AN INTRODUCTION TO REFLECTANCE SPECTROSCOPY FOR DRY PHASE REAGENT CHEMISTRY

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INTRODUCTION

Many of the diagnostic tests produced by the Miles Diagnostics Division rely on the principles of reflectance. In this article, there is a brief overview of some aspects of reflectance spectroscopy which are relevant to dry phase reagent strip measurements. An exact theoretical treatment of reflectance can be found, for example, in Kortüm (1). Aspects of reflectance instrumentation and radiometry are discussed by Grum and Becherer (2).

In analytical chemistry, we have become accustomed to absorption spectroscopy. According to Beer's Law, the amount of light transmitted through a sample at a particular wavelength can be related to the concentration of a species within a solution. In reflectance spectroscopy, we use the amount of light reflected off our reagent strips to measure the concentration of a substance in a blood or urine sample. For example, the GLUCOSTIX® and GLUCOFILM® Reagant Strips are used for measurement of blood sugar by the diabetic, the MULTISTIX® Reagent Strips for measurement of multiple components of urine in hospital laboratories, and the various SERALYZER® Reagent Strips for measurement of blood components by the physician. The instruments used for making these measurements are all based upon reflectance.

Typically, a specimen is applied to a dry reagent strip. Through a reaction involving the component of interest, some species is generated or destroyed which alters the amount of light absorbed at each wavelength. The change in absorbed light within the reacting strip causes the amount of reflected light to change. A light source illuminates the reagent strip in a fixed geometry. The light reflected by the sample towards the detector is measured in a limited wavelength range. The reflected light signal is digitized and processed numerically through an equation or algorithm which relates reflectance to concentration.

Although this article does not present methods and techniques necessary for optimization of chemistry or instrument performance, there are several factors which are important in a measurement of reflectance. We will consider these next.

A major difference between absorption and reflectance spectroscopy is that a measurement of absorption can usually be performed with effective elimination of any signal from scattering sources such as cell walls. In reflectance spectroscopy, the detected light is entirely scattered light.

When you look around, consider what you see. As light enters the eye, it comes either directly from a source of light or indirectly through reflectance or, more generally, scatter. There are relatively few direct light sources in our environment: the sun, the stars, light bulbs, LED's, CRT screens, lasers, and fires account for the majority of them. Most of what we see is via reflected or scattered light.

CHARACTERIZATION OF SCATTERED LIGHT

How do we characterize reflected light? When we use an instrument such as the GLUCOMETER® II Reflectance Photometer to measure the GLUCOSTIX reagent, we get a measure of the reflectance of that reagent pad, that is, one number. Is there just a single reflectance which characterizes a surface or does the GLUCOMETER II Instrument give just one measure among many?

Most things we see are colored. This indicates that a proper treatment of reflectance must account for its variation with wavelength. The GLUCOMETER II Instrument uses an interference filter at 736 nm to select the wavelength, just outside the visible range of 400 nm to 700 nm. Using any other wavelength filter will generally give different results.

When fluorescence or phosphorescence occurs, the wavelength leaving the object is generally quite different from the wavelength striking the object. In our normal environment, this is very common. Brighteners added to paper and textile products as well as laundry powders usually work through absorption of environmental UV radiation with fluorescence in the blue. For our present reagent strips, fluorescence and phosphorescence do not usually occur at the illumination wavelengths used.

Another characteristic of materials which is important in reflectance spectroscopy is glare or specular scattering. Materials such as mirrors exhibit

almost exclusively specular scattering. That is, light is reflected off the surface at the same angle as the incident light. This is illustrated in Figure 1.

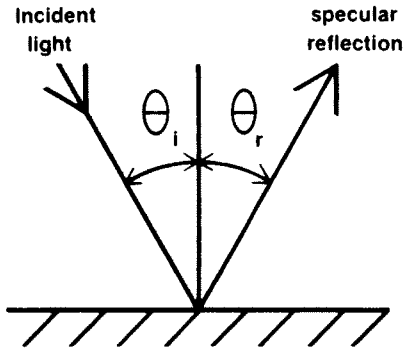

Figure 1. For specular reflections, incident angle θ_i equals reflected angle θ_r.

Specular scattering must be taken seriously because there is always some specular light reflected off the liquid/air interface on a urine (MULTISTIX) or blood chemistry (SERALYZER) strip. In general, the specular light contains no information about the concentration of the substance being analyzed and it is therefore undesirable to allow the specularly scattered light to reach the detector.

Other materials exhibit almost pure diffuse scattering. These include some papers and powders. Perfect diffuse scattering follows the Lambert Law. This kind of scattering is independent of the illumination angle. In addition, the intensity of the scattered light falls off with the cosine of the angle relative to the sample normal. This is illustrated in Figure 2.

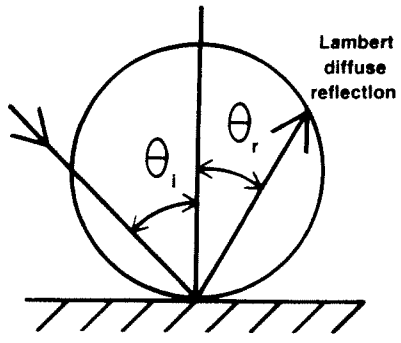

Figure 2. For a surface characterized by the Lambert Law, diffuse reflections are independent of incident angle θ_i and proportional to the cosine of the reflected angle θ_r, as indicated by the length of the arrow.

Most materials are characterized by neither pure specular scattering nor pure Lambert Law diffuse scattering. There is some glare associated with most surfaces, making the non-Lambertian scattering a function of illumination angle. This type of scattering is illustrated on Figure 3 for a typical drawing paper. The conclusion is that the dependence of reflectance on illumination angle and scattering angle must be explicitly considered and experimentally determined.

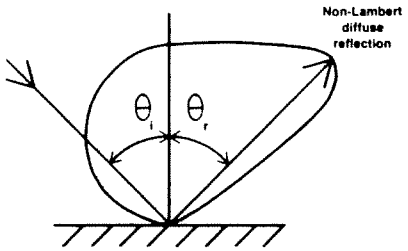

Figure 3. For a typical dry drawing paper, there is glare such that the scattering at reflected angle θ_r is a maximum when it equals incident angle θ_i.

A final observation to consider is that when a surface such as paper or a translucent plastic is illuminated with a narrow beam of light such as a laser, other parts of the surface are sometimes seen to light up. There is scattering within the material which causes light entering at one location to leave at another.

Wavelength, angle and displacement are thus three factors of importance when considering how light is reflected off a surface. As a first approximation, we will ignore fluorescence. Note that variables such as time and temperature have also been ignored. They may be important for a clinical assay but are not essential for our discussion.

The function which describes how light as a function of incident wavelength, angle and displacement is reflected at the same wavelength, into all angles and from all positions was first developed by Nicodemus et al. (3). It is called the Generalized Bidirectional Reflectance Distribution Function. When subsurface scatter is ignored or does not occur, the function is simplified and known as the Bidirectional Reflectance Distribution Function or BRDF. There is not just one number which characterizes the reflectance of a surface. There is a function. In addition, virtually all measures of reflectance can be obtained from the BRDF since it contains all scattering information about the surface.

When an instrument measures reflectance, it has been shown that the radiant power scattered off a surface to a detector is the product of the BRDF, the incident power reaching the sample, and a geometric factor which accounts for sample orientation and for distance to the detector (4). Different geometries produce a measure of different reflectance functions primarily because the BRDF is not a constant. Therefore, reflectance measurements can not generally be compared between instruments using different wavelengths or having different geometries.

Measured BRDFs are unknown for most surfaces and are difficult to obtain. Improved instrumentation is now under development to allow determination of reagent strip BRDF values at Miles.

CALCULATION OF CLINICAL CONCENTRATIONS

How does the measurement of reflectance allow us to determine the concentration of a substance in blood or urine? In absorption spectroscopy, it is not the transmission T which is most closely related to the concentration of a substance, but rather the optical density (OD = $\log(T/T_o)$ where T_o is the transmission at concentration = 0.)

Likewise, in reflectance spectroscopy it is not the reflectance R which is most closely related to concentration, but rather the Kubelka-Munk K/S function which is defined as follows:

$$K/S = \frac{(1 - R)^2}{2R}$$

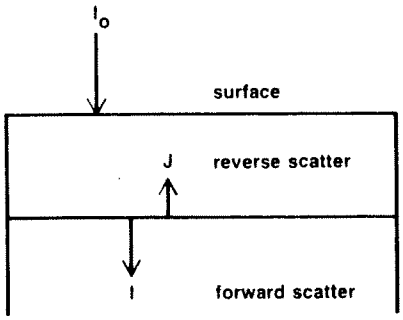

Figure 4. For the Kubelka-Munk model, light of intensity I_o strikes the surface of a sample. The sample is analyzed as of an infinite number of parallel planes, each characterized by an absorption coefficient k and a scattering coefficient s, which produce a net forward scatter I and reverse scatter J.

This equation comes from a particular model of scattering, as illustrated on Figure 4. Light strikes the surface of a translucent sample. For analysis, the sample is divided into an infinite number of parallel planes, each of which is characterized by a scattering coefficient per centimeter s = (S/2) and an absorption coefficient k = (K/2). For isotropic scattering and an infinite thickness, solution of the coupled differential equations describing the system produces the equation given above.

When the Kubelka-Munk equation can be applied, concentration linearity is achieved because, (1) the scattering coefficient S is generally constant for a reagent paper, and (2) the absorption coefficent K will linearly increase with the concentration of the absorbing material. Therefore, K/S will be linearly related to concentration.

There are some instances in absorption spectroscopy where the optical density (OD) fails to be a function linear with concentration. In reflectance spectroscopy, K/S often fails to be a function linear with concentration. In each case, however, the OD and K/S functions are generally chosen to initially analyze the data.

To get an idea of why K/S can fail, let us consider a typical strip cross section. This is illustrated on Figure 5. On some reagent strips, there is a standing dome of liquid. About 3-4% of the light striking the liquid will be specularly reflected at the air-liquid interface, the other light will be refracted into the liquid. Within the dome, absorption can take place if any chromophores have entered the solution. If solid matter has entered the liquid, scattering can also occur. Once light reaches the reagent pad, it can be absorbed and scattered in accordance with the Kubelka-Munk model. However, since the pad is not infinitely thick, light can strike the tape, backing, and support table to be absorbed and/or scattered with different coefficients. With all of these events occurring, it is not surprising that the simple K/S model can fail. In addition, factors such as interfering substances, reagent formulation, and temperature can contribute to a nonlinearity in K/S.

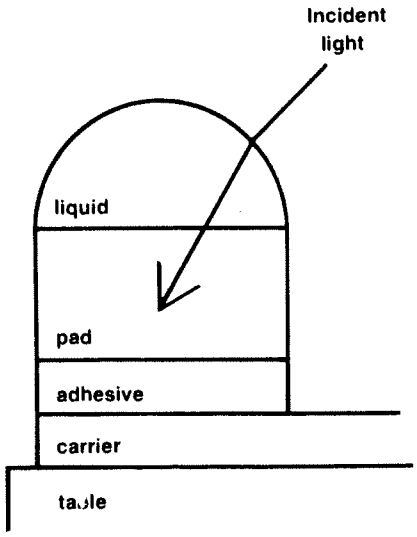

Figure 5. Cross section of a typical strip (not to scale), placed on the instrument table, with a standing drop.

Where K/S fails, functions of K/S or other functions of R can be considered for the algorithm which linearizes concentration relative to the measured reflectance. Selection of the algorithm for a clinical test involves many factors, including the spectral reflectance as a function of concentration, the sources of error in the system, and the medical fitness for use criteria.

EXAMPLES

As an example of reflectance systems, we will consider the GLUCOSTIX Reagent Strip and GLUCOMETER Instruments. In use, the blood is blotted off before this strip is placed in the instrument. A spectral reflectance scan for the GLUCOSTIX Reagent Strip at several concentration levels is shown on Figure 6. These data were collected on a Rapid Scanner reflectance instrument with angular illumination and near normal detection.

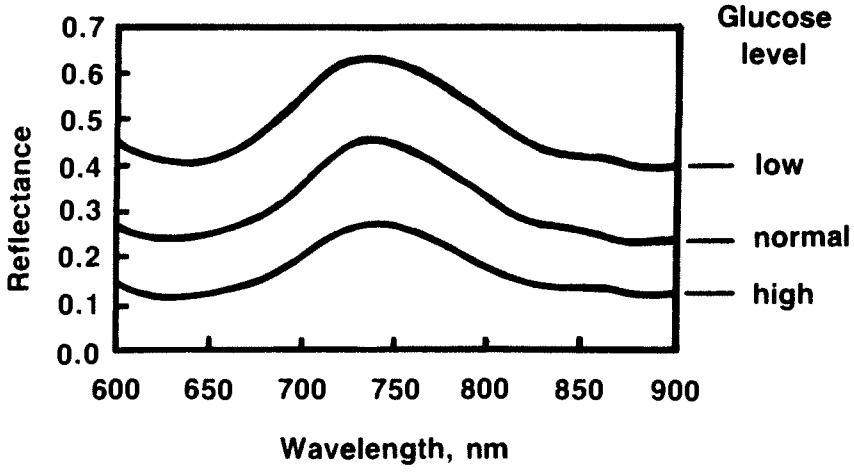

Figure 6. Reflectance measured on the Rapid Scanner is plotted as a function of wavelength for the GLUCOSTIX reagent at various concentrations.

Several different instrument configurations have been implemented to measure the GLUCOSTIX Reagent Strip. As examples let us consider the GLUCOMETER M and GLUCOMETER II Instruments. Simplified optical layouts are shown for these instruments on Figures 7 and 8, respectively.

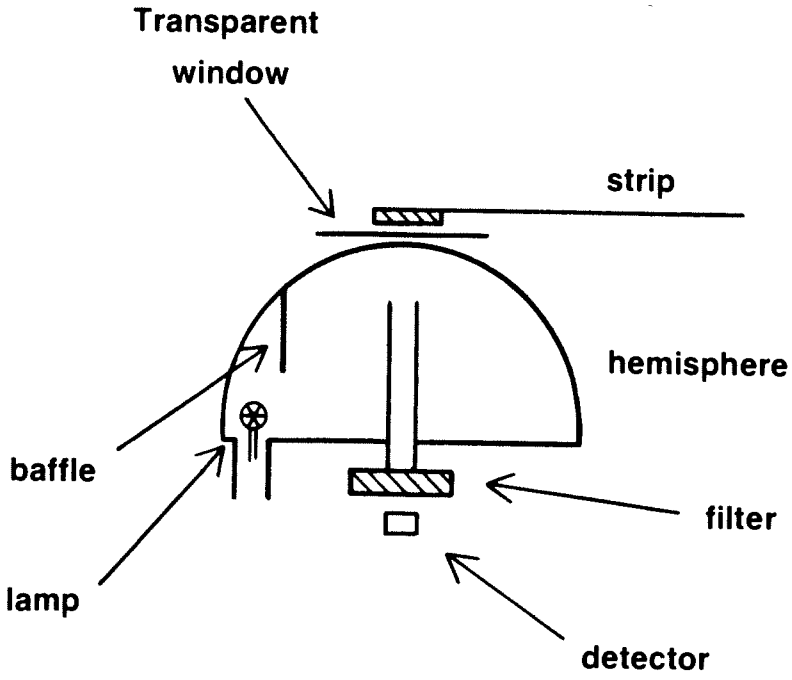

Figure 7. GLUCOMETER M optical layout.

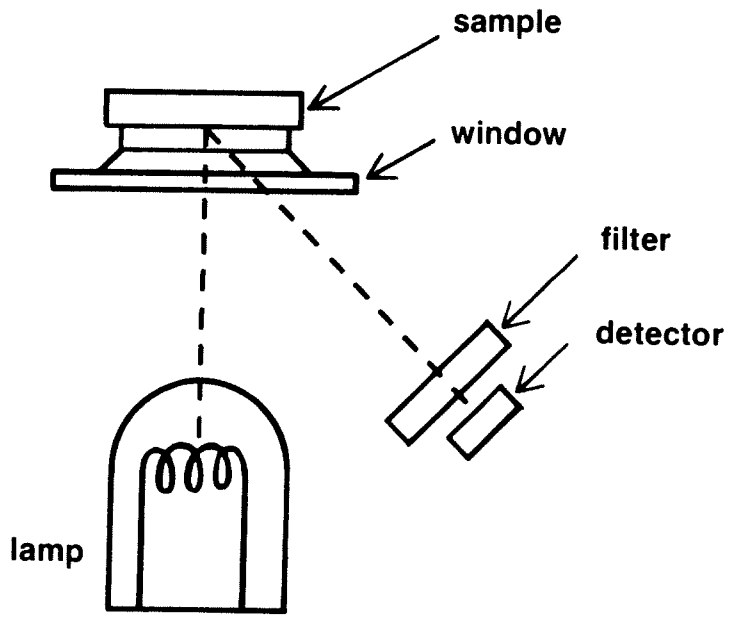

Figure 8. GLUCOMETER II optical layout.

The GLUCOMETER M Instrument uses a tungsten bulb to illuminate the walls of an integrating hemisphere, which in turn diffusely illuminate the sample. Light reflected normally from the reagent strip goes through a 736 nm filter to a detector.

The GLUCOMETER II Instrument uses a tungsten bulb to illuminate the sample along the normal, which means perpendicular to the sample surface. Light reflected at 45 degrees goes through a 736 nm filter to a detector.

The GLUCOMETER M and GLUCOMETER II use different geometries but the same wavelengths. It was found that different algorithms were required although each was linear with K/S. Different algorithms are expected since different reflectance functions of the reagent strip are being measured.

This article has identified several of the factors which play a significant role in the determination of the concentration of clinically important substances in blood and urine through reflectance spectroscopy. In particular, the BRDF and K/S functions were presented as fundamental to the understanding and interpretation of reflectance readings generated through the interaction of the chemistry, the reagent matrix, and the instrument configuration.

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Willis E. Howard, III, also known as Bill, was born in Oklahoma City in 1948, although much of his childhood was spent in Huntsville, Alabama. He obtained a B.S. from Samford University and then a Ph.D. from Northwestern University in 1976. Much of his thesis work in physical chemistry and laser spectroscopy was performed at the Technical University of Munich in Germany. Bill did postdoctoral work at the University of California in Irvine and then came to Miles in 1979. At Miles he has worked in the areas of fluorescence and reflectance spectroscopy, algorithm development, software validation and optical engineering. He is currently Chairman of the Miles Science Forum. In his free time, Bill can be found reading, listening to music, eating Chinese food, enjoying his home computer, or racing his boat up and down the river.