were performed; the optimal switch volume (wash volume) test and the dilution series reproducibility test. There is a trade off between to avoid systematic errors and to minimize the extra consumption of material. The switch volume test was performed in order to investigate what the optimal balance between these are. To test the switch volume, a 96-well plate was loaded with both pyranine and buffer while the switch volume varied from 0 µl to 100 µl. The fluorescence was measured in each well to see the difference in expected value between different switch volumes. To test the reproducibility, a 96-well plate was loaded with four replicates of a linear pyranine dilution series to test the accuracy and precision of the dispenser machine. Three dilution series of pyranine (100%-0%, 10%-0% and 1%-0%) were made to test if there was any difference in accuracy and precision depending on the dilution series range. The reproducibility tests showed that the dispenser machine have both very good accuracy and precision. The larger dilution series range, the better accuracy and precision in the dispensed volumes. The optimal switch volume turned out to be 50 µl since it was the smallest volume that yet showed small enough deviation from the expected value, which means that almost all contaminants were washed away.

When the dispenser machine had been validated, it was used in an enzyme kinetic experiment with the enzyme alcohol dehydrogenase in order to reveal kinetic properties of this enzyme. First a proper NAD^+ concentration was revealed through measuring the absorbance in a spectrophotometer to get absorbance within the range 0,1-1,0 units. Then a dilution series of the enzyme was loaded onto a 96-well plate to investigate what enzyme concentration yielded a suitable reaction rate for studying a Michaelis Menten curve. Last a dilution series of the substrate ethanol was loaded onto a new 96-well plate to generate a Michaelis Menten curve when the absorbance was measured. The kinetic parameters for when the ethanol concentration was 0,2 M were determined to as follows: K_M 10,5 mM, V_{max} 6,74 nM/s, k_{cat} 1,06 s^{-1} and the catalytic efficiency 100,7 $M^{-1}s^{-1}$. When the ethanol concentration was 0,1 M, the kinetic parameters were determined to as follows: K_M 7,48 mM, V_{max} 13,1 nM/s, k_{cat} 0,39 s^{-1} and the catalytic efficiency 51,5 $M^{-1}s^{-1}$.

Introduction

The dispenser machine

Many techniques can be used for transferring liquids in chemistry and the most common way is by pipetting the liquid with different kinds of pipettes. There are many types of pipettes on the market and many different techniques that are suitable to be used for different volumes and that gives various accuracy. However, when it comes to very small volumes it gets more difficult to retain the precision and to dispense relatively accurate volumes.[1] In an attempt to optimize the precision and accuracy of small dispensed volumes, a dispenser machine named Gradis was built. The dispenser machine can be seen in figure 1.

Figure 1: *The dispenser machine, Gradis that was used in the experiments. The channels A and B start with small tubes that are placed in the selected samples. The machine pumps the liquid from the sample containers (in front of the machine) into the syringes (on both sides of the machine) while loading them and then selected volumes of the samples are pumped through the dispenser head (on top of the machine) and dispensed into the wells of a 96-well plate (on the black rotating platform). The large Falcon tube (in front of the machine) is the waste collector, where flush volumes and switch volumes are dispensed.*

Gradis has two channels, A and B, that leads to one syringe respectively. When loaded, the syringes pump various volumes of the liquids, depending on the program settings, toward a dispenser head where the two channels are joined together and ends in a very small capillary. The dispenser head moves over the 96-well plate, that is placed on the rotating platform, in a pattern that has been selected in the settings for the machine. A drawing of the dispenser head can be seen in figure 2.

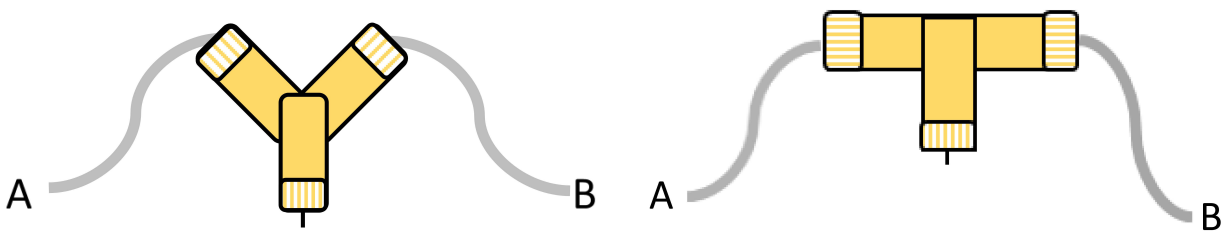

Figure 2: *Two shapes of the dispenser head where the two substances, loaded in channel A and B respectively, are mixed and dispensed according to the settings for the program. To the left the dispenser head has a Y-connection and to the right it has a T-connection. Different geometry of the dispenser head can affect dispensed volumes differently.*

Gradis is loaded with two different substances in channel A and B respectively, for example a protein in channel A and a buffer in channel B. By selecting different settings in the program, one can create many types of dilution series and by regulating the switch volume one can adjust the accuracy of the dispensed volumes.

Flush volume is the volume of liquid that is pumped through the system into the waste collector in order to wash or equilibrate the system before loading the syringes with samples. There is a trade off between to avoid systematic errors and to minimize the extra consumption of material. The switch volume can be adjusted in order to get the optimal balance between these. Switch volume is the volume of the samples in channel A and channel B that is dispensed into the waste collector before the dispensing of a new sample mixture series in the wells. This is in order to wash the dispenser head during the loading of the 96-well plate and to prevent residues of the previous sample mixture series from contaminating the new sample mixture series that is dispensed in the following wells. A good switch volume is large enough to wash the dispenser head in order to reduce contamination due to remaining residues but at the same time small enough to waste as little of the samples as possible. The optimal switch volume might differ between different geometry of the dispenser head.

Precision and accuracy

The reproducibility of a result is described by precision and how close a measured value is to the true value is described by accuracy. For a measurement to be precise, the values of a quantity that has been measured several times must agree closely to one another. To have good accuracy, the measured values must have nearness to the true value. An ideal procedure is both precise and accurate.[2]

Uncontrollable variables in the measurement can cause indeterminate error, which always is present and cannot be corrected. Indeterminate error can result from electrical noise in an instrument. Fluctuations, both positive and negative, occur with approximately equal frequency and cannot be completely eliminated.[2]

Fluorescence

Fluorescence occurs in certain molecules called fluorophores and fluorescent dyes. Fluorescence consists of three stages: excitation, excited-state lifetime and fluorescence emission. In the first stage, the molecule gets excited by absorbing a photon which supplies energy so that the molecule gets to a higher energy level. In the second stage, the molecule exists in the excited state for a very short time (often only 1-10 ns). The molecule undergoes conformational changes during this state and then, in the third stage, a photon is emitted which

causes the molecule to return to its ground state. However, the emitted photon has longer wavelength because it has lower energy than the photon that was absorbed due to the energy dissipation in the previous stage. The sensitivity of fluorescence techniques depends much on the Stokes shift. The Stokes shift is the difference in energy between the absorbed and emitted photon. This makes it possible to detect emission photons against a low background which is isolated from excitation photons.[3]

When fluorescence intensity is quantitatively measured it is dependent on the molar extinction coefficient, the optical pathlength and the solute concentration. These are the same parameters as for absorbance, defined by the Beer-Lambert law which is shown below.[3]

$$A = C \times l \times \varepsilon$$

The absorbance (A) is the product of the molar extinction coefficient (ε), the optical pathlength (l) and the solute concentration (C). The fluorescence intensity is linearly proportional to these parameters in dilute solutions. This makes it easier to see the ratio between the concentration of the fluorescent dye and its fluorescence intensity. However, when the absorbance of the sample exceeds 0,05 in a 1 cm pathlength, the ratio becomes nonlinear. This is because artifacts like self-absorption may distort the measurements.[3]

Background fluorescence must be taken into account when it comes to the sensitivity of fluorescence detection. One type of background fluorescence is autofluorescence. Autofluorescence originates from sample constituents that are within the sample. When bulk measurements which means measuring the overall signal from (almost) the whole solution, are done, none-specific binding is not a factor. By selecting filters that reduces the transmission of one emission spectra relative to another emission spectra, the detection of autofluorescence can be minimized. To get increased resolution of emission spectra, one can narrow the fluorescence detection bandwidth and this also compromises the general fluorescence intensity detected.[3]

8-Hydroxypyrene-1,3,6-trisulfonic acid, also called pyranine, is a fluorescent dye that has a yellow-green color. In basic environment, it absorbs at the wavelength 454 nm and it emits at the wavelength 511 nm. Pyranine has a pKa of 7,3 in aqueous buffers and the molar extinction coefficient is 24000 $lmol^{-1}cm^{-1}$ in basic solutions.[3]

Alcohol dehydrogenase

Saccharomyces cerevisiae, also known as Baker's yeast, has two major pathways in its energy metabolism, which are glycolysis and aerobic respiration. Ethanol has a critical role in the yeast energy metabolism since it is the end product of glycolysis and ethanolic fermentation. It is also serving as a carbon substrate in aerobic respiration, where the enzyme alcohol dehydrogenase catalyzes the interconversion of ethanol and acetaldehyde. Fermentative and oxidative carbon metabolism is linked in yeast because of the alcohol dehydrogenase reaction and it allows optimal use of sugar carbons for the yeast.[4]
Alcohol dehydrogenase is a metalloenzyme that catalyzes the reversible transformation of alcohols to aldehydes or ketones.[5] In yeast, one of the reactions catalyzed by alcohol dehydrogenase is as follows:

$$NAD^+ + Ethanol \rightarrow Acetaldehyde + NADH + H^+$$

The reaction is equimolar which means that for each NAD^+ molecule that is used, a molecule of NADH is formed. The amount of NADH formed is the same as the amount of acetaldehyde

formed. By measuring the absorbance of the reaction mixture at 340 nm, one can calculate the concentration of NADH in the sample by using the Beer-Lambert's law and that concentration is the same as the concentration for the product acetaldehyde.[6]
The proposed mechanism of alcohol dehydrogenase reaction can be seen in figure 3. The active site of the enzyme contains a zinc atom that is important for the catalysis. The enzyme promotes the hydride transfer which causes NAD^+ to be reduced to NADH and the alcohol to be oxidized to the corresponding aldehyde.[7]

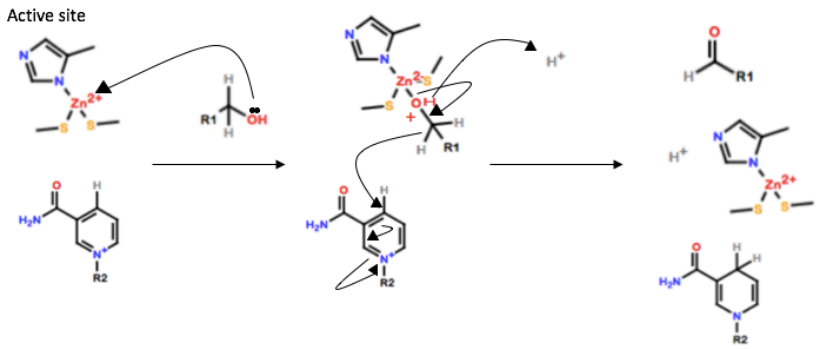

Figure 3: *The proposed mechanism for alcohol dehydrogenase. The positively charged zinc atom (II) plays a central role in the catalysis. The active site of the enzyme combines with the alcohol to promote the hydride transfer to NAD^+. NAD^+ reduces to NADH while the alcohol oxidizes to the corresponding aldehyde.*

The molecular weight of Alcohol dehydrogenase is 141-151 kDa (146 kDa in between).[8] The extinction coefficient for Alcohol dehydrogenase is 213160 $lcm^{-1}mol^{-1}$ (E%=14,6 at 280 nm)[8] while the extinction coefficient for NADH is 6220 $lcm^{-1}mol^{-1}$ (at 340 nm).[9] The optimal pH of the enzyme is 8,6-9,0 (8,8 in between).[8] The specific activity is ≥300 units/mg protein, one unit will convert 1,0 µmol of ethanol to acetaldehyde per minute at pH 8,8 at 25 °C.[10]

Michaelis Menten kinetics

Leonor Michaelis and Maud Menten proposed a model for enzyme kinetics that serves to explain how an enzyme can increase kinetic rate of a reaction and explain how reaction rates depends on the concentration of both the enzyme and the substrate. This model is known as Michaelis Menten kinetics.[11]
The mathematical formula they proposed is as follows:

$$E + S \rightleftharpoons_{k_{-1}}^{k_1} ES \rightarrow^{k_2} E + P$$

The enzyme (E) combines with the substrate (S) to form a complex (ES) with the rate constant k_1. The complex can either dissociate to enzyme and substrate with the rate constant k_{-1}, or it can proceed to form the product (P) with the rate constant k_2. This equation proposes that the product formation is negligible so that no back reaction takes place, hence k_{-2} equals zero, because the rate of reaction at times close to zero (V_0) is considered.[11]
By measuring the rate of product formation at early times before the product accumulates, one can determine V_0 for each substrate concentration. The steady-state assumption is used to facilitate the description of enzyme kinetics and to relate the rate of catalysis to the concentrations of substrate and enzyme. The steady-state means that the concentration of intermediate stay constant even if the concentration of reactants and products are changing.[11]

The Michaelis Menten equation states that when the substrate concentration is much less than the Michaelis constant, the reaction is of the first order with the rate directly proportional to the substrate concentration and when the substrate concentration is greater than the Michaelis constant, the reaction rate is maximal. When the rate is maximal, the reaction is of the zero order which means that it is independent of the substrate concentration. The Michaelis Menten equation can be seen below.[11]

$$V_0 = V_{max} \times \frac{[S]}{[S] + K_M}$$

In the equation the relation of the substrate concentration ([S]), the maximal reaction velocity (V_{max}), the Michaelis constant (K_M) and the initial reaction velocity (V_0) is shown.[11]
The Michaelis Menten curve show V_0 as a function of the substrate concentration and V_{max} is approached asymptotically for enzymes that obeys Michaelis Menten kinetics. The substrate concentration that yields half of the maximal reaction velocity is called K_M or the Michaelis constant. By studying the Michaelis Menten curve, one can obtain kinetic information about the enzyme. Information like the maximum velocity, the optimal substrate concentration and the catalytic efficiency. k_{cat} is the number of substrate molecules that each enzyme site converts to product per unit time and it is calculated by the V_{max} divided by the enzyme concentration in the cuvette. Catalytic efficiency is a measure of the efficiency of the reaction and it is calculated by the k_{cat} divided by the K_M. An example of a general Michaelis Menten curve can be seen in figure 4.[11]

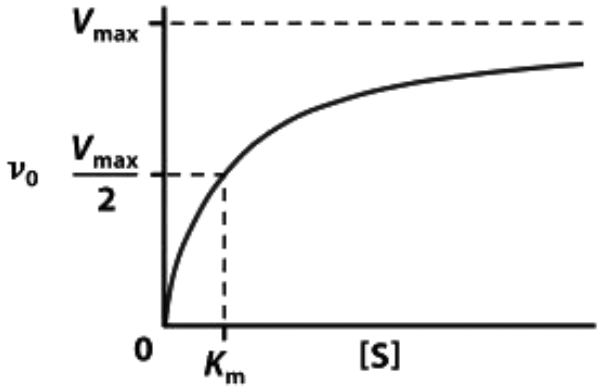

Figure 4: *An example of a general Michaelis Menten curve. The figure show the initial reaction velocity (V_0) as a function of the substrate concentration [S] and the maximal reaction velocity (V_{max}) is approached asymptotically for enzymes that obeys Michaelis Menten kinetics. The substrate concentration that yields half of the maximal reaction velocity is called K_M or the Michaelis constant.*

Experimental procedures

Preparations for dispenser machine validation

Bicine was chosen as buffer since it has the appropriate pH-range (7,0-9,0) for studying pyranine. A bicine buffer (50 mM, 500 ml) with pH 9,0 was prepared by dissolving 4,07 g of bicine in 500 ml of milliQwater. A pyranine stock solution (191 µM, 10 ml) was prepared by dissolving 1,08 mg of pyranine in 10 ml of the bicine buffer.
The spectrophotometer's best precision range was 0,1-1,0 absorbance units and in order to get 0,5 absorbance units in a 1 ml solution of pyranine, 109 µl of the pyranine stock solution was diluted in 891 µl of bicine buffer. The absorbance was measured at 25 °C in the wavelength range 300-600 nm and averages was set to 20. The purpose of measuring the absorbance was to determine how accurate the concentration of the pyranine stock solution actually was.

Switch volume test

For the first run in the dispenser machine Gradis, a 96-well plate was loaded with 100% of pyranine (2,0 µM from channel A) in the wells 3-6 for the rows A-D with the switch volume set to 0 µl. 100 % of bicine buffer (50 mM from channel B) were loaded in the wells 7-10 for the rows A-D with the switch volume set to 0 µl. The mean value of the fourth well in each part (the number 6 on row A-D for pyranine, the number 10 on row A-D for bicine buffer) were used for calibration when studying the obtained results. The switch occurs during the transition from 100% pyranine to 100% bicine buffer and vice versa and it is the first well after a switch that is analyzed. Each well on the plate was loaded with a total volume of 100 µl. The loading scheme can be seen in figure 5.

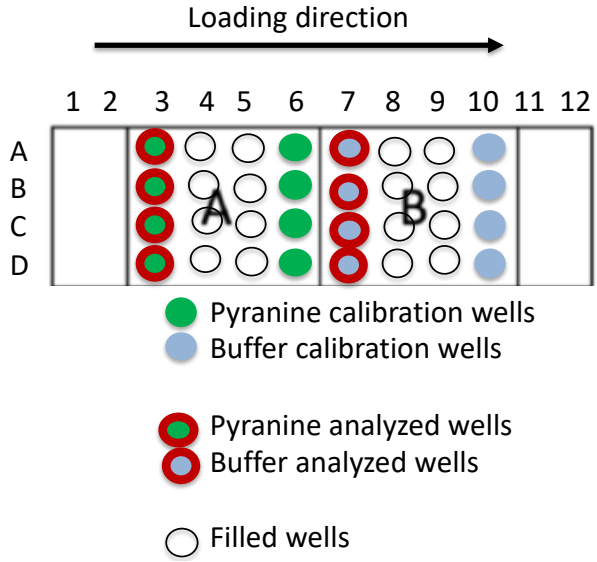

Figure 5: *The loading scheme for the 96-well plate. A represents the wells containing 100% pyranine (2,0 µM) and B represent the wells containing 100% bicine buffer (50 mM). Wells 1, 2, 11 and 12 were empty due to the program in Gradis.*

Other plates were loaded the same way as the first one, but with the switch volumes set to 10 µl, 25 µl, 30 µl, 40 µl, 50 µl, 75 µl and 100 µl. The plates were then equilibrated to 25 °C in the CLARIO STAR plate reader for 8 min before the fluorescence for each well was measured 20 times. The excitation wavelength was set to 450 nm and the emission wavelength was set to 510 nm. The obtained results were then studied in order to see what the optimal switch volume

was. The test was performed with two different shapes of the dispenser head, a Y-connection and a T-connection, to investigate how the geometry affect the dispensed volumes. The Y-connection was used in further experiments since it turned out to be the best shape.

Dilution series reproducibility

A 96-well plate was loaded with four replicates of a linear dilution series in the dispenser machine Gradis. The pyranine solution was loaded to channel A and the bicine buffer was loaded to channel B. The dilution series goes from 100% to 0% pyranine (2,0 µM) and the switch volume was set to 50 µl. Each well on the plate was loaded with a total volume of 100 µl. The loading scheme can be seen in figure 6.

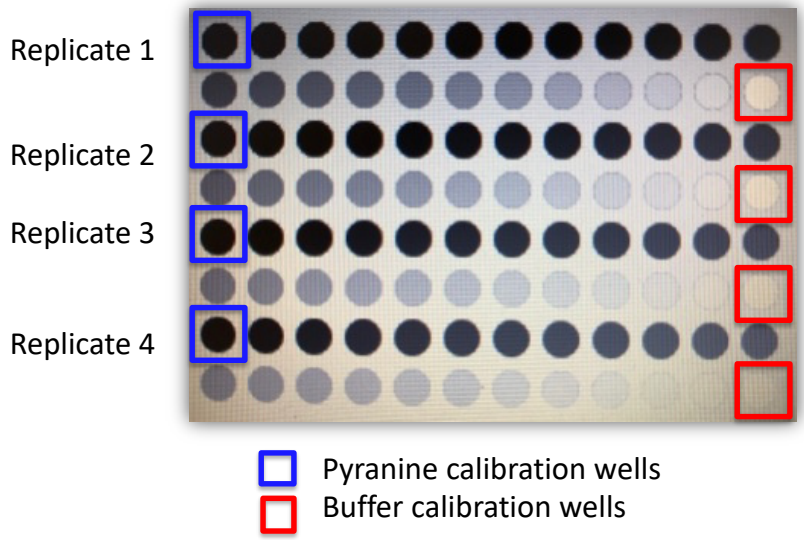

Figure 6: *The loading scheme for the 96-well plate. Four replicates were made for the same linear dilution series and each replicate is two full rows. The dilution series goes from 100% to 0% pyranine (2,0 µM). The picture is taken from the interface of the robot. The darkest wells contain 100% pyranine and the lightest wells contain 0% pyranine.*

The plate was then equilibrated to 25 °C in the CLARIO STAR plate reader for 8 min before the fluorescence for each well was measured 20 times. As calibration wells, the mean value of the 100% and 0% pyranine wells were used when studying the obtained results. Two more plates were loaded the same way to investigate the reproducibility of the dilution series. The same experiment was carried out with the dilution series from 10% to 0% pyranine (20 µM) and from 1% to 0% pyranine (190 µM) to test the limits of Gradis.

Preparations for enzyme kinetics

Bicine was chosen as buffer since it has the appropriate pH-range (7,0-9,0) for studying alcohol dehydrogenase kinetics. A bicine buffer (50 mM, 500 ml) with pH 8,8 was prepared by dissolving 4,07 g of bicine in 500 ml of milliQwater. The enzyme stock solution (0,34 µM, 39,2 ml) was prepared by dissolving 2,06 mg of alcohol dehydrogenase in 39,2 ml milliQwater. The always fresh NAD^+ stock solution (0,1 M, 1,0 ml) was prepared by dissolving 66,3 mg of NAD^+ in 1 ml of the bicine buffer.

In a cuvette, a 500 µl reaction mixture was prepared by first adding NAD^+ (44 mM, 45,5 µl), ethanol (1,0 M, 100 µl) and bicine buffer (50 mM, 301,6 µl) during magnetic stirring. A blanc

was measured before the enzyme (0,34 μM, 52,9 μl) was added to the cuvette and the absorbance of the reaction mixture was then measured in the spectrophotometer over time in 10 min.
Different enzyme concentrations were tested to see what concentration that gave the reaction an appropriate speed for studying. The spectrophotometer's best precision range was 0,1-1,0 absorbance units and a NAD^+ concentration was chosen in order to get absorbance within that range for the first 10 min of the reaction. The absorbance was measured at 25 ºC in the wavelength range 338-342 nm. Figure A11 in the appendix section show the concentration of NADH produced during the reaction in the cuvette as a function of the time the reaction has taken place. The starting concentration of NAD^+ was 4,4 mM and this was the concentration NAD^+ that was used in further experiments.

The choice of enzyme concentration

Two solutions named A and B were prepared. In A, NAD^+ (44 mM, 1009 μl) and alcohol dehydrogenase from Baker's yeast (0,34 μM, 1633 μl) were mixed with the bicine buffer to a total volume of 10 ml. In B, NAD^+ (44 mM, 1009 μl) was mixed with the bicine buffer to a total volume of 10 ml.
A 96-well plate was loaded with four replicates of a linear enzyme dilution series in the dispenser machine Gradis. Solution A was loaded to channel A and solution B was loaded to channel B. The dilution series goes from 100% to 1% alcohol dehydrogenase (0,05 μM) and the switch volume was set to 50 μl. Each well on the plate was loaded with a total volume of 90 μl. The loading scheme can be seen in figure 6, with the exception that the last well in each replicate was hand pipetted with solution B. By using a 96-channel pipette the substrate ethanol (2,0 M, 10 μl) was added to all the wells at the same time just before the plate was placed in the CLARIO STAR plate reader at 25 ºC and the absorbance for each well was measured at wavelength 340 nm during the reaction 1000 times. As calibration wells, the mean value of the hand pipetted last well in each dilution series replicate was used when studying the obtained results.

Enzyme kinetics

A 96-well plate was loaded with four replicates of a linear ethanol dilution series in the dispenser machine Gradis. This was done the same way as previously but with the A solution containing NAD^+ (44 mM, 1009 μl) and ethanol (2,0 M, 1111 μl) that were mixed with the bicine buffer to a total volume of 10 ml. The dilution series goes from 100% to 1% ethanol (0,2 M). By using a 96-channel pipette the enzyme alcohol dehydrogenase (0,5 μM, 10 μl) was added to all the wells at the same time and the measurement was done as previously. This procedure was repeated but with ethanol (1,0 M, 1111 μl) added to the A solution and four replicates of a logarithmic dilution series from 100% to 1% ethanol (0,1 M) was made instead.

Results

Optimal switch volume

Eight switch volumes (0 µl, 10 µl, 25 µl, 30µl, 40 µl, 50 µl, 75 µl and 100 µl) were tested for both 100% pyranine (2,0 µM) filled and 100% buffer (50 mM) filled wells on 96-well plates loaded in the dispenser machine Gradis. The results are presented as the deviation from the expected value of fluorescence intensity (%) as a function of the switch volumes (µl). It is the first well after a switch from 100% pyranine to 100% bicine buffer and vice versa that is analyzed. Figure 7a show the mean value results for the wells containing buffer when the dispenser head had a Y-connection and figure 7b show the results for the wells containing buffer when the dispenser head had a T-connection.

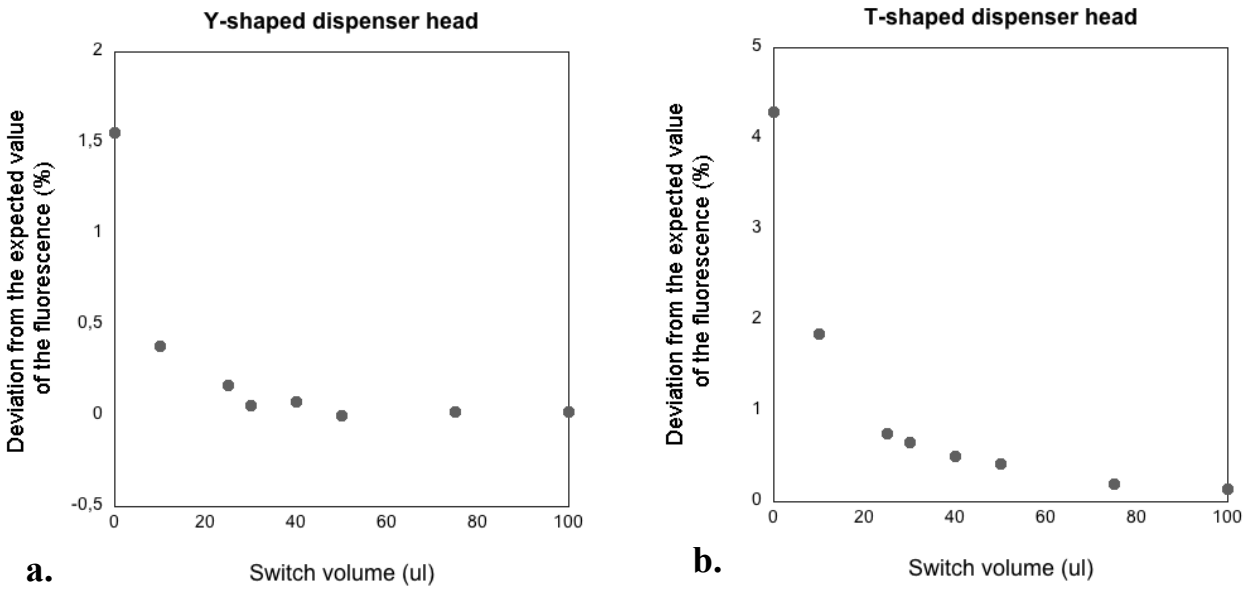

Figure 7: *The deviation from the expected value of fluorescence intensity in % for every switch volume tested in µl. The figure* **a)** *show the mean value data from the wells that were expected to be filled with 100% bicine buffer (50 mM) when the dispenser head had a Y-connection. The figure* **b)** *show data from the wells that were expected to be filled with 100% bicine buffer (50 mM) when the dispenser head had a T-connection.*

From figure 7, one can see that the deviation in the wells follow the same pattern, the larger switch volume the smaller systematic error. One can also see that the Y-connection had smaller systematic errors than the T-connection had in every switch volume. The Y-connection was selected as the best and it was used in the other experiments. From these results, the switch volume 50 µl was chosen as the optimal switch volume since it was the smallest volume that still generated low enough deviation from the expected value. Since the values goes a little up and down 50 µl was selected to be on the safe side. The replicates that figure 7a is based on can be seen in figure A1 in the appendix section. The results for the wells containing pyranine when the dispenser head had a Y-connection can be seen in figure A2 in the appendix section. More detailed results from the switch volume test can be seen in figures A3-A10 in the appendix section. In the appendix section, table A1 show how the volumes of sample needed differs between different selected switch volumes.

Reproducibility

Three linear dilution series for pyranine (100%-0%, 10%-0% and 1%-0%) were tested in the dispenser machine Gradis. The results are presented as the normalized value of the fluorescence intensity (%) as a function of the volume pyranine added (µl). Figure 8a show the results for the linear dilution series 100%-0% pyranine (2,0 µM), figure 8b show the results for the linear dilution series 10%-0% pyranine (20 µM) and figure 8c show the results for the linear dilution series 1%-0% pyranine (190 µM).

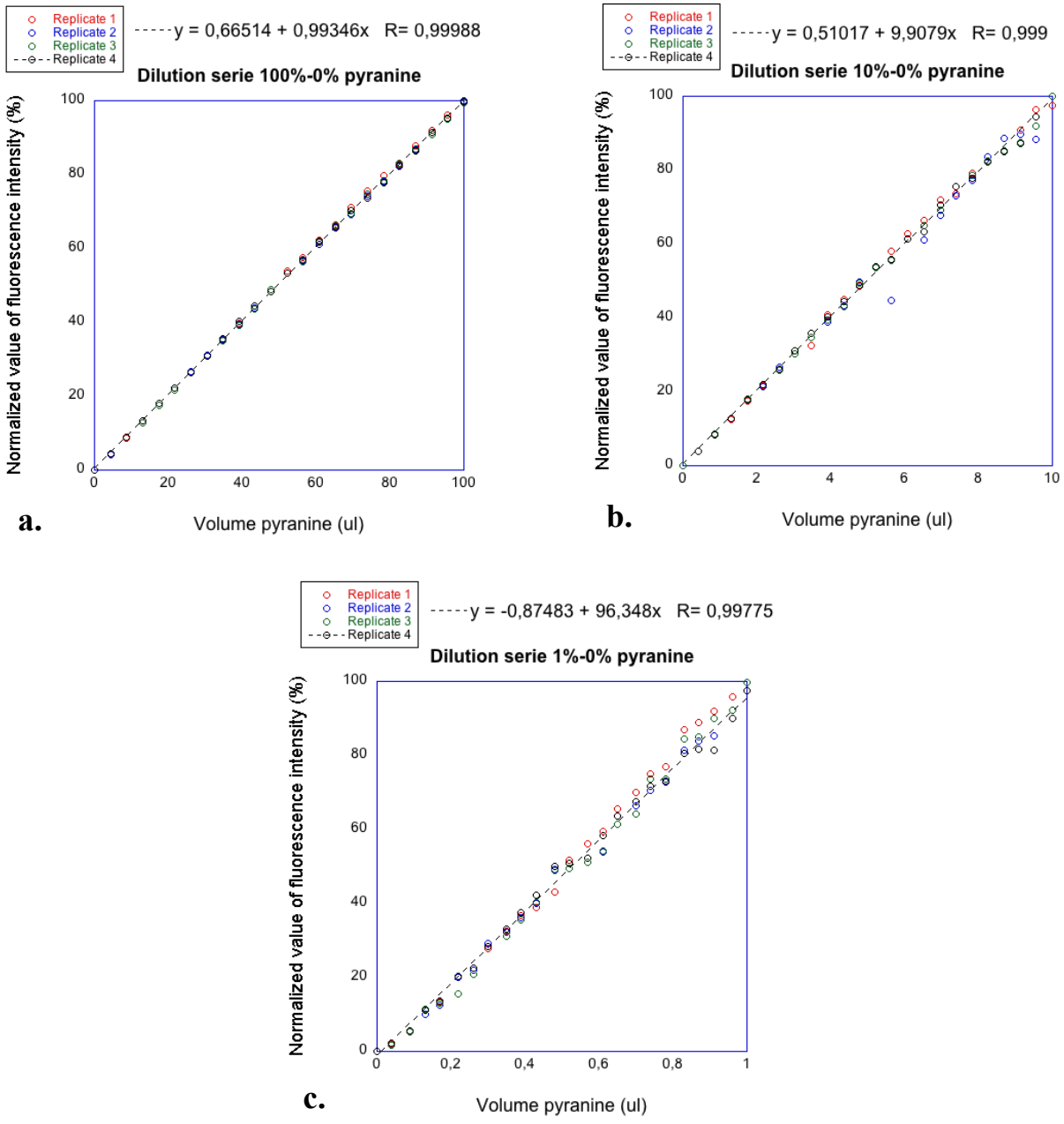

Figure 8: *The normalized value of the fluorescence intensity in % for every volume pyranine added to the wells in µl. The figure* **a)** *show data from the linear dilution series 100%-0% pyranine (2,0 µM). The figure* **b)** *show data from the linear dilution series 10%-0% pyranine (20 µM). The figure* **c)** *show data from the linear dilution series 1%-0% pyranine (190 µM).*

From the figure 8, one can see that all the measured values of the linear dilution series are relatively close to the linear regression they have been fitted to. According to the R-value, the 100%-0% dilution series has the best accuracy and the 1%-0% dilution series has the least accuracy. The deviation looks greater among the wells that contains larger volume pyranine and this is most expressed in the smallest linear dilution series. However, it is the same error in % through the whole dilution series and it just looks bigger when the values are higher. The results are still very close to accurate in all the dilution series.

The variance among the measurements of the fluorescence intensity is shown for the three linear dilution series as the coefficient of variance (%) as a function of the volume pyranine added (µl). Th coefficient of variance was calculated by the standard deviation of the fluorescence for each well in the dilution series divided by the mean value of the fluorescence for each well in the dilution series. The resulting values were multiplied with 100 to get the coefficient of variance in %. Figure 9a show the variance in the dilution series 100%-0% pyranine (2,0 µM), figure 9b show the variance in the dilution series 10%-0% pyranine (20 µM) and figure 9c show the variance in the dilution series 1%-0% pyranine (190 µM).

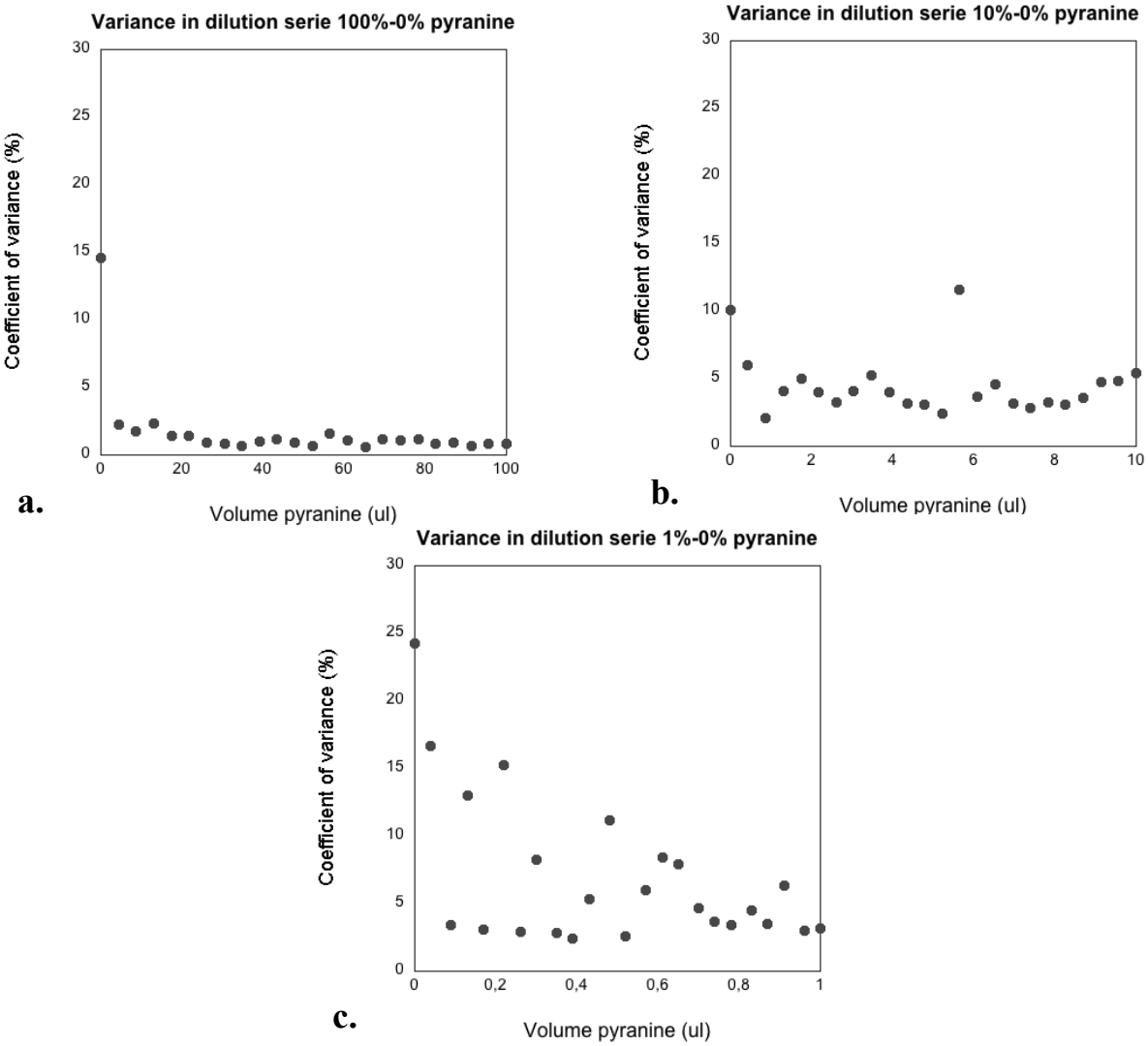

Figure 9: *The coefficient of variance in % for every volume pyranine added to the wells in µl. The figure* **a)** *show data from the linear dilution series 100%-0% pyranine (2,0 µM). The figure* **b)** *show data from the linear dilution series 10%-0% pyranine (20 µM). The figure* **c)** *show data from the linear dilution series 1%-0% pyranine (190 µM).*

From the figure 9, one can see that the variance between the fluorescence measurements becomes larger when the dilution series is starting with a smaller concentration of pyranine than it is when the dilution series is starting with a larger concentration of pyranine. However, the accuracy is still relatively good in each case.

Michaelis Menten curves and kinetic parameters

The four replicates of the linear and the logarithmic ethanol dilution series generated absorbance values that were calculated to concentrations to see the reaction velocity at different ethanol concentrations. The results are presented as the reaction velocity (nM/s) as a function of the ethanol concentration (mM). After the curve from the linear dilution series was obtained and most of the data points were at the plateau of the curve, a logarithmic dilution series was made in order to get more data points in the beginning of the curve. Figure 10a and 10b show the Michaelis Menten curves as the resulting curves for the linear and the logarithmic dilution series 100%-1% ethanol (0,2 M and 0,1 M respectively).

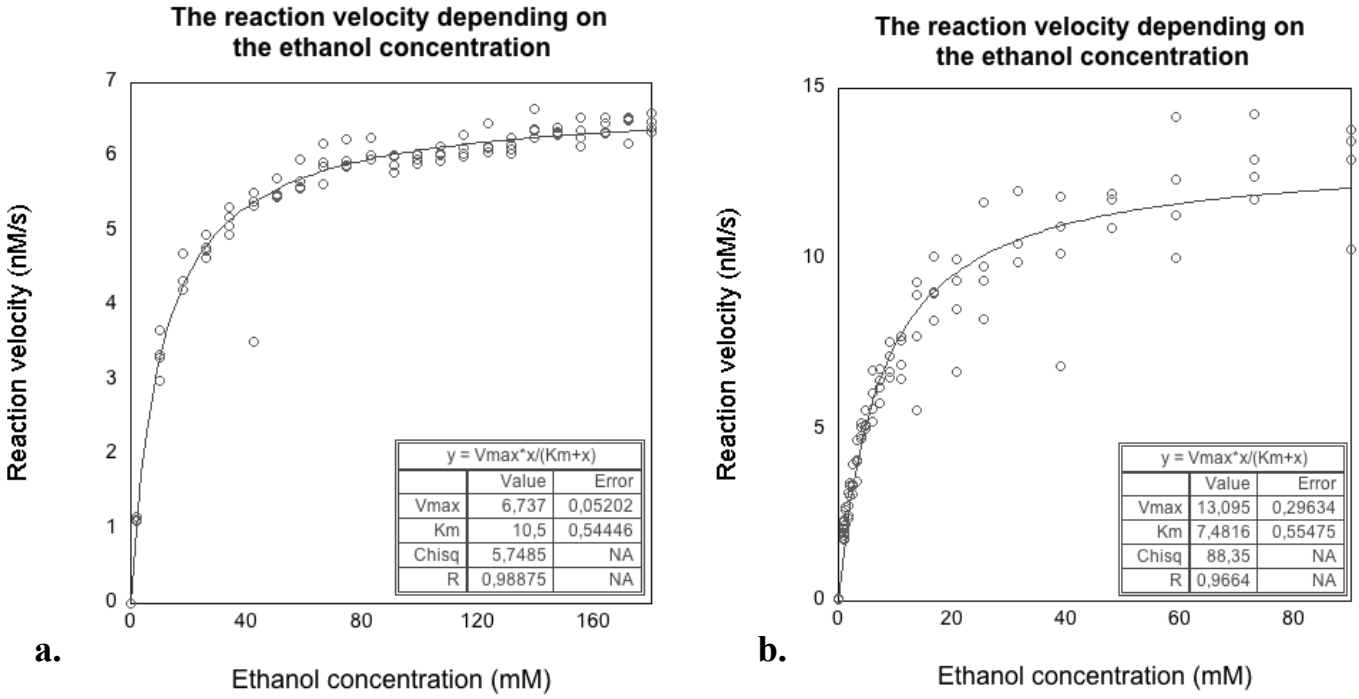

Figure 10: *The reaction velocity in nM/s for every ethanol concentration in the dilution series in mM. The figure* **a)** *show data from the linear dilution series 100%-1% ethanol (0,2 M). The K_M is 10,5 ±0,544 mM and the V_{max} is 6,74 ±0,052 nM/s. The figure* **b)** *show data from the logarithmic dilution series 100%-1% ethanol (0,1 M). The K_M is 7,48 ±0,555 mM and the V_{max} is 13,1 ±0,296 nM/s.*

From figure 10 of the Michaelis Menten curves, one can see that the reaction velocity increases the larger ethanol concentration that is present in the reaction mixture. The calculated values are relatively close to the fitted Michaelis Menten line. Figure 10a has better accuracy and precision in the later part of the curve than figure 10b has but in the early part of the curve both figures show good accuracy and precision. From figure 10a K_M obtained was 10,5 mM and V_{max} obtained was 6,74 nM/s, while from figure 10b K_M obtained was 7,48 mM and V_{max}

obtained was 13,1 nM/s. Table 1 show the kinetic parameters for alcohol dehydrogenase that was obtained from figure 10.

Table 1: *The kinetic parameters K_M, V_{max}, k_{cat} and catalytic efficiency for alcohol dehydrogenase obtained from figure 10 respectively.*

Kinetic parameters	**Figure 10a**	**Figure 10b**
K_M	10,5 mM	7,48 mM
V_{max}	6,74 nM/s	13,1 nM/s
k_{cat}	1,06 s^{-1}	0,39 s^{-1}
Catalytic efficiency	100,7 $M^{-1}s^{-1}$	51,5 $M^{-1}s^{-1}$

Discussion

Switch volume

Figure 7a and 7b show the same pattern, that the larger switch volume the smaller systematic error. However, there is a difference between the Y-connection and the T-connection when it comes to the size of the systematic errors. The Y-connection has smaller systematic errors than the T-connection has. This show that the angle of the entering samples matters. The T-connection most likely causes more swirls and larger dead volume which affect the accuracy of the dispensed volumes. The Y-connection, on the other hand, show better accuracy since the dead volume becomes smaller and therefore it was chosen as the optimal shape of the dispenser head.

By looking at the results from the pyranine filled wells that are shown in figure A2 in the appendix section, one cannot see much of a correlation between the deviation and the switch volume. The results from the buffer filled wells that are shown in figure 7a, on the other hand, reveals a pattern that the larger switch volume the smaller deviation. This seems to be accurate since the more the dispenser head is washed the less residues should be left to contaminate the wells.
Why the pyranine wells differed might be because of fluctuation when measuring the fluorescence. There might have been small bubbles or some dust in the 96-well plate that affected the fluorescence measurements of the wells and that this error was more expressed when higher fluorescence was measured. However, by looking at figure 7a switch volume 50 µl was selected to be the optimal switch volume since it is a volume large enough to wash away most of the contaminants from the dispenser head and yet it is small enough so that as little of the samples as possible is wasted.

Accuracy

Figure 8a show that the linear dilution series 100%-0% of 2,0 µM pyranine was close to the linear regression according to the R-value 0,9998 so this is a very accurate dilution series. The 10%-0% linear dilution series of 20 µM pyranine that is shown in figure 8b, with the R-value 0,9990 it is still very accurate to the linear regression. The smallest linear dilution series 1%-0% of 190 µM pyranine in figure 8c, on the other hand, show a little more deviation from the linear regression with the R-value 0,9976 but it is still very close to accurate. The smaller dilution series the smaller concentration differences between the wells and the less accuracy of the dispensed volumes. The precision show the same pattern as the accuracy.
It is the same error in % through the whole dilution series and it just looks bigger when the values are higher. That is why the wells containing a larger volume of pyranine looks like they have greater deviation than the wells containing smaller volume pyranine. The results are still very close to accurate in all the dilution series.

When studying the coefficient of variance for the three dilution series made, it becomes even more obvious that the larger dilution series the better accuracy and precision. The results from the 100%-0% of 2,0 µM pyranine in figure 9a show the smallest coefficient of variance pattern with all well values below 5%. The 10%-0% dilution series of 20 µM pyranine in figure 9b show a coefficient of variance pattern with well values around 5% and the results from the smallest dilution series 1%-0% 190 µM pyranine in figure 9c show a coefficient of variance pattern with well values up to 25%. The variance among the values of the coefficient of variance becomes greater when the dilution series range becomes smaller.

However, a very skilled chemist could possibly obtain the accuracy and precision showed in the 100%-0% dilution series but the accuracy and precision in the 1%-0% dilution series is beyond human capacity. This makes the dispenser machine Gradis a very helpful tool when creating dilution series of reaction mixtures for experiments.

Kinetic parameters

The Michaelis Menten curves in figure 10 indicates that alcohol dehydrogenase is an enzyme that follows Michaelis Menten kinetics because V_{max} is approached asymptotically. This is consistent with the literature.[11] An enzyme that does not follow Michaelis Menten kinetics, for example allosteric enzymes, display sigmoidal plots of the reaction velocity versus substrate concentration instead of hyperbolic plots predicted by the Michaelis Menten equation and this is not the case.

The obtained results are relatively close to the fitted Michaelis Menten line, the R-values are 0,989 and 0,966 respectively. One can see that figure 10a has better accuracy and precision than figure 10b when it comes to the later part of the curve but at the early part of the curve both of the figures have good accuracy and precision. The two figures compensate each other since one has many accurate measurements in the beginning and one has many accurate measurements in the end. Figure 10b has a narrower ethanol concentration range than figure 10a. What caused the distribution in the later part of the curve in figure 10b is unknown.

From the curve in figure 10a, V_{max} turned out to be 6,74 nM/s and K_M turned out to be 10,5 mM. While in figure 10b, V_{max} was 13,1 nM/s and K_M was 7,48 mM. K_M in the figures does not differ so much and the small difference is most likely due to some measuring fluctuations. The literature value for K_M is 20 mM[12] which is twice as high as the obtained value. Why the values differ might be if the reaction was measured at different pH. In fact, these experiments were measured at pH 8,8 while the literature value was obtained from measurements at pH 7,3.[12] The enzyme might have catalyzed slower at lower pH, which another study proposes. According to that literature source K_M is 21-26 mM at pH 7,05 and around 10 mM at pH 8,9.[13] These literature values are well matched by the obtained results from the experiments.

The substrate concentration is greater than the Michaelis constant, hence the reaction is of the zero order in this case. From figure 10a, k_{cat} was calculated to be 1,06 s^{-1} and the catalytic efficiency was calculated to be 100,7 $M^{-1}s^{-1}$. From figure 10b, k_{cat} was calculated to be 0,39 s^{-1} and the catalytic efficiency was calculated to be 51,5 $M^{-1}s^{-1}$.

Conclusions

From the reproducibility test it was shown that both the accuracy and the precision were very good, especially when the dilution series is over a larger range. The dispenser machine Gradis is trustworthy when it comes to both accuracy and precision of linear dilution series and when it comes to the optimal switch volume, it was revealed to be 50 µl. It was the smallest volume that yet showed the least deviation from the expected value.

Alcohol dehydrogenase from Baker's yeast follows Michaelis Menten kinetics. For the ethanol concentrations 0,2 M and 0,1 M respectively; K_M was determined to be 10,5 mM and 7,48 mM, V_{max} was determined to be 6,74 nM/s and 13,1 nM/s, k_{cat} was determined to be 1,06 s^{-1} and 0,39 s^{-1} and the catalytic efficiency was determined to be 100,7 $M^{-1}s^{-1}$ and 51,5 $M^{-1}s^{-1}$.

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Appendix

Figure A1 show the results for the wells containing buffer in the switch volume test when the dispenser head had a Y-connection. Data from the three replicates are shown and the mean values in figure 7a in the results section is based on these values.

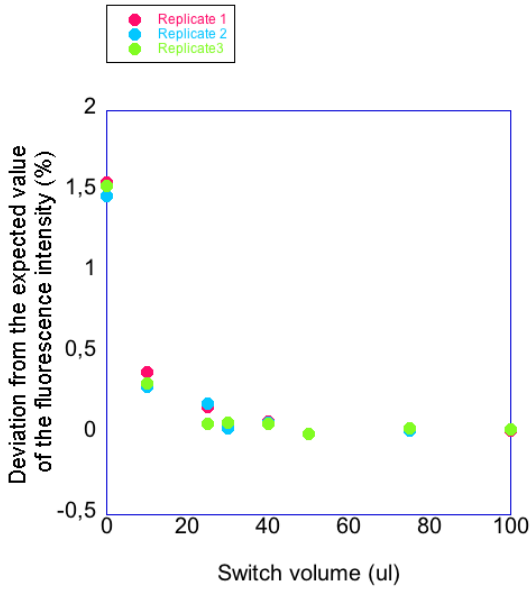

Figure A1: *The deviation from the expected value of fluorescence intensity in % for every switch volume tested in µl. The figure show the three replicate data that figure 7a is based on. The wells were expected to be filled with 100% bicine buffer (50 mM) and the dispenser head had a Y-connection.*

Figure A2 show the results for the wells containing pyranine in the switch volume test. The deviation from the expected value of the fluorescence intensity (%) is shown as a function of the switch volume (µl).

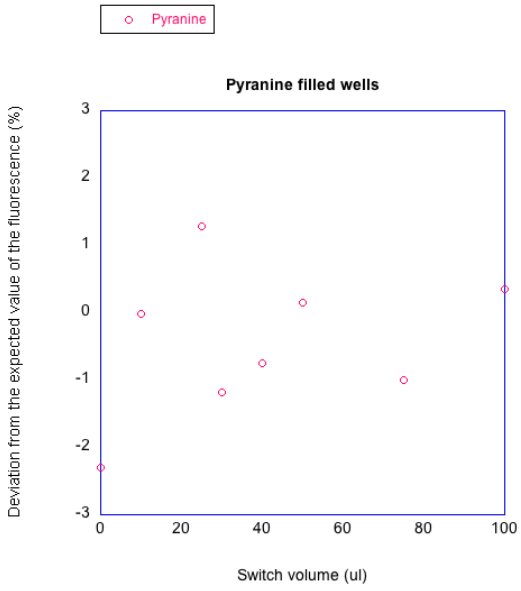

Figure A2: *The deviation from the expected value of fluorescence intensity in % for every switch volume tested in µl. The figure show data from the wells that were expected to be filled with 100% pyranine (2,0 µM).*

Figures A3-A10 show more detailed results from the switch volume test. The deviation from the expected value of the fluorescence intensity (%) is shown for each well on each row on the 96-well plate when the switch volumes 0 µl, 10 µl, 25 µl, 30 µl, 40 µl, 50 µl, 75 µl and 100 µl were used.

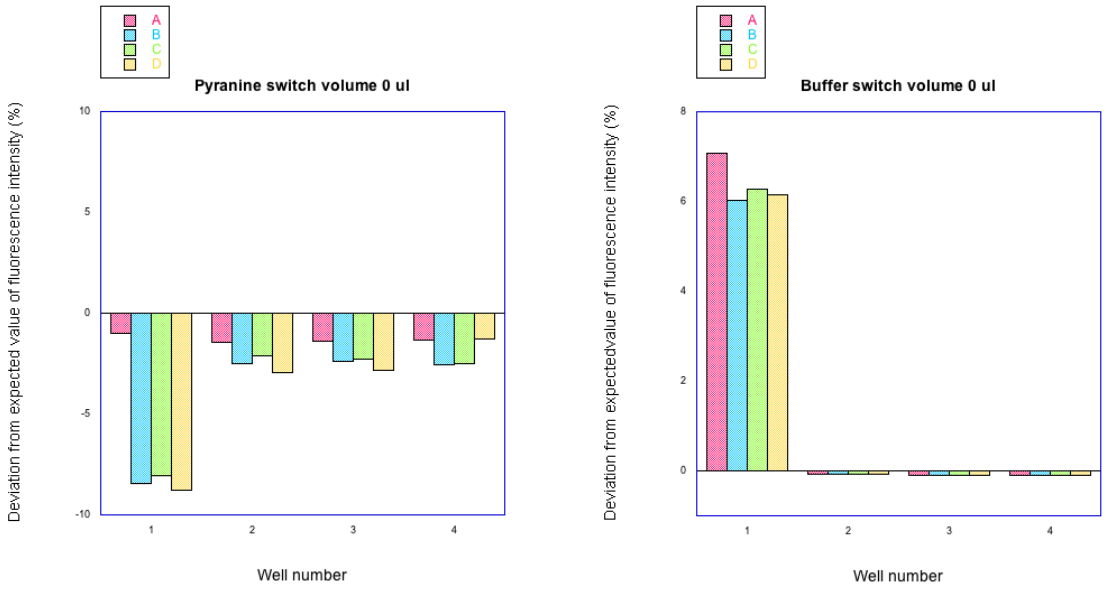

Figure A3: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 0 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

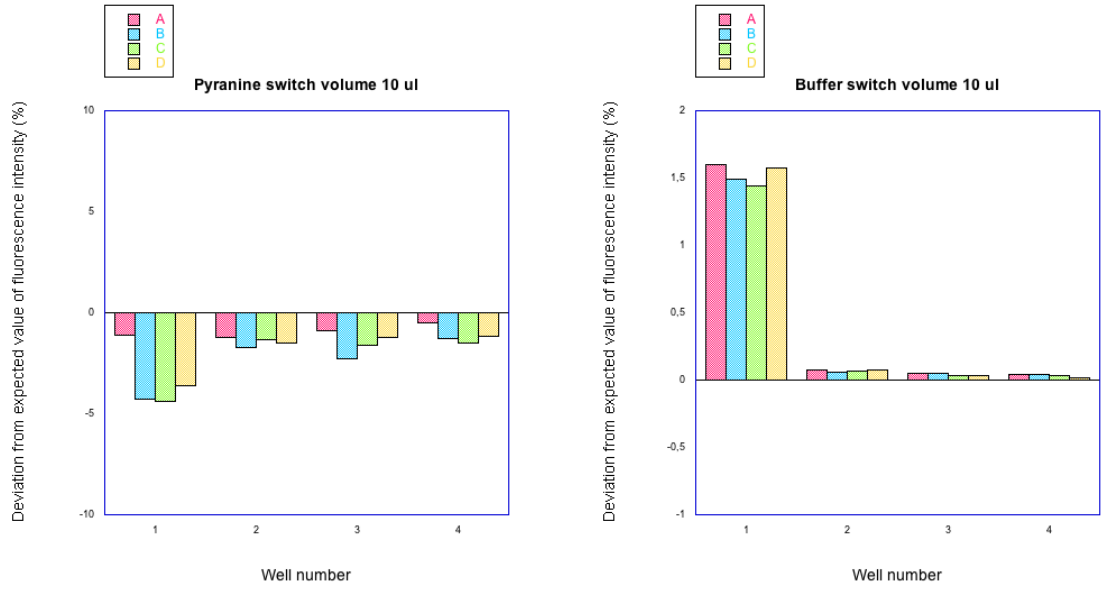

Figure A4: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 10 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

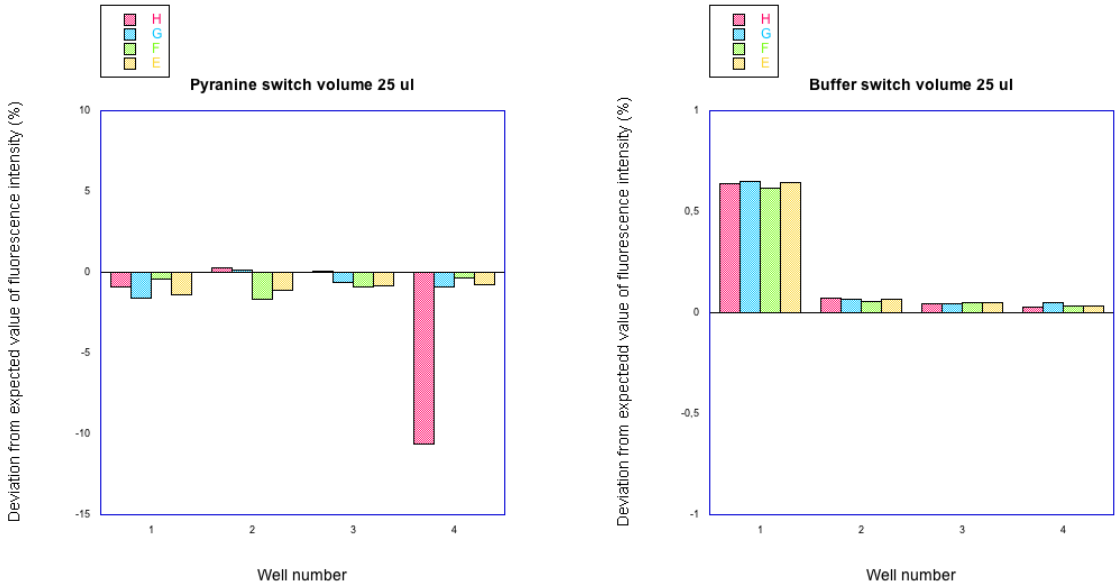

Figure A5: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 25 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

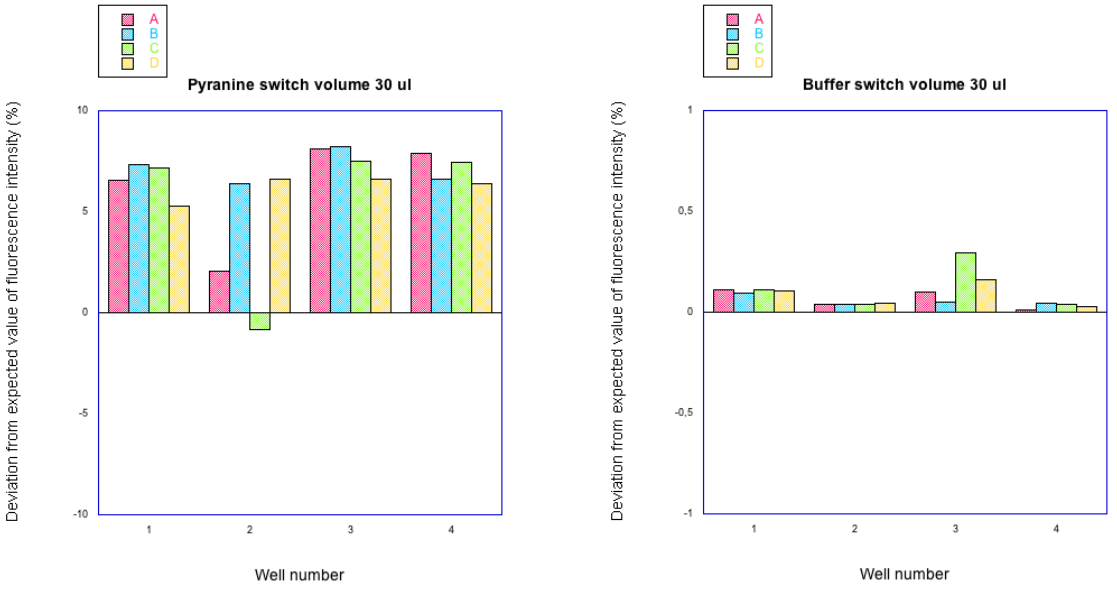

Figure A6: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 30 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

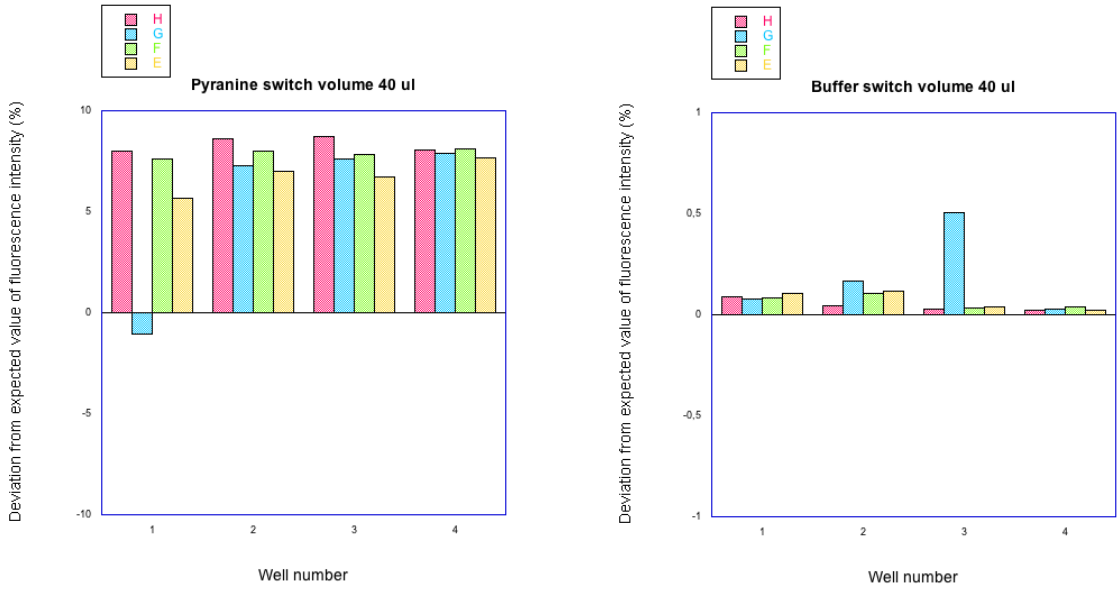

Figure A7: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 40 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

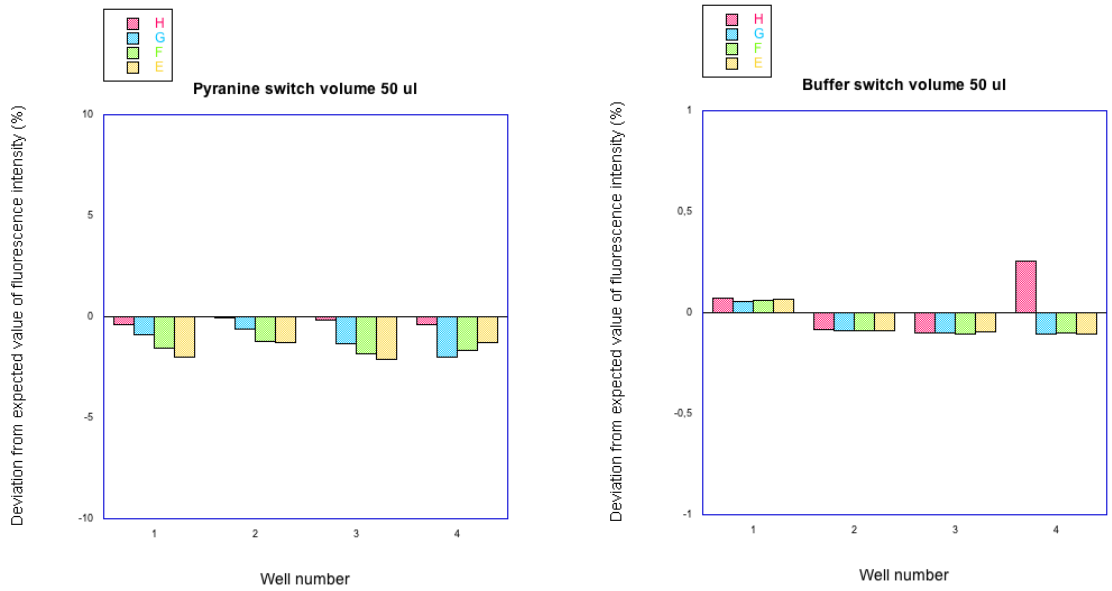

Figure A8: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 50 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

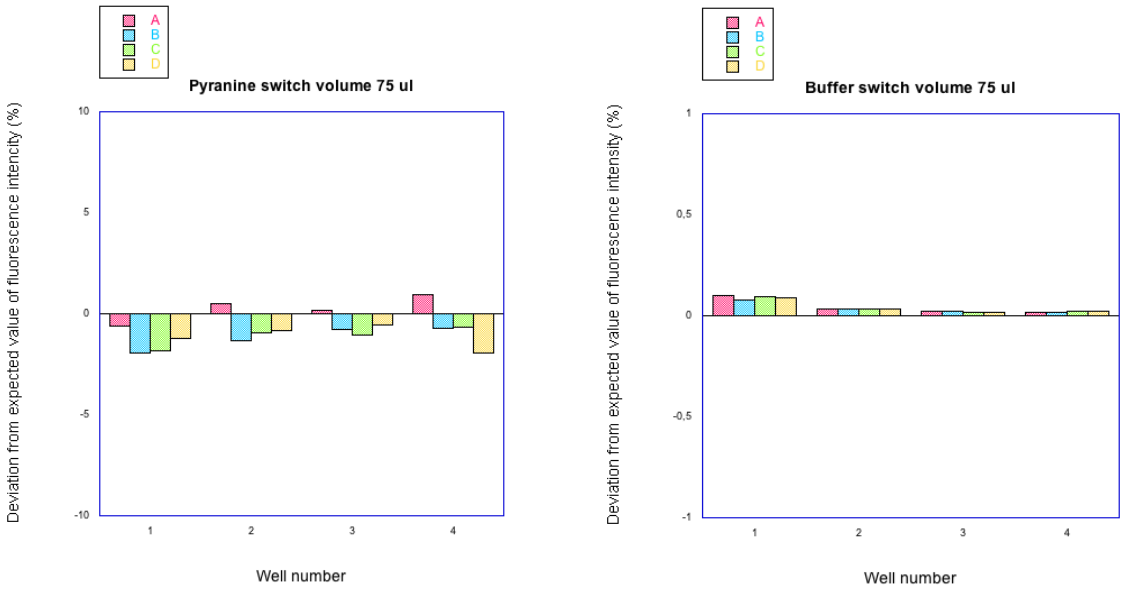

Figure A9: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 75 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

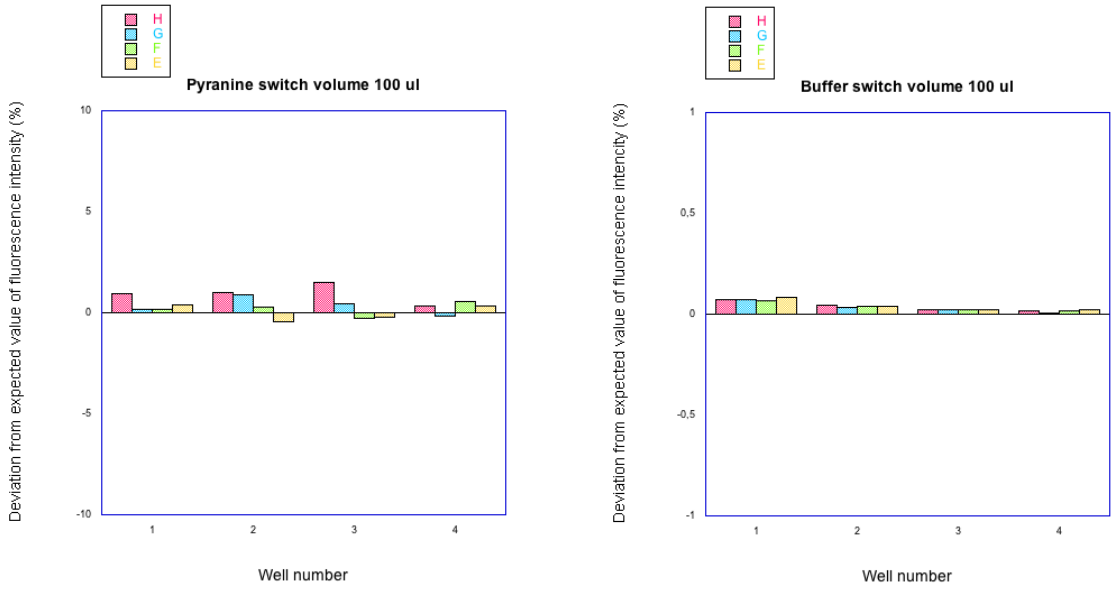

Figure A10: *The deviation from the expected value of fluorescence intensity in % for each well on each row when the switch volume was set to 100 µl. The figure to the left is for the wells filled with pyranine and the figure to the right is for the wells filled with buffer.*

Table A1 show the volumes of samples in channel A and channel B respectively that the dispenser machine needs to complete a dilution series when different switch volumes are used in a 100%-0% dilution series. This is good information when one wants to know how much extra material is needed in order to minimize the systematic errors.

Table A1: *The different volumes of sample in channel A and channel B respectively that is needed to complete four replicates of a 100%-0% dilution series when different switch volumes are used. Larger switch volume needs more samples but gives smaller systematic error. The systematic error for each switch volume can be seen in figure 7a in the results section.*

Switch volume (µl)	Volume A (µl)	Volume B (µl)
0	5189	5189
10	5229	5229
25	5289	5289
30	5309	5309
40	5349	5349
50	5389	5389
75	5489	5489
100	5589	5589

Figure A11 show the concentration of NADH produced during the reaction in a cuvette when searching for a good NAD^+ concentration. The concentration NADH (µM) is shown as a function of the time the reaction has taken place (min). The starting concentration of NAD^+ was 4,4 mM and the absorbance of NADH was measured during 10 min. The absorbance of NADH was then calculated to NADH concentration.

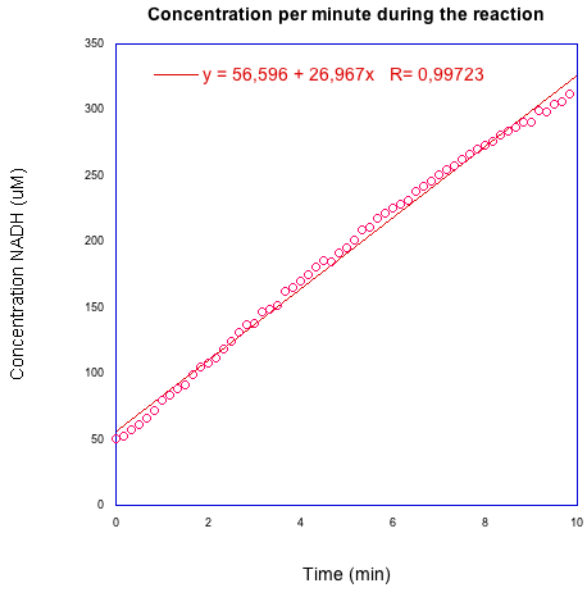

Figure A11: *The concentration of NADH produced (µM) as a function of the time the reaction has taken place (min). According to the linear regressions R-value, the measured values of the absorbance are pretty close to a linear connection. The NAD^+ concentration in the cuvette was 4,4 mM at the start.*

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