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Femtogram detection of horseradish peroxidase by a common desktop scanner

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We report an image-based detection of horseradish peroxidase (HRP) by different color spaces. The results show excellent correlation between color saturation and absorbance (Pearson correlation coefficient; 0.9868) with respect to HRP. The present method can detect 185 and 46.45 fg/ml of HRP using *o*-phenylenediamine dihydrochloride and 3,3′,5,5′-tetramethylbenzidine as chromogenic substrates respectively.

[**Key words:** Image-based assay; Horseradish peroxidase; Color space; 3,3′,5,5′-Tetramethylbenzidine; *o*-Phenylene diamine dihydrochloride; Microtest plate]

Horseradish peroxidase (HRP) is one of the most widely used enzymes in analytical applications due to its specificity in reaction, flexibility in assay, stability, sensitivity in analyte detection, as well as availability in pure form at reasonable cost. HRP is well suited for the preparation of enzyme-conjugated antibodies (1) and antigens (2) due to its relatively good stability and relatively small molecular size, but especially due to its ability to yield chromogenic products with high turnover numbers (3). HRP conjugates have been extensively used in immunoassays, such as enzyme-linked immunosorbent assays (ELISA), Western-Blotting and immuno-histochemistry (IHC) techniques. HRP is also used as a labeling agent for nucleic acids or to track the movements of labeled cells in culture, tissues or intact organisms (4). Besides the enzyme, the chromogenic substrate is also important for analytical accuracy. Currently, color developed after enzyme–substrate interaction is quantified on a microtiter plate by a spectrophotometer which is an expensive instrument. Also, it requires substantial quantity of an analyte which is often precious or not affordable. Therefore, it is desirable to have a device or assay system that is cheap, specific, and sensitive, requires small amount of protein and most importantly, does not require costly instrument. Image-based biochemical assay could be a potentially useful solution for low cost assay system. However, one of the important aspects of the image-based assay is the analysis of the image. Recently, digital imaging in diagnostics has become highly advanced technology due to tremendous progress made in electronics and computational science (5–9). Several image analytical tools have been developed to study the color properties of an image. Color properties can be measured by different color space parameters such as CMYK, RGB, gray scale, Lab color, color saturation and so on which are reported in the literature for quantification of an assay color (10–20). Choice-of-color space has become very important for image-based quantification as different researchers used different color spaces. Martinez et al. have done protein assay by converting the image to CMYK color and measuring the intensity of cyan (10). Paper-based glucose assay was done from the image in gray scale (11). RGB-a popular color space was used for identifying leaves for different plant and animal diseases (12–15). Non invasive detection of HE4 in urine was done by using red value from RGB color space (16). Macedo-Cruz et al. (17) quantified the damage caused by frost in oat crops by converting the image from RGB to the L*a*b* representation. Sannakki et al. (18) also presented a method to quantify disease symptoms based on Fuzzy logic by converting the images to the L*a*b* color space. Yet, in another work Xu et al. (19) developed a method to detect nitrogen and potassium deficiencies in tomato plants by taking b* component of the L*a*b* color space. However, for a biochemical assay accurate quantification of color with respect to concentration of a biochemical is utmost important. Therefore, it is necessary to analyze the color of an experiment by different color spaces for better comparison.

In this report, we have for the first time analyzed the image of a colored assay solution by different color spaces for detection of HRP. We have used HRP as a model analyte because of it's widespread applications as a label in ELISA-based diagnostic assays. The study of different color spaces with respect to HRP concentrations enable us to find out the most suitable color space for a highly sensitive biochemical assay.

Microtest plates were made from polypropylene sheet having dimension of 9 cm in length and 3 cm in width. On the strip an array of cavities was made by slightly pressing the sheet with blunt end iron rod. Each cavity was separated by 0.5 cm. Cavity of the microtest plate (polypropylene microtest plate: PPμTP) has an average diameter of 3.5 mm and a depth of around 1 mm. All the dimensions were measured by an electronic caliper.

Absorbance-based colorimetric quantification of HRP is a well tested method used in biological or clinical laboratories; hence, we have compared our image-based assay with absorbance-based

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assay. The challenge of image-based or photocolorimetric assay is the quantification of the scanned image and its correlation with absorbance-based assay. Color properties can be measured by different color space parameters mostly used in digital imaging. We have analyzed different color space parameters to find out the most suitable one for quantification of a scanned image. For this, HRP is used as a model enzyme as it can be assayed easily. Briefly, conventional spectrophotometric analysis of HRP (Sigma, USA) was carried out by taking serially diluted HRP [390–1.5 pg/well/5 μl of PBS (0.85% NaCl in 0.01 M phosphate buffer, pH 7.2)] into triplicate wells of a microtiter plate. The color was developed at ambient temperature (26 ± 2C°) by adding 95 μl of the substrate-dye buffer which was prepared by adding 6 mg of *o*-phenylene diamine dihydrochloride (OPD; Sigma) and 8 μl H_2O_2 in 6 ml of citrate buffer (pH 5.2, 0.2 M). After 1 min the reaction was stopped by adding 20 μl of stop solution (5% H_2SO_4). Absorbance of the color solutions were recorded in an ELISA reader (Biorad iMark Microplate Reader, USA) at 490 nm.

For comparison between spectrophotometric and photocolorimetric assay results, it was necessary to do the analysis with the same colored assay solution. Therefore, for photocolorimetric analysis 10 μl of the colored solution from each of the above microtiter wells, was taken out and loaded into a cavity of a microtest plate. The image of the colored solutions was then scanned by placing the microtest plate upside down on a desktop scanner (HP Photosmart C6388) connected to a computer. Due to surface tension solution remained in the cavities without any spillage. The scanned image was then opened in Adobe Photoshop. From the photoshop, the mean intensity of each test zone was obtained from the histogram of different color space parameters (CMYK, lab color, gray scale, RGB). Individual as well as combined luminosity for each of the color space parameters were recorded. Individual values of hue, saturation, and brightness were obtained by converting RGB to HSB color mode using freely available Macbeth color calculator software. Combined intensity represents the intensities of all the colors taken together in a particular color space such as RGB. Similarly, individual colors of cyan, magenta, yellow and black (CMYK); L, a and b (Lab color); red, green and blue (RGB); hue, saturation and brightness (HSB) and gray scale were obtained. Graphs were plotted to correlate values of different color space parameters and HRP concentrations. Fig. 1 shows correlation between different color space parameters and HRP concentrations. Among all the color spaces studied, color saturation excellently correlates with the increase in the amount of HRP. Intensity of the color increases as the color of the assay solution increases which in turn depends on the amount of HRP. For photocolorimetric and spectrophotometric comparison, we have plotted a graph (Fig. 2A) with color-saturation (primary *y*-axis) and absorbance (secondary

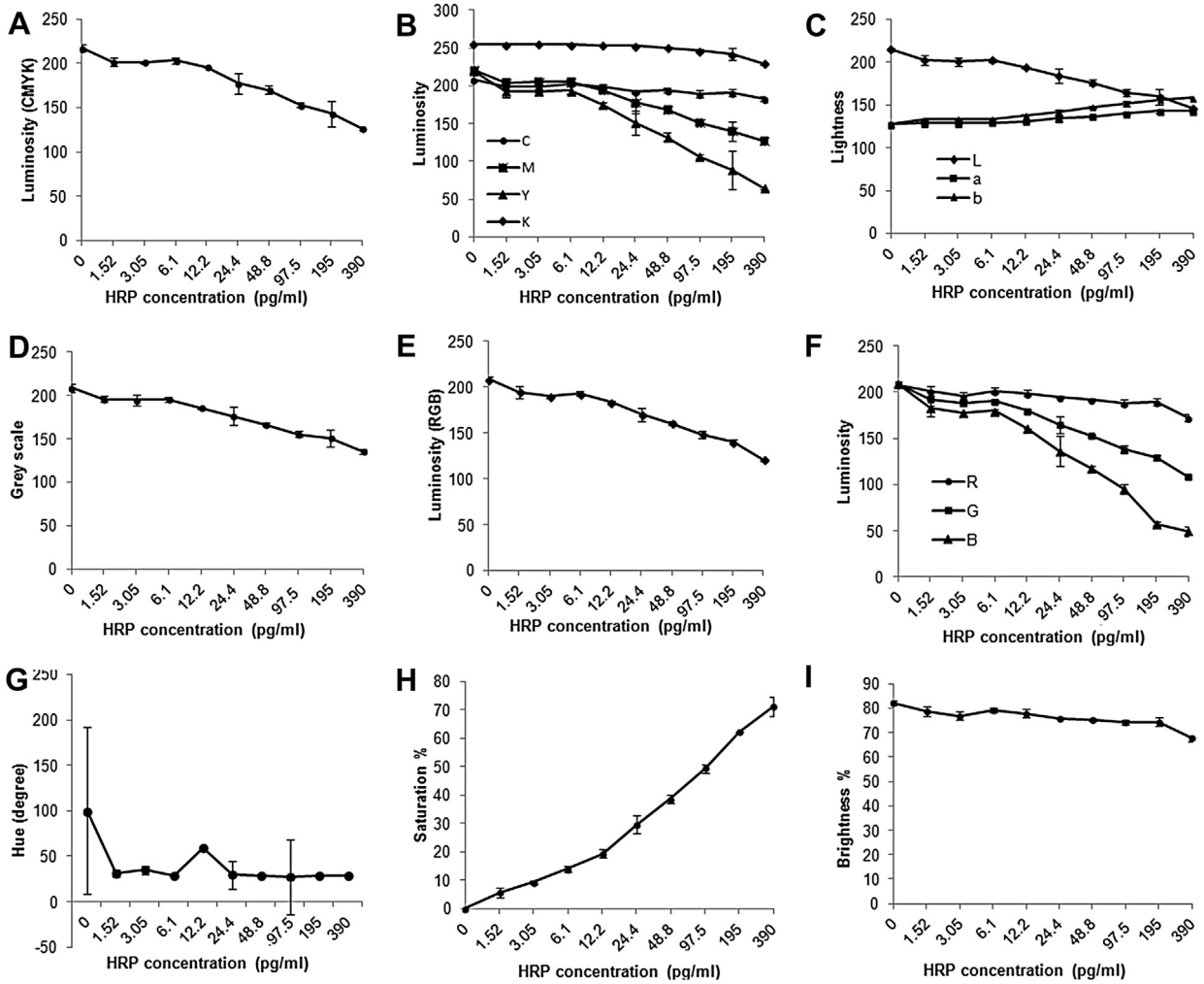

FIG. 1. Correlation between HRP concentrations and different color space parameters such as: (A) combined luminosity of CMYK, (B) individual luminosity of cyan [C], magenta [M], yellow [Y] and black [K], (C) individual luminosity of L, a and b, (D) gray scale, (E) combined luminosity of RGB, (F) individual luminosity of red, green, and blue, (G) hue, (H) saturation and (I) brightness.