CHAPTER 7

VALIDATION OF EXPERIMENTS AND RESULTS

7.1 BLOOD GLUCOSE MEASUREMENT EXPERIMENTS AND RESULTS

Validation of experiments in order to check the validity of the measurements through the newly developed Colorimeter with RGB Color Sensor were conducted at “Karaikudi Computerized Clinical Laboratory” under the aegis of Raghavakumar Clinic in Karaikudi. This Clinical Laboratory is utilizing the Absorbance Colorimeter for most of its Clinical diagnosis. Readings of their existing Colorimeter and the newly developed Colorimeter with RGB Color Sensor have been compared and painstakingly analyzed herewith. Over a period, blood samples from 203 patients were tested for the measurement of Glucose and Haemoglobin. The patients were from a 1 day old baby to 73 year old elderly persons taken from both genders.

Blood samples of 103 patients were meticulously tested for the measurement of their blood glucose concentration and blood samples of 100 patients were scrupulously tested for the measurement of their Haemoglobin concentration. These experiments were conducted under the guidance of the Senior Pharmacist of the Clinical Laboratory. Colorimeter with RGB Color Sensor is a modified Absorbance Colorimeter only. Hence sample holder remains identical in both equipments. Owing to this reason, same test tube can be used for measurement of blood chemical in both the equipment. The readings have been taken in both equipments side by side.
In the Clinical Laboratory, popularly used *Glucose Oxidase method* as explained in Chapter 4.1 has been used for the measurement of glucose in blood plasma. The Glucose (GOD-POD, End point and Kinetic assay) reagent was supplied by M/s. Span Diagnostics Ltd., Sachin, India.

### 7.1.1 Initial Settings

The test tubes used for the Clinical measurements should have path length of 1 cm and clean and dry inside and outside. Marker lines are provided in both test tube and sample holder of the instrument to ensure similarity in all measurements.

1 ml of clear GOD – POD reagent was taken in the test tube and inserted into the sample holder of 8-filter Digital Colorimeter available in the Clinical Lab and the Absorbance was set to null value in the Digital display. Marker lines in both test tube and Colorimeter must coincide while setting this initial condition.

Similarly, this test tube was inserted into the sample holder of Colorimeter with RGB Color Sensor and the RGB data displayed in the LCD display was adjusted to R=255, G=255 and B=255 by adjusting the RGB gain knobs provided in the instrument. This process replicated the incidence of White source with maximum intensity over the RGB Color Sensor.
7.1.2 Measurement Procedure

After setting the initial condition, 10µl of glucose standard of concentration 100 mg/dl was added to 1 ml of reagent. Glucose Oxidase (GOD) oxidized glucose to Gluconic acid and Hydrogen Peroxide. In the presence of enzyme Peroxidase (POD), already released Hydrogen Peroxide got coupled with Phenol and 4-Aminoantipyrine (4-AAP) to form colored Quinoneimine dye. The intensity of the color of the assay thus formed was directly proportional to the concentration of glucose added to the reagent. After adding the standard glucose to the reagent, the entire solution was kept in an incubator at the temperature of 37°C (i.e., Body temperature). Wavelength of 520 nm was selected in the 8-filter Digital Colorimeter of the Clinical Laboratory and colorimetric measurement was done exactly after 10 minutes of adding glucose standard to the reagent. Absorbance thus measured in the 8-Filter Digital Colorimeter was $A_{\text{standard}}$. The test tube was again inserted into the sample holder of the Colorimeter with RGB Color Sensor and the RGB data that was displayed was noted. Using the software for 'Color measurement using Chromaticity Diagram', the hue and purity equivalents of the RGB data were found. This purity was noted as $P_{\text{standard}}$. After taking the readings, the test tube was thoroughly cleaned with deionized water and dried in an incubator.

Next, the 10 µl of plasma separated under the aseptic conditions from the blood sample of the patient was added to another 1 ml of reagent and its Absorbance was also measured in the 8-filter Digital Colorimeter as $A_{\text{sample}}$. Then, the equivalent RGB data of the color of the assay was obtained from the Colorimeter with RGB Color Sensor and its equivalent purity was noted as $P_{\text{sample}}$. 
The glucose Concentration in the blood sample was calculated as per the equation (7.1) in the 8-filter Digital Colorimeter.

\[
\text{Concentration of glucose} = \frac{A_{\text{sample}}}{A_{\text{Standard}}} \times 100 \text{ (mg/dl)} \quad (7.1)
\]

The glucose Concentration in the blood sample was calculated as per the equation (7.2) in the Colorimeter with RGB Color Sensor.

\[
\text{Concentration of glucose} = \frac{P_{\text{sample}}}{P_{\text{Standard}}} \times 100 \text{ (mg/dl)} \quad (7.2)
\]

Blood samples of 103 patients were tested in this way in Absorbance Colorimeter and then with Colorimeter with RGB Color Sensor. The results thus obtained are shown in Figure 7.1.

![Glucose measurement Analysis](image)

**Figure 7.1** Blood glucose measurement analysis
From this chart we can understand very well that there is a measurement difference between the readings taken from the Absorbance Colorimeter and the newly designed Colorimeter with RGB Color Sensor. The % differences between the readings of both equipments were calculated as per the equation (7.3).

\[
\text{Absorbance Colorimeter reading} - \frac{\text{Colorimeter with RGB Color Sensor reading}}{\text{Absorbance Colorimeter reading}} \times 100 \quad (7.3)
\]

The differences between their readings were ranging from 0.17% to 38.88%. The differences in the readings between both the instruments in the 103 samples are shown in Figure 7.2. This figure indicates that in most of the experiments, the error percentage is less than 15% only.

\[\text{Figure 7.2 Percentage differences between the blood glucose readings of two instruments}\]

Out of 103 experiments, 73 experiments (70.87%) were having difference of less than 10%. 25 experiments (24.27%) exhibited 10% to 20%
of difference and only in 5 cases (4.85%) the difference in measurements was above 20%. According to ISO/FDIS 15197 (5) and the NCCLS (7), the number of measurements deviating more than ±20% should be less than 5%. This condition was achieved in the newly designed instrument. The mean difference in the measurement of this glucose range was 7.79 and the Correlation coefficient between the results obtained from both the equipment was 0.9738. The Standard Deviation of difference in the readings was found to be 6.698 and the Variance of difference between the readings of both Colorimeters hover around 44.43981.

Performance of Colorimeter with RGB Color Sensor was strenuously analyzed further by dividing the glucose ranges into three categories. They were:

i. 70 – 126 mg/dl, which is the normal glucose Range of human beings as per the American Diabetes Association and World Health Organization standards

ii. 126 – 200 mg/dl, where it is identified that the patients have the trace of diabetes and

iii. greater than 200 mg/dl, where the glucose level in blood is in the alarming level.

7.1.2.1 Normal blood glucose Range 70 – 126 mg/dl

Blood samples of 61 patients were declared to have the glucose values between 70 – 126 mg/dl by the Clinical Laboratory after conducting experiment in their 8-filter Digital Absorbance Colorimeter.

The results from the Absorbance Colorimeter and the Colorimeter with RGB Color Sensor are shown in Figure 7.3.
Figure 7.3 Comparison of results in the range from 70 to 126 mg/dl of blood glucose

Out of 61 samples, 49 (80.3%) samples were identified to have the glucose level in the range of 70 – 126 mg/dl by the Colorimeter with RGB Color Sensor and surprisingly 7 (11.5%) were identified to have the trace of diabetic. And 5 samples (8.2%) had the glucose level less than 70 mg/dl, which may lead to Hypoglycemia i.e., Low Sugar level and perennial complications like Shivering, Fainting, Blurredness, and Rapid Heart beat, etc. As it has been proved in Chapter-6 that the Colorimeter with RGB Color Sensor has exhibited more accuracy in measurement, this new instrument can very accurately detect the Low Sugar as well as High Sugar conditions of the patients in the reduced FPG (Fasting Plasma Glucose) threshold (to 126mg/dl from 140 mg/dl) situation.

Measurement differences between the Absorbance Colorimeter and the Colorimeter with RGB Color Sensor were less than 10% in 43 out of 61 (70.5%) and greater than 10% in the remaining 18 cases (29.5%). The mean difference in the measurement of this glucose range was 7.16 only and
the Correlation Coefficient between the results obtained from both the equipment was 0.89415. The Standard Deviation of difference in the readings of both the Colorimeters was 5.77 and the Variance of difference between the results of both Colorimeters was 32.7114.

7.1.2.2 Blood glucose range 120 – 200 mg/dl

27 patients’ blood samples were declared to have the glucose values between 126 – 200 mg/dl by the Clinical Laboratory after conducting experiment in their Absorbance Colorimeter. The results from the Absorbance Colorimeter and the Colorimeter with RGB Color Sensor are shown in Figure 7.4.

![Glucose Range 126 - 200 mg/dl](image)

Figure 7.4 Comparison of results in the range from 126 to 200mg/dl of blood glucose

Out of 27 samples, 23 (85.2%) samples were identified to have the glucose level in the range of 126 – 200 mg/dl by the Colorimeter with RGB Color Sensor and surprisingly 4 (14.8%) were identified to have more than 200 mg/dl, which may lead to Hyperglycemia i.e., High Sugar level and
perennial complications like *Heart attack, Brain attack, Strokes, Blindness, and Kidney failure*, etc.

Measurement differences between the Absorbance Colorimeter and the Colorimeter with RGB Color Sensor were less than 10% in 20 out of 27 (74.1%) and greater than 10% in the remaining 7 cases (25.9%). The mean difference in the measurement of this glucose range was 9.00 only and the Correlation Coefficient between the results obtained from both the equipment was 0.769. The Standard Deviation of difference in the readings of both the Colorimeters was 8.75 and the Variance of difference between the results of both Colorimeters was 73.836.

### 7.1.2.3 Blood glucose range over 200 mg/dl

15 patients’ blood samples were declared to have the glucose values over 200 mg/dl by the Clinical Laboratory after conducting experiment in their Absorbance Colorimeter. The results from the Absorbance Colorimeter and the Colorimeter with RGB Color Sensor are shown in Figure 7.5.

All the 15 samples were identified to have the glucose level over 200 mg/dl by the Colorimeter with RGB Color Sensor also. Measurement differences between the Absorbance Colorimeter and the Colorimeter with RGB Color Sensor were less than that of 10% in 10 out of 15 (66.7%) and greater than 10% in the remaining 5 cases (33.3%). The mean difference in the measurement of this glucose range was 8.47 only and the Correlation Coefficient between the results obtained from both the equipment was 0.893. The Standard Deviation of difference in the readings of both the Colorimeters was 5.966 and the Variance of difference between the results of both Colorimeters was found to be 33.2266.
Figure 7.5  Comparison of results in the blood glucose range over 200 mg/dl

Comparisons of Mean, Correlation Coefficient, Standard Deviation and Covariance between the results of Absorbance Colorimeter and Colorimeter with RGB Color Sensor are shown in Figure 7.6.

Figure 7.6  Comparison of results of colorimeters
Figure 7.6 clearly shows that the Colorimeter with RGB Color Sensor can perform exceedingly well in the crucial range of above 126 mg/dl and less than 200 mg/dl. As far as the diabetic patients are concerned, this range is the critical range where the measurement of blood glucose must be done accurately. The Correlation between the results of both the Colorimeters is far from the actual values. However, newly designed Colorimeter with RGB Color Sensor performs reasonably well in the range of 70 – 400 mg/dl as it has been vindicated in Chapter-6 and hence this instrument can very well replace the existing absorbance Colorimeters in the Clinical Laboratories.

7.2 HAEMOGLOBIN MEASUREMENTS AND RESULTS

Haemoglobin is the other blood chemical that is being measured from the blood samples in the Clinical Laboratories using Absorbance Colorimeter. The principle of Haemoglobin measurement is Cyanmethemoglobin method. In alkaline medium Haemoglobin and its derivatives are oxidized in presence of potassium ferricyanide and became converted to methemoglobin which then reacts with potassium cyanide to form purple red colored Cyanmethemoglobin complex. The intensity of which is measured at 546 nm or green filter.

Drabkins solution of M/s. Beacon Diagnostics Pvt. Ltd., Navsari, India was used as the reagent for the measurement of Haemoglobin in the Clinical Laboratory. Cyanmethemoglobin standard was having the value of 15.06 gm% and the same was used during the conduct of experiments.

In the Clinical Laboratory experiment procedure, 5ml of working Drabkins solution was taken in a test tube and 20µl of blood plasma was added to the reagent. Both were mixed well and the test tube was kept in the room temperature for 5 minutes. The available 540 nm filter close to 546 nm
wavelength was selected in the Absorbance Colorimeter to carry out measurements. After 5 minutes, color would have developed in the reagent and its absorbance value in the Absorbance colorimeter can be measured and noted as $A_{\text{sample}}$. Previously, in the same method, the Absorbance $A_{\text{standard}}$ of the Cyanmethemoglobin standard must have been measured. Now, using the equation (7.4), the concentration of Haemoglobin in the blood sample can be calculated.

$$\text{Concentration of Haemoglobin} = \frac{A_{\text{sample}}}{A_{\text{Standard}}} \times 15.06 \text{ (gm %)} \quad (7.4)$$

For measurement of Haemoglobin through Colorimeter with RGB Color Sensor, Assay with Cyanmethemoglobin standard and its equivalent RGB output was converted to its equivalent hue and purity and the purity was noted as $P_{\text{sample}}$. Then the assay with blood plasma sample and its equivalent RGB output was converted to its equivalent hue and purity and the purity was noted as $P_{\text{standard}}$. The concentration of Haemoglobin in the blood plasma can be calculated from equation (7.5), as

$$\text{Concentration of Haemoglobin} = \frac{P_{\text{sample}}}{P_{\text{Standard}}} \times 15.06 \text{ (gm %)} \quad (7.5)$$

Blood samples of 100 patients were tested in both Absorbance Colorimeter of the Clinical Laboratory and the newly designed Colorimeter with RGB Color Sensor. The results of those experiments are shown in Figure 7.7. Out of 100 cases, in 57 cases (57%) the differences between the readings of Absorbance Colorimeter and Colorimeter with RGB Color Sensor were less than 10%. But in the remaining 43 cases, the differences in measurements were above 10%. Only in 11 out of 100 cases the difference in
measurements was greater than 20%. Surprisingly, in 95 out of 100 samples, the newly designed Colorimeter with RGB Color Sensor gave the concentration of Haemoglobin less than the value measured by the Absorbance Colorimeter. Hence, the credibility of using the existing Absorbance Colorimeter for the measurement of Haemoglobin in blood sample is dicey. The reasons are the imperative need for having a monochromatic source and the spectral bandwidth of the filters used in the Absorbance Colorimeter.

![Hemoglobin measurement comparison](image)

**Figure 7.7 Haemoglobin measurement analysis**

Differences in the results obtained from the existing colorimeter and the colorimeter with color sensor are shown in Figure 7.8.

The mean percentage difference between the readings of both the colorimeters was 10.88. The standard deviation of percentage difference in measurements was 6.515 and the variance of differences in the measurements using both the colorimeters was 42.02. The correlation coefficient between the results obtained from both the colorimeters was 0.88449.
Figure 7.8 Percentage differences between the haemoglobin readings of colorimeters

Performances of Colorimeter with RGB Color Sensor are further analyzed by dividing the Haemoglobin range into three categories.

i. Low Haemoglobin range i.e., less than 11 gm %
ii. Normal Haemoglobin range i.e., 11 – 16 gm % and
iii. Haemoglobin over 16 gm %.

7.2.1 Low Haemoglobin Range ( < 11 gm %)

Out of 100 blood samples, only 3 samples were identified by the Absorbance Colorimeter to have Haemoglobin less than 11 gm%, which is a low Haemoglobin value that may lead to Anaemia Symptoms such as headache, fatigue, weakness, difficulty in thinking, shortness of breath and rapid heartbeat.

Responses of both Colorimeters are shown in Figure 7.9.
7.2.2 Normal Haemoglobin Range 11 – 16 gm %

Out of 100 blood samples, 57 samples were identified by the Absorbance Colorimeter to have Haemoglobin in the range from 11 gm% to 16 gm%, which is the normal Haemoglobin value as per the Drabkins solution. Results obtained from both the colorimeters are shown in Figure 7.10. In 54 out of 57 cases (94.7%) the results obtained from the Colorimeter with RGB Color Sensor were less than the results offered by the Absorbance Colorimeter.
In 35 out of 57 cases (61.4%) the differences in the results of both colorimeters were less than 10% and in the remaining 22 cases (38.6%) the differences between the results were above 10%. The mean percentage difference between the readings of both the colorimeters was 10.11. The standard deviation of percentage difference in measurements was 7.091 and the variance of differences in the measurements using both the colorimeters was 49.4. The correlation coefficient between the results obtained from both the colorimeters was 0.731.

7.2.3 Haemoglobin over 16 gm %

Out of 100 blood samples, 40 samples were identified by the Absorbance Colorimeter to have over 16 gm% of Haemoglobin. Results obtained from both the colorimeters are shown in Figure 7.11. In 39 out of 40 cases (97.5%) the results obtained from the Colorimeter with RGB Color Sensor were less than the results offered by the Absorbance Colorimeter.
In 16 out of 40 cases (40%), the differences in the results of both colorimeters were less than 10% and in the remaining 28 cases (60%) the differences between the results were above 10%. The mean percentage difference between the readings of both the colorimeters was 12.23. The standard deviation of percentage difference in measurements was 5.53 and the variance of differences in the measurements using both the colorimeters was 29.808. The correlation coefficient between the results obtained from both the colorimeters was 0.79.

7.3 INFERENCE

Blood glucose measurements done in both Absorbance Colorimeter and Colorimeter with RGB Color Sensor have correlation in most of the experiments and only in 4.85% of the cases only the differences were exceeding 20%. This has satisfied the ISO/FDIS 15197 (5) and the NCCLS (7) standard of the number of measurements deviating more than ±20%
should be less than 5%. Moreover, the Colorimeter with RGB Color Sensor has exhibited measurement error of less than 10% in all measurements as per Chapter 6.

In the Haemoglobin measurements, the readings with more than 20% of differences were 11%. However, the Clinical Laboratory has given a green signal to the new equipment viz., Colorimeter with RGB Color Sensor because the existing Colorimeter had gone more erratic especially during the measurement of Haemoglobin, because there was no filter with the wavelength of exactly 546 nm, in the Colorimeter used in the Clinical Laboratory. The 540 nm green filter was used in the place of 546nm filter, for the measurement of Haemoglobin whose spectral width was also high. Hence as per the Clinical Laboratory measurements, the Colorimeter with RGB Colorimeter has also produced relevant results during the Haemoglobin measurements as well.

Similarly, this new equipment can be used in the place of Absorbance Colorimeter for any sort of Color related measurements for any other applications.