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I, ALLA SURESH KUMAR,

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This work and its defense approved by:

Chair: Dr Fred R Beyette

Dr Hal Carter

Dr Joseph F Clark

Implementation of an Optical Spectroscopy System for Quantification of Bilirubin in a Complex Media

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By
Alla Suresh Kumar

B.Tech, JNTU, College of Engineering, India, 2003

Committee Chair: Fred R Beyette, Ph.D.

Implementation of an Optical Spectroscopy System for Quantification of Bilirubin in a Complex Media

Suresh Kumar A

ABSTRACT

Neonatal jaundice is a condition in new born babies, where a high level of bilirubin is present in blood. Bilirubin is a by-product of the breakdown of hemoglobin (the oxygen-carrying substance in red blood cells). Before an infant is born, the mother's liver system processes Bilirubin. After birth, the neonate liver has to process the bilirubin independently. In many cases, due to the slow development of the liver organ, a condition of excess bilirubin production occurs in neonates. At low to moderate levels, the presence of bilirubin leads to yellowing of the neonates' skin. At elevated levels (> 20mg/dL), a condition known as hyperbilirubinemia, bilirubin can enter the brain cells and lead to a catastrophic brain damage. With advances in the field of optical spectroscopy, a reflectance measurement on the skin can give the spectral signature of all the absorbing chromophores in the skin (including bilirubin). The focus of this thesis is to develop the data collection procedure and analysis techniques necessary to quantify bilirubin levels in neonates using optical spectroscopic method. A mock skin model is prepared using the primary absorbing chromophores which shape the spectral signature of skin. Mock skin samples with varying concentrations of bilirubin/hemoglobin and a single concentration of melanin were prepared as a layered model resembling that of skin. The transmission spectroscopy system was used to capture the spectral information of each mock skin sample which represents a particular composition of hemoglobin; bilirubin and melanin. Bilirubin concentrations used in this model are in the range of 1-20 mg/dL. The software part developed in this thesis performs signal to a noise analyzed quantifying the bilirubin signal from the hemoglobin/melanin noise signals. A multiple regression analysis has been developed to predict the concentrations of bilirubin in presence of hemoglobin and melanin using the molar extinction coefficient property of the chromophores which constitute the sample. A novel method is

designed in the process of preparing mock skin samples. The spectral signature of the mock skin samples is the inputs to the algorithm which process the spectral data to extract information of bilirubin signal.

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CHAPTER1

1. INTRODUCTION

This chapter puts forth an overview of the research work. This chapter focuses on the problem statement, background work and research impact.

1.1 OBJECTIVE

Objective of this research is to test the hypothesis that quantification of bilirubin in the skin model can be achieved and also enable us to lay down the foundation for the development of the hardware and software platforms for a noninvasive biomedical optical system. Such a system would assist pediatricians in diagnosing the condition of jaundice. It would assist in reducing the need for collecting blood samples from neonates which is a traumatic and painful procedure.

1.2 OVERVIEW AND PROBLEM STATEMENT

Jaundice is derived from the French word ***jaune***, meaning yellow [1]. Jaundice, also known as icterus (adjective:"icteric"), is a yellowing of the skin, sclera (the white of the eyes) and mucous membranes that is caused by elevated levels of bilirubin in the human body **[1]**. For the coloration to be easily visible and perceived the normal concentration of bilirubin in the blood should be in the range of at least 2–3mg/dL. Bilirubin is a pigment which is formed as a natural process when hemoglobin (the oxygen-carrying component of blood) is metabolized. Any disease affecting the liver, the bile ducts or the breakdown of red blood cells, can cause an accumulation of bilirubin and lead to jaundice.

Jaundice in newborn babies is very common and must be kept within normal limits. It is normal for the body to break down red blood cells and thus form a large amount of bilirubin which is cleared in the liver. Further a newborn's liver is underdeveloped or immature and therefore may not be able to process the bilirubin as quickly as an adult. In most cases, an infant's jaundice will resolve within a few days of birth often without the need for any treatment **[2]**. Jaundice is also associated with hyperbilirubinemia which is the excess bilirubin in the blood. This kind of jaundice is called physiologic jaundice, because it is part of a normal body process. Once the newborn's bilirubin-disposal system matures and the excess red blood cells diminish, the jaundice subsides usually within a week or two and causes the baby no permanent harm **[2].** Jaundice is more common in premature infants, whose livers are less able to cope with excess bilirubin.

In some cases, the bilirubin levels can rise high enough to damage the baby's brain **[2].** For this reason, if the doctor suspects that something more than normal physiologic jaundice is the cause of a baby's yellow color, bilirubin levels will be monitored more closely, using blood samples.

In Infants the heel stick is used to collect blood samples. Simple blood tests help doctors distinguish from the normal physiologic jaundice. Generally two blood tests are done concurrently. One blood test measures the total amount of bilirubin in the blood and the other measures the bilirubin that has been conjugated. The "free bilirubin", is in a lipid-soluble form that must be made water-soluble to be excreted. The free, or unconjugated, bilirubin is carried by albumin to the liver, where it is converted or conjugated and made water soluble. Once it is conjugated into a water-soluble form, bilirubin can be excreted in the urine **[2].** The difference between these two measurements is used to calculate the unconjugated bilirubin. When the level

of bilirubin is high the fat soluble, unconjugated bilirubin may, cross the blood-brain barrier and cause brain damage (kernicterus) **[2].** Another method used to measure total bilirubin in the blood uses a device held onto the skin and does not require a needle stick. The drawback to this method is that is can only be used as a screening tool and cannot replace the blood test in infants whose jaundice is getting worse **[3]**

There are devices which estimate the total serum bilirubin by measuring through skin. There are some interfering factors which lead to inaccuracy in predicting the bilirubin measurements. Some of the interfering factors are variations in skin pigment, hemoglobin level, and variable skin bleaching resulting from the ambient lighting. There has not been much study on these interfering factors and their affect on estimation of bilirubin. This thesis describes the method of bilirubin estimation in the skin model system and explains the influence of hemoglobin and melanin on the quantification of bilirubin.

1.3 BACKGROUND WORK

From a signal and noise perspective the identification of bilirubin signal in the presence of hemoglobin signal (noise) has been studied and quantified for early diagnosis of SAH (Subarachnoid Hemorrhage). A cerebral aneurysm is a weakened portion of an artery in the brain. When cerebral aneurysm ruptures a specific type of bleeding known as a subarachnoid hemorrhage occurs. A spinal tap is performed to extricate CSF fluid which is tainted with blood and as time proceeds following the rupture the blood starts converting to bilirubin. The CSF fluid is analyzed using an optical spectrophotometer. There are chances of blood being introduced into the CSF fluid when a blood vessel is accidentally punctured in the process of performing a spinal tap. Many mathematical algorithms have been developed to solve the

problem of extracting bilirubin signal and also differentiate blood associated with traumatic tap from a CSF spinal tap. **[4]**. All these algorithms have not taken into consideration the role that the molar extinction coefficient of substances plays in determining the spectral shape and how is it correlated to concentrations of the substances present in the mixture. The approach of using the molar extinction coefficients to predict concentration is utilized in the estimation of hemoglobin and melanin concentration from the diffuse reflectance spectra of skin **[5].** Using this approach an algorithm can be developed to quantify the bilirubin signal in the presence of melanin and hemoglobin signal from the spectral signature of skin. The goal is to separate the bilirubin signal in the presence of hemoglobin and melanin which are the absorbing substances in the subcutaneous layers of the skin. The objective is to establish a relationship between the molar extinction coefficient (characteristic property of the substance) and the concentration within the mixture. The development of any algorithm is based on the quality of the data. With the complexity associated in generating the spectral signatures which resemble that of skin, an experimental protocol is developed and optimized.

1.4 IMPACT OF THE RESEARCH

The development of a noninvasive system for quantifying bilirubin in the jaundiced patient will have a profound effect in clinical applications. This thesis work can be applied to the development of a noninvasive biomedical system which can help us in quantifying bilirubin in blood. The development of the algorithms for the computation of bilirubin (i.e. unconjugated bilirubin binded to albumin and free bilirubin). This noninvasive system may bring a change in the medical community and reduce the need for collection of blood samples.

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CHAPTER 2

MEDICAL RESEARCH DESCRIPTION

This chapter describes the substances which play an important role in the medical condition jaundice. The role each substance plays is clearly illustrated and their presence in different parts of the body is put forth. The conversion process of hemoglobin to bilirubin is also explained for the cases of both jaundice and bruise. Once the role played by each of the substances in the medical condition jaundice is understood, knowledge of their physiological ranges and their presence in the human body can helps us in the preparation of samples for the study presented in this thesis. This section starts off explaining the terms which have their role in understanding the medical condition Jaundice. The first thing which comes to mind when a physician orders a test for jaundice is obtaining blood from the human body. An explanation of the components or substances which makeup the blood is provided below. The conversion process from hemoglobin to bilirubin is then described.

2.1 BLOOD

Blood is a circulating tissue composed of plasma and cells (red blood cells, white blood cells, platelets). The main function of blood is to supply nutrients (oxygen, glucose) and constitutional elements to tissues and remove waste products.

Plasma is the liquid portion of the blood. Dissolved in plasma are electrolytes, nutrients and vitamins, hormones, clotting factors, and proteins such as albumin. Plasma distributes the substances it contains as it circulates throughout the body. The cellular portion of blood contains red blood cells (RBCs), white blood cells (WBCs) and platelets.

Red blood cells (RBCs), also known as erythrocytes, are by far the most abundant cells in the blood. RBCs give blood its characteristic red color. This percentage of blood made up of RBCs is a frequently measured number and is called the hematocrit. An RBC contains hemoglobin, a molecule specially designed to hold oxygen and carry it to cells that need it. White blood cells also known as leukocytes are responsible for defense of the organism and help in defending the body against infectious diseases and foreign materials. In the blood, they are much less numerous than red cells. Platelets are irregularly-shaped, colorless bodies that are present in blood. When bleeding from a wound suddenly occurs, the platelets approach at the wound and attempt to block the blood flow.

2.2 HEMOGLOBIN

The name **hemoglobin** is the concatenation of heme and globin, a generic term for a globular protein. A single subunit of hemoglobin is made of a heme embedded in a globular protein. The globin consists of four subunits, which each bind a heme (or haem) groups. The heme groups are organic molecules, and containing one iron atom each that is important for the binding of oxygen to the molecule**.** The normal life span of red blood cells is on average 120 days **[1, 2]**.

2.3 HEMEOXYGENASE

Hemeoxygenase degrades heme to carbon monoxide, iron and biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase and most common example is the formation of bruise and the changing colors develop during its sequential disappearance **[3]**.

2.4 BILIVERDIN

Biliverdin is the green pigment formed as a byproduct of hemoglobin breakdown. The resulting biliverdin is then reduced to bilirubin, a yellow pigment, by the enzyme biliverdin reductase. The changing color of a bruise from deep purple to yellow over time is a graphical indicator of this reaction [3].

2.5 BILIRUBIN

Bilirubin is a yellow breakdown product of hemoglobin. It is one of the main pigments in jaundice and bruises. The bilirubin which is relatively insoluble in water (Unconjugated bilirubin) has to be carried by some carrier in the blood so that it can be excreted out through the liver where it mixes with acid groups and is converted into a soluble form (conjugated). The three forms of bilirubin are free bilirubin, bound bilirubin (bound to proteins) and the conjugated bilirubin The bound bilirubin is bounded to a protein molecule called albumin

Albumin is a protein binding molecule. This protein binding molecule has specific binding sites for bilirubin. The unconjugated bilirubin in the blood at normal levels is binded to this protein molecule. Once the concentration of the unconjugated bilirubin starts increasing due to the inability of the liver to process the bilirubin. This unconjugated bilirubin which is beyond the binding capacity of the albumin spills into the tissue which makes the skin appear yellow.

2.6 JAUNDICE

In the case of neonatal **jaundice**, bilirubin levels rise to a concentration beyond the binding capacity of the serum albumin protein molecule in the blood. The Unconjugated bilirubin is not processed due to the immaturity of the liver. This leads to the build up of unconjugated bilirubin

in the blood vessels. Unconjugated bilirubin then leaks into the surrounding tissues (including the skin). Bilirubin build up in the skin is thus associated with the yellow skin tone of neonates with jaundice.

In the case of jaundice it is the accumulation of the free bilirubin which is found in the tissues. Bilirubin concentrations are elevated in the blood either by increased production, decreased conjugation, and decreased secretion by the liver, or blockage of the bile ducts. In cases of increased production, or decreased conjugation, the unconjugated or indirect form of bilirubin will be elevated. The rise in unconjugated bilirubin levels in the blood are of critical importance in neonatal care. The brain which is the core of the central nervous system is not mature enough to prevent the unconjugated bilirubin from crossing the blood brain barrier **[4]**. The blood brain barrier (BBB) consists of a continuous lining of endothelial cells connected by tight junctions that restrict intercellular diffusion. The BBB selectively excludes most water-soluble substances, proteins, macromolecules, but is permeable to low molecular weight lipid soluble substances that are not highly protein bound **[5]**.

2.7 KERNICTERUS

Kernicterus is defined as the grossly visible yellow staining of the basal ganglia and other characteristic nuclei of the neonatal brain **[5]**. Kernicterus is a severe neurological condition associated with high levels of unconjugated bilirubin in the blood. Kernicterus occurs when free bilirubin crosses the blood brain barrier. Free bilirubin exists when the amount of unconjugated bilirubin exceeds the binding capacity of albumin. Bilirubin can enter the brain if it is not bound to albumin or is Unconjugated or if there has been damage to the blood brain barrier **[5]**. Once inside the brain, precipitation of bilirubin at low pH may have toxic effects. Neurons

undergoing differentiation are particularly susceptible to injury from bilirubin, suggesting that prematurity predisposes infants to bilirubin encephalopathy **[5]**.

The unconjugated bilirubin is present in different compartments of the human body. It first starts building up in the blood and as the levels start rising above the binding capacity of albumin they diffuse into the skin. This unconjugated bilirubin moves back and forth between these compartments. With the underdeveloped nature of the blood brain barrier associated with the premature neonates the unconjugated bilirubin may start crossing the blood brain barrier and lead to neural disorders **[5]**. The unconjugated bilirubin in the skin is a clear indicator or a precursor for prevention of neural disorders. Clearly there is a need for measurement of unconjugated bilirubin in the skin and also in the blood.

The process of conversion from hemoglobin to bilirubin occurs in every cell but the conversion process at each stage can be visible to the naked eye when a bruise occurs. The next section discusses the physiological processes associated with the bruise recovery.

2.8 BRUISE

A **bruise** occurs when a blunt force to the skin causes blood vessels to break. Blood leaks into areas under the skin and causes skin discoloration. The predictability of the bruise color helps in estimating when the injury occurred. Initially, a bruise will be red, the color of the blood which is due to the hemoglobin part of the red blood cells under the skin. After one to two days, the red blood cells begin to break down, i.e. hemoglobin starts being converted and the bruise will darken to a blue or purplish color. This fades to green followed by a brown or yellowish appearance. Finally the skin will gradually diminish back to its normal color **[6]**. A bruise can last from one to two weeks. Bruises sometimes take a long time to appear because the damage can occur deep in the body tissues. The body under the skin is not an amorphous mass. It has

discrete muscles and organs, separated by planes of fibrous tissue. When blood leaks from damaged vessels it is often prevented from reaching the skin's surface quickly by these planes of tissue. Over a period of time the blood will seep into the surrounding tissue while capillaries are repaired. The striking colors of a bruise are due to hemoglobin and its breakdown products, biliverdin (which is green) and bilirubin (which is yellow). It is the same process as the disposal of red cells once their life cycle ends.

Researchers have been using the principle of the breakdown of the hemoglobin in the blood to predict the age of the bruise. In the process of breakdown of hemoglobin many chemical interactions occur in vivo (i.e. within the body) which affect the conversion process. As the process of conversion starts we perceive different colors with a specific sequence. Each color has its explanation in the conversion stage within the process of hemoglobin conversion to bilirubin. Some of the conversion process cannot be visualized with a naked eye at an earlier stage. Thus using the naked eye as a source of judging the age of bruise is not a reliable mechanism. It would be difficult for a person to judge how old a bruise is and how far the conversion process of hemoglobin to bilirubin had has proceeded. It is also difficult to identify what factors are limiting the chemical processes of hemoglobin breakdown i.e. some chemical process happen fast and some slow. These processes vary from person to person with factors such as age and physiological condition of the person. Using a technology called reflection spectroscopy the bilirubin signal can be picked up earlier than is possible with the naked eye. Using noninvasive analysis methods described in this thesis may help us in predicting the age of the bruise as well as diagnosis of conditions which limit the healing process.

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CHAPTER 3

SAMPLE PREPARATION

This chapter puts forth the novel methods employed to make the samples of different chromophores which are the primary chromophores found in skin. Skin is a complex substance whose color is dominantly determined by the presence of different chromophores and the range in which they are present in the skin. Any variations in the chromophore concentrations are visible in the skin.

The main chromophores which are of interest are

1. Melanin
2. Hemoglobin
3. Bilirubin

Table 1 lists the wavelength at which the maximum absorbance occurs for each chromophores and the concentration range of the chromophores used in the sample preparation of mock skin samples

Chromophores	λ_{MAX} (nm)	Concentration Range
Melanin	380nm	0.001% -0.1%(wt/vol)
Bilirubin	420 – 440 nm	1 – 20 mg/dL
Hemoglobin	410	0.1 – 2 mg/mL

This section starts with a basic definition of the significant chromophores in the skin and their sample preparation process adopted in the lab. A chromophore is the part of a molecule

responsible for its color. Light that hits the chromophore can be absorbed by exciting an electron from its ground state into an excited state. When a molecule absorbs certain visible wavelengths in this way, but transmits or reflects others, the molecule has color.

3.1 Sample Preparation

Transmission spectroscopy is initially used to study of the characteristics of individual chromophores. To facilitate the study the optical properties for chromophores important in skin, novel methods has been adopted in the preparation of samples which are analogous to skin. These samples are analyzed using the uQuant microplate spectrometer. The individual solutions of these chromophores are placed in the wells of the micro well plate for spectral generation.

3.1.1 Melanin

The color of human skin is due primarily to the production of melanin by specialized cells in the skin called melanocytes. Melanin produced by these cells both absorbs and scatters UV radiation and thus protects skin from the damaging and mutagenic effects of the solar radiation. [1] Using the protocol established below, different concentrations of melanin solutions are prepared from melanin (powder) acquired from Sigma Chemical Co. Absorbance spectra of different melanin solutions have been analyzed by pipetting a known volume into the micro-well plates. Since the micro well plates are optically scanned from the bottom,care is taken that the volume of material placed in each well is constant thus the path length which the light traverses in each well is nearly constant.

Melanin Protocol

Objective: To prepare melanin solutions of known concentration.

Materials:

a) 1gm of melanin b) 100ml of distilled water

Equipment

a) Beaker 250ml b) Balance

General precautions

a) Melanin has to be properly handled using gloves

b) No spilling of melanin

Conclusion

a) 1% (w/v) melanin is prepared

b) This melanin solution in the later process is used in the sample skin model by injecting this solution into the gelatin or the jello which acts as an epidermal layer of the skin

Melanin preparation 10 mgs of melanin is weighted on the weight balance. The weighted 10 mgs of melanin in transferred into 1ml of distilled water contained in the round bottle. A magnetic stirrer bar is placed inside the bottle and placed on a stirrer plate at 600 rpm for a period of 24 hrs to allow all the melanin particles to be dissolved in the water. The concentration of melanin of 1 %(w/v) is prepared. About the highest practical concentration with synthetic sigma melanin other melanin sources such as squid melanin will be higher in solubility.

3.1.2. Optical Property of Melanin

The absorbance of Melanin doesn't have a peak in the visible region. Its absorbance decreases with increase in wavelength as shown in Figure1. A relation is established between the slope of the melanin line and melanin concentration. Choosing the optimum zone (i.e. range of wavelengths for the calculation of slope) varies from the experiments being performed in vivo or

in vitro. Figure1 shows the absorbance spectrum for melanin (concentration 0.025% i.e. 0.25 mg in 1 ml) in the wavelength range from 400 to 800 nm.

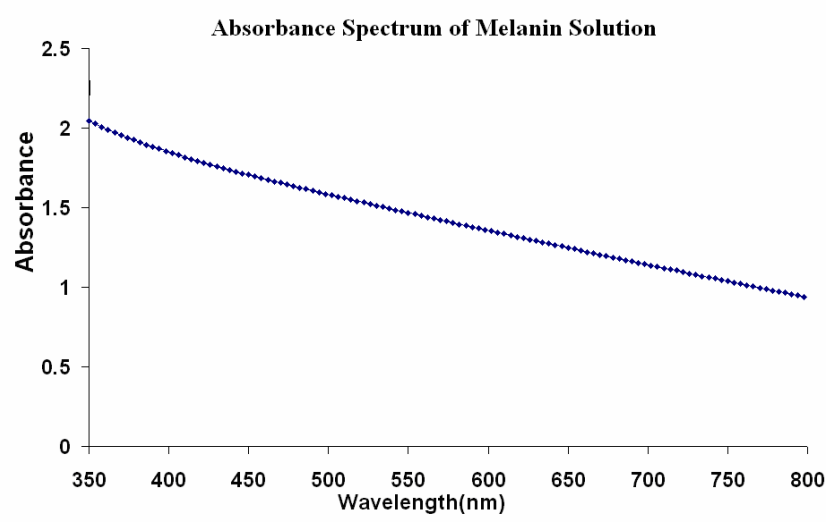


Figure 1 shows the absorbance spectrum of melanin solution of 0.025 %(0.25mg/ml) and the absorbance of melanin is higher at shorter wavelengths i.e. in the UV region and decreases linearly as the wavelength increases.

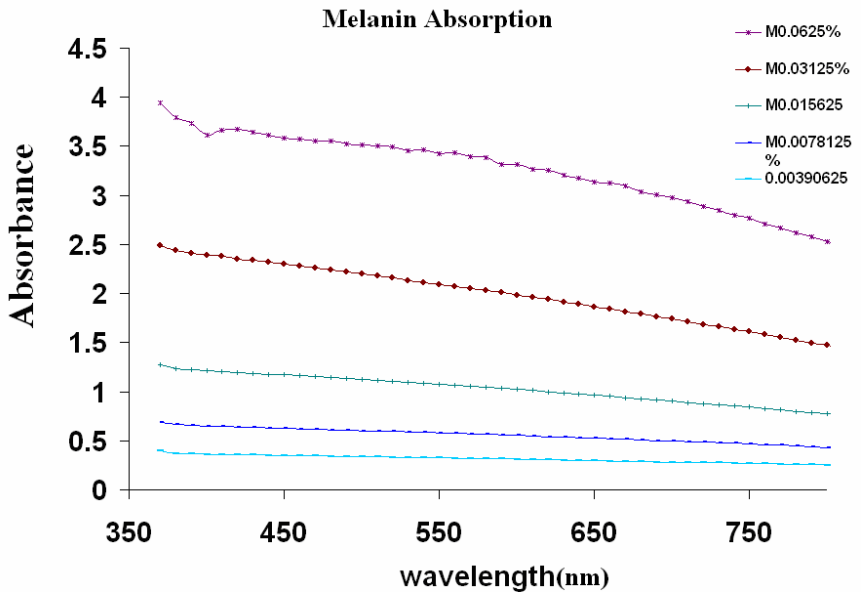


Figure 2 shows as the concentration of melanin is increased the absorbance increases in the shorter wavelength region and flattens out at longer wavelengths. The values indicated in the legend are the different concentrations of melanin solutions prepared. From the spectral signatures of melanin solutions of different concentrations, the slope of the line from 600 – 800 nm can be correlated with concentration.

3.1.3 BILIRUBIN

Bilirubin is a waste product formed when red blood cells die and hemoglobin is broken down. Since bilirubin is poorly soluble in water, it is bound to albumin (a protein molecule) and then carried to the liver by the blood stream. Bilirubin is made water soluble in the liver. As described in Chapter 2 jaundice is the rise of bilirubin in the skin. The free, unconjugated bilirubin has the potential of crossing the blood brain barrier leading to an increased potential of neural disorders.

Unconjugated Bilirubin Protocol

Objective: To prepare Unconjugated bilirubin solutions in a known concentration

Materials:

a) 10gms NaOH

b) 100mg Bilirubin

c) 50ml Distilled water

d) Concentrated Hcl

Equipment:

a) 50ml Culture Bottle with Orange lid

b) Stir bar

c) pH meter

d) Pasteur Pipette

e) Pasteur Pipette Bulbs

f) Graduated Cylinder

g) Aluminum Foil

h) Stir Plate

General Precautions

a) When using Concentrated HCl perform the experimental steps in the fume hood

b) Clean the pH electrode with distilled water

Sample preparation procedure

A 250 ml bottle with cap is taken and filled with 50 ml of distilled water 10g of NaOH is weighted and is transferred into the bottle containing 50 ml of distilled water. The bottle is shaken well to make sure that all the NaOH solid particles are dissolved. Then 100mg of Bilirubin is weighed and transferred into the bottle having NaOH dissolved in distilled water. A stir bar is placed inside the bottle and the bottle is covered with aluminum foil and is placed on magnetic stirrer plate for 24 hrs. The solution is green in color. The contents in the bottle are transferred into a 250 ml beaker along with a stirrer and placed on magnetic stirrer plate. A 20 ml of concentrated hydrochloric acid is transferred into the flask of solution. The flask is covered with aluminum foil and the top opening surface is three fourth covered with aluminum foil. The solution is stirred for an hour and then the pH is adjusted to 7.5. The final volume is measured by transferring the contents from 250 ml beaker to a graduated cylinder. The volume of the solution in the graduated cylinder is noted.

Conclusion

a) The bilirubin solution of known concentration had been prepared.

b) This known concentration of bilirubin is the stock solution for our experiment

c) Using this stock solution and a dilution procedure we prepare solutions of different concentrations.

Theoretical calculations to estimate the bilirubin stock solution concentration

a. Once the volume of the solution is measured using the graduated cylinder

b. Let us assume the volume is x ml

c. The initial amount of bilirubin taken was 100mg

d. Concentration is calculated as follows

e. ((100mg/x ml)/584.6) is the Bilirubin concentration in Molar

Molecular weight of Bilirubin is 584.6 gms

1Molar solution = 584.6 gms/1Litre

Protect bilirubin from exposure to light. Bilirubin is photo sensitive and will breakdown if exposed to light over extended periods.

3.1.4 Optical Property of Bilirubin

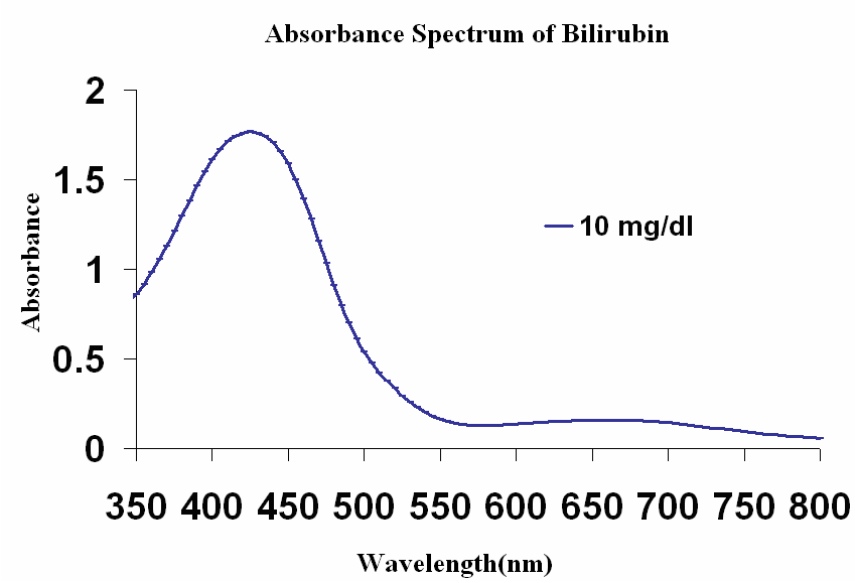
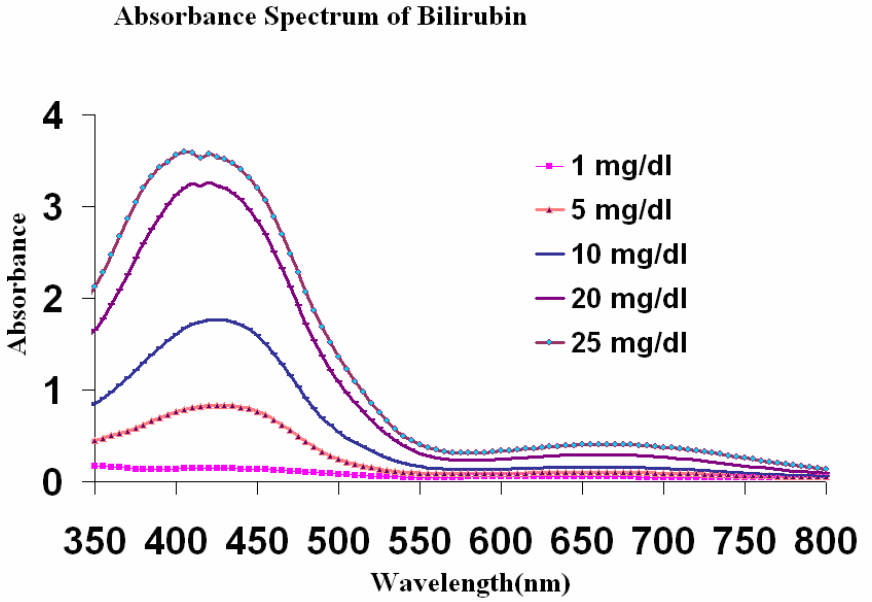


Figure 3 shows the absorbance spectrum of bilirubin solution of concentration of 10mg/dl shown in figure 3 has a broad peak in the wavelength range of 400-460 nm with a peak in the range of 430-445 nm. As the concentration of bilirubin is increased the absorbance value at 435 nm is increasing in a linear fashion as shown in Figure 4. The reason for choosing the

concentrations in the range of 1 mg/dL to 25 mg/dL is to cover the physiological range of



concentrations

Figure 4 shows the absorbance spectra of bilirubin solution of concentration of 1 to 25 mg/dl. The peak absorbance occurs at 430nm and the absorbance increases as the concentration of bilirubin increases.

3.1.5 Hemoglobin

The Hemoglobin which is present in the red blood cells of the capillaries and veins is one of the interesting chromophores which play an important role in the diagnosis of Medical condition such as (jaundice). Hemoglobin may be present under some conditions outside the red blood cells in the plasma and may interference with bilirubin. This interference may arise due to binding between hemoglobin and bilirubin. Hemoglobin powder which is made available from Sigma is used in the sample preparation and analysis.

Hemoglobin Protocol:

Objective to prepare hemoglobin in a known concentration

Materials:

a) 1mg of hemoglobin from Sigma

b) 1ml of distilled water

Equipment:

a) Balance

b) Plastic cylinder

General Precautions: When using hemoglobin, universal precautions should be used. Human hemoglobin is a biohazard.

Conclusion:

The hemoglobin of a known concentration has been prepared.

Preparation procedure:

A 1mg of hemoglobin powder is weighted and is transferred into a bottle containing of 1ml of distilled water. A magnetic stirrer bar is placed in the bottle for the hemoglobin to dissolve in water. The bottle is placed on the magnetic stirrer plate for 4 – 6 hrs and this completes the preparation of stock solution of hemoglobin of 1 mg/mL. Different dilutions of the master solution are prepared. The maximum solubility which could be achieved using the sigma hemoglobin is 1mg in 1 mL.

3.1.6 Optical Property of Hemoglobin

The hemoglobin absorbance spectra as shown in Figure 5 have three significant peaks. The dominant peak is located at 410nm. The two smaller peaks are located at 548 nm and 577 nm. These two smaller peaks in the 500 nm – 600 nm range are attributed to the presence of oxy hemoglobin. These peaks form a '**W**' pattern in this zone. The spectral signatures shown in figure 6 are the spectra of hemoglobin solutions of concentrations in the range 0.1 to 1 mg/mL.

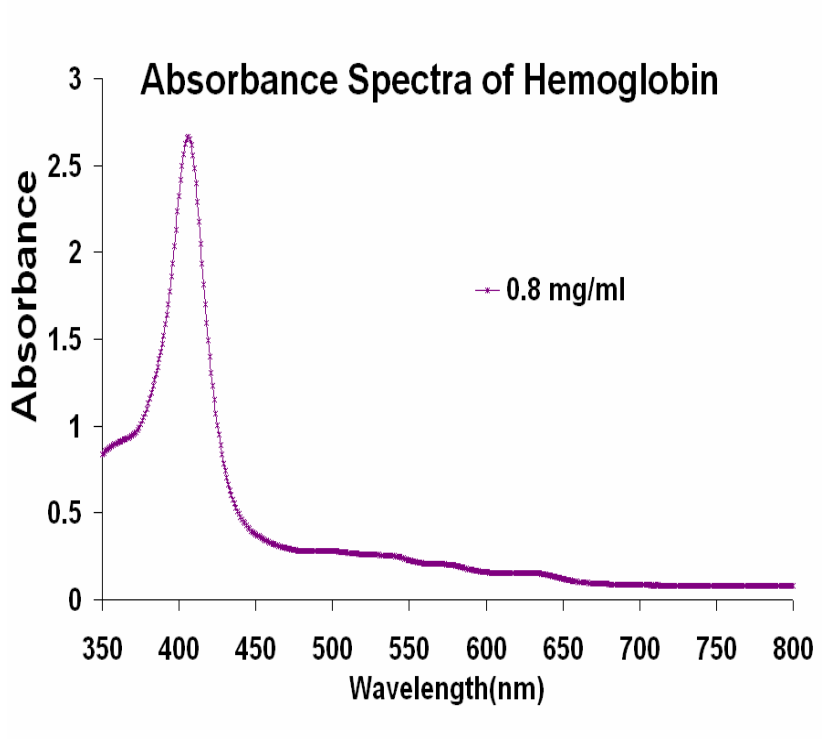


Figure 5 shows the absorbance Spectrum of hemoglobin solution with a significant peak absorbance at 410 nm and small peaks in the wavelength range of 500 to 600 nm

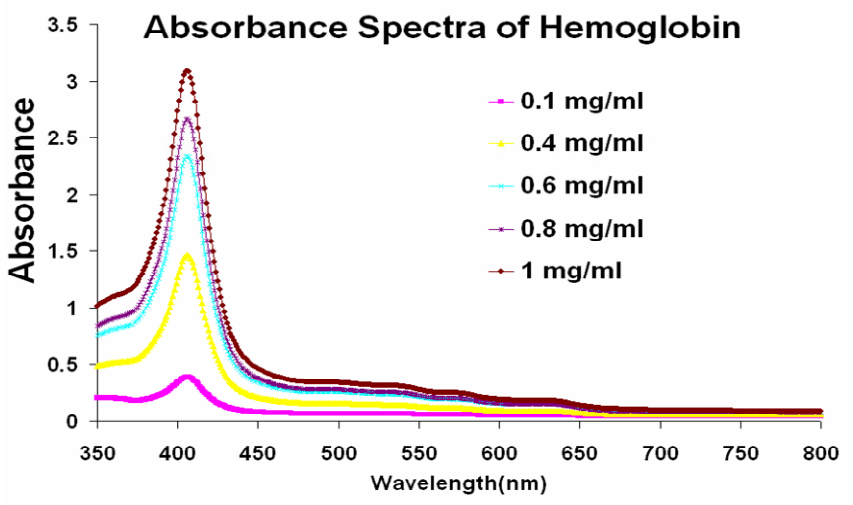


Figure 6 shows the absorbance spectrum of hemoglobin solutions of different concentrations and the absorbance value at 410 nm increases as the concentration of hemoglobin increases and the absorbance of the small peaks observed in the wavelength range of 500 to 600 nm.

3.1.7 Gelatin

Gelatin is a translucent brittle solid substance, colorless or slightly yellow, nearly tasteless and odorless, which is created by prolonged boiling of animal skin and connective tissue.

Gelatin Protocol:

Materials:

1. ¼ ounce of gelatin
2. 1 and ½ cup of water
3. 500ml cylindrical flask
4. Hot plate stirrer
5. Magnetic stirrer
6. Thermometer

Volume of 350 ml of water is poured into a 500ml beaker and placed on the hot plate.

The temperature of the water is monitored as it is heated to 60-70 degrees Centigrade

Magnetic stirrer is placed in the beaker and gelatin powder (1/4 ounce) is added. The solution is stirred until the gelatin dissolves. Cool to room temperature (25-30 degrees Centigrade). Now the solution is ready to be used. A known volume of gelatin was pipetted.to all the wells in the 96-well plate. The absorbance measurements of the gelatin solution are obtained. Figure 7 shows a typical absorbance spectrum for gelatin

3.1.8 Optical property of Gelatin

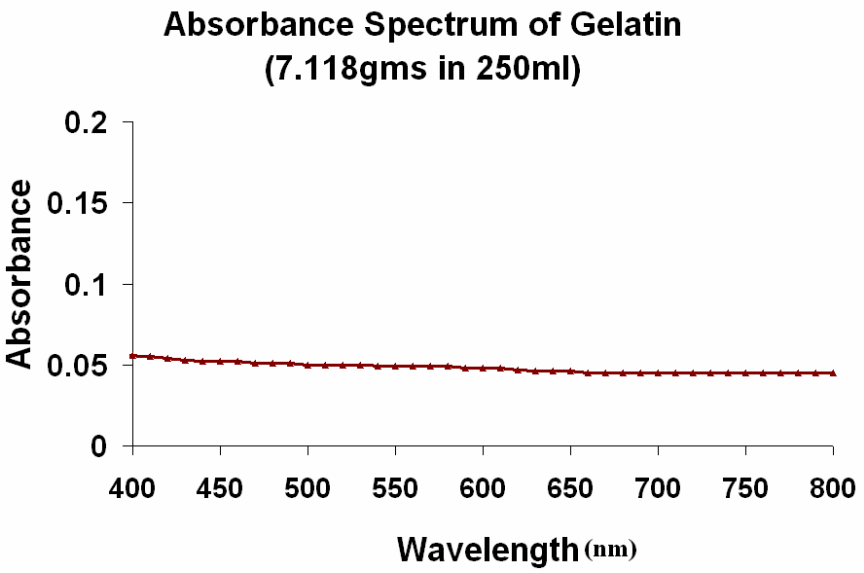


Figure 7 shows the absorbance spectra of gelatin solution

The absorbance spectrum of gelatin show in Figure 7 is uniform in the visible range of wavelength. The process of pipeting the gelatin solution into one of the wells is repeated 10 times and so there are ten wells in the micro plate which hold the gelatin solution. The absorbance measurements are taken for each of the solution in the plate. The objective of doing this experiment of repeating the process 10 times is to calculate the randomization error. The standard deviation involved in this process is 0.001. The same Gelatin solution which is prepared through this process can also be solidified into rectangular blocks.

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CHAPTER 4

SPECTROSCOPY

Spectroscopy is a technique used for the detection of various molecular or chemical species present in a solution. Spectroscopy is used in biology for the identification of substances or molecules based on the emitted spectrum, transmitted or absorbed spectrum. A device for recording this spectrum is a spectrometer. Spectroscopy can be classified according to the physical quantity which is measured or calculated or the measurement process **[1]**. The preferred method for analyzing the molecules in skin is through a noninvasive procedure. **[2].** The term non-invasive implies that the procedure does not harm the skin or body which is the material of interest to be studied, where this procedure doesn't involve any method of incision or pricking the veins or any other part of the body for analysis of the state of the medical symptom. Among many non-invasive methods of assessing the skin structure, the method adopted as a part of this research is to put forth the diffuse reflectance spectroscopy which is also known as elastic scattering spectroscopy **[3]**.

4.1 Reflectance Spectrometer

When light strikes a substance, it can be absorbed, transmitted, scattered or reflected. Reflectance spectrometers are used to measure this reflected Light. Reflected light carries the information of the concentration of the substance being evaluated. To understand the optical properties of substances like skin, reflectance spectroscopy would be the best approach. The reason for choosing the reflectance spectroscopy is it is a non-invasive approach with no harmful radiations and no interaction with the skin or its chromophores. It is also a rapid diagnostic procedure. The skin being a complex substance composed of different materials which lead to different colors under different medical conditions. For example in the case of jaundice the skin

appears to show off more of yellow color compared to the normal condition. This noninvasive approach of analyzing the substance for diagnosis of medical conditions will lead to many interesting applications of the use of reflectance spectroscopy. **[2]**

4.2 Diffuse Reflectance Spectroscopy

The diffuse reflectance spectroscopy is also known as elastic scattering spectroscopy. When a white light source is scanned over a visible range the reflected light due to back scattering conveys a lot of information about the presence of the different chromophores **[3].** It should. be noted that there is no shift in the wavelength as in the case of Raman scattering.

The Principal components of the diffuse reflectance spectroscopy system include

a) White light source

b) Reflectance probe

c) Spectrometer

d) Sampling Optics

A white light-source has a uniform broad spectrum in the visible range. This source of light is coupled into a fiber-optic bundle, the end of which is connected to a reflectance probe. Light leaves the probe and enters the sample which is to be analyzed. After the processes of scattering and absorption, light that leaves the sample is collected by read fiber, and directed into a spectrometer. The spectrometer gives the reflectance measurement of the sample at each wavelength and the spectrum can be further processed to determine the characteristics of the medium.

The use of this diffuse reflectance spectroscopy system reduces the complexity of the equipment needed to diagnose a condition. A noninvasive biomedical photonics system is utilized to study the optical properties of skin and their constituents which utilizes the principles and construction mechanism of diffuse reflectance spectroscopy.

4.3 Noninvasive Biomedical Photonic System (Reflectance spectrometer)

- Non-Invasive Biomedical Photonic system consists of four core elements\
- USB2000 Spectrometer
- OOIBase32 operating software
- Light source
- Sampling optics

Figure1 shows the arrangement of the core elements in building the noninvasive system. Light from the source is coupled into the reflection probe. The reflection probe has 1 illumination fiber and 6 read fibers. The light coupled from the light source to the illumination fiber interacts with the sample. When the light interacts with the skin (sample under consideration) part of the light is absorbed, part is transmitted and a part of it backscattered or reflected back. The reflected light is collected using the read fibers in the reflectance probe. The reflectance probe is a bundle which houses the illumination and read fibers. The reflectance probe is connected in a bifurcated fiber bundle where one end is connected to the source (white light) and the other end is coupled to the spectrometer which computes the reflectance measurement at each wavelength **[4]**.

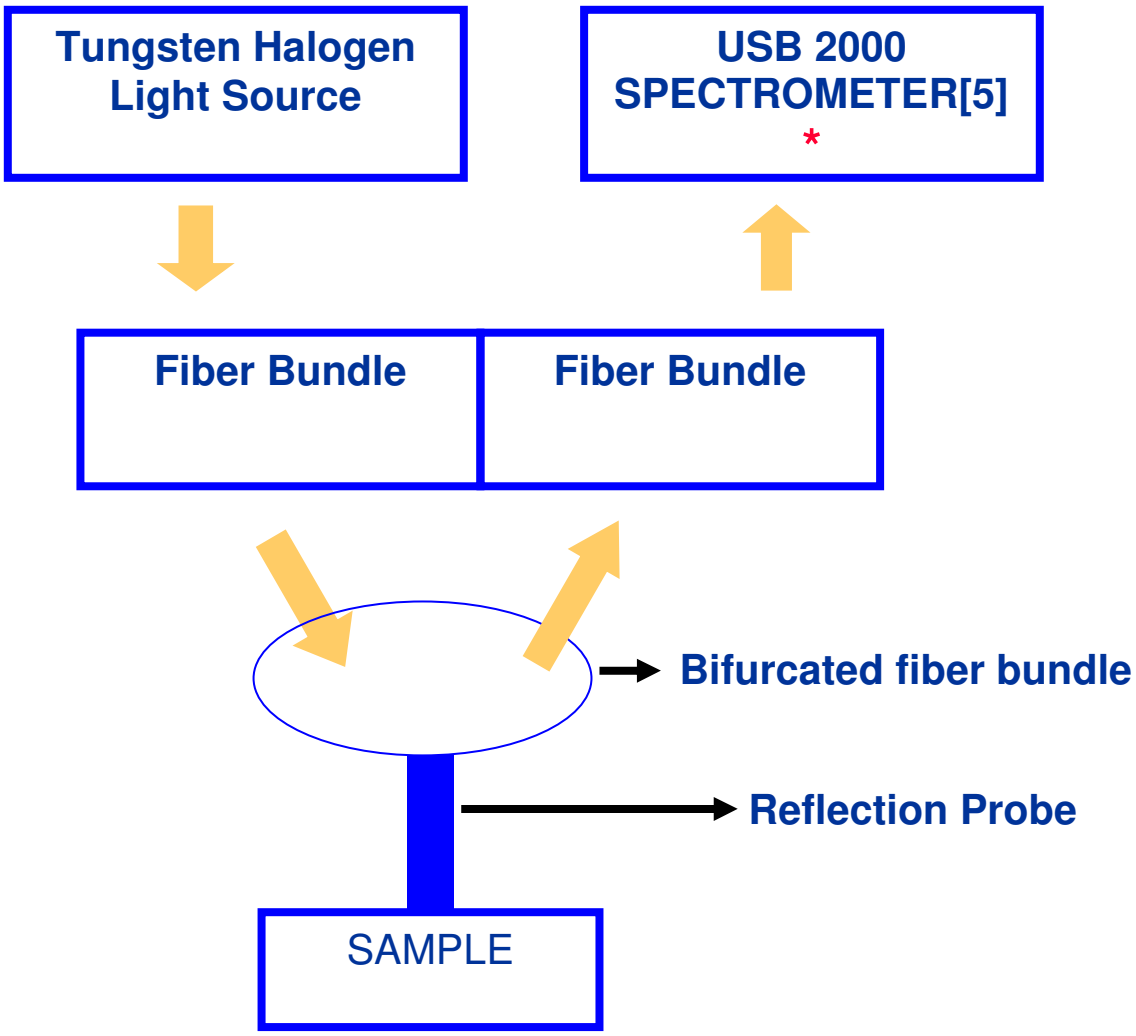

Figure 1 shows the optical construction of the reflectance spectrometer system used to capture the diffuse reflectance of sample. The skin samples can be analyzed using the system shown in figure1 with the reflectance probe placed in close proximity with the skin.

Optical properties of skin reflect the structure and chemical composition of the skin. When a beam of light strikes the skin surface, it reflects, refracts and transmits. Light in the near infrared region (700 - 1000 nm) penetrates relatively deep into tissue when compared to visible light (450-700 nm) making the detection of deeper tissue structures, like hemoglobin, possible. Therefore, it is possible to perform spectral noninvasive real-time analysis of skin properties.

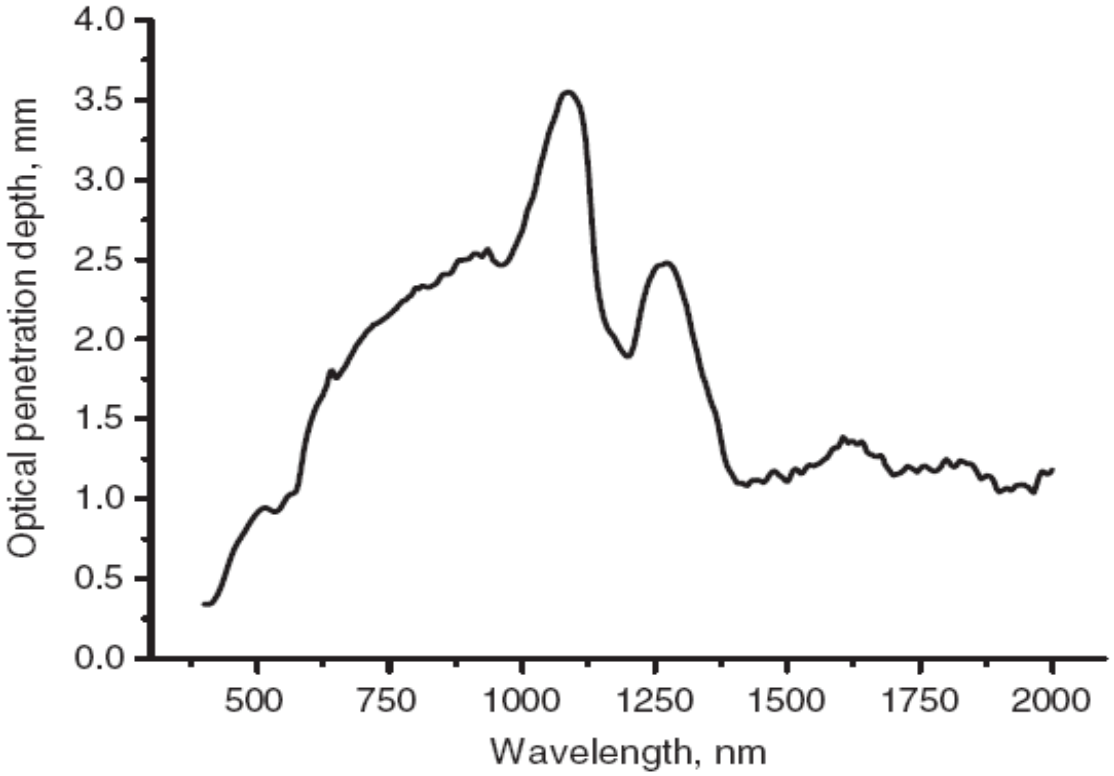

Figure 2 shows the optical penetration depth of light into the skin over a wavelength range of 400 to 2000nm **[6]**

The penetration depth of light into the skin tissue at different wavelengths is shown in figure1. The penetration depth increases as the wavelength increases in the visible range which is of interest in the diagnosis of bilirubin through noninvasive method.

4.4 TRANSMISSION SPECTROSCOPY

In the Initial phase of understanding the optical properties of the different chromophores the reflectance measurements needed robust sample preparation procedures to replicate the different layers of skin with clear separation between the layers. To reduce the complexity of the sample preparation initial research is done by using a transmission model of skin. For the transmission model measurements we have used the uQuant transmission spectrometer. The construction and working principle is explained in the following sections. The advantage of opting for the

transmission measurements for initial analysis comes from the ease of using transmission spectroscopy in characterizing the different chromophores individually and also collectively all the chromophores in a mixture. Biological samples were characterized using the transmission spectrometer.

Beer Law

Beer's Law states that the absorbance, A(λ), of a species in a material at a particular wavelength of electromagnetic radiation, λ, is proportional to the concentration, c, of the absorbing species in the material and to the path length *,l, (*i.e. the length the electromagnetic radiation has traversed through the material containing the absorbing species). This can be written in a form listed below:

$$A = \in \times c \times l$$

- A = Absorbance (dimensionless)
- ε = Molar Extinction Coefficient (liter mole-1 cm-1)
- l = path length (cm)
- c = concentration (moles/liter)

Different Configurations of Transmission Spectrometer

As mentioned in the Beer's law equation the path length term, l = 1 cm, as the length the light has traversed while interacting with the material. The two different ways of arrangement of light traversing through the sample is described below

Horizontal Configuration

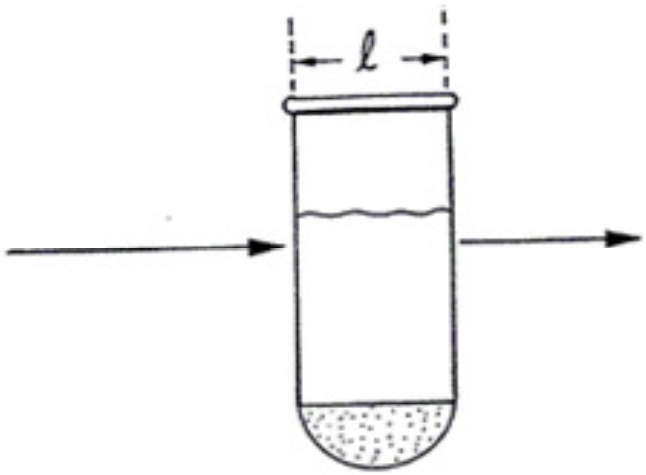

Figure 3 shows the path the light traverses as it interacts with the sample in the horizontal configuration of transmission spectrum measurement.

The horizontal arrangement of transmission spectroscopy shown in figure 3 is used when the sample volume is of the order of 1 ml. The path the light travels traverses are shown by the arrows. This arrangement is useful when the number of samples to be analyzed is relatively small.

Vertical Configuration

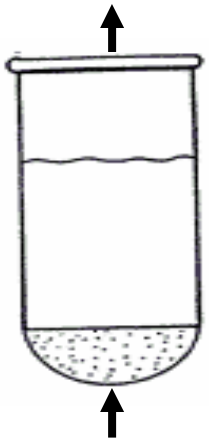

Figure 4 shows the path the light traverses as it interacts with the sample in the vertical configuration of transmission spectrum measurements.

The light traverses from the base to the top and interacts with the biological fluid under analysis in the arrangement shown in Figure 4. The transmitted light is collected by photo detectors on the top. The path length depends on the volume to which the fluid has been filled. The Transmission Spectrometer used for biological fluid analysis in this research is the µQuant (shown in figure 5). The µQuant is an innovative microplate reader with a high- powered xenon flash lamp, tunable monochromator and low UV capability. that reads 6-, 12-, 24-, 48-, 96-, and 384-well microplates.

Figure 5 is the photograph of the µQuant micro plate reader using which the. spectral scans of the mock skin samples in the wavelength range of 350 to 800 nm are captured **[7]**.

Figure 6 shows the micro well plate .The well depth is 10.5mm with a slightly tapered sidewall shape, while the maximum well volume is 190ul; the recommend working volume is in the range of 25 to 125 uL.

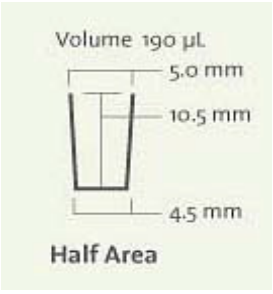

Figure 6 shows the dimensions of the micro well in 96 well microplate **[8]**

4.4.1 Experimental setup of the transmission spectrometer

Wavelength selection is performed by monochromator in 1nm or greater increments. The 96 well micro plate is positioned on the reader platform. Then using the KC4 software communication is established with the instrument. The light beam in the wavelength range of 350 to 800nm is passed through the base of the plate and interacts with the sample before it is collected by the photo detectors placed on top of the platform.

The time required to scan a complete micro plate (96 samples) from 350 nm to 800 nm in increments chosen by the user varies based on the precision level of the measurement and the number of data points (i.e. depends on the number of specific wavelengths from which absorbance is computed)

4.4.2 Principle of operation

The process flow of absorbance measurements in μ–Quant Micro plate Reader used for Transmission Spectrometer is as follows:

a) The 96 well microplates with saline solution is loaded into the dock which holds the microplate.

b) Once the 96 well microplates is loaded. Using KC4 a software which establishes communication with the instrument.

c) In the software we select the wavelength range for absorbance measurements.

d) The wavelength range chosen for the experimental analysis of the three chromophores which are bilirubin, melanin, hemoglobin are 350 to 800 nm mostly over the visible range.

e) Before loading the micro plate which has the biological fluid in the wells we load a micro plate with saline solution

f) The spectral scan is run over the microplate with saline solution. This is referred to as blank plate reading.

g) Once the blank plate reading is completed we load the microplate with biological samples in it is loaded for analysis

h) Absorbance spectra of the biological fluids are generated

4.5 References

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7. http://www.biotek.com/products/detection.php

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CHAPTER 5

In this chapter we use the transmission spectroscopy (horizontal alignment) method of calculating the molar extinction coefficient of the three significant chromophores bilirubin, hemoglobin and melanin. The basic law in analytical chemistry is Beer's Law which states:

$$A = \varepsilon cl$$

A – Absorbance

ε – Molar extinction Coefficient

c – Concentration

l - Path Length

The molar extinction coefficient of a chemical substance is a measure of how strongly the species absorbs the photons at a given wavelength. The absorbance depends on the product of the molar extinction coefficient, the concentration and the path length. The normal way of representing the absorbance characteristics of any biological substance is to plot absorbance vs. wavelength. A plot of absorbance vs. wavelength can be converted into a plot of molar extinction coefficient vs. wavelength by dividing the absorbance by the product of concentration and path length. If the path length is fixed, generally 1cm, a plot of absorbance vs. concentration should be linear. From the plot of absorbance vs. concentration the slope of the line joining the points on the plot gives the extinction coefficient. In a case where path length is not known, in the plot of absorbance vs concentration the slope of the line is a product of 'ε' the molar extinction coefficient and the path length.

5.1 USB-ISS-VIS System

Experiments to measure the molar extinction coefficient were performed on the USB-ISS-VIS system. As shown in Figure1, the USB-ISS-VIS system is an integrated sampling system which houses the light source and sample compartments. This system has advantages where no external fibers are used to couple the light from a light source to the sample or to collect the transmitted light into the spectrometer.

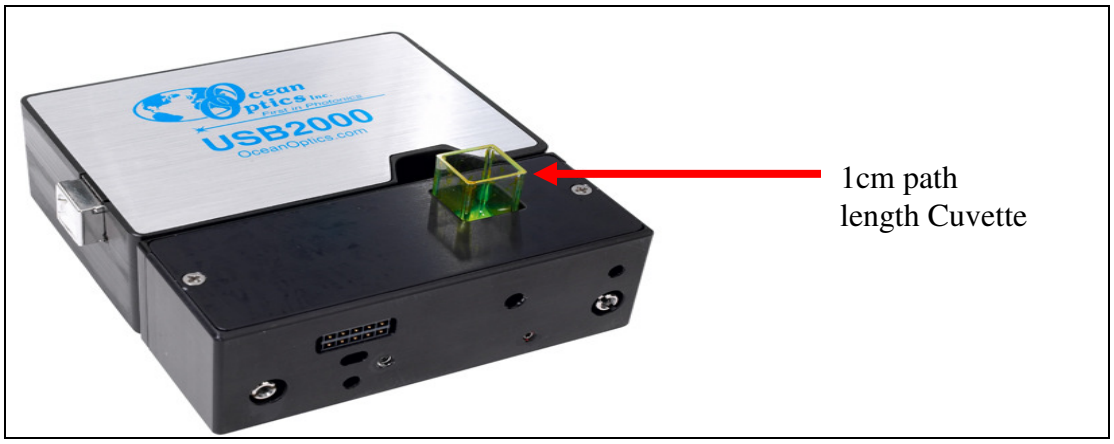

Figure 1 shows the photograph of the USB-ISS-VIS Spectrometer, an integrated sampling system used to study the spectral scans of the samples placed in the cuvette of 1 cm path length The light traverses in the horizontal direction making the path length fixed as 1 cm.[1]

The USB-ISS-VIS holds 1cm square cuvette. This fixes the path length the light interacts with the biological sample to 1cm. The sample is scanned over a wavelength range of 350 – 900 nm.

5.2 Measuring the Molar Extinction Coefficient

The USB-ISS-VIS is a spectrometer hardware system with integrated optics and A/D interface. The OOIBASE32 software package works with the hardware to provide computer controlled data acquisition. A USB communication protocol has been chosen for exchange of data between the hardware and software. A standard protocol has to be adopted for absorbance measurements. The protocol is a step by step process explained below

1. The parameters such as integration time, average and box car smoothing are fixed

2. The reference measurements are taken in the scope (intensity counts in scope mode is fixed at 3500 counts)
3. Reference Measurement:
    a. The reference measurement is used to measure the transmission properties of the cuvette
    b. The LED is turned on and the cuvette holder hold the cuvette with no sample in it
    c. Reference measurement is recorded.
4. Dark Measurement:
    a. The dark measurement takes care of the background noise of the environment
    b. The cuvette is placed in the cuvette holder with the LED off
    c. This measurement form the dark spectral signature
5. The design parameters are fixed.
6. System is ready to capture Absorbance measurements of the chromophores in the solution form.
7. Spectral signatures i.e. Absorbance's are calculated using the equation listed below **[1]**

$$A_\lambda = -\log_{10}\left(\frac{S_\lambda - D_\lambda}{R_\lambda - D_\lambda}\right)$$

where, S is the sample intensity, D is the dark intensity and R is the reference intensity at a given wavelength λ.

8. Using the absorbance and known concentration mathematical processing for calculation of molar extinction coefficients is done.

The flow chart which explains the process of the data acquisition is explained is shown in Figure2. The process of data acquisition is a sequential process. Once the dark and reference measurements are taken the parameters are fixed.

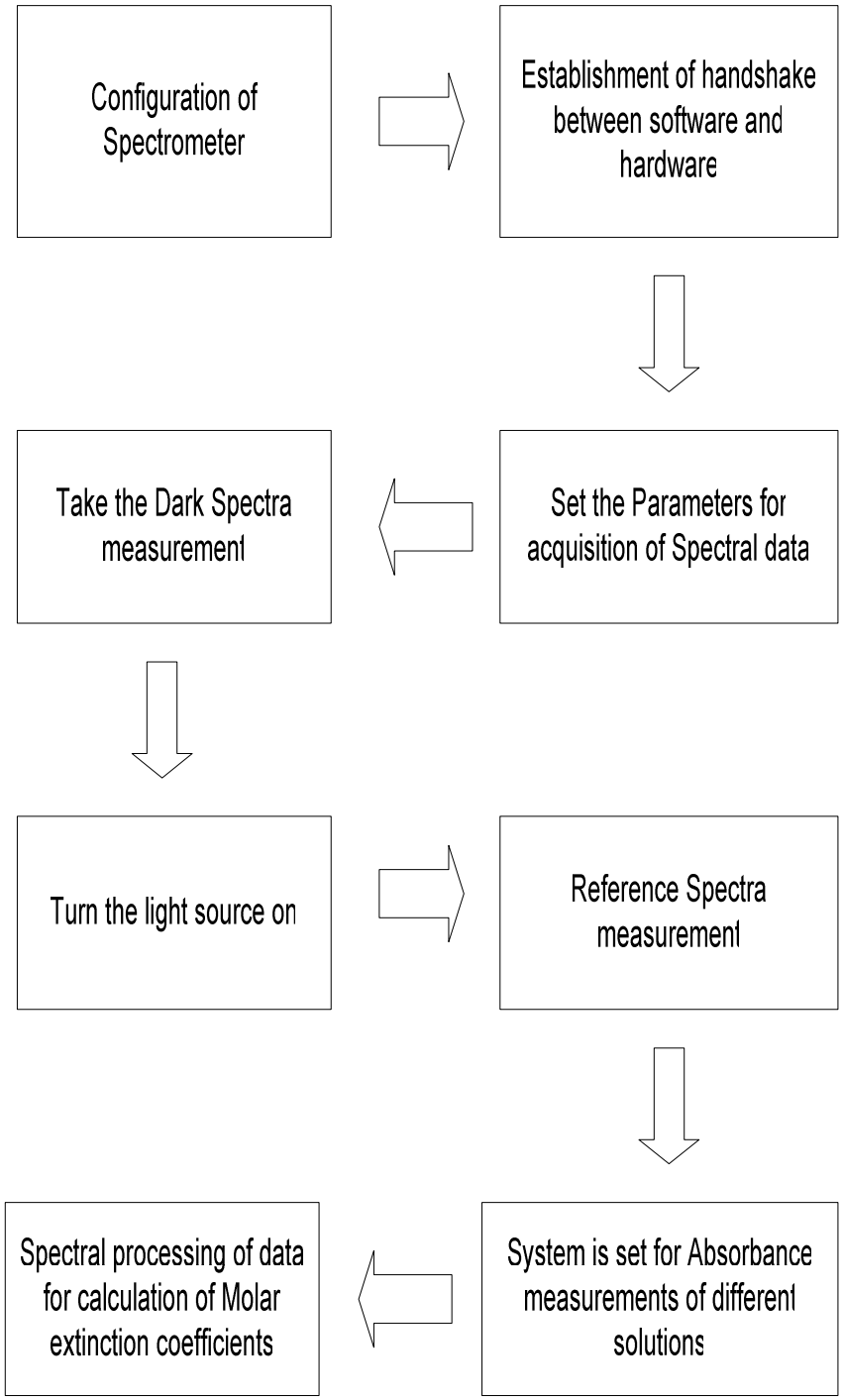

Figure 2 shows the flow chart describing the process of data acquisition

5.3 Molar extinction coefficient of Bilirubin

A known concentration of bilirubin solution is pipetted into the cuvette and using the data acquisition software a plot of absorbance vs. wavelength is captured. From this plot of absorbance vs. wavelength dividing the absorbance with the concentration of the known solution gives the plot of molar extinction coefficient vs. wavelength (from Beer's Law).

Solving for the molar extinction coefficient gives

$$\mathbf{e}(\lambda) = \mathbf{A}(\lambda)/\mathbf{c}$$

Converting bilirubin concentration from mg/dl to molar concentration is done as follows

1. Molecular weight of bilirubin is 584.68 g/liter**[2]**
2. For calculation of e we have used 10 mg/dL bilirubin concentration
3. 1 Molar solution implies 584.68 gms dissolved in 1 liter of solution
4. 10 mg/dl = 0.1 g/L
5. 0.1 g/L = 0.1/584.68 Molar
6. This value of (0.1/584.68) is the c value in the equation of A/c = e.

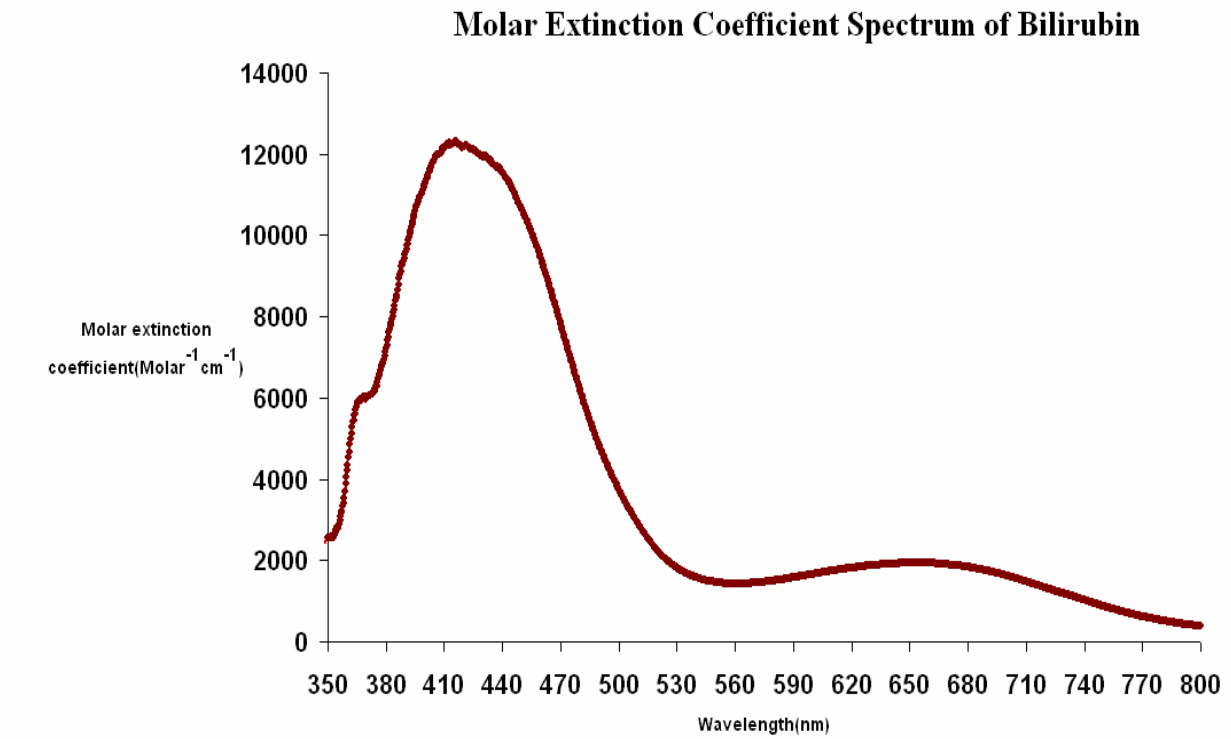

Figure 3 shows the molar extinction coefficient of bilirubin calculated using the transmission spectroscopy system. The bilirubin solution is placed 1cm path length cuvette and the spectral scans are captured. The spectral scans are used in the process of calculating the molar extinction coefficient.

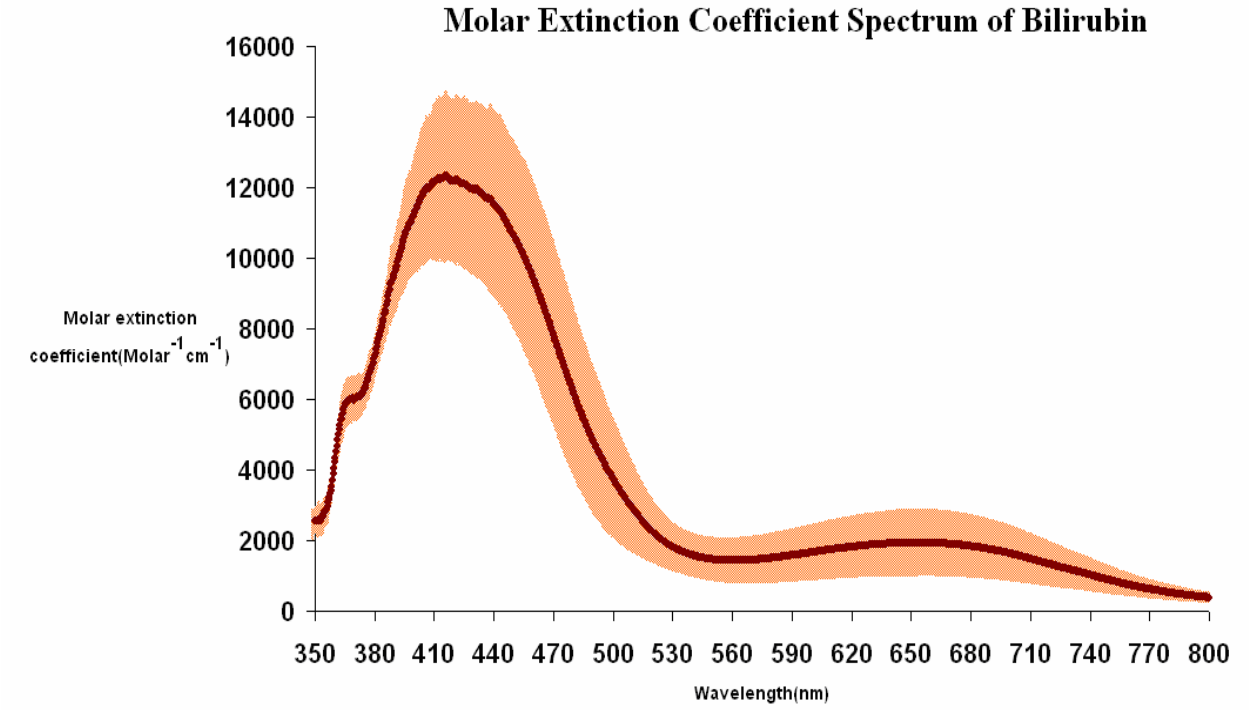

Figure 48 shows the standard deviation curves on either side of the mean value in the measurement of molar extinction coefficient of bilirubin.

5.4 Molar extinction coefficient of Hb

A Known amount of hemoglobin is dissolved in water on a stir plate for 2-4 hrs to insure that it is completely solubilised in water. This forms the aqueous solution of hemoglobin. Using the data acquisition software a plot of absorbance vs. wavelength is captured. As was done with the bilirubin, dividing the absorbance with the concentration and path length of the known solution gives a plot of molar extinction coefficient vs. wavelength.

Mathematical Calculations

1. Molecular weight of hemoglobin is 64,500 g/l.**[3]**
2. For calculation of e we have used 1 mg/ml hemoglobin concentration
3. 1 Molar solution implies 64 500 gms dissolved in 1 liter of solution
4. 1 mg/ml -> 1/64, 500 (Molar)
5. This value of (1/64500) is the c value in the equation of A/c = e

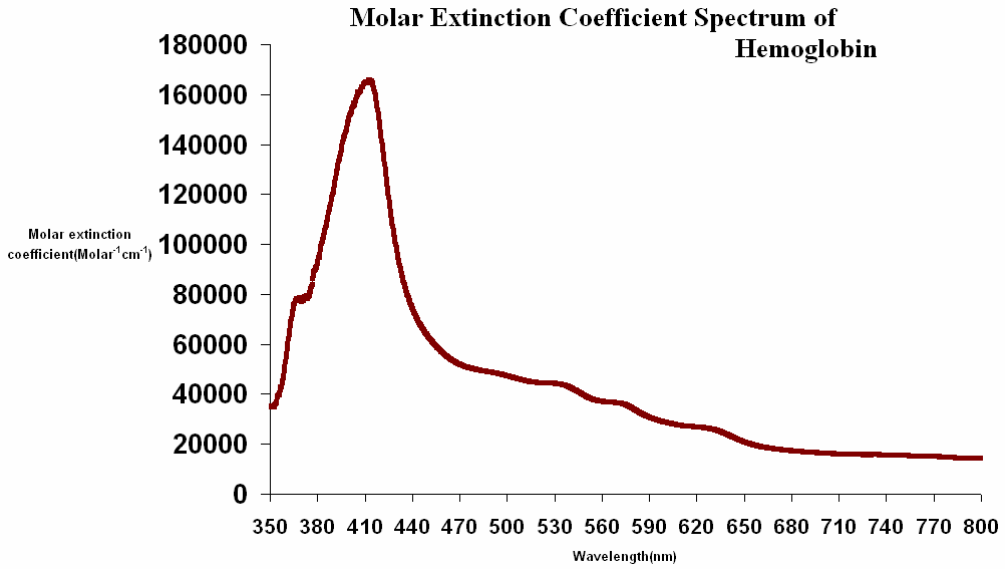

Figure 59 shows the molar extinction coefficient of hemoglobin calculated using the transmission spectroscopy system. The hemoglobin solution is placed 1cm path length cuvette and the spectral scans are captured. The spectral scans are used in the process of calculating the molar extinction coefficient

The experiment is with same concentration of hemoglobin prepared on three different days

The standard deviation measured in the process of calculating the molar extinction coefficient is calculated and plotted as shown in Figure 6.

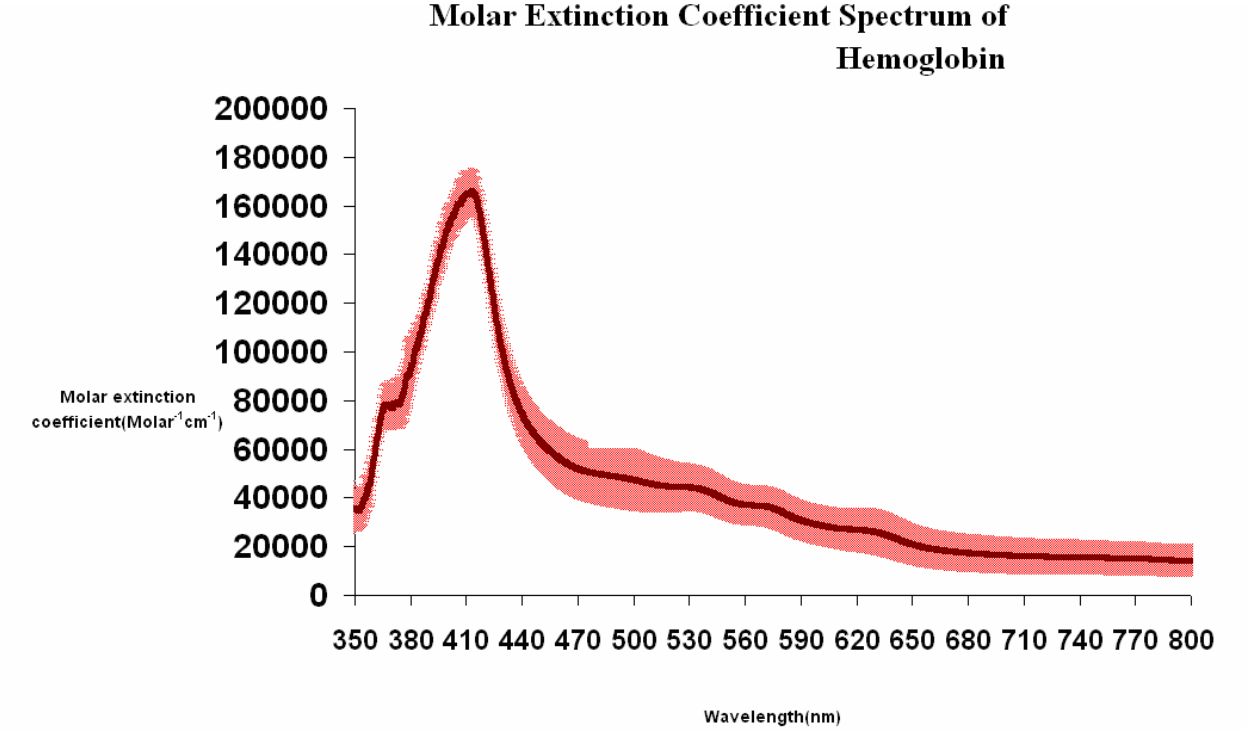

Figure 6 shows the standard deviation curves on either side of the mean value in the measurement of molar extinction coefficient of hemoglobin.

5.5 Molar extinction coefficient of Melanin

Solution Preparation

A known amount of melanin is mixed with deionised water. The aqueous solution of melanin is placed on a stirrer plate for 12- 24 hrs to make sure that all the melanin dissolves in water. This forms a known concentration of melanin. A known concentration of melanin solution is pipetted into the cuvette and using the data acquisition software a plot of absorbance vs. wavelength is captured. As with the previous section dividing the absorbance with the concentration and path length of the known solution gives the plot of molar extinction coefficient vs. wavelength.

Black melanin has been used for the preparation of known concentrations of melanin for spectral measurement of molar extinction coefficient and also used in the process of mock skin sample preparation. Black melanin is used and the molecular weight of black melanin from the literature is reported as 178 g/mole **[4,5].**

1. 1 unit implies dissolving 178 g in 1 litre of water.
2. 0.025 % melanin concentration is 0.025 gms dissolved in 100 mL of water
3. This gives 0.25 g/L
4. Molar equivalent of 0.25 g/L = 56.17

The absorbance spectra generated by the spectrometer is recorded and divided by the concentration.

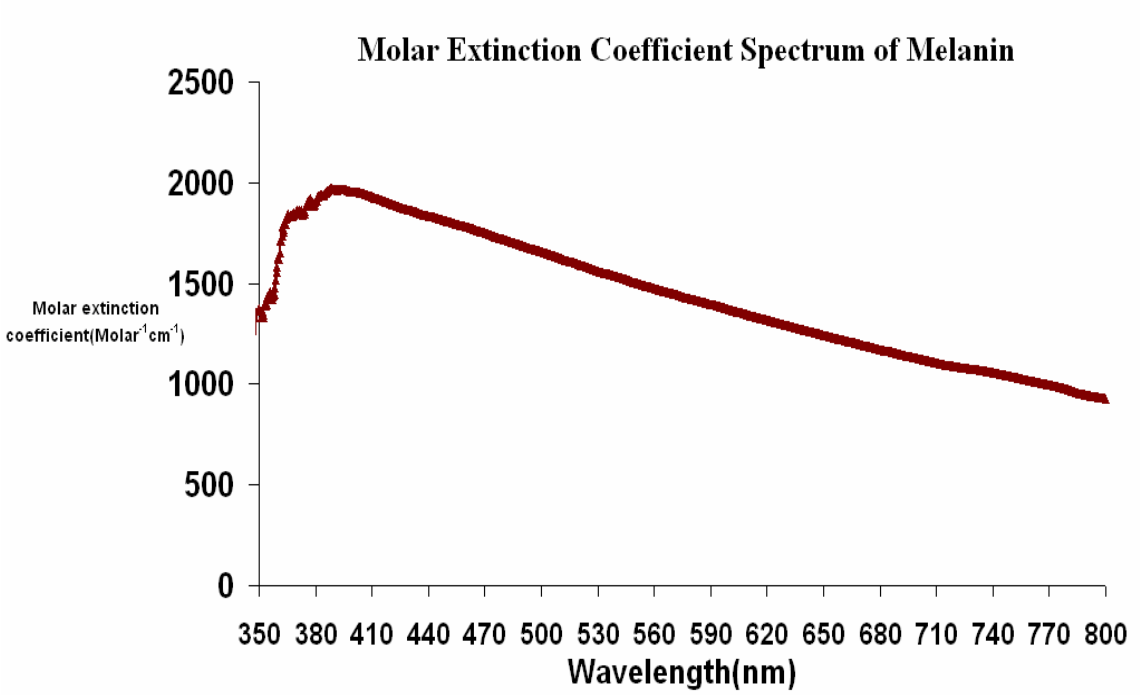

Figure 7 shows the molar extinction coefficient of melanin calculated using the transmission spectroscopy system. The melanin solution is placed 1cm path length cuvette and the spectral scans are captured. The spectral scans are used in the process of calculating the molar extinction coefficient

The experiment is with same concentration of melanin prepared on three different days

The standard deviation measured in the process of calculating the molar extinction coefficient is calculated and plotted as shown in Figure 8

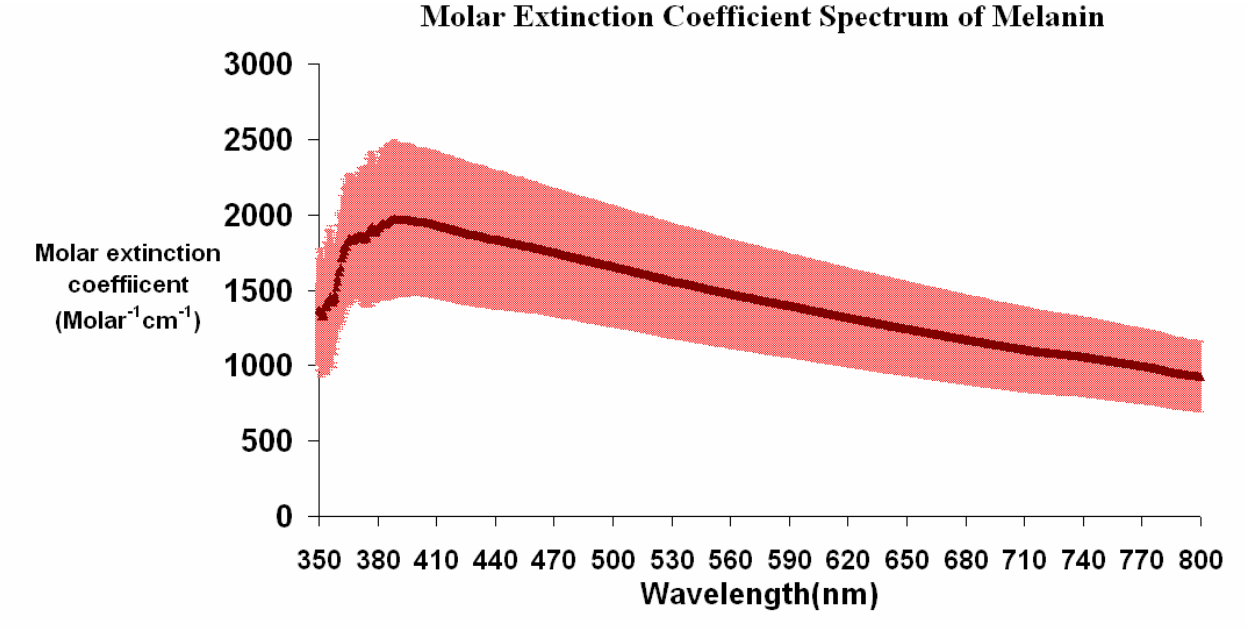

Figure 8 shows the standard deviation curves on either side of the mean value in the measurement of molar extinction coefficient of melanin

5.6 References

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3. http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/H7379
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CHAPTER 6

This chapter describes how bilirubin interactions with the other chromophores when present in different compartments i.e. (in the blood, in the skin tissue). In the plasma of the blood bilirubin will be present along with hemoglobin. As the bilirubin levels increase in the blood the bilirubin might diffuse into the skin where bilirubin is present along with melanin. To clearly detect and extract achemical means to chemically remove the bilirubin signal in the presence of noise signals hemoglobin and melanin, a study of interaction between bilirubin and melanin and bilirubin and hemoglobin are considered in this chapter.

In the design of Experiments Section we have chosen the factorial experiments model to study the interactions. The factorial experiment is a statistical study in which the experiment allows the user to study the effects of more than one factor on the response variable and at different levels. This design methodology also studies the effects of interactions between factors on the response variable **[1]**. The factorial experiment used in this thesis is a 2^2 factorial experiment. It is so called as we are considering only two levels and two factors. Results from 2^2 factorial designs can be analyzed using the statistical analysis package R software.

In the factorial design, factors which have little effect on the variability of the output are not considered in the process of building a model.

6.1 Interaction between Melanin and Bilirubin

Matrix Generation

The following matrix shows a design of experiment created to study the interaction between bilirubin and melanin at different concentrations of bilirubin and melanin.

Table 1 Matrix template used to study interactions between bilirubin and melanin

	Br0	**Br1**	**Br2**
M0	**A1**	**A2**	**A3**
M1	**B1**	**B2**	**B3**
M2	**C1**	**C2**	**C3**

M0	No Melanin	Br0	No Bilirubin
M1	Melanin Concentration 0.1%	Br1	Bilirubin Concentration 1mg/dl
M2	Melanin Concentration 0.5%	Br2	Bilirubin Concentration 5 mg/dl

The matrix template designed for studying the interactions between melanin and bilirubin is repeated three times to account for random error associated with the experiments. Each of the values in the matrix is a column data of absorbance of the mixture of the substances. The column data has absorbance values in the wavelength range from 350 to 800 nm at an interval of 4 nm. Absorbance at specific wavelengths is chosen to study the interactions. As a procedure to study the interactions using the factorial design a data set has been prepared Bilirubin and Melanin are two different factors. Bilirubin solutions at two concentrations signify two different levels. They are 1 and 10 mg/dl as they are stated in the column under B in table 2. Melanin solution at two concentrations one at 0.25 %(0.25gms/100mL) and the second one at 1 % (1gm/100mL).

Table 2 Data set used to study the interactions between bilirubin and melanin at 432 nm, 380nm and 600nm

B	**M**	**Absorbance at 432 nm**
1	**0.25**	**0.339**
10	**0.25**	**2.126**
1	**1**	**1.189**
10	**1**	**2.493**

As shown in Table 2 the values in the B column and M column are different concentrations and the values under the column absorbance at 432 nm are the responses. The data is stored in a .csv format file and all the analysis is done using the R statistical package. A detailed description of the R statistical analysis is given in Appendix1.From the R statistical analysis conducted to study the effect of interaction between bilirubin and melanin the percent variation is about 2% which is insignificant.Thus we conclude that melanin has no significant interactions with bilirubin.

6.2 Interaction between Hemoglobin and Bilirubin

Bilirubin is present in the plasma part of blood. The concentration ranges of hemoglobin in plasma would be low (i.e. in the range of 0.1 – 2 mg/ml).As hemoglobin and bilirubin chromophores are present together in a system and their optical signatures overlap. To study interactions between the Hemoglobin and Bilirubin a set of experiments have been designed and statistical results establish the contribution of each of the factors (i.e. hemoglobin, bilirubin, interaction term between bilirubin and hemoglobin) contribution to the spectral signature of a mixture of BR and HB

From a stock solution of bilirubin of pH 7.4 using a dilution procedure, different bilirubin solutions of varying concentrations are prepared. Hemoglobin solutions in the range of 0.1 mg/ml to 2 mg/ml are prepared. Using a 96 well micro plate and a micro plate reader setup the transmission measurements are taken on the samples that have been pipetted into the micro well plate. A bilirubin solution of 100 uL (micro liter) is mixed with a 100 uL of hemoglobin solution corresponding to the row and column heading assigned to the well in Table 3. In the process of developing the experiments to study the interactions, the factorial design approach is used.

In the factorial design approach used here we have two factors (i.e. bilirubin and hemoglobin) and the levels are the concentrations of each of the factors. Two levels are chosen for each of the factor. The factorial design is replicated 4 times and the matrix template used is shown in Table3

Br0	No Hemoglobin	Hb0	No Bilirubin
Br1	Bilirubin Concentration 1mg/dl	Hb2	Hemoglobin 0.4 mg/ml
Br3	Bilirubin Concentration 10 mg/dl	Hb4	Hemoglobin 1 mg/ml

Table 3 shows the matrix template used to study the interaction between bilirubin and hemoglobin from the spectral scans. The Columns headed with Hb0, Hb2, Hb4 stands for different concentrations of hemoglobin and Br0, Br1 and Br3 stands for the concentrations of bilirubin.The names assigned at the intersection of a particular row or a column are the names of the wells.

	Hb0	Hb2	Hb4	Hb0	Hb2	Hb4	Hb0	Hb2	Hb4	Hb0	Hb2	Hb4
Br0	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**
Br1	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**
Br3	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**

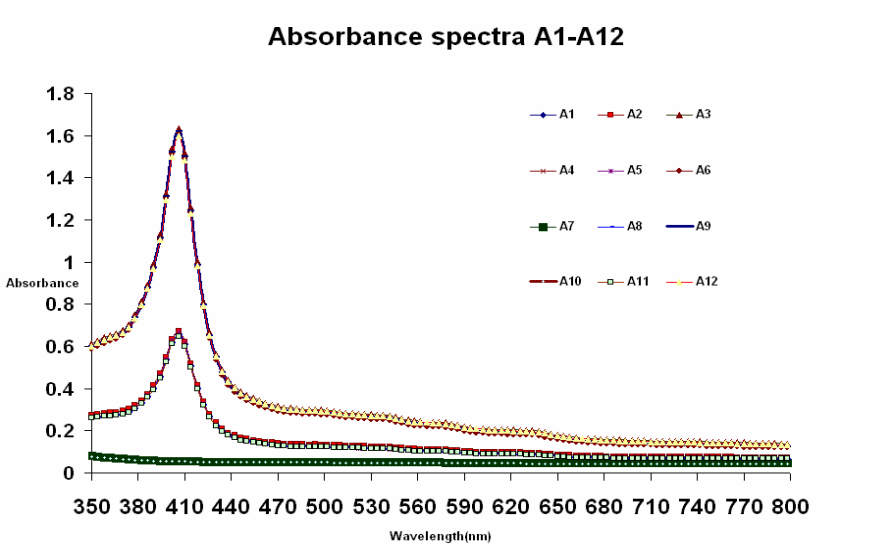

Figure 10 shows the absorbance spectra of the wells from A1 – A12. The spectral scans of the wells have only hemoglobin solutions in the wells and there is no bilirubin in each of the sample

Figure 1 shows the spectral scans of the wells A1-A12 in the row heading Br0 in table 3. The Absorbance spectra of wells A1, A4, A7 and A10 in Figure 1correspond to blank (empty well). The spectral signatures of wells A2, A5, A8 and A11 have a hemoglobin concentration of 0.4 mg/ml and an absorbance peak at 410 nm. The spectral signature of wells A3, A6, A9, A12 have a hemoglobin concentration of 1 mg/mL.

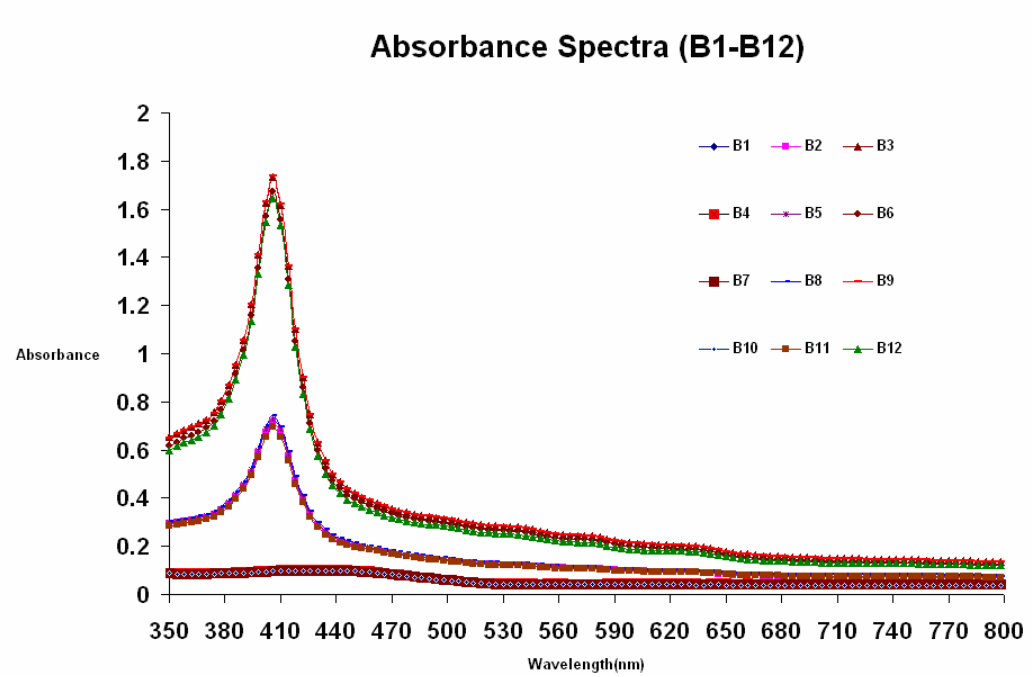

Figure 2 shows the absorbance spectra of the wells from B1 – B12. The slight hump in the region from 430 – 470 nm in these spectra is due to the addition of bilirubin to the hemoglobin spectra.

Figure 2 shows the spectral scans of the wells B1-B12 in the row heading Br1 in table 3. The Absorbance spectra of wells B1, B4, B7, and B10 have a bilirubin concentration of 1 mg/dl. The spectral signatures of wells B2, B5, B8 and B11 have a bilirubin concentration of 1 mg/dl with a hemoglobin concentration of 0.4 mg/ml. The spectral signature of wells B3, B6, B9, B12 have a bilirubin concentration of 0.4 mg/ml with hemoglobin concentration of 1 mg/mL. The addition of bilirubin to hemoglobin solution, had a slight increase in the slope of the line in the

wavelength region of 430 – 470 nm, the slope of the line being proportional to the amount of bilirubin present

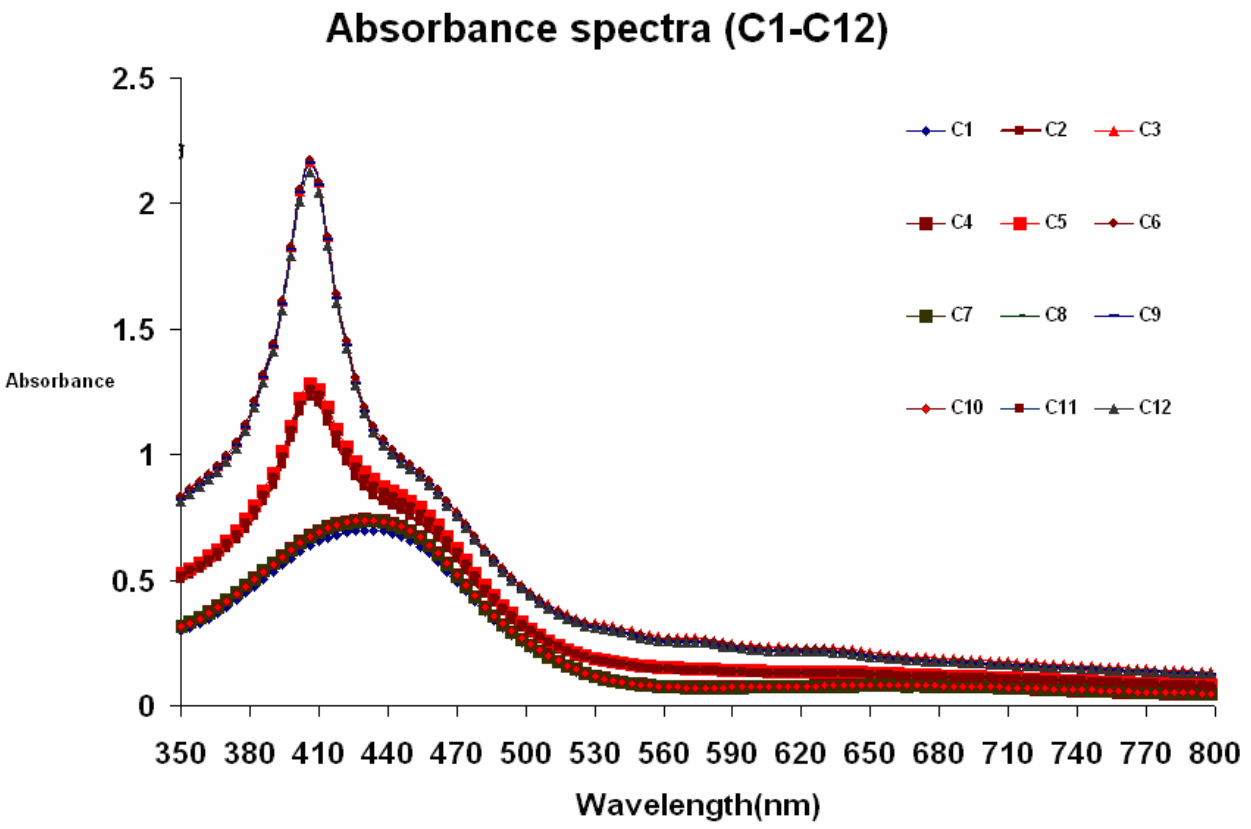

Figure 3 shows the absorbance spectra of the wells from C1 – C12. The hump in the region from 430 – 470 nm in these spectra is due to the addition of bilirubin to the hemoglobin spectra

Figure 3 shows the spectral scans of the wells C1-C12 in the row heading Br2 in table 3. The Absorbance spectra of wells C1, C4, C7 and C10 have a bilirubin concentration of 10 mg/dl

The spectral signatures of wells C2, C5, C8 and C11 have a bilirubin concentration of 10 mg/dl with a hemoglobin concentration of 0.4 mg/ml. The spectral signature of wells C3, C6, C9 and C12 has a bilirubin concentration of 10 mg/dl with hemoglobin concentration of 1 mg/ml. The addition of bilirubin to hemoglobin solution had a further slight increase in the slope of the line in the wavelength region of 430 – 470 nm.

Statistical Analysis on Interactions between Br and Hb

Design of Experiments (Factorial approach)

From Table3 a selected set of data is used for 2^2 factorial design experiments. The Responses B2, B3, C2 and C3 are a part of the study

Table 4 Design of Matrix for 2^2 factorial design experiments

	Hb0	**Hb2**	**Hb4**
Br0	**A1**	**A2**	**A3**
Br1	**B1**	***B2***	***B3***
Br3	**C1**	***C2***	***C3***

Br0	No Bilirubin	Hb0	No Hemoglobin
Br1	Bilirubin Concentration 1mg/dl	Hb2	Hemoglobin 0.4 mg/ml
Br3	Bilirubin Concentration 10 mg/dl	Hb4	Hemoglobin 1 mg/ml

The values in the matrix i.e. A1,A2,A3,B1,B2,B3,C1,C2 and C3 in table 4 is a column data of absorbance of the mixture of the substances formed by the combination of corresponding row heading and column heading concentrations are shown in the above matrix template. The column data has absorbance values in the wavelength range from 350 to 800 nm at an interval of 4 nm. Absorbance at specific wavelengths are chosen to study the interactions As a procedure to study the interactions using the factorial design a data set has been prepared with bilirubin and hemoglobin are two different factors

Bilirubin solution at two concentrations which signify two different levels They are 1 and 10 mg/dl, as they are stated as Br1 and Br3. Hemoglobin solution at two concentrations one at 0.4 and other at 1 mg/ml, stated as Hb2 and Hb4.

Table 5 shows the absorbance at wavelengths stated under the column headings for particular combinations of bilirubin and hemoglobin concentrations.

B	H	A410	418	430	442	450	462	542	550	562	570
1	0.4	0.671	0.471	0.29	0.22	0.2	0.2	0.1	0.1	0.1	0.1
10	0.4	1.204	1.043	0.87	0.8	0.76	0.7	0.2	0.2	0.1	0.1
1	1	1.618	1.101	0.63	0.47	0.42	0.4	0.3	0.3	0.2	0.2
10	1	2.084	1.638	1.19	1.02	0.96	0.9	0.3	0.3	0.3	0.3

The column values under B are the bilirubin concentrations of 1 and 10 mg/dl and the values under the H are the hemoglobin concentration of 0.4 and 1 mg/ml. The column headers A410,418,430,442,450,462,542,550,562,570 are the wavelengths at which the absorbance is measured for each combination of bilirubin and hemoglobin concentration. Using the factorial design approach the Absorbance at different wavelengths for the combination of bilirubin and hemoglobin are taken. The analysis of variance is conducted on this data set repeating the same process as adopted for the study of bilirubin and melanin interactions. In the analysis of variance the Absorbance values are the dependent variables and the two factors bilirubin and hemoglobin are the independent variables and a study of interactions are conducted using the tilde symbol in the ANOVA expression as detailed in Appendix1. The results are tabulated as shown in table 6.

Table 6 shows the percent variation contributed by each factor and also the contribution from the interaction term to the absorbance at a specific wavelength

Wavelength	Bilirubin	Hemoglobin	Interaction
410	22.99	76.9	0.1
418	45.02	54.93	0.04
430	74.5	25.45	0.03
442	85.37	14.59	0.02
450	87.21	12.76	0.01
462	86.31	13.68	0.002
542	5.72	94.16	0.1
550	5	94.85	0.14
562	4.59	95.26	0.13
570	4.73	95.12	0.14

The hemoglobin has a significant absorbance peak at 410 nm and minor peaks at 542 and 570nm. The bilirubin has a broad absorbance spectrum in the 400 – 470 nm regions with a peak at 435 – 440 nm. From Table 6 the percent variation contribution by hemoglobin, bilirubin and the interaction term are tabulated at specific wavelengths in the range of 400 to 600 nm. With an emphasis on the percent variation in hemoglobin the most significant wavelengths to study the characteristics of hemoglobin signal in the presence of bilirubin would in the range of 540 to 580

nm. The contribution from the bilirubin signal is not significant. As we look from table6 for the bilirubin percent variation is high in the range of 430 to 460nm.This would be the important range to study the characteristics of bilirubin with the objective to remove the interference from hemoglobin by subtracting the contribution of hemoglobin signal at those wavelengths. A look on the interaction term in table 6 shows that there is a fluctuation in the percent variation contributed by interaction.The interaction term effect has to be considered in building a robust model for prediction of concentration of bilirubin signal in the presence of hemoglobin and melanin noise signals.

Transmission Spectra Experiment

The ISS-UV-VIS system is used to study the effect on the absorbance spectrum of hemoglobin by adding bilirubin and also the effect on the absorbance spectrum of bilirubin by adding hemoglobin.

Experiment 1

The objective to study the spectral changes observed in the hemoglobin spectrum by addition of bilirubin, using the experimental procedure is explained in this section. The horizontal configuration of transmission spectroscopy is used and the protocol described for absorbance measurements in Chapter 5 is followed. The cuvette of 1 cm path length is used. The cuvette is filled with 1ml of 0.4 mg/mL of hemoglobin solution. The spectral scan in the wavelength range of 350 to 800 nm is captured with only hemoglobin solution in the cuvette. The spectral scan of the hemoglobin solution has shown a significant peak at 410 nm and small peaks at 540 and 570 nm. These absorbance peaks at 540 and 570 nm correspond to the oxy hemoglobin peaks. To study the effect of addition of bilirubin on hemoglobin spectral signature, to the hemoglobin solution in the cuvette a 100ul of 10mg/dL of bilirubin solution is added and stirred well. The spectral scan is captured. To the existing solution in the cuvette, 100ul of 10mg/dl of bilirubin

solution is added and spectral scan is again captured. This process is repeated and the spectral scans are taken till the final volume of bilirubin added is 500ul. The spectral scan captured after each addition of bilirubin solution is shown in Figure 4.

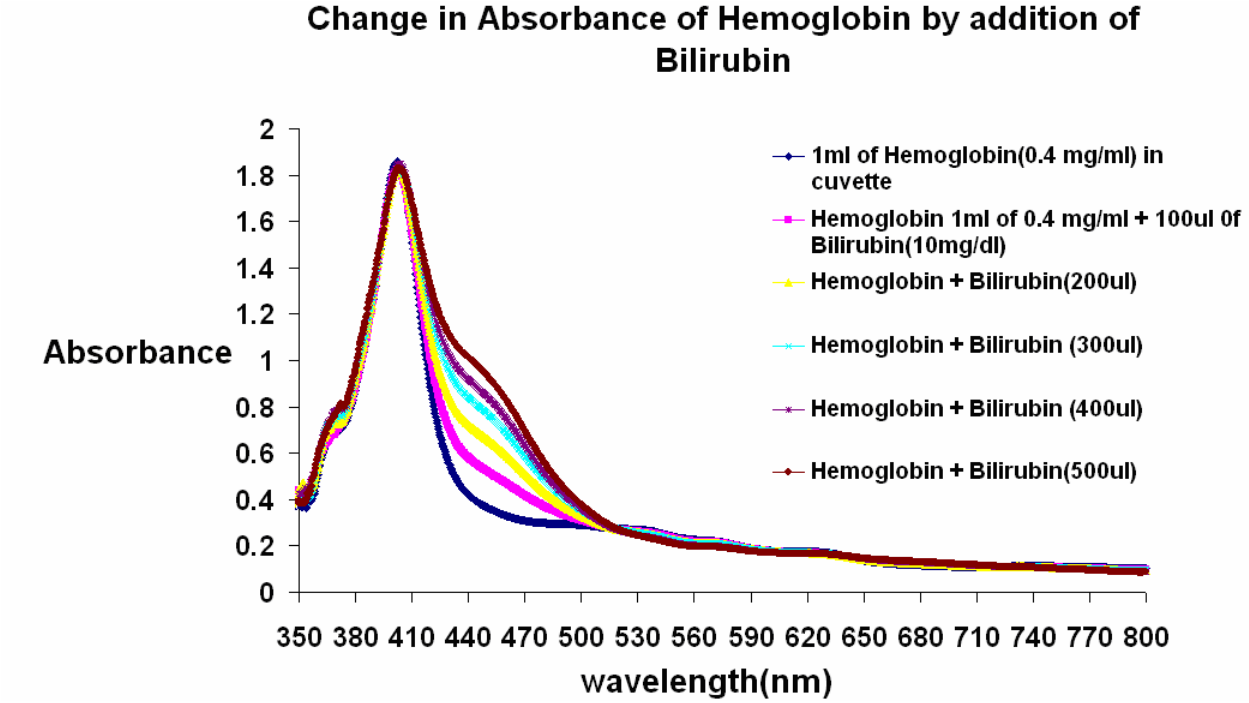

Figure 4 shows the increase in absorbance in the wavelength range of 410 to 460 nm as the bilirubin is added to hemoglobin solution. As the bilirubin solution is added, the hemoglobin solution is diluted and the absorbance at wavelengths 540 and 570 nm decreases.

Figure 4 shows an increase in absorbance in the 430-470 nm regions as the concentration of bilirubin is added to a fixed concentration of hemoglobin. The absorbance values at 540 and 570 nm are decreasing in a linear fashion as the bilirubin solution is added. This change in absorbance at these two peaks is due to the dilution of hemoglobin solution which changes the concentration of the hemoglobin in the mixture. As expected these two peaks have no influence from the bilirubin signal.

Experiment 2

A study on the spectral changes observed in the bilirubin spectrum by addition of hemoglobin is conducted using the transmission spectroscopy method. The horizontal configuration of transmission spectroscopy is used and the protocol described for absorbance measurements in Chapter 5 is followed. The cuvette which is of 1 cm path length is used. The cuvette is filled with 1ml of 10 mg/dl of bilirubin solution. The Spectral scan in the wavelength range of 350 to 800 nm is captured with only bilirubin solution in the cuvette. The spectral scan of the bilirubin solution has shown a broad peak in the region of 400 to 470 nm with a .maximum in the range of 430-440nm. To study the effect of addition of hemoglobin on bilirubin spectral signature, to the bilirubin solution in the cuvette a 100ul of 1mg/mL of hemoglobin solution is added and stirred well. The spectral scan is captured. To the existing solution in the cuvette, 100uL of 1mg/mL of bilirubin solution is added and spectral scan is again captured. This process is repeated and the spectral scans are taken till the final volume of hemoglobin added is 500ul. The spectral scan captured after each addition of hemoglobin solution is shown in Figure 5.

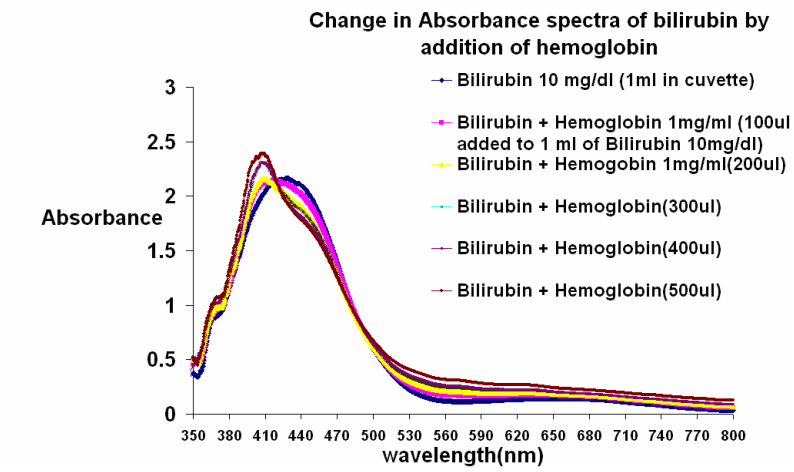

Figure 5 shows an increase in absorbance at 410 nm as the concentration of hemoglobin is added to a fixed concentration of bilirubin. The absorbance values at 540 and 570 nm are increasing in a linear fashion as the hemoglobin solution is added.

This change in absorbance at these two peaks is due to the increase in the concentration of hemoglobin solution as the concentration of the bilirubin is changing in the mixture. As described in experiment #1 these two significant peaks have no other significant influence from the bilirubin signal. The broad peak in the region of 400 to 470 nm is decreasing as the bilirubin concentration decreases due to dilution by hemoglobin significantly increasing the peak at 410 nm due to hemoglobin

6.3 References

1. http://en.wikipedia.org/wiki/Factorial_experiment

CHAPTER 7

ALGORITHM DEVELOPMENT

The development of a robust algorithm to extract the information of bilirubin present in a complex mixture of mock skin model is the goal of this chapter. With the knowledge of the molar extinction coefficient of the different chromophores and pigments, a relationship is established between the absorbance spectrum and molar extinction coefficients.Using the principle of multiple regression analysis an algorithm is developed to predict the concentration of bilirubin present in the mock skin model.

7.1 BEER LAMBERT LAW

Absorbance spectrum of a homogeneous scattering medium containing an absorber is expressed as

$$A = \in (\lambda) \times c \times l(\lambda, c)$$

The skin tissue mainly consists of epidermis containing the melanin and dermis containing the hemoglobin and bilirubin.The absorbance spectrum of skin tissue is given by the sum of the absorbance of epidermis, *Aepi* and dermis *Ader.*

$$A(\lambda) = A_{epi}(\lambda) + A_{der}(\lambda) \qquad \textbf{-- (a)}$$

Absorbance in the epidermis layer of the skin using beer's law is stated in equation (b)

$$Aepi\ (\lambda) = C_m \times l_{epi}(\lambda, C_m) \times \in_m (\lambda) \qquad \textbf{-- (b)}$$

Absorbance in the dermis layer of the skin using beer's law is stated in equation.(c)

$$A_{der}(\lambda) = C_h \times l_{der}(\lambda, C_h) \times \in_h (\lambda) + C_b \times l_{der}(\lambda, C_b) \times \in_b (\lambda)$$

-- (c)

As expressed in equation (a) the Absorbance spectrum of skin is sum of the absorbance of the epidermal layer and dermal layer of the skin. Using equation (b) and (c), equation (a) is rewritten as

$$A(\lambda) = C_m \times l_{ept}(\lambda, C_m) \times \in_m + C_h \times l_{der}(\lambda, C_h) \times \in_h (\lambda) + C_b \times l_{der}(\lambda, C_b) \times \in_b (\lambda) \quad \textbf{-- (d)}$$

Where the subscripts m, h and b stand for melanin, hemoglobin and bilirubin respectively.

7.2 MULTIPLE REGRESSION ANALYSIS MODEL

The multiple regression equation takes the form $\mathbf{y = b_1x_1 + b_2x_2 + ... + b_nx_n + c.}$ The b's are the regression coefficients, which represent the change in the dependent variable i.e. 'y' for a corresponding change in independent variable i.e. 'x'. The absorbance spectra of the mock skin samples in the range of 350-800 nm is the dependent variable also called the response variable. The molar extinction coefficients of melanin, hemoglobin and bilirubin are the independent variables [1]. By performing multiple regression analysis on one sample of the absorbance spectrum of the mock skin a set of four regression coefficients are generated. Equation (e) shows the multiple regression model applied to absorbance spectrum of skin

$$A(\lambda) = a_m \times \in_m (\lambda) + a_h \times \in_h (\lambda) + a_b \times \in_b (\lambda) + a_0 \quad \textbf{-- (e)}$$

The regression coefficients $\mathbf{a_m}$ $\mathbf{a_{hb}}$,$\mathbf{a_{br}}$ express the degree of contribution of each molar extinction coefficient to the absorbance spectrum and also the three regression coefficients are related to the concentrations of melanin, hemoglobin and bilirubin respectively. Comparing the third

terms in equations (d) and (e) the regression coefficient associated with bilirubin is nonlinearly related to bilirubin concentration.

The process of building a calibration model to establish a relationship between the concentrations of bilirubin present in the sample to the regression coefficient is started with the mock skin sample preparation in 96 well micro plates. The absorbance spectra of each of the mock skin samples are recorded. The multiple regression analysis is performed on the absorbance spectra, using the molar extinction data calculated in chapter 5 of this thesis. Regression analysis on each absorbance spectra has generated a regression coefficient corresponding to bilirubin. As the concentration of bilirubin present in each of the spectra are known, a relationship is established between concentrations and regression coefficients. The regression coefficients in the regression equation developed are known as conversion factors. The formation of regression equation and the conversion factors marks the end of the calibration model. Once the calibration model is developed, it is the time to test the model. Mock skin samples are prepared and their absorbance spectra are recorded. Multiple regression analysis is performed on each of the spectra. Using the conversion factors the regression coefficient corresponding to bilirubin from each spectrum is converted into concentration of bilirubin.

The flow chart of the calibration model starts with using a known set of concentrations of bilirubin, hemoglobin and melanin to generate absorbance spectra of mock skin samples. Using the molar extinction coefficients calculated in chapter 5 for each of the chromophores a multiple regression analysis is performed on the absorbance spectra as the response variable and the molar extinction coefficients as independent variables. The regression coefficients generated as a result of the previous step forms the input to the next step in the flow chart. With the Concentration of each of the chromophores as response variable and regression coefficients as

independent variable a regression analysis is performed to calculate the conversion factors. The complete calibration model process is shown below as a flow chart model in figure1.

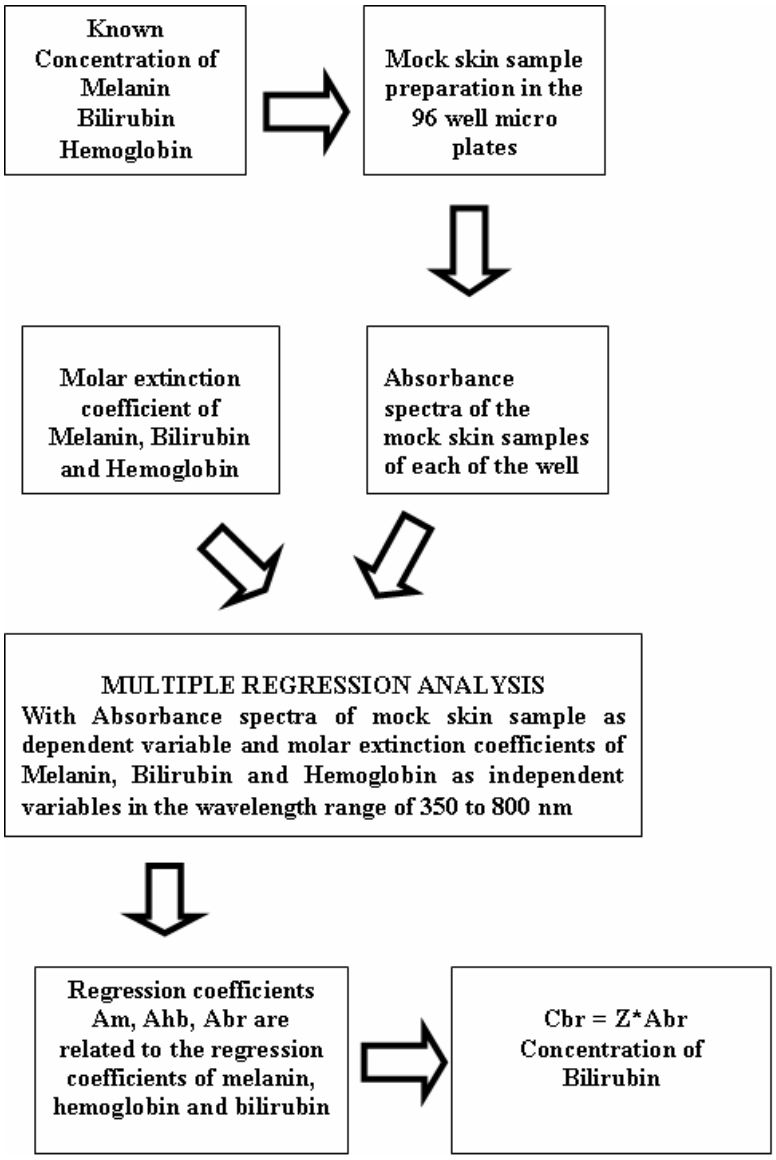

Figure 11 describes the procedure adopted to build a calibration model used to establish a relation between concentration of bilirubin solutions present in the mock skin samples and regression coefficients related to bilirubin calculated from multiple regression analysis.

7.4 References

1. *"Estimation of melanin and hemoglobin in skin tissue using multiple regression analysis aided by Monte Carlo simulation",* Izumi Nishidate,Yoshihisa Aizu and Hiromichi, Mishina Journal of Biomedical Optics, July 2004 Volume 9, Issue 4, pp. 700-710

Chapter 8

As an approach to mimic the optical properties of skin a preliminary mock skin model system is developed. Skin is a layered structure. The Epidermal layer of the skin has melanin which is the primary absorbing chromophores. Dermis has the blood vessels where bilirubin and hemoglobin are present. The interface between the two layers should be a clear interface. The reason for having a clear interface is to replicate the condition of skin in the case of a normal neonate. This chapter explains the experimental protocol adopted to create a mock skin model in the micro well plate.

8.1 Epidermal layer of the skin

The top most layer of the skin is the epidermis. The main absorbing substance in the epidermis part of the skin is the melanin. Melanin keeps excessive ultraviolet rays from burning the skin. Exposure to sunlight causes the skin to produce more melanin, causing suntan, a temporary change in skin color. Melanin-rich cells continually move toward the surface, where they die and are sloughed. Skin color results from the presence of melanin, carotene (yellow to orange pigment), and underlying hemoglobin reflected through skin **[1].** The experimental approach is designed to mimic the optical properties of epidermal layer.

8.1.1 Preparation process of melanin-gelatin layer which acts as an epidermal layer

The first step in the process of preparing a two layer mock skin sample starts with the epidermal layer. The main absorbing chromophores in the epidermal layer of the skin are the melanin.
The preparation process of forming an epidermal layer with melanin is explained in this section. A known amount of melanin (i.e1mg is placed in a 1mL of deionised water) to form 0.1 %melanin solution. The container holding the melanin solution is placed on a magnetic stirrer

with a stir bar in it. This process allows all the melanin particles to be dissolved completely in water. The normal time for the melanin to dissolve completely is 18-24 hrs. To form a solid epidermal layer with melanin in it, melanin solution is mixed with gelatin. The gelatin solution is prepared as per the protocol mentioned in Chapter 3. Melanin solution of known volume is mixed with a known volume of gelatin solution. Gelatin solution is at 60 degrees Centigrade when it is mixed with melanin solution. This ensures that gelatin is still in liquid form. The mixture of melanin and gelatin solution is placed on a vortex for a proper mixing. The mixture is now ready to be pipette into the micro well plates. The process of pipeting the melanin-gelatin mixture is to be done with an hour from the time the mixture is ready as gelatin starts forming a solid substance which is difficult to pipette. 100uL of Melanin-gelatin mixture is pipetted into each of the micro well plates. The micro well plate is sealed with paraffin film and covered with aluminum foil. The plates are placed at 4 degree centigrade for 12 hrs. The melanin-gelatin mixture pipetted into the micro well plates form a solid layer.

A trial of 100 samples was pipetted and studies were conducted to analyze the system error associated with pipe ting of gelatin at 60^{o}C. The standard deviation measured from the absorbance measurements at 5 specific wavelengths on the melanin-gelatin solid layer formed is 0.05. In the process of building a model skin, the first layer is melanin gelatin layer. The light first interacts with the melanin and gelatin layer and the transmitted light then interacts with the bilirubin and hemoglobin solutions which are stacked on top of the melanin gelatin layer. The model is being built in a micro well plate. With the consideration that light reaches the hemoglobin and bilirubin solutions stacked on top of melanin gelatin layer the concentration of melanin used in the preparation of melanin gelatin layer is limited to 0.1%. The parameter which can be varied is the concentration of melanin. The variation in skin pigmentation is due

to presence of different concentrations of melanin. So by varying the amount of melanin in the process of preparation of melanin gelatin layer mock skin models can be prepared which resemble the different skinned individuals i.e. light skin, dark skin, Asian etc.

8.2 Bilirubin and Hemoglobin Solutions Stacked on the Epidermal Layer of the Skin-Optical Properties

The Dermis layer of the skin consists of blood vessels of which the primary absorbing substances are hemoglobin and bilirubin in the visible range of electromagnetic spectrum. Hemoglobin and bilirubin stock solutions are prepared as per the protocol stated in chapter 3. Five different concentrations of hemoglobin and of bilirubin are prepared from their respective stock solutions.Using the matrix described in Table 1 the different concentrations of hemoglobin and bilirubin are pipetted into a 96 well plate. To study the effect of increasing concentration of hemoglobin on the epidermal layer, Absorbance measurements of A1, A2…A6 showed an increase in absorbance peak at 410 nm. Hemoglobin concentrations used in this study are in the range of 0.1 mg/ml to 2 mg/ml.

Hb1 - 0.1 mg/ml

Hb2 - 0.4 mg/ml

Hb3 - 0.8 mg/ml

Hb4 - 1 mg/ml

Hb5 - 2 mg/ml

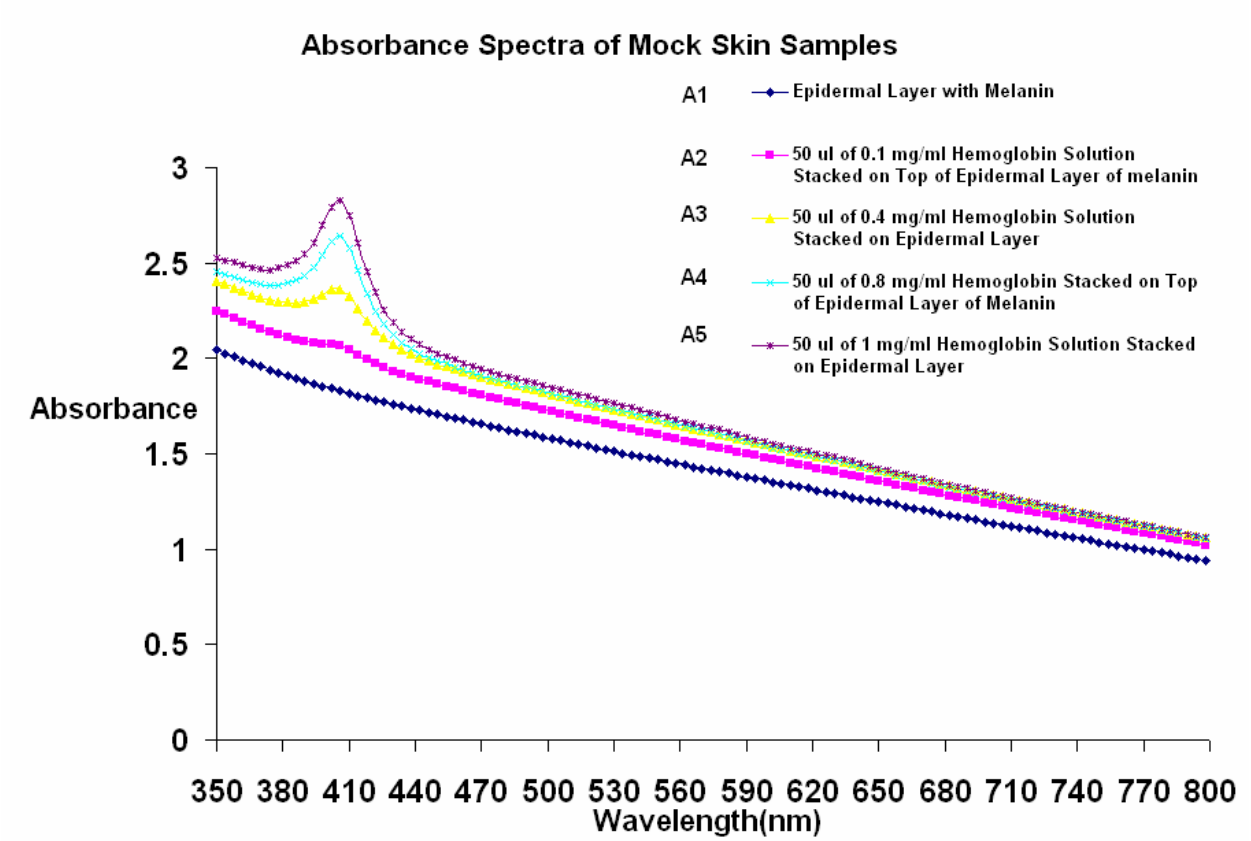

Figure 12 absorbance spectra of different concentration of hemoglobin stacked on melanin layer

In Figure1 the absorbance spectrum of epidermal layer has a fixed concentration of melanin which is represented as A1. Hemoglobin solution each of 50uL with concentrations in the range of 0.1 to 2 mg/ml is pipetted.to on top of the solid layer. The absorbance spectrum of A2 has a hemoglobin concentration of 0.1 mg/ml and an absorbance peak is absorbed at 410 nm and as the concentration of hemoglobin solution is increased with 0.4 mg/ml in A3,0.8 mg/ml in A4,1 mg/ml in A5,the absorbance peak at 410 nm has increased. As hemoglobin and bilirubin is the primary absorbing substances or chromophores in the dermal layer of the skin five different concentrations of bilirubin solution are stacked on the melanin-gelatin solid layer with a fixed volume of hemoglobin solution of a known concentration.

Absorbance measurements were taken for a single concentration of melanin-gelatin layer and a fixed concentration of hemoglobin and varying concentration of bilirubin. As the bilirubin concentration has increased there is a significant increase in the absorbance in the range of 400 - 460 nm, which is the bilirubin absorption range. As the concentration of Bilirubin increased the Absorbance values at around 410 nm has increased in spite of a fixed concentration of hemoglobin in each of the wells due to overlapping absorbance spectrum of bilirubin. The bilirubin concentrations used are in the range of 1 mg/dl to 20 mg/dl and the notation used for each concentration is as follows

Br1 - 1 mg/dl

Br2 - 5 mg/dl

Br3 - 10 mg/dl

Br4 - 15 mg/dl

Br5 - 20 mg/dl

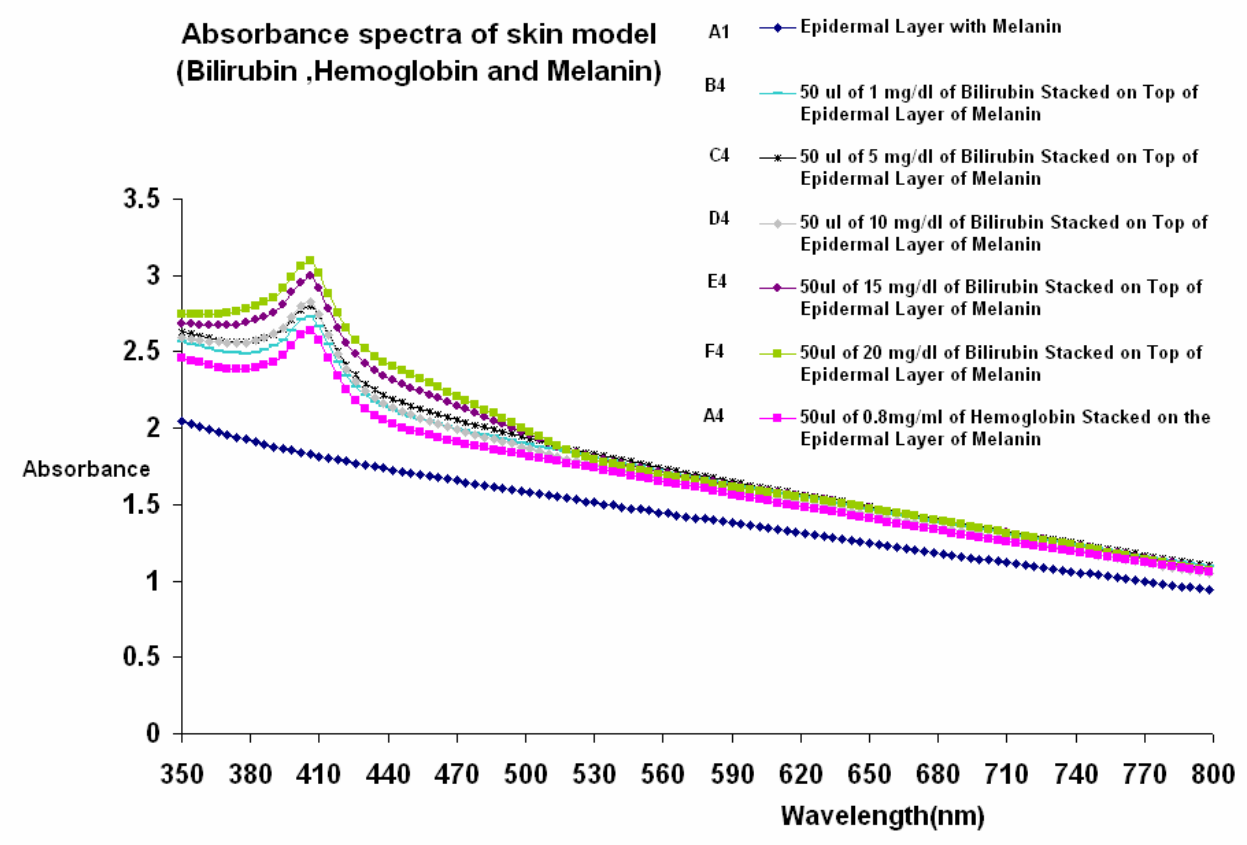

Figure 2 Absorbance spectra of skin model of different concentrations of bilirubin stacked on top of epidermal layer of melanin along with a fixed concentration of hemoglobin

In Figure 2 the absorbance spectrum of epidermal layer has a fixed concentration of melanin which is represented as A1.A4 is the absorbance spectrum of the well with 50uL of hemoglobin solution of concentration 0.8 mg/mL stacked on top of the epidermal layer. The absorbance spectrum of B4 has 50uL of hemoglobin solution with concentration of 0.8 mg/mL and 50uL of bilirubin solution with concentration of 1 mg/dl pipetted on the epidermal layer. The absorbance spectrums of C4, D4, E4, and F4 have bilirubin concentration of 5,10,15,20 mg/dL in each well along with hemoglobin solution of 0.8mg/mL respectively with bilirubin and hemoglobin solutions each of 50 uL.

8.3 Spectral Data Generation

The 96 well micro plates are used in the formation of a 6*6 matrix. Two 6*6 matrices are formed which uses 72 wells of a 96 well matrix. The remaining 24 wells are used to analyze the optical properties of the chromophores to test the Beer's Law. The architecture of a 96 well micro plate is as shown in figure 3. Each well is assigned the name corresponding to the row associated with it and followed by the column number corresponding to the well. The top left corner well is assigned as A1.

The Table 1 is formed to study the absorbance spectra of mock skin model. The mock skin model is formed with a uniform concentration of melanin in each of the wells and varying hemoglobin and bilirubin concentrations. The hemoglobin concentrations are increasing from 0.1 mg/mL (Hb1) to 2 mg/mL (Hb5) along the horizontal direction. The bilirubin concentrations are increasing from 1mg/dl to 20 mg/dL in the vertical direction. The alphanumeric characters inside the boxes at the intersection of a particular row and column are the absorbance spectra of mock skin sample with the concentrations of hemoglobin corresponding to the column head and concentration of bilirubin corresponding to the row head. The melanin concentration is uniform (0.05%)

Each 6*6 matrix consumes 36 wells. Two 6*6 matrices are prepared in this process in a single 96 well micro plate. The experiment of preparing mock skin spectra on 96 well micro plates is replicated 3 times i.e. the samples are prepared on different days.

Table 1 Matrix formed as a part of studying the optical absorbance spectra of mock skin samples

	Hb0	**Hb1**	**Hb2**	**Hb3**	**Hb4**	**Hb5**	**Hb0**	**Hb1**	**Hb2**	**Hb3**	**Hb4**	**Hb5**
Br0	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Br1	B1	***B2***	***B3***	***B4***	***B5***	***B6***	***B7***	***B8***	***B9***	***B10***	***B11***	***B12***
Br2	C1	***C2***	***C3***	***C4***	***C5***	***C6***	***C7***	***C8***	***C9***	***C10***	***C11***	***C12***
Br3	D1	***D2***	***D3***	***D4***	***D5***	***D6***	***D7***	***D8***	***D9***	***D10***	***D11***	***D12***
Br4	E1	***E2***	***E3***	***E4***	***E5***	***E6***	***E7***	***E8***	***E9***	***E10***	***E11***	***E12***
Br5	F1	***F2***	***F3***	***F4***	***F5***	***F6***	***F7***	***F8***	***F9***	***F10***	***F11***	***F12***
	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Table1 shows the procedure used to name the wells. The spectral scans of all the wells in the micro plate are recorded. The spectral scans recorded are one of the inputs to the model. Using the model developed in chapter 7 the concentration of bilirubin present in each spectral scan is predicted.

8.4 Implementation of the Algorithm

The algorithm developed in Chapter 7 is implemented on the spectral data generated.The goal of the algorithm development is the creation of a model which takes as input the absorbance spectra of a mock skin sample generated as a part of skin model and predict the concentration of bilirubin present in the sample.

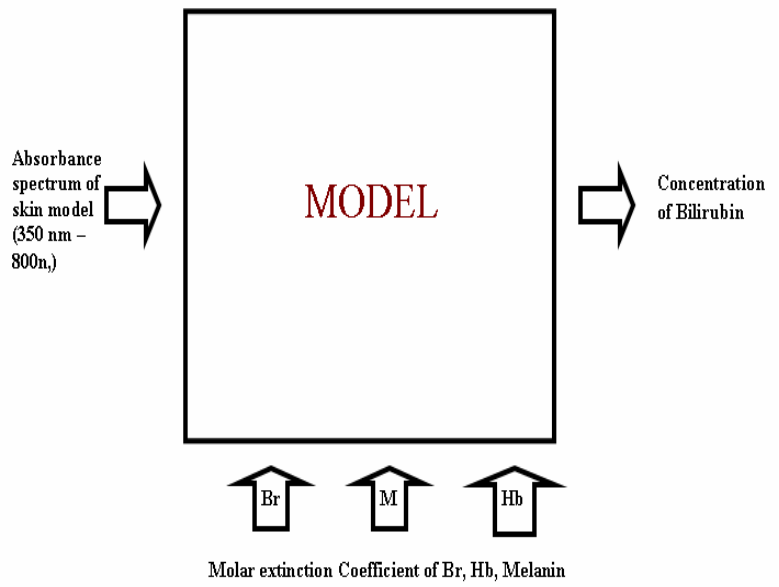

Figure 3 The block diagram of the system used to predict the bilirubin concentration which is the output of the system, inputs being the absorbance spectrum of mock skin samples and the molar extinction coefficient spectra of Br(bilirubin),M(melanin) and Hb(hemoglobin).

8.5 Stages of Algorithm Implementation

An explanation of the algorithm is described in chapter 7.The flow of the algorithm is explained using the data collected earlier. In the 6*6 matrix, we use the 5*5 spectral data information as these spectral data have all the three chromophores i.e. melanin, hemoglobin and bilirubin. A calibration model is built to establish a relationship between regression coefficients and concentrations present in the samples of skin model

8.5.1 Step 1

The spectral scan of the wells B2 to B6, C2 to C6, D2 to D6, E2 to E6 and F2 to F6 are the input dataset to the calibration model. The reason for choosing the data sets of these wells in the micro plate is that these wells have all the three chromophores. With the Absorbance spectrum of each of the well in the wavelength region of 350 to 800 nm as the response variable and the molar extinction coefficients of all the three chromophores (melanin, hemoglobin and bilirubin) as independent variables, the regression coefficients are calculated. A set of four regression

coefficients are generated for each spectral scan. The regression coefficients generated are related to the concentration of each of the chromophores present in the sample. These regression coefficients form the basis for step 2 in the algorithm. The experiment of generating Table 1 is repeated on three different days to generate three data sets using which a calibration model is built. The regression analysis performed on three data sets generated on three different days and the regression coefficients generated for each data set i.e. a spectral scan of 36 wells are tabulated in table2, 3 and 4.

Table 2 shows the regression coefficients generated corresponding to each well .The relation between regression coefficient and concentration of a particular chromophores present in the mock skin samples is established.

	Hb	M	Br
A1	1.46E-07	0.001	-1.09E-05
A2	7.38E-07	0.001	-1.25E-05
A3	2.02E-06	0.001	-1.47E-05
A4	4.45E-06	0.001	-1.90E-05
A5	5.61E-06	0.001	-2.07E-05
A6	1.20E-05	0.001	-3.14E-05
B1	2.27E-07	0.001	-1.06E-05
B2	6.21E-07	0.001	-1.12E-05
B3	2.42E-06	0.001	-1.35E-05
B4	3.23E-06	0.001	-1.53E-05
B5	4.98E-06	0.001	-1.83E-05
B6	8.57E-06	0.001	-2.46E-05
C1	5.67E-07	0.001	-3.25E-06
C2	1.17E-06	0.001	-4.54E-06
C3	2.12E-06	0.001	-5.55E-06
C4	4.09E-06	0.001	-9.18E-06
C5	4.95E-06	0.001	-1.03E-05
C6	9.20E-06	0.001	-1.66E-05
D1	1.06E-06	0.001	2.96E-06
D2	1.78E-06	0.001	-3.68E-08
D3	3.40E-06	0.001	-9.37E-06
D4	5.47E-06	0.001	-1.19E-05
D5	6.11E-06	0.001	-1.12E-05
D6	1.01E-05	0.001	-1.49E-05
E1	1.36E-06	0.001	1.78E-05
E2	2.12E-06	0.001	1.60E-05
E3	3.39E-06	0.001	1.62E-05
E4	5.44E-06	0.001	1.42E-05
E5	6.61E-06	0.001	9.41E-06
E6	1.20E-05	0.001	4.33E-06
F1	1.36E-06	0.001	2.33E-05
F2	2.04E-06	0.001	2.09E-05
F3	3.60E-06	0.001	1.60E-05
F4	5.42E-06	0.001	1.27E-05
F5	6.79E-06	0.001	8.43E-06
F6	1.15E-05	0.001	8.49E-06

Table 2 has columns headed with Hb, M and BR which stand for hemoglobin, melanin and bilirubin respectively. The rows are headed with the names of the wells in the 96 well micro-plate shown in table1.The method adopted to generate the dataset tabulated in table 2,3 and 4 is explained in the Appendix 2. As mentioned in step 1, a regression analysis is performed on the spectral scan on each well and a set of regression coefficients corresponding to hemoglobin, melanin and bilirubin are generated. The values listed for each row i.e. each well under the column header Hb correspond to regression coefficient term related to hemoglobin. In the same manner the values listed under the column named M correspond to regression coefficient term related to melanin and similarly for the column listed under Br corresponds to regression coefficient related to bilirubin. The regression analysis procedure is adopted on each well.

Table 3 shows the regression coefficient corresponding to each of the 36 mock skin samples prepared on a day2 as a part of the study

	Hb	M	Br
A1	2.33E-07	0.001	-1.19E-05
A2	8.40E-07	0.001	-1.36E-05
A3	2.49E-06	0.001	-1.68E-05
A4	4.74E-06	0.001	-2.04E-05
A5	6.01E-06	0.001	-2.27E-05
A6	4.86E-06	0.001	-1.49E-05
B1	3.02E-07	0.001	-1.24E-05
B2	8.48E-07	0.001	-1.33E-05
B3	2.42E-06	0.001	-1.56E-05
B4	4.73E-06	0.001	-2.03E-05
B5	6.09E-06	0.001	-2.29E-05
B6	4.93E-06	0.001	-1.53E-05
C1	2.73E-07	0.001	-8.60E-06
C2	6.08E-07	0.001	-1.03E-05
C3	2.32E-06	0.001	-1.16E-05
C4	4.61E-06	0.001	-1.6E-05
C5	5.98E-06	0.001	-1.81E-05
C6	4.89E-06	0.001	-1.23E-05
D1	1.62E-07	0.001	1.09E-06
D2	1.16E-06	0.001	-6.30E-06
D3	2.73E-06	0.001	-1.12E-05
D4	5.46E-06	0.001	-1.64E-05
D5	6.88E-06	0.001	-1.73E-05
D6	5.64E-06	0.001	-1.39E-05
E1	3.23E-08	0.001	8.08E-06
E2	8.32E-07	0.001	5.68E-06
E3	2.55E-06	0.001	1.73E-06
E4	5.05E-06	0.001	-2.04E-06
E5	6.57E-06	0.001	-3.27E-06
E6	5.50E-06	0.001	1.93E-06
F1	-1.15E-07	0.001	1.88E-05
F2	7.31E-07	0.001	1.85E-05
F3	2.49E-06	0.001	1.27E-05
F4	5.11E-06	0.001	7.72E-06
F5	6.61E-06	0.001	5.91E-06
F6	5.68E-06	0.001	1.06E-05

The data set generated in Table3 is the result of the regression analysis performed on the spectral scans of the wells in the 6*6 matrix generated using Table1. The set of three regression coefficients related to Hb, M and BR are generated and analyzed further in step2 of the algorithm.

Table 4 shows the regression coefficient corresponding to each of the 36 mock skin samples prepared on a day3 as a part of the study

	Hb	M	Br
A1	2.34E-07	0.001	-7.25E-06
A2	7.72E-07	0.001	-7.78E-06
A3	3.03E-06	0.001	-1.26E-05
A4	5.22E-06	0.001	-1.57E-05
A5	6.64E-06	0.001	-1.78E-05
A6	1.37E-05	0.001	-2.60E-05
B1	2.84E-07	0.001	-7.20E-06
B2	8.49E-07	0.001	-8.36E-06
B3	2.61E-06	0.001	-1.20E-05
B4	4.46E-06	0.001	-1.34E-05
B5	6.19E-06	0.001	-1.61E-05
B6	1.30E-05	0.001	-2.59E-05
C1	9.08E-08	0.001	7.93E-06
C2	9.70E-07	0.001	3.98E-06
C3	2.54E-06	0.001	1.16E-06
C4	4.36E-06	0.001	8.62E-07
C5	6.00E-06	0.001	-6.05E-07
C6	1.23E-05	0.001	-9.54E-06
D1	-4.53E-08	0.001	8.56E-06
D2	8.56E-07	0.001	4.35E-06
D3	2.43E-06	0.001	2.69E-06
D4	4.45E-06	0.001	2.08E-06
D5	6.02E-06	0.001	-1.17E-06
D6	1.22E-05	0.001	-7.12E-06
E1	5.72E-07	0.001	4.11E-05
E2	1.72E-06	0.001	3.43E-05
E3	3.90E-06	0.001	2.36E-05
E4	6.11E-06	0.001	2.07E-05
E5	7.17E-06	0.001	2.34E-05
E6	1.42E-05	0.001	1.42E+05
F1	1.35E-09	0.001	2.56E-05
F2	1.12E-06	0.001	2.09E-05
F3	2.87E-06	0.001	1.48E-05
F4	5.43E-06	0.001	8.58E-06
F5	7.10E-06	0.001	8.89E-06
F6	1.25E-05	0.001	1.28E-06

The data set generated in Table4 is the result of the regression analysis performed on the 6*6 matrix generated using Table1. The set of three regression coefficients form the basis for the further analysis on the spectral data.

8.5.2 Step 2

As the concentrations of each of the chromophores present in each of the wells are fixed a relation is established between regression coefficient calculated and the known. concentration of

each of the chromophores present in the well. The experiments are performed with a fixed concentration of melanin (0.05% i.e. 0.5 mg/ml) and varying concentrations of hemoglobin and bilirubin. The concentration of hemoglobin and bilirubin are known for each of the wells. Using these known concentrations as response variable and the regression coefficients as independent variables a relation is generated which establishes a conversion factor between regression coefficient and concentration. The conversion factor here implies the regression coefficient generated by running a linear regression analysis between the concentrations and regression coefficients. The generation of conversion factor is done separately on bilirubin and hemoglobin.

From Table1 the data set A1, B1, C1, D1, E1, F1 are in the order of increasing concentration of bilirubin with no hemoglobin present in the sample. The data set A2 ,B2,C2,D2,E2,F2 have the concentration of bilirubin in the sample increasing from B2 to F2 with the concentration of hemoglobin fixed at 0.1 mg/mL. These regression coefficients corresponding to bilirubin are calculated on the data sets with increasing concentration of bilirubin in the sample in the vertical direction for a single concentration of hemoglobin are tabulated in Table 5. One important observation from Table 5 is that as the concentration of bilirubin is increased in the sample for a fixed concentration of hemoglobin the regression coefficient also increased in a linear fashion and a plot of the relationship between regression coefficient and concentration of bilirubin present in sample is shown in Figure 4

Table 5 Data used for the plot of Figure 4

0	A1	-1.19E-05	A2	-1.36E-05	A3	-1.68E-05	A4	-2.04E-05	A5	-2.27E-05	A6	-1.49E-05
1	B1	-1.24E-05	B2	-1.33E-05	B3	-1.56E-05	B4	-2.03E-05	B5	-2.29E-05	B6	-1.53E-05
5	C1	-8.60E-06	C2	-1.03E-05	C3	-1.16E-05	C4	-1.6E-05	C5	-1.81E-05	C6	-1.23E-05
10	D1	1.09E-06	D2	-6.30E-06	D3	-1.12E-05	D4	-1.64E-05	D5	-1.73E-05	D6	-1.39E-05
15	E1	8.08E-06	E2	5.68E-06	E3	1.73E-06	E4	-2.04E-06	E5	-3.27E-06	E6	1.93E-06
20	F1	1.88E-05	F2	1.85E-05	F3	1.27E-05	F4	7.72E-06	F5	5.91E-06	F6	1.06E-05

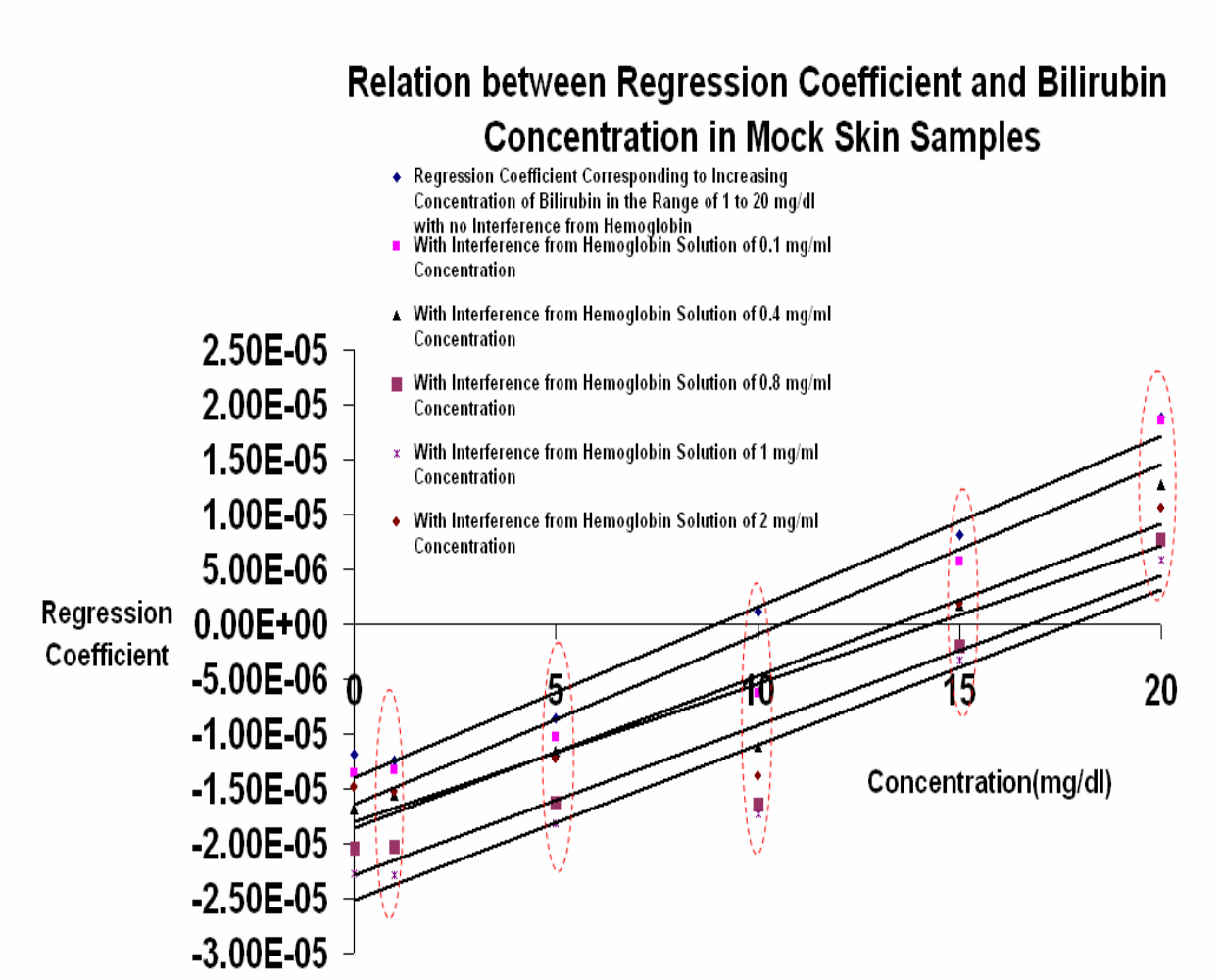

Figure 4 increase in Regression coefficient as the concentration of bilirubin increases and also the change in regression coefficient as the hemoglobin concentration increases increasing the interaction effect.

Figure 4 establishes the proof of the relationship which exists between the concentration and regression coefficients. As the concentration of bilirubin is increased from 1 to 20 mg/dl the regression coefficients corresponding to bilirubin are fitting into a second order polynomial function. As the concentration of hemoglobin is increased the regression coefficient is decreasing with the amount of decrease assumed to be proportional to the concentration of hemoglobin present in the sample. This accounts for the interference of hemoglobin in bilirubin measurements.

Table 6 Variation observed in the Bilirubin (Br) Column as the hemoglobin concentration is increasing from B2 TO B6. This accounts for the interference.

	Hb	M	Br
B1	3.02E-07	0.001	-1.24E-05
B2	8.48E-07	0.001	-1.33E-05
B3	2.42E-06	0.001	-1.56E-05
B4	4.73E-06	0.001	-2.03E-05
B5	6.09E-06	0.001	-2.29E-05
B6	4.93E-06	0.001	-1.53E-05

The dataset for Table 6 is extracted from the regression coefficients generated in table 3. Variation observed in the column headed with Br1 of datasets B1, B2, B3, B4, B5, and B6 which have the same concentration of bilirubin with increasing concentration of hemoglobin from 0.1 mg/mL to 2 mg/mL account for the interference of hemoglobin spectra on bilirubin spectra. The increase in the regression coefficients under column headed Hb bears a relationship to the concentration of hemoglobin present in the sample. As the concentration of bilirubin is constant there is slight deviation in the regression coefficients calculated for bilirubin. This deviation is expected due to interference of hemoglobin concentration on bilirubin.

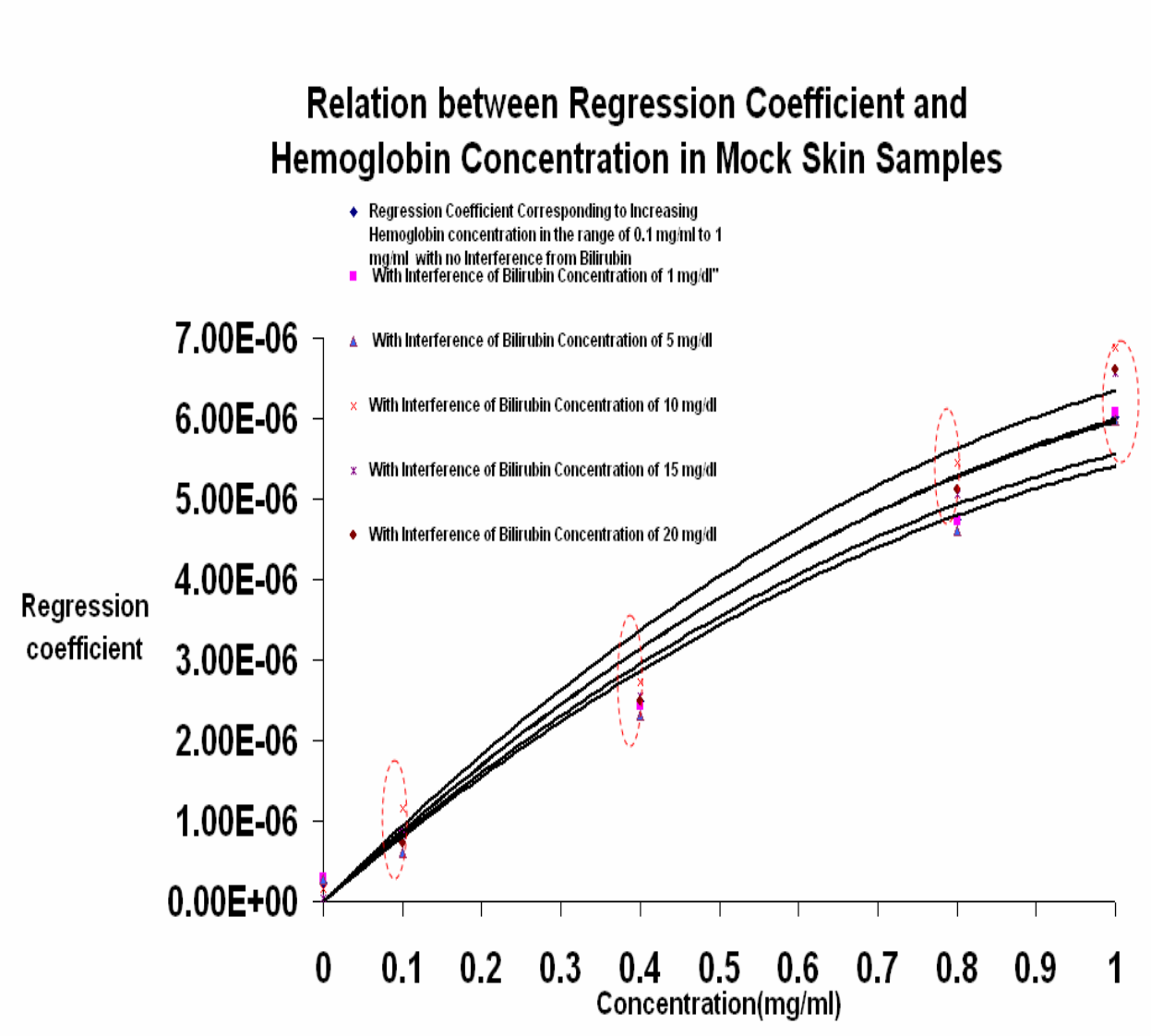

Figure 5 Increase in Regression coefficient as the concentration of hemoglobin increases and also the change in regression coefficient as the bilirubin concentration increases accounting for the interaction effect

Figure 5 establishes the relationship which exists between the concentration and regression coefficients. As the concentration of hemoglobin is increased from 0.1 to 1 mg/mL the regression coefficients are increasing in a linear fashion. As the concentration of bilirubin is increased there is a change in the regression coefficient. This accounts for the interference of bilirubin in hemoglobin measurements. A relationship is established between regression

coefficients headed under the column heading Br and concentration of bilirubin in the samples. A regression analysis is performed and a regression equation with the regression coefficient and the intercept value are shown in figure 6. The regression equation y = 441917*x+4.6097 is the relation which converts the x value which is the regression coefficients calculated in step 1 related to bilirubin.and the estimated y value is the concentration of bilirubin present in the spectral scan of the well. With a need to validate a model the next step is to test the model with input as absorbance spectra of mock skin in the wavelength range of 350 nm to 800 nm.

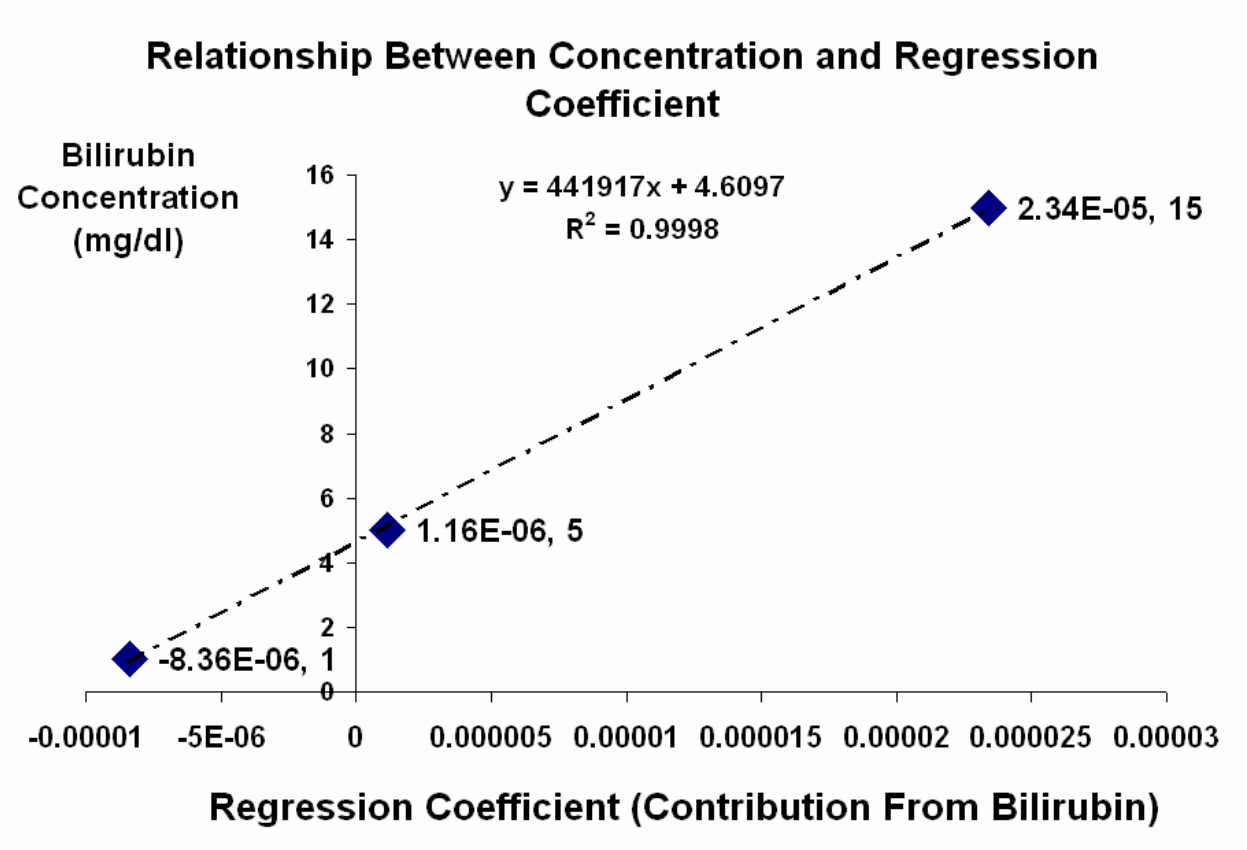

Figure 6 shows the relationship established between concentration and regression coefficients. The concentration of bilirubin present in each of the mock skin sample is known and the regression coefficients corresponding to bilirubin are calculated using the multiple regression analysis. Using the relation y = 441917*x+4.6097 where y is the concentration of bilirubin in the sample and x is the regression coefficient of bilirubin. The values of this equation are called the calibration model parameters.

A linear regression analysis is performed with the concentrations of bilirubin in the mock skin sample as the response variable (dependent variable) and the regression coefficient as independent variables. The regression analysis gave an expression which converts the regression coefficients calculated in Step 1 of the algorithm into the concentration of bilirubin in the mock skin sample.

Observations

From Figure 5 the response equation which is the slope of the line is changing as the hemoglobin concentration is changing from 0.1 mg/ml to 1 mg/ml which is not the behavior of a first order model of multiple regression analysis.This accounts for a study of interactions which necessitates for the inclusion of interactions terms as a part of the model to predict the concentrations more accurately which can take care of the nonlinearity occurring between the concentrations and regression coefficients. There is a need to introduce higher order terms in the multiple regression analysis which accounts for the interactions

8.5.3 Step 3

Once the calibration model has been developed which involves the development of conversion factor which convert the regression coefficients generated in step1 into a corresponding concentration of chromophores which are present in the sample. To validate this model, 8 random samples are picked from 30 data samples to test the validity of the model. The concentrations predicted using the calibration model i.e. the regression equation shown in figure 6 is tabulated with the actual concentrations present in the sample. The row heading indicates the well chosen for the analysis. The performance of the model is shown in figure 7 with a regression fit between the predicted concentration on x axis and the actual concentration on y

axis. Figure 8 shows a plot of predicted vs. actual bilirubin concentration for the mock skin samples prepared. The coefficient of variation R^2 value is 0.9844 which indicates a good fit.

Table 7 The performance of the model.

	Predicted Concentration	Actual Concentration
B1	1.43	1
C3	5.12	5
C4	4.99	5
C5	4.34	5
D1	8.39	10
E3	15.05	15
E4	13.76	15
E5	14.96	15

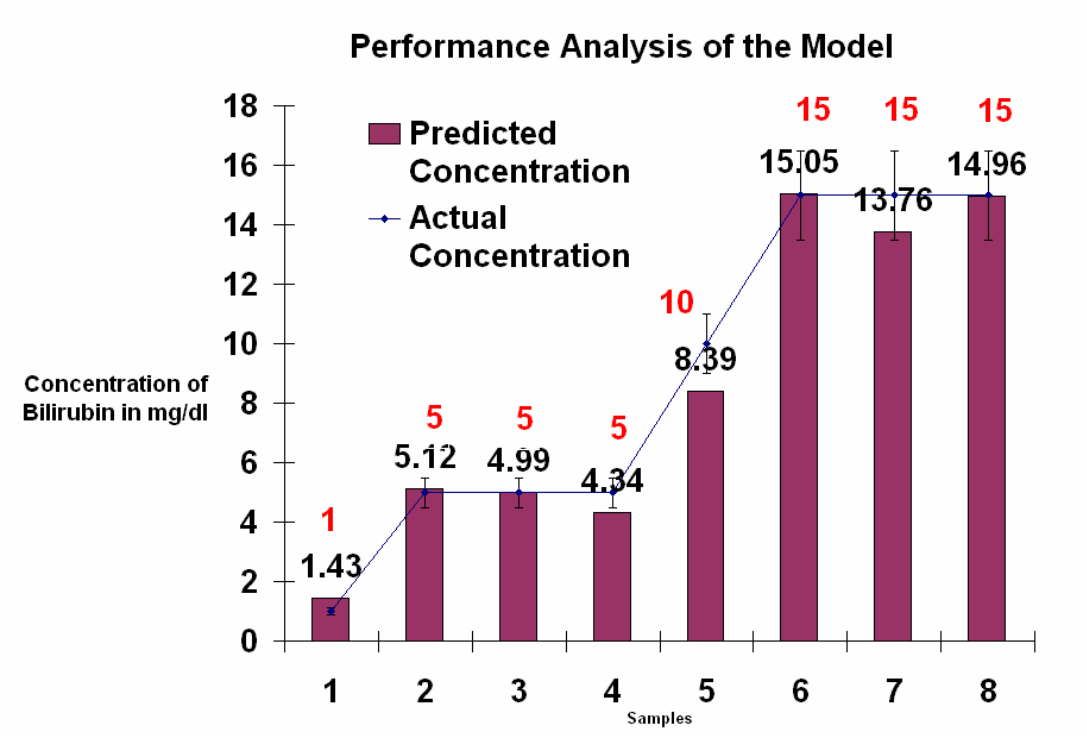

Figure 7 is the performance of the model with most of the predicted concentrations falling within 10% error from the actual concentration.

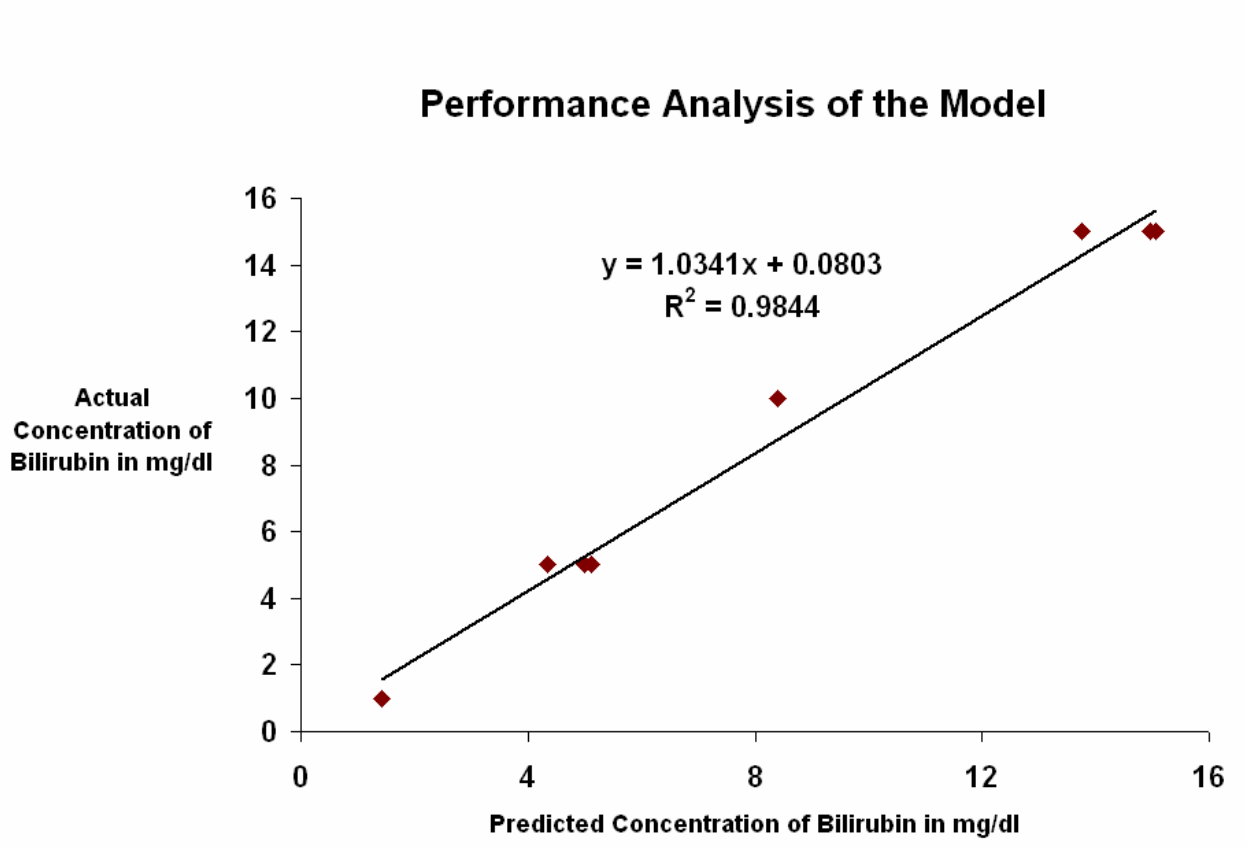

Figure 8 is the result of linear regression analysis used to establish the correlation between actual and predicted values using R^2 value.

8.6 References

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9 CONCLUSION AND FUTURE WORK

In this chapter significant results of the work and the objectives achieved at different stages starting from the process of sample preparation, spectral generation and algorithm development. This chapter outlines the future work areas for the development of a noninvasive biomedical system for quantification of bilirubin the skin sites.

9.1 Overview

In this thesis work, a novel approach has been developed to develop mock skin samples.

The process of developing a model to predict the concentration of bilirubin in a complex media like skin starts off with the sample preparation. An optimized process of developing a mock skin model with a consideration of the primary absorbing chromophores such as melanin in the epidermal layer as the base and the bilirubin and hemoglobin present in the dermis layer. A two layered structure of skin is developed in the micro well plate and using the transmission spectrometer the light interacts first with the melanin particles embedded in the gelatin matrix which form the epidermal layer and part of the light that is not absorbed interacts with the hemoglobin and bilirubin which form the dermal layer. The spectral signature generated using this two model structure has resemblance to that of the spectral signature taken on the skin site. In this thesis, in the process of developing a software platform for spectral processing of data, the novel approach of using the molar extinction coefficient of the substances as factors which determine the contribution of each of the substance present to the spectral signature of mock skin which is a mixture of substances like melanin, hemoglobin and bilirubin. A factorial design of experiments are conducted for a study on the interactions between bilirubin and melanin and bilirubin and hemoglobin.

9.2 Algorithm Development

In the process of developing a software platform to process the raw spectral data to extract the information of bilirubin signal in the presence of noises like hemoglobin and melanin. In this thesis multiple regression analysis model is used to establish a relation between the regression coefficients (extracted from the relationship between absorbance spectra and molar extinction coefficients of the chromophores which constitute the mock skin model) and the concentrations of the chromophores which are present in the spectra. This part of the model is called the calibration model and to validate the model developed we test it on different spectra generated. In this thesis a linear relationship is observed between the regression coefficients corresponding to bilirubin and bilirubin concentrations in the mock samples of skin. This linear relationship is affected by the presence of hemoglobin solution which concludes that there are interferences by the hemoglobin on extracting the spectral signature. The deviation in the regression coefficients in absence and presence of hemoglobin have a linear relationship with the increase in hemoglobin concentration which are explored in Chapter8.This suggests for an improvement in the calibration model which can account for the nonlinearity and interferences by introduction of higher order terms which take care of the interactions. A more extensive study on the interactions between hemoglobin and bilirubin is needed which has to be explored as a part of the future work.

9.3 Future Work

As the concern for the kernecticurus which is bilirubin induced brain neural disorders is increasing due to lack of continuous monitoring of bilirubin and also due to early discharge of babies from Neonatal care unit. The concern for kernecticurus is more of concern in preterm

babies. Bilirubin which is of two forms of unconjugated bilirubin i.e. one which is bound to the protein molecule albumin and one which is unbounded to protein has different spectral signatures. The free bilirubin (unconjugated bilirubin not bound to albumin) is a primary indicator for the onset of kernecticurus. The future work involves the research in the field of exploring the spectral signatures of the two forms of bilirubin with varying amounts of albumin present in the system. The future work involves the development of a robust noninvasive system for measuring the diffuse reflectance of the skin. Different optical methods of extracting the information present at different layers of the skin are to be explored. As the absorbing chromophores or pigments like melanin, bilirubin and hemoglobin are present at different sites in the skin i.e. at different layers from the skin surface. To extract the information of melanin which is present in the epidermal layers i.e. the top 100 um of the skin the distance between the light illumination probe and light collection probe should be order of 0.5 mm.In the case of extracting the information of bilirubin which is different compartments, it is present along with the blood and also outside the vascular system in the skin. So an optical setup has to be designed to extract the spectral information contributed by bilirubin in different compartment to the overall spectral signature of the skin. The optical setup of reflectance measurements opens a new area of research in the field of forensics. Using the optical setup reflectance measurements of the bruise helps us to predict the age of the bruise. Extracting the spectral information on different days from the bruise has formed a calibration model would be developed to predict the age of bruise. With the interactions and interferences taken care of in the software part and with a robust optical setup a novel biomedical photonic system for quantification of bilirubin present in the skin and blood can be estimated.

Appendix 1

R Software Analysis for 2^2 factorial designs

The following is a set of protocol developed for studying the interactions

Data are from a 2X2 design with the factors being bilirubin and hemoglobin. Read the data file containing this data.

Reading the excel file in a .csv format from working directory

Step 1. data = read. csv ("Analysis1.csv")

> Data

	B	M	Absorbance at 432 nm
1	1	0.25	0.339
2	10	0.25	2.126
3	1	1.00	1.189
4	10	1.00	2.493

Once the data is read, names are assigned to the factors whose interactions we are studying.

Step2 B=factor (B)

M=factor (M)

Using the factors B for bilirubin and M for melanin.

Two levels for Bilirubin (1 – low and 10 – high) and two levels for Melanin (0.25% - low and 1 % -high) are used for this study.

There are two independent variables in this design separated by an asterisk *. The asterisk indicates to R that the interaction between the two factors is interesting and should be analyzed. Run the analysis on the interactions

Step 3 Analysis=aov (A~B*M)

The first argument inside the parenthesis is always the dependent variable (Absorbance). It is followed by the tilde symbol (~) and the independent variables. Analysis is the name of the structure you want the analysis to store.

Step 4 Analysis

Call:

aov(formula = A ~ B * M)

Terms:

	B	M	B: M
Sum of Squares	2.3885703	0.3702722	0.0583223
Deg. of Freedom	1	1	1

Estimated effects may be unbalanced

Step 5 Summary (analysis)

The results of the ANOVA can be seen with the summary command:

	Df	Sum Sq	Mean Sq
B	1	2.38857	2.38857
M	1	0.37027	0.37027

B: M 1 0.05832 0.05832

Step 6 SSx=c (2.38857, 0.37027, 0.05832)

SSx is the data set which has the variation due to each factor

Step 7 SST=sum (SSx)

SST is the total variation as a sum of all the individual variations of each of the factors are considered

Step 8 Percent. Variation = 100*SSx/SST

The contribution of each of the factor is calculated using the formula (SSx/SST)*100

Step 9 Percent. Variation

84.78645 13.14338 2.07017

Appendix 2

The following analysis is the set of protocols developed for calculating the regression coefficient of each mock skin sample spectrum

Regression analysis is performed on each well of the 96 well micro plate. The inputs to the regression model are molar extinction coefficients of melanin, bilirubin, hemoglobin and the absorbance spectrum of each well.

Well name: B4

The input variables are listed under Variables Entered which are

MEL – Melanin

BIL – Bilirubin

HAE – Hemoglobin.

The dependent variable is the absorbance spectrum of the mock skin sample in the well B4 in the wavelength range

Variables Entered/Removed[b]

Model	Variables Entered	Variables Removed	Method
1	MEL, BIL, HAE[a]	.	Enter

a. All requested variables entered.

b. Dependent Variable: B4

Summary of the model

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.941[a]	.885	.882	.158620

a. Predictors: (Constant), MEL, BIL, HAE

ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	21.070	3	7.023	279.149	.000[a]
	Residual	2.742	109	.025		
	Total	23.813	112			

a. Predictors: (Constant), MEL, BIL, HAE

b. Dependent Variable: B4

SIG

The ANOVA also signifies a sig column here again explaining the significance of the model again with alpha as .05. We go on to test for the significance of the model.Any value <=.05 tells us that the model is significant, in other words there is a significant relationship between the variables in the model and any value >.05 tells us vice versa.

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	.071	.112		.634	.527	-.151	.293
	HAE	4.734E-06	.000	.389	5.136	.000	.000	.000
	BIL	-2.03E-05	.000	-.166	-2.212	.029	.000	.000
	MEL	.001	.000	.735	10.948	.000	.001	.001

a. Dependent Variable: B4

ALL 3 ARE SIG

From the above table named the coefficients, the column headed with B in the unstandardized coefficients are coefficients corresponding to HAE which is hemoglobin, BIL refers to bilirubin and MEL refers to melanin. The coefficients at the intersection of the row header named HAE and column header named B is the value of regression coefficient related to hemoglobin and signifies the contribution of hemoglobin extinction coefficient to the absorbance spectrum of the well B4. In the same way the regression coefficients related to bilirubin and melanin are listed in the column under B with row heading BIL and MEL respectively.

The same process is repeated for the regression coefficients for each well listed in the 96 well micro plates. The Tables 2,3 and 4 are tabulated using the data generated in the coefficients section of the analysis of each well.