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Design of a pixel-based quantification method for *in vitro* colorimetric enzyme assay to evaluate enzyme inhibition in a dose-dependent manner.

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Biochemical and biophysical assays *in vitro* are often valuable assets to evaluate new compounds as inhibitors that can be developed into potential drugs. With rising expenses for laboratory research these days, designing an alternative economic method is highly desirable. In this study we evaluate the inhibitory potencies of two compounds, sodium azide (SA) and hydroxylamine (HA) in a dose-dependent manner using the standard horseradish peroxidase (HRP) enzyme. HRP is commonly conjugated to secondary antibodies that allows for colorimetric detection of proteins in western blotting technique. We chose to perform the colorimetric quantification using the pixel count with Image J software instead of the routine colorimetry/spectrophotometry. For both SA and HA the area under the curve (AUC) was quantified using the pixel count. Changes in the AUC with respect to the inhibitor concentration were carefully plotted to obtain the dose-dependent curves that were further used to calculate the individual potencies of SA and HA. This method was found to be at least 50 times more economical than the standard methods that use colorimetry/spectrophotometry.

Keywords: Colorimetry, HRP, enzyme inhibitors assay, pixel count, area under the curve, Image J.

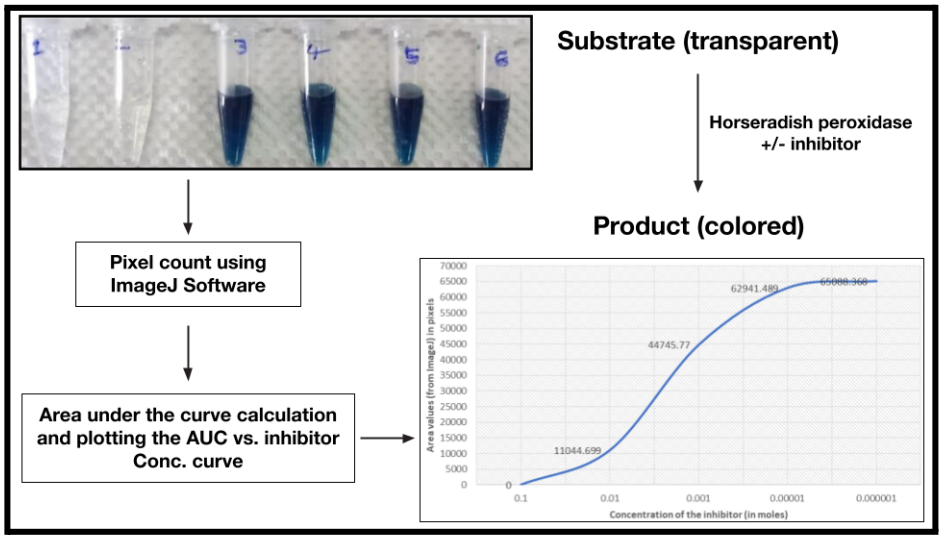

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Figure 1. Overview of the *in vitro* colorimetric enzyme assay quantification using pixel count to calculate inhibitor potency.

Enzyme kinetics are an integral part of many drug discovery programs in which candidate compounds (usually inhibitors of enzymes) are evaluated for their potency *in vitro* using biochemical assays and are ranked accordingly. Design of such assays is simple if a chromogenic or fluorescent substrate is available. Typically colorimeters and/or spectrophotometers are used to evaluate the absorbance of the reaction mixture at a given wavelength in the presence of various concentrations of inhibitor molecules. Drug discovery projects are often well funded. However, in startup companies and many academic laboratories, in order to generate preliminary data, one has to identify economic ways to evaluate the compounds towards preclinical drug discovery projects.

In this study we propose using the commonly used horseradish peroxidase (HRP) as a model enzyme along with a chromogenic substrate in the presence and absence of two HRP [1] inhibitors, sodium azide (SA) and hydroxylamine (HA). HRP is found in the roots of horseradish and is used extensively in biochemical applications. It is a metalloenzyme. It catalyzes the oxidation of various organic substrates by hydrogen peroxide [2-4]. HRP catalyzes the conversion of chromogenic substrates (e.g., TMB, DAB, ABTS) [5] into coloured products. The chromogenic substrate is expected to yield different shades of green depending on the inhibitor concentration.

In order to quantify the color change, we used the ImageJ software [6-8] that can quantify the pixels in the image. High resolution camera was used to take the pictures of the reaction mixtures containing various concentrations of either SA or HA. Once the pixels were read in, the area under the curve (AUC) was calculated for each measurement. These AUC values were then plotted against the inhibitor concentrations in order to determine the enzyme inhibition. We further made an attempt to calculate the 50% inhibitory concentrations (IC_{50}s) for both SA and HA. Our method completely bypasses the usage of colorimeter and/or spectrophotometer thus making this method more economical.

Figure 2. Sliced chicken liver pieces taken in a petri plate.

Materials & Methods:

Preparation of stock solutions for inhibitors: In this study, 2 inhibitors which inhibit the enzyme HRP were considered namely, Sodium azide & Hydroxylamine. Six different concentrations/ dilutions of stock solution of both inhibitors i.e., 0.1M, 10mM, 1mM, 100µM, 10µM & 1µM are needed for the enzyme assay. For this, two concentrations i.e., 1M & 1mM stock solutions, 1ml of each of both the inhibitors are prepared. To prepare 1ml of 1M solution, the given formula is used:

$$M = \frac{wt\ (gm)}{Mol.Wt.} \times \frac{1000}{Vol\ (ml)}$$

For example, in the case of Sodium Azide (molecular weight: 65.01) the total weight required was calculated to be 0.065 grams in order to prepare a stock solution of 1M concentration and 1ml volume. Similarly, a 1ml stock solution of 1M hydroxylamine was also prepared by weighing the required amount of the inhibitor and dissolving it in deionized water. Further dilutions were made in order to obtain the required concentrations of each inhibitor.

Preparation of Substrate & Enzyme: The substrate is prepared by mixing 10ml of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) with 2µl of hydrogen peroxide. Five ml of horseradish peroxidase (HRP) enzyme is taken.

Colorimetry-based Enzyme Assay: The total reaction mixture volume i.e., substrate + enzyme + inhibitor + distilled water should be 300µl in volume. The volumes of substrate (269µl), enzyme (1µl) and the inhibitor (30µl) remain the same. A total of 6 different concentrations of inhibitors are prepared.

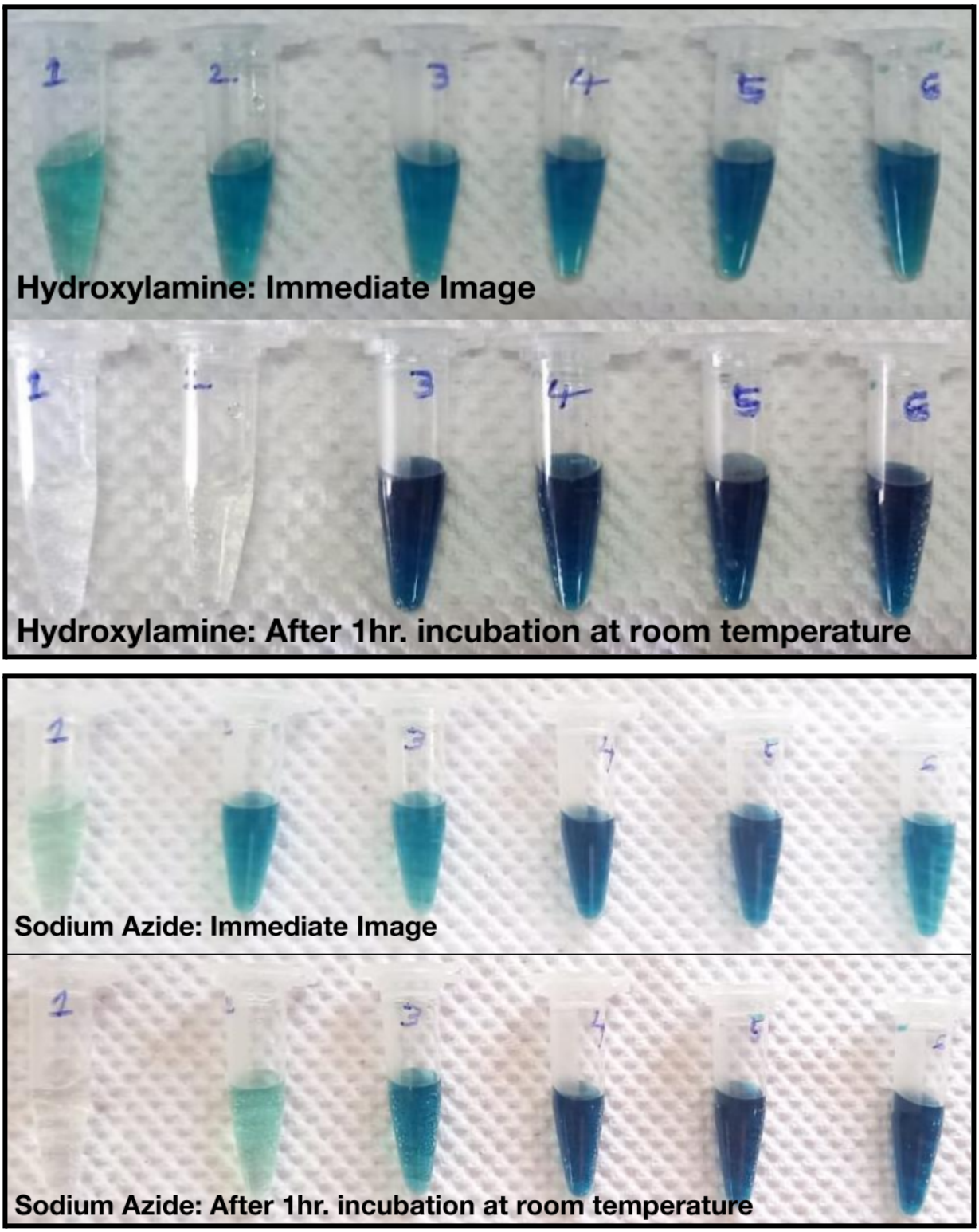
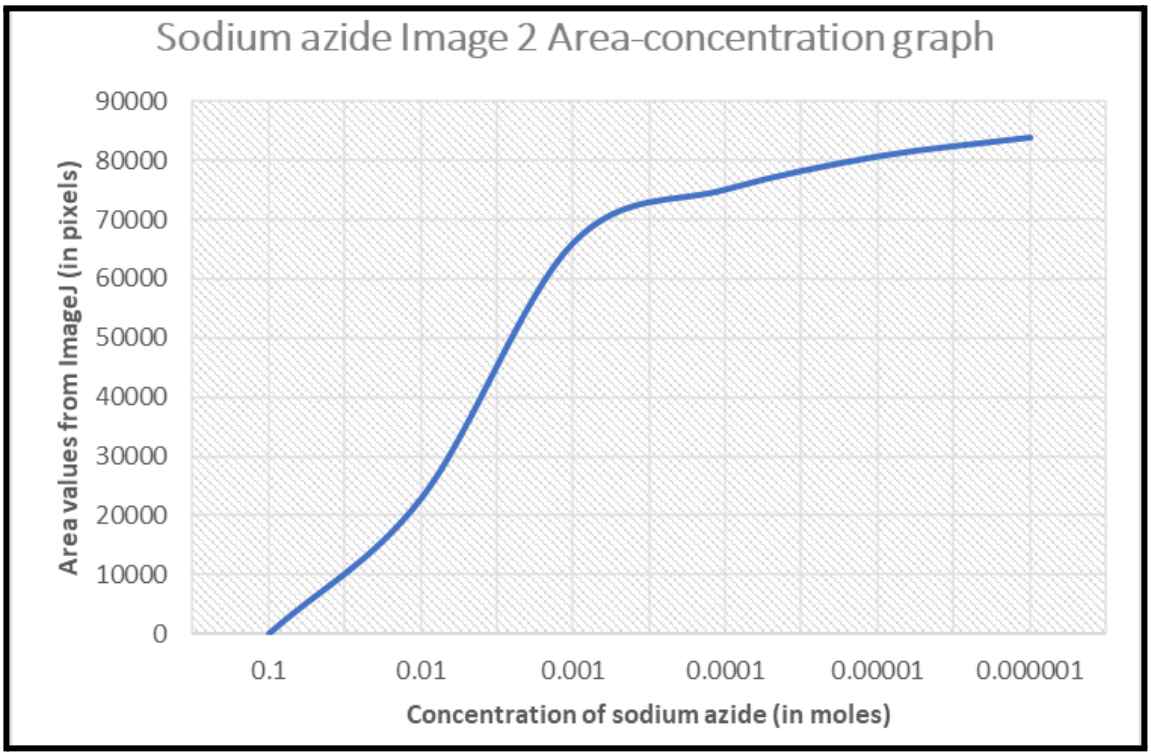


Figure 2. Reaction tubes showing different shades of green.



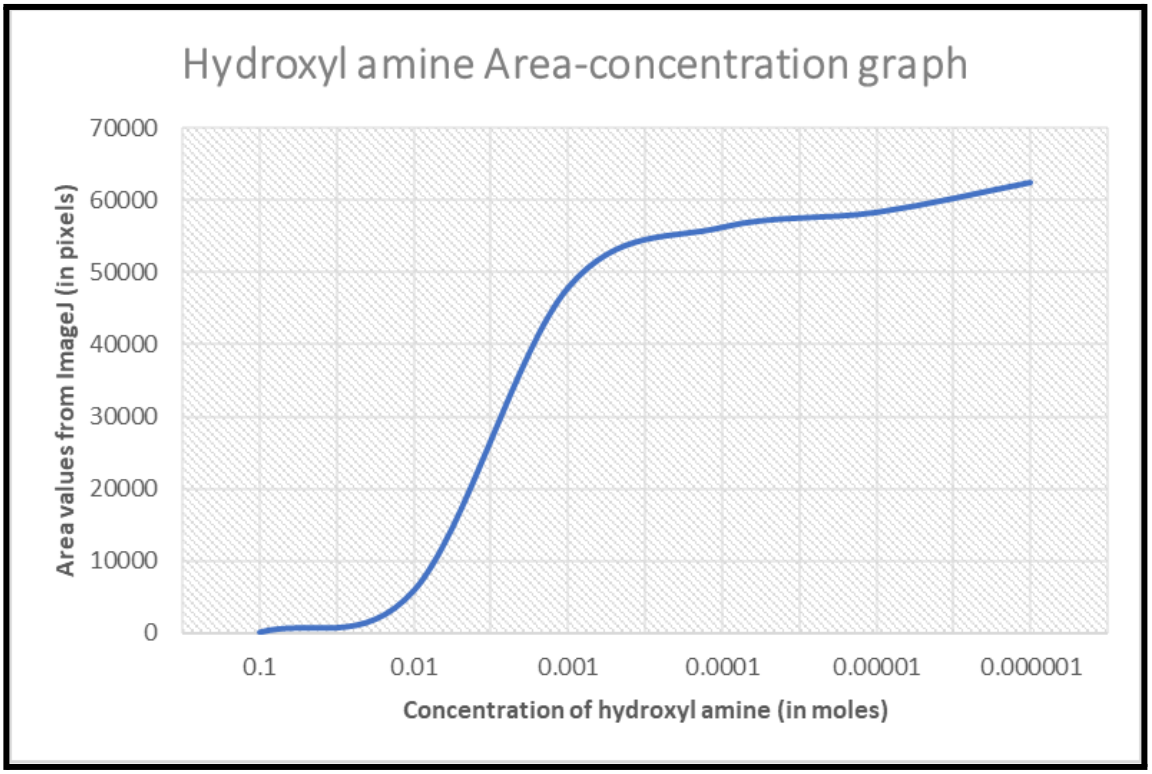


Figure 3. AUC vs. [I] curve plotted for 6 different values.

Six polypropylene tubes of 2ml capacity were taken for the 5 different concentrations of the inhibitors, 0.1M, 0.01M, 0.001M, 0.0001M, 0.00001M & 0.000001M. These 6 concentrations were chosen to obtain a clear inhibition curve of the enzyme activity that would be helpful in calculating the IC_{50} values. Using a micropipette, substrate is added first into all the tubes. Following it, deionized water is added into the tubes. Next, the enzyme is added into each of the tubes. The inhibitor is added in the last into all the tubes according to the values given in the above table. After adding all these, an immediate image is taken. Then, the tubes were incubated for 1 hour at room temperature. After incubation, another image is taken to determine the color change. This process is performed for both the inhibitors in similar manner. Repeats were performed in order to obtain at least two values for each.

Pixel quantification using ImageJ software: ImageJ is a Java-based image processing program developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin). It can display, edit, analyze, process, save, and print 8-bit color and grayscale, 16-bit integer, and 32-bit floating point images. It can calculate area and pixel value statistics. It can measure distances and angles. Using Image J, enzyme activity is determined based on color intensity & pixel values. Image J application is opened and image is selected and uploaded. After uploading the image, a rectangle tool is selected and a solution containing the area in each tube in the image is outlined using the tool. To do this, "Analyze" option is selected and in the "gels" option, "select first lane" option is clicked. Then, after selecting the 2nd tube, "select next lane" option is clicked and so on. After selecting all the tubes, go to "Analyze" & "gels" & then click "Plot Lanes". After obtaining the plots, using the "Straight" tool, the curve area is plotted. Then, "Wand" tool is used to select the area of the plot and measure it.

Results and Discussion:

Colorimetric enzyme assay: The images of all tubes before incubation and after incubation were compared to see any changes in the color for both inhibitors. As shown in Figure 2, the color change was observed in the first tube where the highest tested concentration of HA was present compared to the other tubes. Similar trend was observed for SA as well. With decreasing inhibitor concentrations in both cases (SA and HA) the green color was more intense indicating the increased activity of the HRP in the presence of lower concentrations of inhibitor. The colors were further quantified using imageJ.

Inhibitory potencies of SA and HA: The IC_{50} values for both SA and HA were arbitrarily calculated from the curves plotted through manual inspection of the curves with the help of MS-Excel sheets. In each case, the corresponding AUC value was considered for each concentration point of the inhibitors. In the case of SA, the two experiments yielded 5.25 mM and 6.0 mM IC_{50} values of HRP. Hence the average of both experiments including the standard deviation was calculated to be: 5.625 mM $\pm$ 0.53. Similarly, the two values for HA evaluation were used to obtain a final average IC_{50} value of 4.25 mM $\pm$ 2.0. Based on the standard deviation values for both SA and HA, we conclude that this assay can further be used to evaluate inhibitors of other enzymes that act on chromogenic substrates.

Applications of this method in future: The current study evaluates SA and HA at 6 different concentrations using HRP as a model system in the presence of ABTS substrate. This economical method is very much useful especially when HRP is cloned in frame with other enzymes such as PETase in plastic degradation analysis. Further, this assay can also be used to design to test the environment pollutants that can be detected by using HRP without the requirement of colorimeter and/or spectrophotometer. Especially for field

studies, miniaturization of this assay would significantly help because one can simply quantify the pixels instantaneously without any waiting period for data acquisition and/or analysis.

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Conflict of interest: This research article is an ongoing project currently at TCABS-E, Rajahmundry, India.