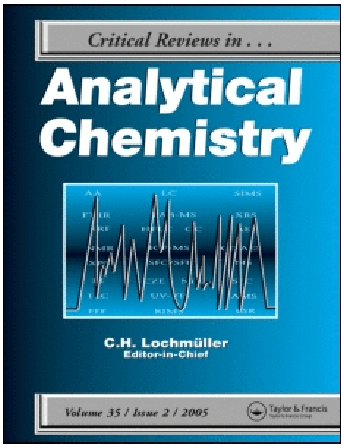

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Frei, Roland W. and Zeitlin, Harry(1971) 'Diffuse Reflectance Spectroscopy', Critical Reviews in Analytical Chemistry, 2: 2, 179 — 246

To link to this Article: DOI: 10.1080/10408347108542764

URL: http://dx.doi.org/10.1080/10408347108542764

DIFFUSE REFLECTANCE SPECTROSCOPY

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INTRODUCTION

The basic difference between transmission and reflectance spectroscopy is that in the former the spectral properties of samples are determined by transmitting monochromatic light through it, whereas in the latter technique specular or diffusely reflected light or both is collected with a suitable device mounted on the same side on which the sample has been irradiated.

Transmission spectroscopy is largely restricted to work in solution, taking advantage of the transparent nature of the medium under investigation. The absorption of dissolved substances is then measured as a function of wavelength. With turbid and colloidal systems it becomes difficult to make measurements in the transmission mode, due to light-scattering phenomena which are responsible for energy losses. Transmission techniques become entirely unsuitable for spectral investigation of substances adsorbed on solid surfaces, and it is for this type of investigation that reflectance spectroscopy can be a valuable tool.

The technique of reflectance spectroscopy is still in a stage of rapid development, but it is not as new as one might think. Diffuse reflectance spectroscopy, for example, found early application in the paper, paint, printing, dye, ceramics, and textile industries. The standard application is routine measurement of color in quality-control processes.[1] In connection with such work, the demand for more sophisticated instrumentation resulted in the design of filter reflectometers, such as those reported by Taylor[2] and Benford.[3] About ten years later the first recording reflectometer was developed by Hardy.[4] The common design feature for these instruments was a device for the collection of diffusely reflected light. This consisted of an integrating sphere, coated inside with a suitable, highly reflecting surface. This sphere was pioneered by Ulbricht[5] and is therefore also known as "Ulbrichtkugel." The potential of specular reflectance technique (in contrast to diffuse reflectance) as an analytical tool was not recognized until the early 1960s, when Fahrenfort published the first paper on attenuated reflectance spectroscopy.[6] This has now developed into an important and fairly wide field by itself and should be treated in a separate review.

Application of diffuse reflectance spectroscopy to the solution of analytical and other chemical problems has recently gained wider interest, due to the ready availability of reflectance attachments for practically all spectrophotometers on the market.

The literature in this area has undergone a rapid growth, with Kortüm being undoubtedly the most prominent contributor. His book on reflectance spectroscopy is also available in an English translation.[7] Wendlandt and Hecht[8] wrote the first comprehensive treatment on this subject, and of further interest are the published proceedings of the 1967 American Chemical Society Symposium on Reflectance Spectroscopy.[9] All these texts include a consideration of specular reflectance techniques and phenomena.

II. THEORY

A. The Kubelka-Munk Theory

The most generally accepted theory concerning diffuse reflectance and the transparency of light-scattering and light-absorbing layers has been developed by Kubelka and Munk.[10,11] It may be said that this theory has acquired an importance in the field of reflectance spectroscopy comparable to the importance of the Bouguer-Beer law in transmittance spectroscopy. Development of the theory for an infinitely thick, opaque layer yields the Kubelka-Munk equation, which may be written as

$$\frac{(1 - R'_\infty)^2}{2R'_\infty} = \frac{k}{s} \qquad (1)$$

where R_∞ is the absolute reflectance of the layer, k is its molar absorption coefficient, and s is the scattering coefficient.

Instead of determining R'_∞, however, it is customary to work with the more convenient relative diffuse reflectance, R_∞, which is measured against a standard such as MgO or $BaSO_4$. In these cases it is assumed that the k values for the standards are zero and that their absolute reflectance is one. However, since the absolute reflectance of the standards exhibiting the highest R'_∞ values never exceeds 0.98 to 0.99, one is actually dealing in such instances with the relationship

$$\frac{R'_\infty \text{ sample}}{R'_\infty \text{ standard}} \equiv R_\infty \qquad (2)$$

and it is essential to specify the standard used. If this expression is introduced into the initial equation it will assume the form

$$F(R_\infty) = \frac{(1 - R_\infty)^2}{2R_\infty} = \frac{k}{s} \qquad (3)$$

which indicates that a linear relationship should be observed between $F(R_\infty)$ and the absorption coefficient k, provided s remains constant. S is rendered independent of wavelength by using scattering particles whose size is large in relation to the wavelength used.

When the reflectance of a sample diluted with a non-or low-absorbing powder is measured against the pure powder, the absorption coefficient k may be replaced by the product $2.30\epsilon C$, where ϵ is the extinction coefficient and C is the molar concentration.[12] The Kubelka-Munk Equation 3 can then be written in the form

$$F(R_\infty) \equiv \frac{(1 - R_\infty)^2}{2R_\infty} = \frac{C}{k'} \qquad (4)$$

where k' is a constant equal to $s/2.30\epsilon$. Since $F(R_\infty)$ is proportional to the molar concentration under constant experimental conditions, the Kubelka-Munk relationship is analogous to the Beer-Lambert law of absorption spectrophotometry.

For a system which conforms to the Kubelka-Munk equation the relationship between the concentration of an adsorbed species, the percent reflectance, and the Kubelka-Munk value are illustrated in Figure 1. If, even at high dilutions, deviations from the linear relationship indicated by the curve in Figure 1b are observed, these, as in the case of true deviations from the Beer-Lambert law, may be taken as an indication of the occurrence of associative or dissociative processes at the boundaries of the particles constituting the sample.

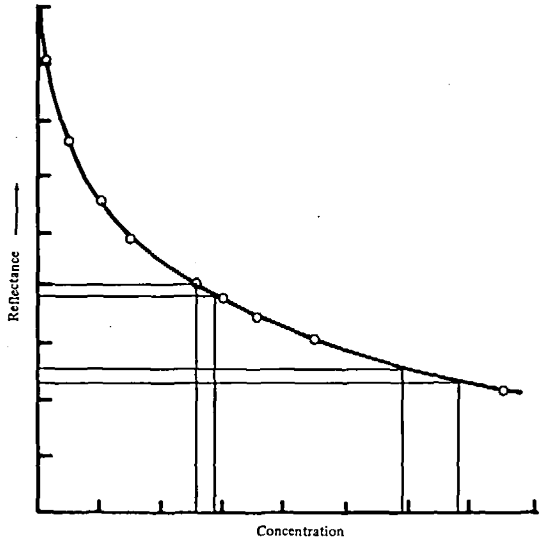


FIGURE 1a. Hypothetical calibration curve percent reflectance vs. concentration.

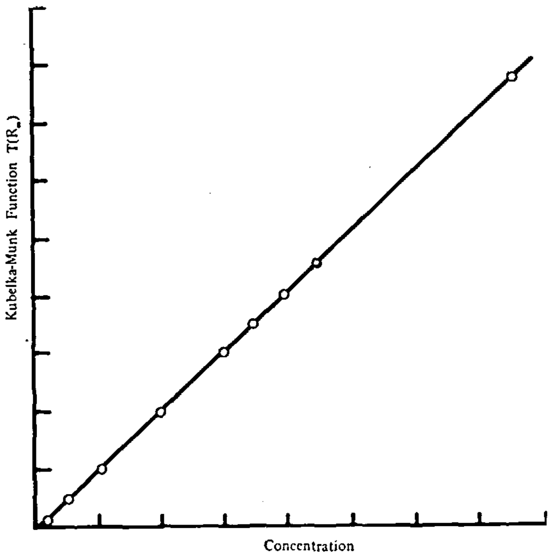


FIGURE 1b. Hypothetical Kubelka-Munk plot $F(R_\infty)$ vs. concentration.

The linear relationship between $F(R_\infty)$ and C also depends on the following conditions. The system has to be weakly absorbing and the grain size of the powders used has to be relatively small (ideally around 1μ in diameter).[13,14] Furthermore, any significant departure from the state of infinite thickness of the adsorbent layer assumed in the derivation of the Kubelka-Munk equation results in background interference, which in turn is responsible for non-ideal diffuse reflectance. When either adsorbents with a large grain size or large concentrations of the absorbing species are used, plots of $F(R_\infty)$ vs. concentration (Figure 1b) deviate from straight lines in that there is a decrease in slope at higher concentrations.

In his explanation of this phenomenon, Kortüm[13,14] postulates that the reflected radiation is the result of both regular and diffuse reflectance. The first can be described as a mirror reflection, whereas the second occurs when impinging radiation is partly absorbed and partly scattered by a system, so that it is reflected in a diffuse manner, i.e., with no defined angle of emergence. Regular reflectance for cases involving normal incidence is described by the Fresnel equation:

$$R_{reg} \equiv \frac{I_{refl}}{I_o} = \frac{(n - 1)^2 + n^2k^2}{(n + 1)^2 + n^2k^2} \qquad (5)$$

where k is the absorption coefficient and n is the refractive index. Since regular reflectance is superimposed on diffuse reflectance, a distortion of the diffuse reflectance spectrum results; this is responsible for the anomalous relationship observed between $F(R_\infty)$ and k at high concentrations of the absorbing species. It is therefore essential to eliminate as far as possible the interference caused by regular reflectance, R_{reg}. This can be accomplished by selecting appropriate experimental conditions. Especially effective is the use of powders having a small grain size, and the dilution of the light-absorbing species with suitable diluents.

Most equations derived by other investigators[1, 15, 16] have proven to be special cases or adaptations of the Kubelka-Munk equation. Kortüm and Vogel[14] have summarized the theory and the derivation of the Kubelka-Munk function for special cases involving infinitely thick, opaque layers, which in the case of fine powders would be those having a depth of ca. 1 mm. Judd and Wyszecki[1] have compiled many of the different forms of the Kubelka-Munk function and they have pointed out some of their specific uses and applications.

The phenomenon of radiative transfer for diffuse incident radiation and isotopic scatter can be treated more rigorously than is done with the Kubelka-Munk equations, particularly in cases where the refractive index of scattering particles is unity. Techniques for such treatment have been given by various authors.[7, 17, 18] Two of the models proposed for the explanation of reflectance phenomena deserve special mention. One is the reflectance model proposed by Johnson,[19] in which the powdered sample is approximated by a stack of parallel plates with thickness d and characterized by a constant index of refraction and surface reflectivity. All the energy radiated on to the plate is assumed to be absorbed or reflected, so that the reflected light would be the sum of the rays passing through n particles (n = 0 to ∞) by means of refraction or reflection at the particle–air interfaces. Phenomena such as multiple reflection and losses due to scattering processes are accounted for by a semi-empirical approach by introducing an adjustable parameter in the expression for the sum of the reflected rays. The shortcomings of the Johnson model, however, were particularly apparent with strongly absorbing samples, or with surfaces with large particle size, due to an inadequate correction for the scattering and multiple reflection losses. In 1963 Melamed[20] proposed a model which explicitly includes multiple reflection. In this model the sum of transmitted, reflected, and scattered rays was considered in an assembly of particles of uniform size but arbitrary shape. Companion[21] discussed and compared the two models and the Kubelka-Munk theory on the basis of experimental data. From her work it can be concluded that the diffuse reflectance models proposed by Johnson and Melamed do not yield absolute absorption coefficients for powder bulk samples, but they do serve to elucidate the reflectance process. The neglection of variations in the index of refraction and surface reflectivity under highly absorbing conditions can attribute to this fact. Improvements of these models requires additional elaborate mathematical treatment, which does not seem justified for analytical purposes.

The author feels that the reflectance function obtained on the basis of the Kubelka-Munk theory is no more in error than the models discussed above, and the deviations encountered by the assumption of different scattering phase functions[22] are just as serious. Therefore, the use of the Kubelka-Munk theory for general analytical applications seems as appropriate now as it was in the early stages of development of diffuse reflectance spectroscopy.

B. Optimum Concentration Range for Analysis

Even with systems which conform to the Kubelka-Munk equation, reflectance analysis is of limited use at high and low concentrations. With high concentrations of absorbing material, very little radiation is reflected; consequently the sensitivity of the spectrophotometer becomes inadequate. On the other hand, with low concentrations the instrument reading error becomes disproportionately large compared to the quantity being measured. The situation is more readily understood with the aid of the curve in Figure 1a, which represents percent reflectance (% R) as a function of concentration. When an arbitrary error amounting to Δ R is plotted at 25, 40, and 90% R, it can easily be seen that the corresponding absolute error in terms of concentration is greatest at 25% R. This in turn leads to a large relative error in the determination of the concentration. At the other extreme, 90% R, the absolute concentration error, though smallest of the three, approaches in magnitude the quantity being measured. This, too, leads to a large relative error in the determination. It is obvious that there must be an optimum region where these two effects can be balanced in such a way as to reduce the relative error to a minimum.

For systems exhibiting no deviation from the Kubelka-Munk equation, the optimum conditions for maximum accuracy can be deduced by computing the relative error dC/C.[23] Thus, Equation 2 can be written in the form

$$C = \frac{k'(1 - R_\infty)^2}{2R_\infty} \qquad (6)$$

where k′ is a constant equal to s/2.303ε. The error in C is

$$dC = \frac{k'(R_\infty^2 - 1)\, dR_\infty}{2R_\infty^2} \qquad (7)$$

and the relative error in C is

$$\frac{dC}{C} = \frac{(R_\infty + 1)\, dR_\infty}{(R_\infty - 1)\, R_\infty} \qquad (8)$$

Assuming a reading error of one reflectance unit, that is $dR_\infty = 0.01$,

$$\frac{dC}{C} \times 100 = \frac{(R_\infty + 1)}{(R_\infty - 1)\, R_\infty} = \%\ \text{relative error in C} \qquad (9)$$

To determine the value of R_∞ which will minimize the relative error in C, d(% error in C)/dR_∞ is equated to zero. The positive solution of the resulting equation,

$$R_\infty^2 + 2R_\infty - 1 = 0 \qquad (10)$$

indicates that the minimum relative error in C, or the optimum value for reflectance measurements, occurs at a reflectance value of 0.414, which corresponds to a reflectance reading of 41.4% R. This is presented graphically in Figure 2, where the relative percent error, computed with the use of Equation 9, is plotted as a function of the % R. As is shown there, the minimum in the resulting curve corresponds to the 41.4% R value obtained by employing Equation 10.

Another approach to the selection of the optimum range for reflectance analysis is that suggested by Ringbom,[24] and later Ayres,[25] who evaluated relative error and defined the suitable range for absorption analysis by plotting the absorbency (1 − transmittance) against the logarithm of the concentration. When this approach is employed in conjunction with the diffuse reflectance technique, plots such as those depicted in Figure 3 result. One curve was obtained with an ideal system (rhodamine B, ●—●) and the other curve typifies a system that does not conform to the Kubelka-Munk equation(aspirin, ▲—▲). If a sufficiently wide range of concentrations is included, the general form of the curves obtained is the same, with an inflection point occurring at 41.4% R with ideal systems and at some other value for non-ideal systems. The optimum range for analyses corresponds to that portion of each curve exhibiting the greatest slope. Since a considerable section of each of the curves in Figure 3 is also linear at about this point, it is apparent that good accuracy can be expected over a wide concentration range. The maximum accuracy can be estimated with the use of the equation

$$\frac{dC}{C} \times 100 = 2.303\ d(\log C) \times 100 = \frac{2.303\ d(\log C)}{dR \times 100\ dR} \qquad (11)$$

Assuming a constant reading error of 1% R, i.e., dR = 0.01,

$$\%\ \text{relative error} = \frac{2.303\ d(\log C)}{dR} \qquad (12)$$

For the two systems under consideration, the

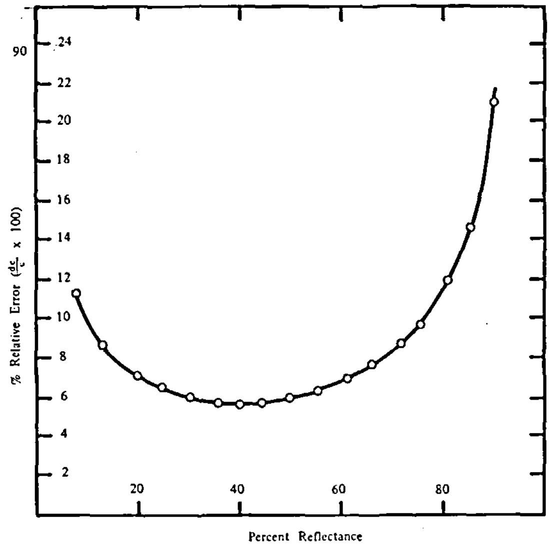


FIGURE 2. Percent relative error, computed with the use of Equation 9 as a function of percent reflectance.[23]

optimum range for analysis can be arrived at from the curves depicted in Figure 3, and the percent relative error resulting from a reading error of 1% R can be computed with the use of Equation 12 and the slope of the appropriate curve, $\Delta\%\ R/\Delta \log C$. When this is done, the data presented in Table 1 are obtained.

It appears that the minimum error in diffuse reflectance spectrophotometric analysis is approximately 6% per 1% R reading error, regardless of whether or not the system in question conforms to the Kubelka-Munk equation. This value can obviously be decreased by reducing the reading error. Although the reading error can be reduced to 0.5% R without too much difficulty, it would be unrealistic to expect a precision better than 0.1 to 0.2% R and, therefore, a smaller minimum error than 1 to 2%.

Kortum[7] has reported similar studies on an error analysis of systems measured by diffuse reflectance spectroscopy.

C. Differential Reflectance Spectrophotometry

As can be seen from the previous discussion, the usual methods for the measurement of diffuse reflectance leave much to be desired with respect to accuracy when one is operating near the ends of the reflectance scale. It is often possible, however, to reduce considerably the error resulting from instrumental uncertainties by modifying the way in which the measurement is carried out. The techniques employed for this purpose, which can be analogous to those used in transmission work, are differential in nature and can be categorized as (1) high-reflectance methods, useful with samples of very low concentration, and (2) low-reflectance methods, useful with more concentrated samples.

Lermond and Rogers[26] were the first to introduce differential techniques for samples of low reflectance, e.g., textiles or inorganic compounds, such as iron (III) oxide. Later, Lieu et al.[27,28] made valuable contributions toward the use of differential diffuse reflectance spectroscopy, particularly in conjunction with chromatographic work.

TABLE 1

Optimum Range for Analysis and Percent Relative Error

Curve	System	Optimum range (% R)	Relative error in concentration per 1% reading error
a	Rhodamine B (ideal)	20-65	6.0
b	Aspirin (non-ideal)	55-85	6.0

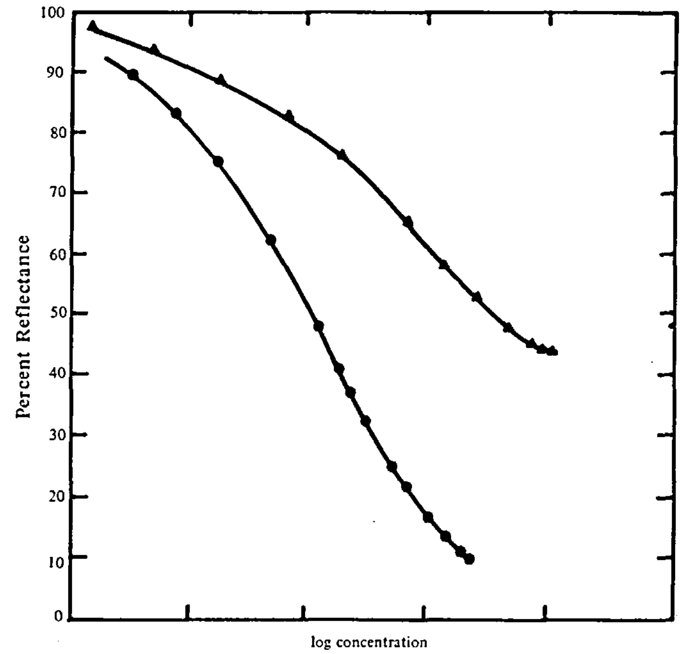


FIGURE 3. Percent reflectance as a function of the logarithm of concentration. ▲, aspirin; ●, rhodamine B.[23]

1. *High-reflectance Method*

This technique is applicable in situations where the concentration of the absorbing species is so low that the reflectance value obtained in the ordinary way lies above the optimum range for analysis.

In the usual procedure for the measurement of reflectance, two preliminary instrumental adjustments must be made to make the full reflectance scale available. These involve setting the instrument to read zero with the photocell in darkness, and 100 when the photocell is exposed to the light reflected from a non- or low-absorbing standard. In this case, expansion of the scale is achieved by setting the instrument to read zero when the photocell is exposed to light reflected from a reference sample somewhat more concentrated than the sample being analyzed; the full-scale adjustment is made in the ordinary way. Usually the zero adjustment is made with the use of the dark-current control, whereas the full-scale setting is achieved by varying the sensitivity and slit width. With some instruments adjustment of the full-scale point may alter the zero setting, and vice versa, so that it is necessary to employ a series of successive approximations to achieve the desired result.

Under the conditions pertaining to the high-reflectance method, the reflectance of a sample relative to the differential standard is given by the following expression:[27]

$$R_{xs} = \frac{(R_{xo} - R_{so})}{(1 - R_{so})} \qquad (13)$$

where R_{xs} is the reflectance of the sample relative to the differential standard; R_{xo} is the reflectance of the sample relative to a non- or low-absorbing standard, and R_{so} is the reflectance of the differential standard relative to a non – or low-absorbing standard. Following rearrangement, Equation 13 can be combined with Equation 6 to give

$$C_x = \frac{k'[(1 - \sigma)(1 - R_{xs})]^2}{[2R_{xs}(1 - \sigma) + \sigma]} \qquad (14)$$

where σ is a constant equal to R_{so} and C_x is the concentration of the analytical sample. If the concentration of the differential standard is such that the incident light is completely absorbed, i.e., $R_{so} = 0$, Equation 14 reverts to Equation 6, which is the more common form of the Kubelka-Munk equation.

Zaye[29] used this technique in the determination of trace amounts of inorganic cations resolved on chromatoplates and found it led to increased accuracy. As can be seen from Figure 4, which presents the percent error arising from a reading error of 1% R (estimated graphically) as a function of the reflectance for various concentrations of nickel-dimethylglyoxime complex adsorbed on cellulose, the use of the high-reflectance method extends the optimum range for analysis toward regions of lower concentration and reduces the relative error in the process.

2. *Low-reflectance Method*

In the low-reflectance method, the scale setting is made with the use of a standard whose concentration is somewhat less than that of the analytical sample. The zero adjustment is made in the usual way. This results in an expansion of the reflectance scale, thus enabling a more accurate reading of reflectance values.

The behavior of an ideal system under the above conditions can be deduced from the Kubelka-Munk equation. If the relationship between R_{so}, the reflectance of the differential standard relative to a non – or low-absorbing standard, and C_s, the concentration of the differential standard, is expressed in the form of Equation 6 and then subtracted from a similar expression for the relationship between R_{xo}, the reflectance of the analytical sample relative to a non – or low – absorbing standard, and C_x, the concentration of the analytical sample, the difference can, following substitution and rearrangement, be put in the form[28]

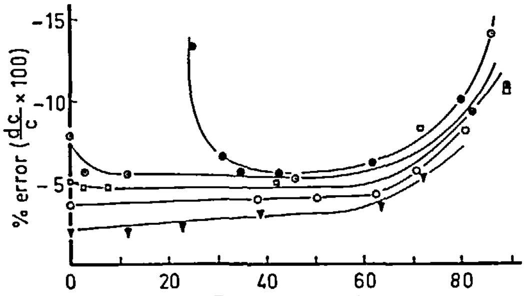


FIGURE 4. Relative error arising from an error of 1% R, as a function of reflectance for nickel-dimethylglyoximate adsorbed on cellulose. Concentration of differential standard, μg Ni/40 mg cellulose: -●- photocell in darkness; -⊙-107; -□-83; -○-59; -▼-5.9.[27]

$$C_x = \frac{k'(1 - \sigma R_{xs})^2}{2\sigma R_{xs} - k'h} \qquad (15)$$

where R_{xs} is the reflectance of the analytical sample relative to the differential standard and σ and h are constants equal to R_{so} and $(1-\sigma)^2/2\sigma\text{-}C_s/k'$ respectively. As indicated, C_x would bear a straight-line relationship to $(1-\sigma R_{xs})^2/2\sigma R_{xs}$

This approach was employed by Labinowich[30] in an effort to increase the accuracy of a procedure designed to determine by diffuse reflectance the amount of copper concentrated on a chromatographic column. Some of his data are presented in Figure 5, which depicts the percent error arising from a reading error of 1% R (estimated graphically) as a function of the reflectance for various concentrations of copper(I)–neocuproine complex adsorbed on magnesium silicate. These data reveal that it is not only possible to extend the optimum range for analysis to higher reflectance values by increasing the concentration of the differential standard, but also to reduce the percent error itself, with the greatest reductions occurring as the concentration of the differential standard approaches that of the analytical sample.

An important factor in the discussion of both differential methods has been the assumption that the reading error associated with the differential reflectance measurement is independent of both the reflectance of the sample relative to the differential standard and the differential standard itself. This assumption has been verified experimentally.[27]

D. Multicomponent Systems

In situations involving a finely divided solid mixture of n light-absorbing components whose reflectance functions are additive, the Kubelka-Munk function $F(R_\infty)$ can also be adapted for simultaneous analysis.[31] The function of the total reflectance $R_{\infty T}$ of the mixture at some wavelength i may then be represented as the sum of all of the individual reflectance functions, or

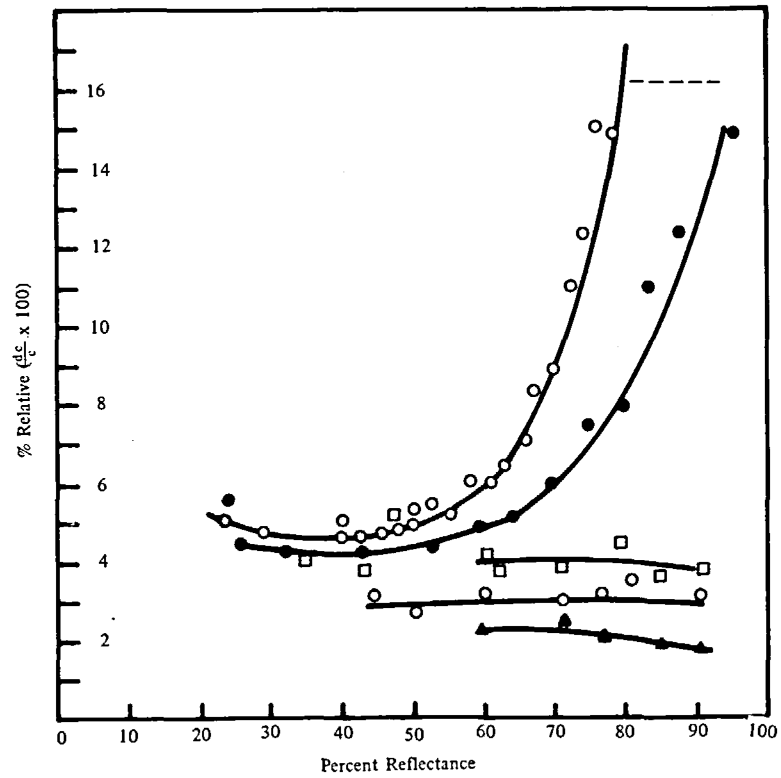


FIGURE 5. Percent relative error arising from a reading error of 1% (estimated graphically) as a function of % R. Copper (I)-neocuproine complex adsorbed on magnesium silicate.[30]

$$F(R_{\infty T})_i = \sum_{j=i}^{n} \tau_{ij} C_j \tag{16}$$

where j refers to the components and τ is the slope of the Kubelka-Munk plot of $F(R_\infty)$ as a function of concentration. Equation 16 can be put in a more explicit form by writing as many equations as there are components in the mixture, or,

$$F(R_{\infty T})_a = \tau_{a1}C_1 + \tau_{a2}C_2 + \cdot\cdot\cdot\ \tau_{an}C_n \tag{17}$$

$$F(R_{\infty T})_b = \tau_{b1}C_1 + \tau_{b2}C_2 + \cdot\cdot\cdot\ \tau_{bn}C_n \tag{18}$$

and so forth. These equations are, of course, valid only for those concentration ranges where adherence to the Kubelka-Munk equation is observed. At higher concentrations interferences due to saturation of the first monomolecular adsorption layer lead to marked deviations from linearity,[32] which, in extreme cases, result in calibration curves asymptotic to the horizontal axis. The useful concentration range, however, can be extended by the use of such semi-empirical relationships as

$$\frac{(1 - R_\infty)^2}{2R_\infty} = k \log C \tag{19}$$

which in one case made an almost fivefold extension possible.[33] Other functions which tend to give linear calibration curves under suitable conditions have been suggested by Lermond and Rogers.[26]

Frei et al.[31] validated the above approach in a study of the simultaneous determination of binary mixtures of dyes adsorbed on silica gel. The spectra of the individual dyes, as well as the spectra of the sample mixture of the dye pair fuchsin–brilliant green, are presented in Figure 6. That the reflectance functions of the dyes are additive was indicated by the fact that the spectra for the dye pair arrived at by the addition of the individual reflectance functions coincided closely with those obtained experimentally. The reliability of the approach in the hands of inexperienced analysts was tested by employing it in an introductory chemical analysis course to determine the composition of dye pair

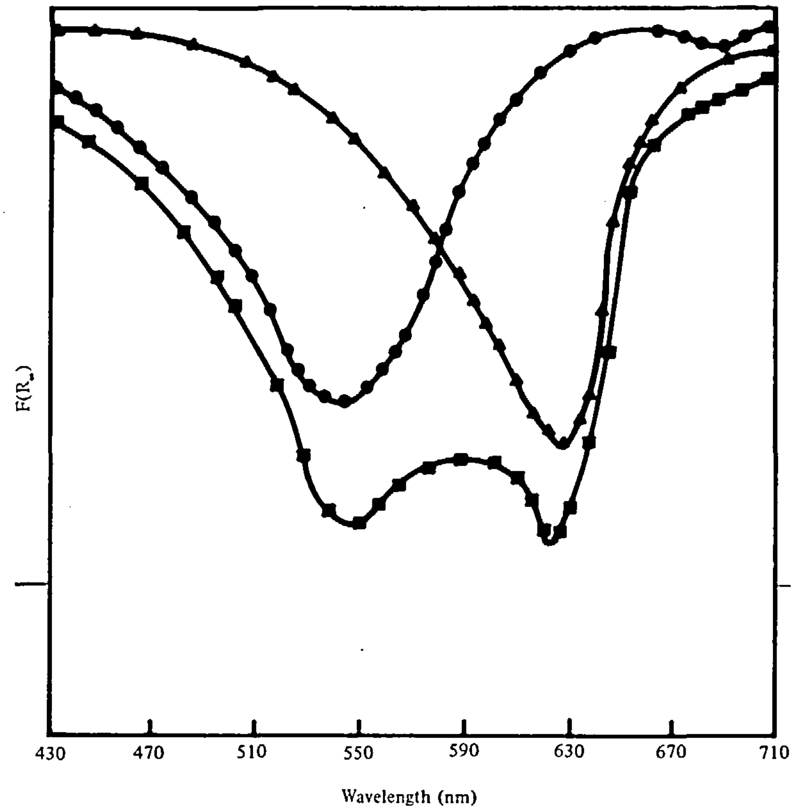


FIGURE 6. Reflectance spectra of ●, fuchsin; ▲, brilliant green; ■, a mixture of the two[31]

"unknowns."[31] In the case of four samples involving the orange G–crystal voilet pair, an average deviation from the true value of 1.9% was obtained for orange G and of 3.3% for crystal violet. The comparable figures for fuchsin and brilliant green were 2.1% and 3.1%, respectively. The average standard deviation achieved with four sets of four samples each was ± 2.4%. As expected, the precision was somewhat poorer than that usually attained in single-component analysis and was limited by sample-preparation processes.

E. The Theory of the Integrating Sphere

The integrating sphere is the best known and theoretically best understood device for the collection of diffuse reflection. It is particularly useful in cases where larger sample areas are illuminated in one operation rather than using a scanning spot[34] or slit procedure and subsequent integration of a large number of very small spot areas, as is customary in chromatographic applications.

Such optical spheres were first described by Sumpner[35] and Ulbricht[5] (Ulbrichtkugel). The device can consist of a sphere or sometimes semisphere-type enclosure with the inner walls coated with a highly reflecting material that can reflect radiation in the wavelength of interest.

Various modes of application as well as a large number of experimental arrangements of optical spheres are known. Two basic designs are depicted in Figure 7. The principle of the substitution method common to reflectance attachment designs for single-beam spectrophotometers is shown in Figure 7a. The diffuse reflectance of the sample is measured relative to a suitable standard, by substituting the sample at the sample port with a reference material and repetition of the measurement under otherwise exactly the same conditions.

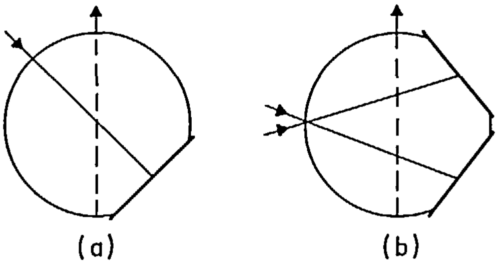


FIGURE 7. Schematic representation of the use of integrating spheres in reflectance spectroscopy. (a) Substitution method. (b) Comparison method.

The comparison method, whose principle is shown in Figure 7b, permits simultaneous irradiation of sample and reference standard and is the common design for double-beam recording spectrophotometers. For most samples with a relatively fine texture, the angle of irradiation does not result in significant changes of the amount of diffuse reflectance measured,[36] as can be seen from Table 2. For samples which possess a definite structure, directional irradiation would produce serious shadow effects and a diffuse irradiation can only be guaranteed with the use of an optical sphere-type setup. This can be achieved in either the substitution or comparison mode, with the optical path in Figure 7 reversed. An external polychromatic source would provide the irradiation energy and the reflected light would then be passed through a monochromator. According to the reciprocity relations $[R_d, (\theta,\phi) = R(\theta,\phi),_d]$, developed by a number of workers,[37-39] the results would remain unaltered. A semisphere[40] or circumferential mirror system could be used for the same purpose.

The principle of radiation flux distribution in an integrating sphere is best illustrated by summation methods for infinite series of interreflected rays.[41-43] A multiple reflection phenomenon occurs at the inner walls of the sphere and an amplification effect can be observed on initial radiation. If we assume that a sample is irradiated at a given angle with a radiation flux intensity of S, R_s would be the reflecting power of the sample and the flux intensity B resulting from specular reflection could be represented by the following equation

$$B_{1s} = R_s S \Delta\omega \tag{20}$$

TABLE 2

Diffuse Reflectance of CaF_2 (Measured Relative to an MgO Standard) as a Function of the Optical Geometry[36]

	${}_{45}R'_{\infty 0}$	${}_{d}R'_{\infty 0}$	${}_{0}R'_{\infty d}$
CaF_2 ;R'_∞	0.970	0.978	0.980
	0.941	0.947	0.950
	0.904	0.906	0.912
CaF_2 ; R'_0	0.885		0.880
	0.856		0.853
	0.827		0.828
CaF_2 ; R'_0	0.897	0.899	
	0.846	0.845	

where $\Delta\omega$ stands for the area of the exit port through which the measurement was made with a suitable detector. The same sample is then mounted against a sample port of the integrating sphere and forms a part of the inner wall lining of the sphere. The sphere possesses an *average reflecting power* R_{sp} and as a result of the multiple reflection on the sphere walls the flux intensity B is amplified to

$$B_s = R_s R_w S \frac{1}{1 - R_{sp}} \Delta\omega \tag{21}$$

where R_w is the general reflection power of the material used for coating the sphere walls and is approximately one. The term $1/1\text{-}R_{sp}$ is often referred to as sphere efficiency factor. An approximately 20-fold amplification of the radiation flux as a result of multiple sphere reflection is quite common. The average reflecting power R_{sp} is computed by multiplying R_w and R_s with the corresponding surface area a. If one adopts the symbol a_s for the surface area of the sample, a_i and a_e for the areas limiting the incidence and emergence flux, and A for total inside area of the sphere, then the following relationship for the average reflection power can be obtained:

$$R_{sp} = \frac{(A - \Sigma a)\, R_w + a_s R_s}{A} \tag{22}$$

In the substitution mode the sample is replaced by the reference standard, and Equation 21 becomes

$$B_r = R_r R_w S \frac{1}{1 - R'_{sp}} \Delta\omega \tag{23}$$

where R'_{sp} is given by a relationship analogous to Equation 22

$$R'_{sp} = \frac{(A - \Sigma a)\, R_w + a_s R_r}{A} \tag{24}$$

The relative difference between R_s and R_r determines the average reflecting power of the sphere. This difference can become negligibly small ($R_s \cong R_r$) when a_s approaches a very small value. The following relationship can then be derived from Equations 21 and 23:

$$\frac{B_s}{B_r} = \left(\frac{R_s}{R_r}\right)\left(\frac{1 - R'_{sp}}{1 - R_{sp}}\right) \tag{25}$$

As can be seen, the ratio B_s/B_r obtained by the substitution method differs from the ratio of the relative reflection powers by the factor

$$\alpha = \frac{1 - R'_{sp}}{1 - R_{sp}} \tag{26}$$

Expression (26) is generally known as "sphere error," and since sphere errors can become quite significant, an equation for the correction of relative reflection terms can be derived from Equations 25 and 26.

$$R' = \frac{R_s}{R_r} = \left(\frac{B_s}{B_r}\right)\left(\frac{1}{\alpha}\right) \tag{27}$$

Sphere errors for a sphere of 12.6 cm diameter coated with MgO ($R_r = 0.98$), with an area $a_s = a_i = a_e = 7\ \text{cm}^2$ and an R'_{sp} value of 0.9526 are presented in Table 3.[43]

The behavior of radiant fluxes in optical spheres can also be considered by other methods. Earlier work,[44-47] notably by Taylor et al.,[44,46] has dealt with perfect spheres, neglecting apertures of any kind. Others have discussed the effect of apertures of certain well-defined dimensions.[48,49] The problem can also be dealt with by means of integral equations.[50,51] The results obtained with these different methods did not vary significantly. In general it can be said that the smaller R_s, the larger the sphere error becomes. Another important factor is the ratio of sample surface a_s to total surface area A. To keep the sphere error small one should choose small values for a_s and large values for A, e.g., large spheres. One has to make a compromise, however, since increasing the sphere size decreases the reflection power R_r.

TABLE 3

Sphere Errors Arising from Reflectance Measurements with an Integrating Sphere by the Substitution Method[43]

R_s	0.900	0.800	0.700	0.600	0.500	0.400	0.300	0.200
R_{sp}	0.9515	0.9501	0.9487	0.9473	0.9459	0.9445	0.9431	0.9417
α	1.025	1.055	1.085	1.114	1.144	1.173	1.203	1.233
$1/\alpha$	0.9753	0.9479	0.9220	0.8975	0.8743	0.8522	0.8313	0.8113

Excellent reviews and discussions on the theory and sources of error of integrating spheres have been given by Wendlandt and Hecht[8] and by Kortüm.[7]

III. INSTRUMENTATION

A. Reference Standards and Sphere-coating Materials

In 1939 the CIE (Commission Internationale de l'Eclairage)[52] recommended MgO as a reflectance standard for photometric and colorimetric measurements by taking the reflectance value of MgO surfaces prepared under clearly specified conditions as 100% at 457 nm. This assumption, however, is quite arbitrary, since the actual value of smoked magnesium oxide surfaces is lower and an experimental value has never been agreed on. A spread of more than 2%, found for values reported by a number of workers,[53] can be attributed to various factors such as different methods of measurement,[48] variations in layer thickness,[54] particle size,[7,51] method of preparation, aging,[55] and density of packing.[56] The difficulties of controlling all these factors sufficiently for the preparation of absolutely reliable standards seem obvious.

Tellex and Waldron[54] showed that the thickness of the MgO layer is very critical and that up to 8 mm of smoked MgO can still possess some transparency. Opinions on the best methods for coating of spheres or MgO plate standards differ widely. Middleton and Sanders[55] have investigated different types of coating procedures. Burning magnesium ribbon rather than burning magnesium turnings as recommended by earlier workers[57] was found to have advantages. The same investigators[55] reported a relatively rapid aging process for MgO, attributed partially to impurities in the material and partially to decomposition of nitrite produced during the smoking process, by UV radiation. [55,58] The effect of compaction of powder samples was investigated by Schatz.[56] Studies were carried out on a number of metal oxides and other compounds, involving also rare earth. It was generally observed that weak absorbers, such as reference materials, show a decrease and strong absorbers an increase in reflectance with increased pressure. This phenomenon could be attributed to the particles moving closer to each other at high pressures, which would result in the occurrence of frustrated total reflection[59] and in transmission of some of the light that otherwise would have been reflected by total internal reflection.

In order to overcome some of these disadvantages of MgO, the use of $BaSO_4$ has repeatedly been suggested.[60–62] Despite the good reproducibility of measurements on $BaSO_4$ within one experiment, results fluctuate again considerably from one worker to another.[53] An important advantage of $BaSO_4$ is its resistance toward aging (< 0.5% after 595 days).[63] Magnesium carbonate, whose reflectance properties are similar to MgO, is occasionally used as a reference material.[53,64] Its main advantage is the convenient block shape in which it is delivered by many instrument manufacturers. For practial applications other more durable materials, such as glasses (Carrara, Didymium, Vitrolite, opal glasses, etc.) are often preferred.[65–68] Vitrolite, a white structural glass, is available from the National Bureau of Standards.[69] Budde[53] has discussed the use of porcelain enamel material for reference purposes. Such a standard is employed as a built-in swivel standard in the Zeiss Chromatogram-Spectrophotometer. Another white glass standard with treated surface is provided for the Zeiss "Elrepho." Reflectance values of these standards relative to MgO are given in Table 4. The absolute values in this table have been determined by Höfert and Loof[70] with the use of a Taylor sphere and are probably the most reliable measurements available at present. A powder press is also available from Zeiss, which permits the reproduction of MgO and $BaSO_4$ standards with a reproducibility of better than ± 0.1%.[71,72]

For quantitative analytical work only relative

TABLE 4

Relative Reflectance of the Zeiss Primary Standard Measured vs. MgO with Absolute Reflectance (Zeiss Information)

Wavelength (nm)	Relative primary standard	Absolute MgO
420	0.992	0.983
460	0.992	0.986
490	0.993	0.986
530	0.994	0.981
570	0.995	0.981
620	0.995	0.988
680	0.996	0.986

measurements are usually required, and a wide range of standards can be chosen. In chromatographic work the plate or column material often serves for reference purposes.[73] Materials such as alumina, cellulose, and silica gel have therefore been used widely as reference standards. Spectra of these standard materials recorded vs. MgO[12] are presented in Figure 8, along with many other substances. In 1959 the CIE recommended that the "perfect diffuser" be adopted as a reference standard for reflectance measurements of opaque specimens.[74] This recommendation did not bring about the immediate adoption of the perfect diffuser, but at the 16th CIE session in 1967 the committee agreed on the following resolution: "The perfect diffuser is recommended as the reference standard. It supersedes magnesium oxide as of January 1, 1969."[75] The implications are that relative reflectances have to be recalculated to absolute values. The need for better instrumentation and more reliable reference materials therefore still exists. It will be increasingly easier, however, to put this new regulation to use as new standard materials and improved automatic calculation techniques become available.[76]

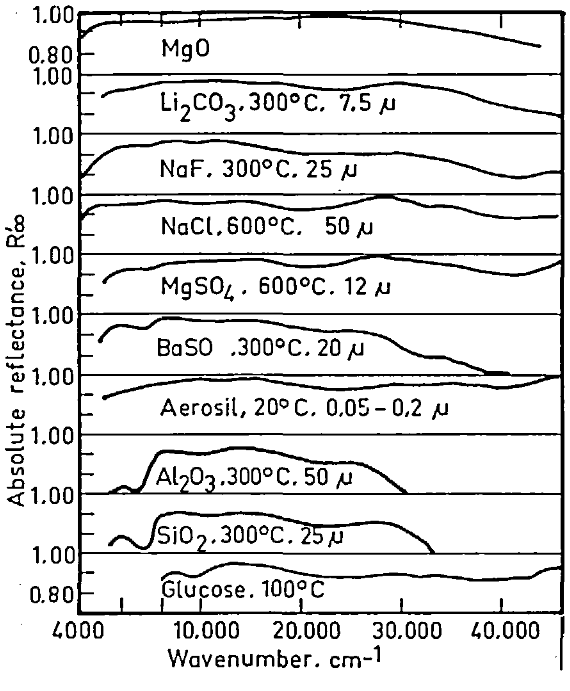


FIGURE 8. Dependence on wavelength of the absolute diffuse reflectance of several white standards (maximum grain size in microns) referred to that of freshly prepared MgO.[12] Temperatures given are drying temperatures.

Obviously some of the materials used for reference standards are also used as coatings in integrating spheres. Such a coating should again be ideally 100% reflecting at all wavelenghts of interest, and an ideal diffuser reflector. Magnesium oxide and barium sulfate are the most widely used materials, although recently a marked shift from MgO to $BaSO_4$ has been noticed for commercial products. A number of methods exist for the coating of optical spheres.[54,57,61,77] They are applied to the sphere either by suitable smoking procedures or in the form of paints. Waterglass and some plastic materials are often used as additives to increase the mechanical stability of the coatings. Detailed coating procedures are usually provided by the instrument manufacturers; for the more recent lines of reflectance attachments the coatings are so stable and resistant to aging that they rarely have to be recoated by the user.

B. Filter Instruments

Akin to transmission spectroscopy, the first instrument that was constructed for the measurement of reflected light used filters to obtain light of a defined wavelength range.[2] Many filter instruments have since been designed both commercially and noncommercially and have been used primarily in the paint, ceramics, paper, and textile industries for defining and matching of colors and for the investigation of properties such as whiteness, brightness, etc. In most cases these instruments were equipped with an integrating sphere for the collection of diffuse radiation;[2,78-81] a few others use light pipes, semispheres, lens and mirror systems, etc. to pick up diffusely reflected light. A typical representative of the line of filter reflectometers is the Elrepho Colormeter (Electric Reflectance Photometer) (Figure 9)

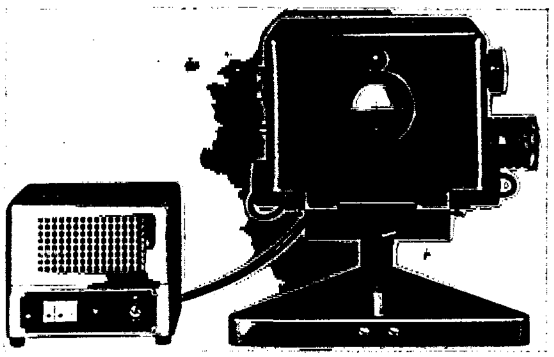


FIGURE 9. The Zeiss Elrepho Colormeter.

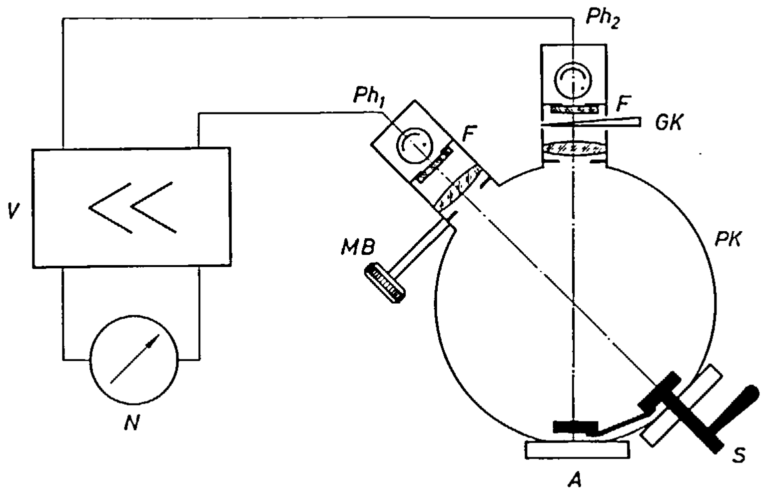
manufactured by Carl Zeiss Inc., Oberkochen, West Germany. Figure 10 shows the optical diagram of the Elrepho Photometer. The sample A is mounted at the proper opening of the integrating sphere and is irradiated in a diffuse manner via the optical sphere by two incandescent lamps, not visible in this drawing. Direct irradiation of the sample is prevented by small screens. A built-in swivel standard S is irradiated by the same system. The light reflected from sample and reference strikes the photocells Ph_1 and Ph_2 at a zero-degree angle to the sample and reference surfaces, respectively. A neutral wedge GK in the measuring beam and a measuring diaphragm MB in the reference beam compensate the two resulting photocurrents which flow in opposite directions.

The RFC-3 Colormeter is a modification of the Elrepho, which permits automatic readout of the color coordinates; it is available from Zeiss. The useful spectral range is the same as for the manual instrument (400 to 700 nm) and the optical geometry can be chosen $_dR_{8°}$; $_{45°}R_{0°}$

The Pretema Spectromat FS-38 (Pretema Ltd., Birmensdorf-Zürich, Switzerland) operates with a similar double-beam arrangement and an integrating sphere. Optical geometries $_dR_{8°}$ and $_{45°}R_{0°}$ are available, and a small built-in digital computer is programmed to produce a direct readout of CIE color coordinates in printed or digital form. Like the Zeiss RFC-3, this instrument is widely used in industries where large-scale color comparisons in quality and production control are essential.

Van den Akker et al.[82] have critically discussed an instrument known as the IDL Color Eye (Industrial Development Laboratories Inc., 67 Mechanics Street, Attleboro, Massachusetts). The instrument also uses an integrating sphere, and simultaneous irradiation of sample and reference standard is achieved with a rotating mirror which reflects the polychromatic incident beam alternately to the reference and sample ports. The instrument permits the measurement of diffuse and/or specular reflectance, absolute or differential.

A line of instruments with somewhat different design features is available from Hunter Associates Laboratory Inc., 9529 Lee Highway, Fairfax, Virginia. The modern Hunter instruments have grown out of some of the earliest known filter-type reflectometers designed and described by Hunter in 1934[83] and 1940.[84,85] All currently available Hunter Color and Color-difference meters operate on the single-beam principle (substitution mode). The solid-state wired electronic unit is the same for all D-25 Models and can be outfitted with three different optical units; D-25A, D-25 M/L, and D-25P. The standard attachment D-25A uses an angle of illumination of 45° and the diffusely reflected polychromatic light is collected at an average viewing angle of zero degrees by means of a light pipe, and directed to a set of three phototubes. Four broad-band spectral filters (tristimulus X, Y, Z amber, and X blue) are placed in front of the detectors for computation of color coordinates. The optical head D-25P has an eight-



Measuring principle of the ELREPHO

Ph_1	photocell
Ph_2	photocell
F	filter
GK	neutral wedge
S	swing-in standard
A	sample
MB	measuring diaphragm
N	balancing instrument
PK	photometer sphere
V	amplifier

FIGURE 10. Optical schematic of the Elrepho Colormeter.

inch diameter optical sphere and the diffuse radiation is viewed at the top of the sphere with the set of phototubes and filters mentioned previously. As in the D-25A unit, the specular reflectance can be excluded, but with the D-25P unit it is possible to measure also total reflectance, by swinging the source or the light sphere about 8° off their common optical axis. The usual geometry for this unit is $0^{\circ}R_d$ The optical head D-25 M/L uses a rather unusual design. A circular mirror system composed of faceted annular rings serves to irradiate rough-textured samples, such as textiles, in a diffuse manner. The light reflected from the sample is viewed at a zero-degree angle and directed through four spectral filters to a set of four phototubes. The line of D-25 Colormeters is available with a built-in computer for computation of color coordinates as described by the ASTM method. A few filter-type instruments have been described in section E.

C. Monochromator Instruments

At present, reflectance attachments are available for almost every commercial spectrophotometer on the market. It is mostly for this reason that diffuse reflectance spectroscopy has become an increasingly useful tool to workers in many branches of chemistry, and the investigator is rarely faced with the problem of having to construct his own instrument for spectral reflectance work. The diffuse reflectance attachments as they exist today were usually designed at a later date for already existing all-purpose spectrophotometers. An exception to this rule is the General Electric Hardy Spectroreflectometer (General Electric Co., West Lynn, Massachusetts), which in 1938 became the first commercially available reflectance spectrophotometer.[86,87]

One of the best known and most widely distributed spectrophotometers in North America is the Beckman Model DU Spectrophotometer (Beckman Instruments Inc., Fullerton, California). This single-beam instrument can be equipped within minutes with a diffuse reflectance attachment whose optical schematic is shown in Figure 11. The light beam passes from source A through the monochromator B. The monochromatic exit beam emerges through mounting block C and is directed upon a deflection mirror D at a zero-degree angle of incidence to the surface of the sample E. The sample drawer F can contain a sample and a reference standard. The diffusely reflected light is collected at a circular ellipsoidal mirror G at angles approximately between 35 and 55°, and focused upon a frosted quartz diffusing screen H which is located in the field of the phototube J in the phototube housing K.

A reflectance accessory is available for the

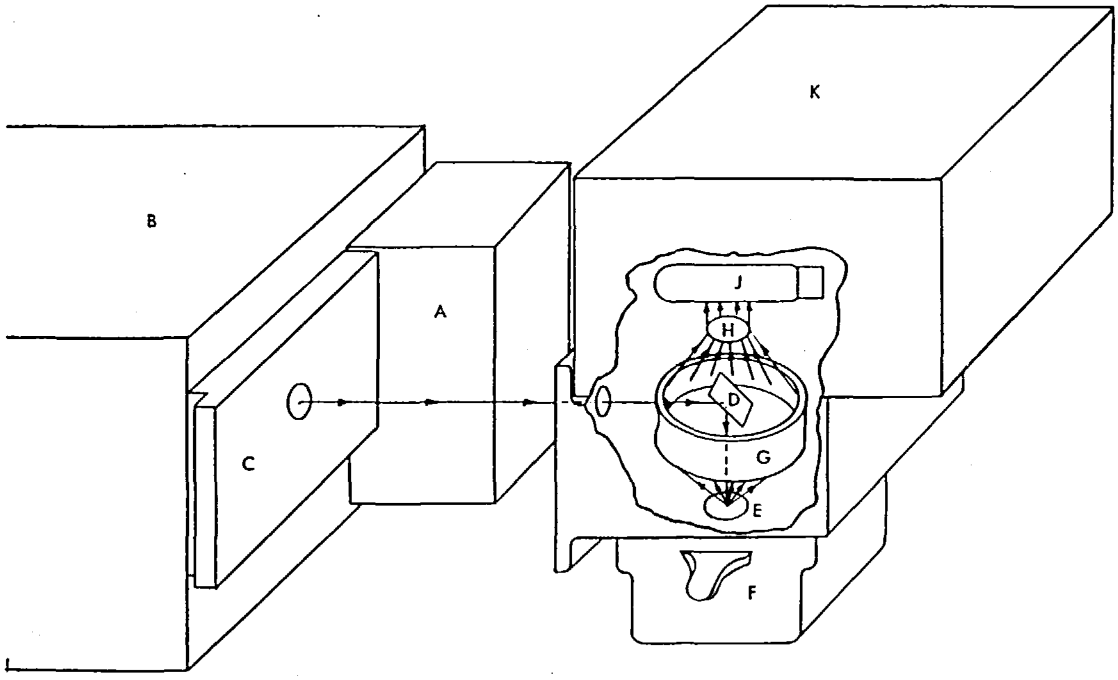


FIGURE 11. Optical diagram of the Beckman Model DU Spectrophotometer with diffuse reflectance attachment.

Model DK-2A Spectrophotometer. This attachment uses an integrating sphere in which both sample and reference are irradiated simultaneously with monochromatic light. An oscillating mirror produces the double-beam effect by alternately deflecting the beam to the sample and the reference ports. In the normal sample position the light impinges at a zero-degree angle and the specular reflectance portion is attenuated. Total reflectance can also be measured by mounting the specimens at a 5° angle to the light beam. The spectral range for these two operation modes is from 220 to 2700 nm. A lead sulfide detector is mounted on top of the sphere for operation in the near-infrared region. For samples of rough texture or gloss it is possible to replace the detector housing on the sphere with a tungsten lamp or hydrogen lamp and irradiate the specimens diffusely via the sphere. The optical path is reversed and the detector is placed in the position of the original lamp housing.

Still another diffuse reflectance attachment with similar operation mode has been introduced for use with the Beckman Models DB and DG Recording Spectrophotometers.

Diffuse reflectance attachments for the Bausch & Lomb instruments Spectronic 20 and Spectronic 505 (Bausch & Lomb Co., Rochester, New York) have also gained wide acceptance. In the Spectronic 20 accessory the monochromatic light impinges at a zero-degree angle on the sample or reference placed at the top of an integrating sphere. The diffusely reflected light is collected by a phototube placed at a 90° angle to the sample surface. An integrating sphere attachment is available for the Model Spectronic 505, which operates in a true double-beam mode. The sample and reference materials are mounted in a vertical position to the sphere. Light traps located at a 90° angle to the irradiated surfaces can be used to eliminate specular reflectance. The standard attachment can be used in the spectral region 400 to 700 nm, but another sphere coated with a $BaSO_4$ paint is available for work in the UV and visible regions of the spectrum. Three reflectance attachments are available for the Cary Models 14 and 15 Spectrophotometers (Cary Instruments, Applied Physics Corp., 2724 South Peck Road, Monrovia, California). One attachment operates with an integrating sphere in a manner similar to the Beckman DK-2 accessory. Another uses a circular mirror instead of a sphere for the collection of diffuse radiation within cones or angles of 45° ± 7°. For this attachment a working range of 220 to 700 nm is recommended and a resolution of 0.13 to 0.7 nm can be expected. The third type of accessory is especially interesting in that it uses a sample sphere and a light-integrating reference box. The sample and reference beams are thus completely separated and interference of the two beams can be eliminated.

A new reflectance accessory for adaptation to the Cary Models 14, 14R, and 14RI has recently become available. With an altered integrating sphere design it can cover the spectral range 220 to 2500 nm. The working principle of this attachment has been discussed elsewhere.[88] Cary Instruments also offer a computer in conjunction with this instrumental setup for the computation of tristimulus values in color measurements.

Worth mentioning is the attachment designed for the Perkin-Elmer Spectrophotometers Models 350 and 450 (Perkin-Elmer Corp., Norwalk, Connecticut). This accessory uses separate spheres for the sample and reference standard, each equipped with its own end-window photomultiplier. The optical geometry is ${}_{16^\circ 15'}R_d$ and specular reflectance can be rejected with a black port located in the sphere wall. This interesting dual-sphere design has previously been discussed in much detail.[89] With the withdrawal of the Models 350 and 450 from the market, manufacturing of this accessory has been discontinued.

Pye Unicam Instruments SP500, SP700, and SP800 (Pye Unicam Ltd., York Street, Cambridge, England) are available with diffuse reflectance attachments. Ellipsoidal mirror systems similar to the one used with the Beckman DU are employed for all three models for the collection of diffusely reflected radiation. The SP500 is a single-beam instrument and can be operated in the reflectance mode in the spectral range 350 to 1000 nm. For reflectance work by the double-beam mode, one can use the SP700 with reflectance attachment Sp. 735. The instrument can be used in the spectral range 200 to 2500 nm. The Model SP800 with reflectance accessory is the latest in the line and covers the UV and visible spectral ranges. It operates essentially as a double-beam instrument, but the reference beam impinges directly on the phototube and the measurements have to be made by the substitution method. In all three versions the optical geometry is ${}_{0^\circ}R_d$ and the diffuse radiation is collected between angles of emergence 35 to 55° by the ellipsoidal mirrors.

A versatile line of reflectance spectrophotometers is available from Carl Zeiss Inc., Oberkochen, West Germany. Three types of reflectance accessories can be chosen for work with the single-beam instrument Model PMQ. In the attachment RA-2 the sample is irradiated with monochromatic light at a 45° angle and the diffuse reflectance is viewed with a suitable lens system built in front of a phototube at right angles to the sample surface. UV, visible and near-infrared work can be performed with this instrument in the reflectance mode.

The directional irradiation of the sample surface via the accessory RA-2 (optical geometry ${}_{45°}R_{0°}$ will lead to systematic errors[47,72,90] with samples of rough texture, such as textiles or certain large-grain powders. In cases such as this, the use of attachment RA-3 in the geometric version ${}_{d}R_{0°}$ is indicated. This guarantees nearly ideal diffuse polychromatic illumination via the integrating sphere of the attachment and eliminates shadow effects. The optical path in this attachment can be reversed to the most widely used geometry ${}_{0°}R_{d}$ in a matter of minutes. The optical diagram for the RA-3 accessory (Figure 12) shows the monochromatic light beam entering the attachment from the monochromator, impinging on the sample surface at a zero-degree angle and after diffuse reflection on the sphere walls entering the phototube, which is placed at a 90° angle to the right hand of the sample. The working range for this modification also includes the UV region.

The third attachment has been designed for very small samples (≥ 0.1 mm^2 surface area) and is well suited for microanalytical work. The total spectral range of the attachment is 250 to 2500 nm and a swivel standard is provided for setting of the reference value without necessitating removal of the sample during the measuring operation.

An attachment for the new double-beam Recording Spectrophotometer Zeiss Model DMR 21 has just become available. Like its predecessor for the earlier Model RPQ 20 it operates with an integrating sphere with spectral range 200 to 2500 nm.

A real breakthrough in the field of diffuse reflectance spectroscopy and color measurements has been achieved with the Zeiss Model DMC Recording and Digital Spectrophotometer. The basic operation principle is very similar to the one explained for the Beckman DK-2 Spectrophotometer, and can best be understood from Figure 13. Light from L passes through monochromator M and is alternately deflected and transmitted by a mirror chopper DS to sample P and standard S. The diffusely reflected light is received by the detector E. Polychromatic and diffuse illumination of sample and reference

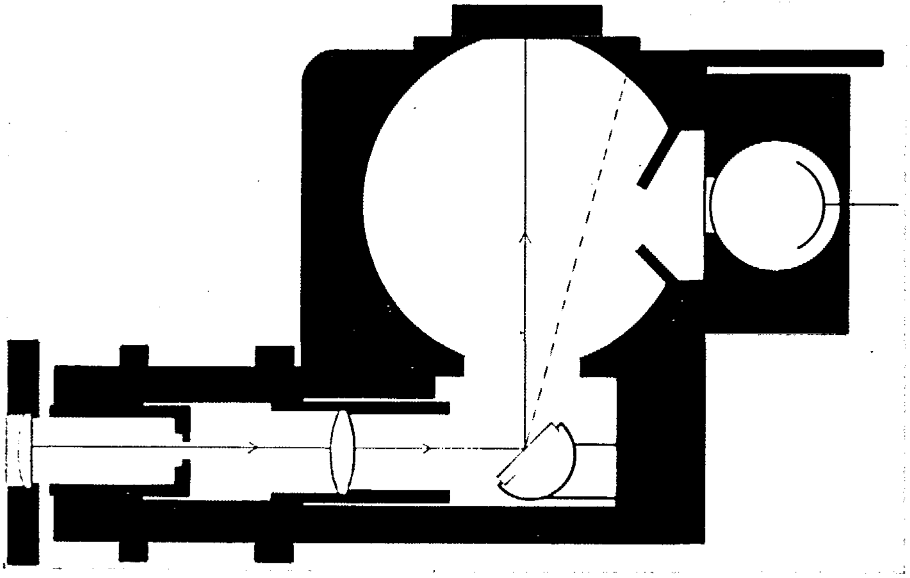


FIGURE 12. Optical diagram of the RA-3 accessory in the ${}_{0°}R_{d}$ optical geometry mode (Zeiss).

material is possible by replacing the detector with a xenon or tungsten lamp. The detector would then occupy the position L and the optical path is reversed. Some of the technical features of this instrument include simultaneous recording of % R and the Kubelka-Munk function or transmittance and absorbance. The recording is done with a twin analogue recorder. Measurements are printed out at preselected wavelength intervals. A built-in digital computer (Davidson & Hemmendinger, Tatamu, Pennsylvania) computes x, y, and z color coordinates for one of the illumants A, C, D, or E. The results are printed or presented on a screen in digital form. Interchangeable prism sets and corresponding detectors for spectral ranges 200 to 650 nm and 380 to 2500 nm can be selected by push buttons. The wavelength reproducibility is better than 0.1 nm. Geometries ${}_{d}R_{8^\circ}$, ${}_{8^\circ}R_{d}$ and ${}_{45^\circ}R_{0^\circ}$ can be selected, as well as two different sizes of integrating sphere (100 and 130 mm diameter). Sample surfaces can vary between 15 x 20 mm and 50 x 38.5 mm, but can be further reduced for special purposes. Seven measuring ranges are available for diffuse reflectance measurements, which is an advantage for differential spectroscopy.

Other, less-common reflectance measuring devices have been discussed by Judd and Wyszecki[1] and by Wendlandt and Hecht.[8]

D. Sample Holders

1. *Commercial and Macrocells*

Measurement of compact samples does not usually pose a problem. The samples can be clamped in horizontal or vertical position with face up or down by means of spring-loaded cups. It would be an impossible task, however, to design one universal sample holder for the variety of liquid or powder samples that can be studied by diffuse reflectance spectroscopy. In many cases the same cells which are used for transmission measurements are also applied to reflectance work, but for powders or slurries there is the problem of packing, removal, and cleaning. For samples placed horizontally with face up, small cups, beakers, or planchets may be used. Few commercial sample holders are available for powder materials. Bausch & Lomb, Zeiss, Hitachi, and others provide windowless macrocells which can be packed with special powder presses. They can only be used, however, with relatively adhesive powders, and are recommended for the preparation of reflectance standards such as $BaSO_4$ or $MgCO_3$. The amount of sample material needed is rather large. A number of noncommerical sample holders have been discussed by Tonnquist.[91] A typical example for a low-cost reflectance cell, for the measurement of slurries and powders in the UV, visible, and near-infrared regions of the spectrum has been designed by Barnes et al.[92] The cell consists of a Bakelite base and cover plate into which a circular quartz window of 1.1 cm has been cemented with epoxy resin. The sample is contained in a planchet in a cavity between cover and base with dimensions 3.2 cm diameter and 0.16 cm deep. This cell has been used extensively for studies of adsorption phenomena by spectral reflectance.

2. *Semimicrocells*

One universal drawback of the cells mentioned

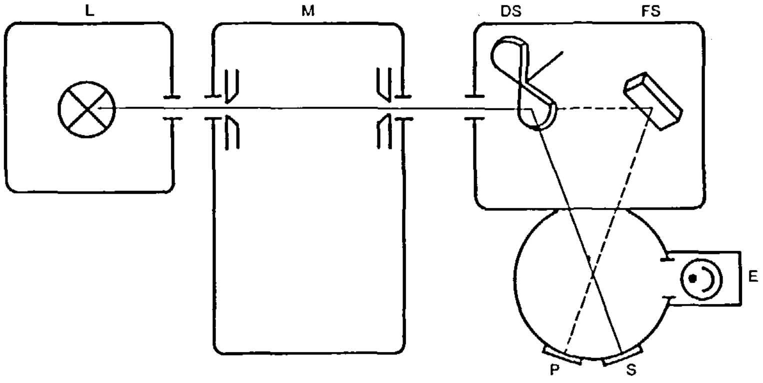


FIGURE 13. Optical diagram of the Zeiss Model DMC-25 Spectrophotometer. L, light source; M, monochromator; DS, chopper mirror; FS, fixed mirror; P, sample; S, standard; E, detector.

so far is the large quantity of sample material needed for complete packing. This ranges from a few grams for the above-mentioned commercial sample holders to 0.5 and 1 g, depending on the material, for the cell described by Barnes et al.[92] A new line of reflectance cells had to be designed when spots scraped off from chromatoplates had to be accommodated for reflectance measurements. Three types of cells were designed by Frodyma and co-workers in conjunction with these problems: (a) a glass window cell,[33] (b) a quartz window cell,[93] and (c) a windowless cell.[94] Cell a is depicted in Figure 14. It consists of a white paperboard to which a microscope cover glass is affixed with masking tape. The paperboard is cut to a size which permits its introduction into the sample holder of the specific apparatus used. In the case of the Beckman DU sample drawer for the reflectance attachment, for which the holder has been designed originally, this would be 4 x 3 cm. In this cell the analytical sample is compressed between the cover glass and the paperboard until a uniform layer of densely packed powder is obtained; care should be taken that no cracks appear on the surface. The main advantages of this cell are its low cost and simplicity, which permit its manufacturing in large quantities needed for the investigation of dilution series. It can, of course, only be used in the visible or near-infrared regions of the spectrum.

Cells b and c are essentially modifications of cell a. A quartz window is used in cell b to make it suitable for work in the UV region. A circular quartz plate is superimposed on a plastic plate and affixed to the backing paper with masking tape. Cell c consists of a plastic plate with circular opening affixed to a paperboard of the same dimensions with masking tape. The cell is packed by introducing the sample into the opening and compressing it with a tamp of suitable size.

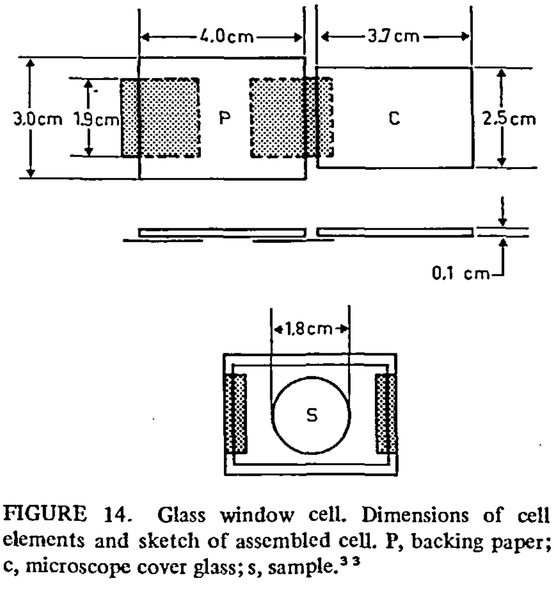


FIGURE 14. Glass window cell. Dimensions of cell elements and sketch of assembled cell. P, backing paper; c, microscope cover glass; s, sample.[33]

3. *Variable-temperature Cells*

Valuable information can often be obtained with reflectance spectra recorded at elevated temperatures. Special sample holders are needed for work at high temperatures, either to keep the sample at a particular temperature (usually between 100 and 300°C) or for gradually increasing the temperature at a uniform rate (dynamic reflectance spectroscopy). Reflectance studies at elevated temperatures have been carried out by several workers (see section IV E) and various types of sample blocks suitable for controlled heating of the sample have been described.[95-100] A sample holder with a cylindrical brass heating block was discussed by Asmussen and Andersen.[96] The temperature regulation was provided by a small bulb located at the bottom of the cylinder. The temperature of the sample, which was contained in a cell at the top of the block, was measured with an imbedded thermocouple. A number of cells were described by Wendlandt et al.[97-101] The first of their sample holders consisted of an aluminum block with a circular indentation (25 x 1 mm) to hold the sample. The heating was performed with a nichrome wire. This heating element was fixed close to the sample indentation, and the sample temperature was monitored with a Chromel-Alumel thermocouple. Modifications of this cell were discussed at a later date.[8,99]

Recently a cell was described which is suitable for high-temperature investigations of small samples.[100] The holder is based upon a design reported previously[33] (Figure 14). It consists of a small aluminum block heated internally by a circular element. The heating block is mounted on a 5 x 5 cm transite block and the sample is compressed between a glass fiber cloth and a cover glass mounted on the heating block. The whole assembly is tightened together by a clamp.

Low-temperature reflectance measurements can also be useful, e.g., in cases where stability of the sample is a problem, for kenetic studies in the adsorbed state or for the investigation of low-temperature reflectance spectra of inorganic systems (see section IV E). Symons and Travalion[102] have designed a low-temperature holder specifically for the reflectance attachment of the Unicam Model SP500. The attachment was cooled with liquid nitrogen transported into the vicinity of the sample by copper rods and coils. There is still a great need, however, for the development of electronically controlled low-temperature cells, which would permit a more flexible operation than the cell mentioned above.

E. Instruments for in situ Measurement of Chromatograms

Direct scanning of paper and thin-layer chromatograms by transmitting light through the chromatograms and measuring the absorbance has been known for some time under the term "densitometry" and has been discussed in a number of publications.[103-105] Diffuse reflectance spectroscopy can be used for the same type of problems, but its development has lagged behind because of a lack of knowledge and suitable instrumentation in this field.

Klaus[106,107] and de Galan et al.[108] have discussed the construction of an accessory suitable for scanning of chromatoplates over the sample aperture of the Zeiss sphere reflectance attachment to the PMQ II Spectrophotometer. Other noncommercial instrument modifications for in situ reflectance measurements on chromatograms were described by Gordon[109] and Hamman and Martin.[110] Recently, Beroza et al.[111] devised a low-cost instrument for the automatic recording of spectral reflectance from thin-layer chromatograms by means of fiber optics. Single- as well as double-beam operation was possible, and with suitable modifications it was possible to use this instrument for the UV as well as the visible regions of the spectrum. The advantage of the fiber optics is its relatively low cost and the possibility of bringing the light to a small defined area on the plate. The validity of the Kubelka-Munk function was determined on the basis of reflectance data gathered from some pesticides. The author believes that fiber optics will have great potential for certain aspects of diffuse reflectance spectroscopy, particularly for low-cost instruments used in routine analyses.

1. Commercial Single-beam Instruments

One of the first commercially available attachments for the direct scanning of thin-layer plates by reflectance spectroscopy was the Zeiss Chromatogram-Spectrophotometer PMQ II[112] (Figure 15). In this instrument the chromatogram is illuminated with monochromatic light at a zero-degree angle of incidence. The diffuse radiation is measured at a 45° angle. A light pipe is used for the collection of the radiation. Specular reflectance can escape through an opening in the light pipe, which admits the incident beam. The chromatograms are placed horizontally on a mechanical stage (see Figure 16) which can be moved in two directions at right angles to each other. In the y axis the movement is mechanical and ten different speeds can be selected. In the x axis adjustments are made manually. The mechanical stage and the lower surface of the measuring head are parallel in any position. The distance between the two can be adjusted with a scale to fit a particular chromatogram. The local resolution in the direction of the motorized feed depends on the dimensions of the scanning spot in this direction. This dimension can be varied between 0.02 and 2 mm. The maximum length of the scanning spot at right angles to the direction of travel is 14 mm but can be reduced to match variations in the diameter of the spot. It is also possible to use a circular scanning spot so as to cover the entire chromatogram spot at the same time. The diameter of this scanning spot is about

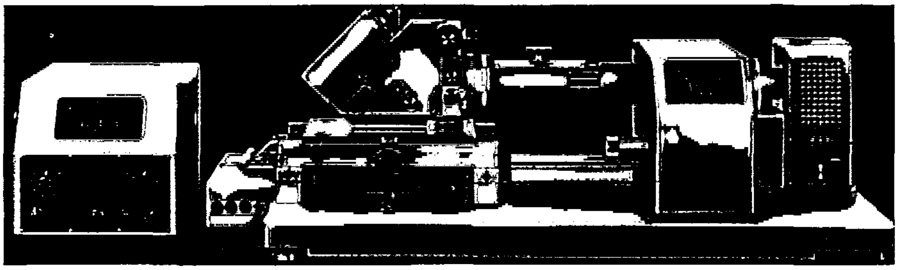


FIGURE 15. Zeiss Chromatogram-Spectrophotometer PMQ II.

30 mm maximum and can be adjusted to fit experimental conditions. A built-in swing-in standard which can be moved into the light path enables test values to be calibrated without removal of the object under investigation. By placing the detector underneath the sample table, transmission measurements through the chromatogram are also possible. The working range for reflectance is 200 to 2500 nm. With small alterations the same instrument can be used for in situ fluorescence measurements.

Recently, Camag Ltd. (Muttenz, Switzerland) has started production of the Camag-Z-Scanner, which can be fitted to the Zeiss PMQ II Spectrophotometer. It can be used for direct measurement of reflectance and fluorescence from thin-layer and paper chromatograms. The Z-Scanner is placed directly adjacent to the monochromator unit of the Zeiss PMQ II and aligned on the triangular rod on the optical bench system of the instrument. From the optical schematic (Figure 17) it can be seen that the thin-layer plate or paper is placed in vertical position and the monochromatic light beam impinges at a zero-degree angle. A lens and photodetector assembly picks up the diffuse radiation. The system can be reversed (Figure 17b), e.g., polychromatic radiation at angle 45° and pickup of the diffuse radiation at zero degree is also possible for certain applications. Specular reflectance can be excluded in both modifications. The working range of this attachment is 220-750 nm. The chromatograms can be scanned at two speeds and the plate holder has a removable bottom plate which permits observation from the back of the chromatogram and facilitates positioning. Slits for scanning can be varied up to a 16-mm maximum width. For manual recording of the spectra a built-in blocking device is provided which permits a reproducible shifting of the plate

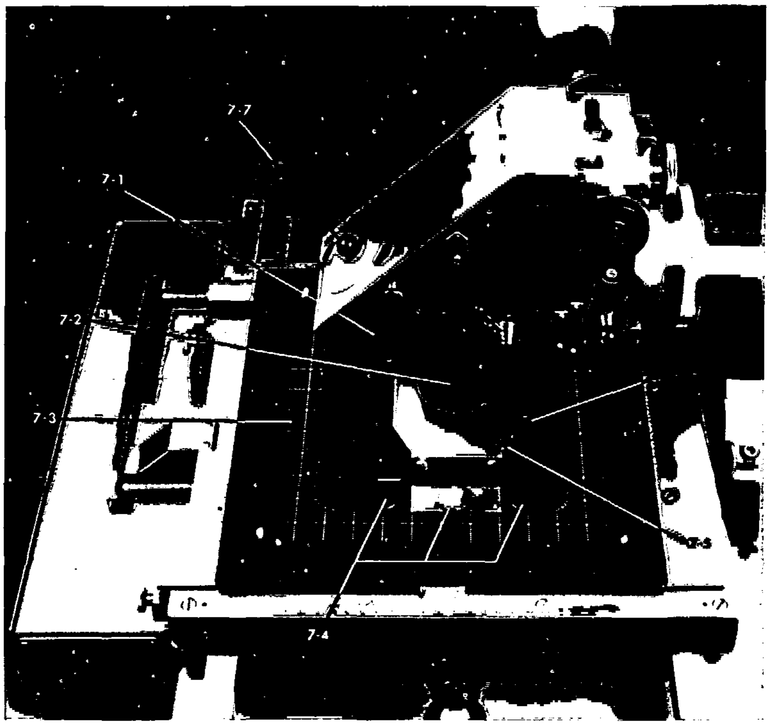


FIGURE 16. Stage and measuring head, arrangement M-Pr (T). M, monochromator; Pr, sample; (T), measurements by transmission.
7-1 Slide with auxiliary finder-illumination
7-2 Light-guiding tube, with detector in housing (the latter is not visible from above)
7-3 Frame-shaped supporting plate
7-4 Positioning screws for carrier of detector housing
7-5 Carrier for detector housing
7-6 Locking screws (2) of detector housing
7-7 Cover for light guide.

holder to an empty region on the chromatogram, and vice versa. This serves to compensate for changes in photomultiplier response at different wavelengths. The Camag-Z-Scanner is available at about one third the cost of the Zeiss attachment, but it is by no means a substitute for it, particularly if high precision and good optical efficiency are required.

Another single-beam instrument, the Vitatron Densitometer TLD 100 (Vitatron Ltd., Dieren, Holland) has been designed for in situ measurements of reflectance as well as transmittance and fluorescence. An interesting feature of this instrument is the "flying spot" scanning device. It was developed by Goldman and Goodall[113] and involves the measurement of a chromatographic spot by an oscillating movement of the scanning table in the X direction. The stroke length of this movement is slightly larger than the spot diameter and at the same time the plate is moved at a uniform speed in the y direction. The integrated values for the optical density measured over the stroke length are registered on a chart recorder, and the corresponding peak areas are computed with an electronic integrator. With this scanning technique the instantaneous spot areas under investigation are very small and have a homogeneous appearance. This would make it particularly suitable to irregularly shaped spots. Unfortunately, at the moment the reflectance mode can only be used in the visible region, but to the author's knowledge, steps are being taken to make UV reflectance possible. This would probably mean replacement of the filters by monochromators. The optical geometry of irradiation and observation is $_{45°}R_{0°}$ At present, the Vitatron TLD 100 is not suitable for the measurement of spectra, and while the "flying spot" technique can be considered the most ideal approach to such scanning problems, it is believed that its inherent impracticalities in comparison to slit scanning will limit a wide adoption of this device.

2. *Commercial Double-beam Instruments*

Several instruments are now available for in situ evaluation of chromatograms by the double-beam mode. The chromoscan with chromatogram accessory (Joyce, Loebl & Co. Ltd., Princesway, Team Valley, Gateshead 11, England) was one of

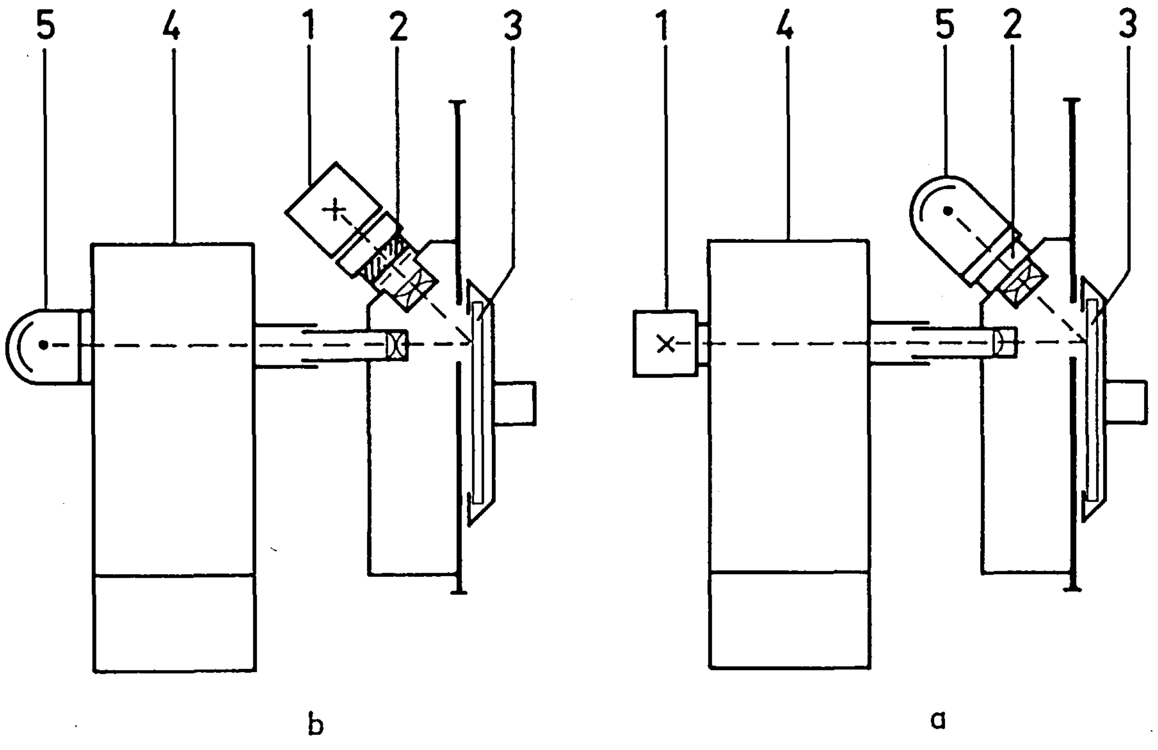


FIGURE 17. Optical diagram of the Camag-Z-Scanner. a. Geometry $_{0°}R_{45°}$ b. Geometry $_{45°}R_{0°}$ 1, deuterium or tungsten lamp; 2, lens system; 3, chromatoplate; 4, monochromator; 5, photomultiplier.

the first commercial instruments used in conjunction with a scanning unit. It was originally designed to measure transmission and fluorescence, but now it can also be used for the direct measurement of spectral reflectance. It is a null point instrument and a grey wedge is balanced against the reflected radiation from the sample. The chromatogram attachment provides its own optical system. A quartz iodide tungsten lamp serves as energy source. UV lamps (deuterium or mercury) can be placed alongside with the tungsten lamp in a dual lamphouse which permits rapid changing from one source to another. At the moment, however, reflectance work can only be carried out in the visible region of the spectrum (400 to 750 nm). Irradiation occurs at a zero-degree angle. For the reflection measurements the radiation reflected from the chromatogram surface is viewed at 45°. Modifications of the chromoscan densitometer have been built by Goldman and Goodall.[34, 113] Again, UV reflectance work becomes difficult with this instrument which uses filters to render the light monochromatic.

A versatile VIS-UV Chromatogram Analyzer is manufactured by Farrand Optical Co. Inc. (Bronx Blvd & 238th Street, Bronx, New York 10470). The instrument (Figure 18) has been discussed in detail by Cravitt.[114] A schematic block diagram is presented in Figure 19. As the name says, the instrument can be used for reflectance work in the UV and visible regions of the spectrum. The light is rendered monochromatic in the exciter leg either with a grating monochromator or, if desired, a set of filters can be used. As can be seen in the diagram the excitation beam is split into a reference and analyzer beam. A sliver of light whose size is adjustable by selection of proper slits illuminates the sample at a zero-degree angle. The central portion of the light beam on the sample is viewed only by the analyzer optics at a 25° angle. Both sides of the beam are viewed by the reference channel at 45° and the two reference signals are averaged. The ratio of the sample and reference branch signals are recorded on a conventional strip-chart recorder. This operation is rather unique and serves to eliminate background fluctuations on both sides of the chromatographic spot. Light sources and optics can be chosen to fit the spectral region of interest. Single-beam operation is also possible and in situ fluorescence can be measured. The scanning table permits manual adjustment in one direction and the choice of nine different scan rates in the other direction. Plates up to 20 x 20 cm can be accommodated.

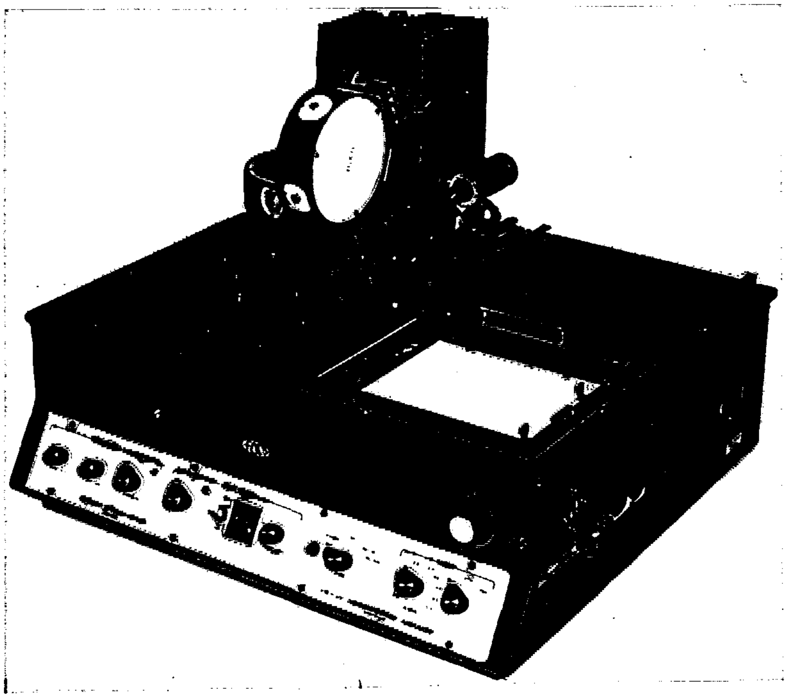


FIGURE 18. The Farrand VIS-UV Chromatogram Analyzer.

From the author's experience this is currently one of the most versatile instruments on the market at a reasonable price. The lack of mechanical monochromator drives for automatic scanning of spectra could be considered a drawback but this is in the process of rectification in future models.

Two Densitometers, Models SD2000 and SD3000, marketed by Schoeffel Instrument Corp. (24 Booker Street, Westwood, New Jersey) have recently been modified for use in reflectance spectroscopy. Both operate on a similar principles, but the SD2000 is a lower-cost filter instrument for routine use, particularly in medical laboratories. The Model SD3000 with the many possible modes of operation could serve as a research instrument for various types of problems. A prism monochromator renders radiation monochromatic. Tungsten-halogen and xenon or xenon-mercury lamps of 150 W and 200 W, respectively, are used for energy sources and focused on to the monochromator by means of a quartz condenser and surface reflector. Monochromatic light can be selected in the range 200 to 700 nm by means of a linearly calibrated wavelength dial. The radiation from the monochromator is magnified by a quartz optics and folded beam-mirror system on to the sample surface at a zero-degree angle of irradiation. The diffusely reflected light is viewed at a 45° angle with two separate phototubes. The same instrument can be operated in the single-beam mode and be used for in situ fluorescence and transmittance work on paper, TLC, and electropherograms. The basic computing readout unit computes log 1/reflectance or transmittance, respectively, which is not too useful for reflectance work, since a nonlinear relationship with concentration is usually obtained. Modifications for optional readout in units of the Kubelka-Munk function are in preparation. On request the same unit is available as a computing/readout/recording system or with a disk integrator. The data can be displayed in digital or printed form, if desired.

It has been claimed and demonstrated by the manufacturers of this instrument that measurements in the visible region of the spectrum are made to advantage in the transmission mode (in situ) rather than the reflectance mode. However, this could not be due to the inferiority of the reflectance technique as such but, the author believes, is due to a poorer design of the reflectance attachment which does not permit a very efficient collection of the diffusely reflected radiation.

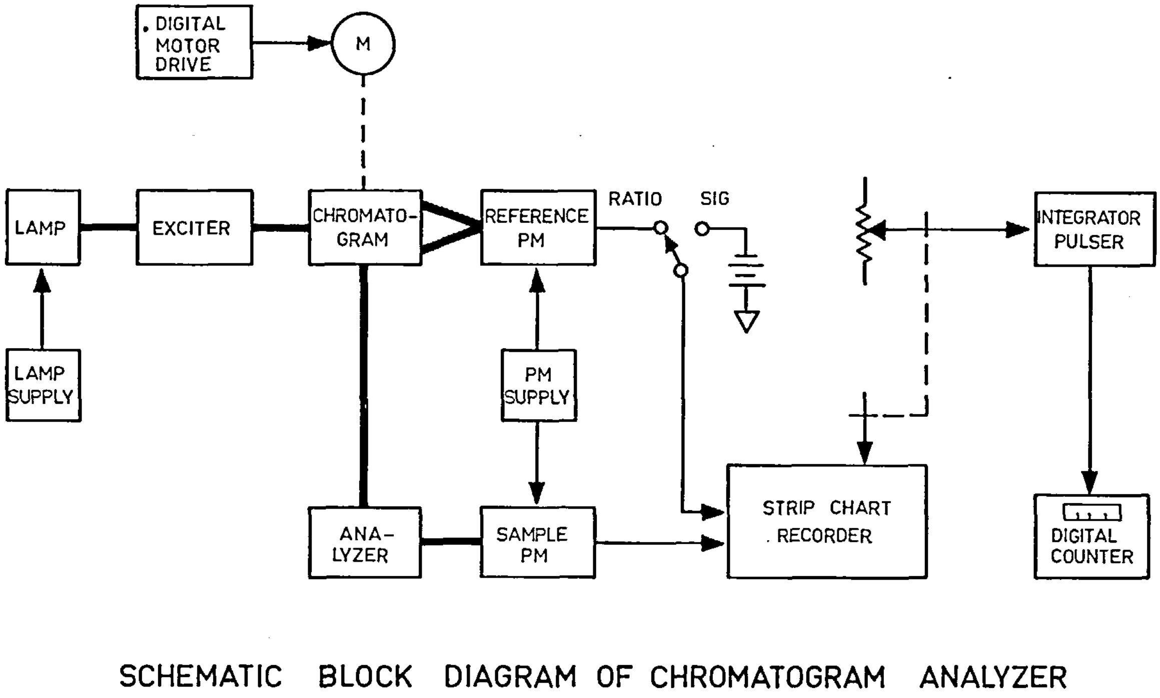


FIGURE 19. Schematic block diagram of the Farrand Chromatogram Analyzer.

Nester/Faust Manufacturing Corp. (2401 Ogletown Road, Newark, Delaware 19711) have recently developed a chromatogram scanner, "Uniscan 900," which permits work in the visible reflectance mode. For UV-active nonfluorescent compounds the fluorescent quenching mode has to be used. For normal operation a double-beam system is used. Rather than splitting the beam, as in most other double-beam instruments, two separate energy sources are used for sample and reference beams. The geometry of illumination and observation is $_{45}{}^\circ R_0{}^\circ$, and a solid-state matched pair detector is provided. The instrument can also be operated in the single-beam mode for cases where space is not available for scanning of the plate surface along with the spot to be analyzed. A set of five standard color filters is available for rendering the radiation monochromatic. The resolution can be enhanced with collimating slits. The instrument can also perform direct fluorescence measurements on chromatograms. An unusual feature is the moving scanning head. Other manufacturers used a moving table and kept the optical head fixed.

The American Instrument Co. Inc. (8030 Georgia Ave., Silver Springs, Maryland 20910) has developed an instrument designed specifically to measure direct reflectance, transmittance, fluorescence, and fluorescence quenching on chromatograms. It can be operated in the single- or double beam mode and covers the UV and visible regions of the spectrum. Illumination of the chromatogram surface occurs at a zero-degree angle with monochromatic light (grating), and the viewing angle is different from zero so that specular reflectance can be avoided.

Some of the above-mentioned instruments have been discussed in a recent paper by Lefar and Lewis.[115]

IV. APPLICATIONS

A. Principle and Method of Color Measurements

A frequent problem in many industries is the objective identification and specification of a color in a form suitable for documenting. Therefore, the use of instrumental rather than visual techniques for recording of colors seems a logical choice, and since color manifests itself in the form of reflected light emerging from the object under observation, it is not surprising that spectral reflectance techniques play a major role in the field of color technology.

A number of color-order systems have been developed,[1,116] but the CIE system (Commission Internationale de l'Eclairage), also known as the ICI system (International Commission on Illumination), is by far the most widely adopted. Many authoritative literature sources are available in this field.[1,117-121]

1. The CIE System

This color system[52,122,123] is based on the concept of the three-dimensional nature of color and on the additivity concept of spectrum colors, first described by Newton (1730) and later by Grassmann (1853). Grassmann's law[124] states that the color-matching functions or *tristimulus values* of the spectrum colors can be calculated for any specified set of primaries. In other words, colors can be specified in terms of three numbers representing relative amounts added from three primary sources. These amounts are known as tristimulus values. The three tristimulus values are designated X, Y, and Z. Y stands for the lightness response (also known as brightness or luminosity); X and Z describe that aspect of color which permits its identification with various regions of the spectrum (hue) and which determines its excitation purity (saturation).

The trichromatic equation for tristimulus values can be represented in the form

$$\text{Color (C)} \equiv x(X) + y(Y) + z(Z) \tag{28}$$

and x, y, and z represent the chromaticity coordinates of C, obtained by the following set of equations:

$$x = \frac{X}{X + Y + Z} \qquad y = \frac{Y}{X + Y + Z} \qquad z = \frac{Z}{X + Y + Z} \tag{29}$$

Since x + y + z = 1, two coordinates are sufficient to describe the color. A chromaticity diagram permits the representation of the colors by means of coordinates x and y which describe hue and saturation. The pair of x and y values is known as chromaticity. A clear specification of the illuminating light source is also required for an unambiguous designation of an object's color. In 1931 the CIE recommended the use of three standard illuminants. The three sources are known as illuminants A, B, and C. A is equivalent to a gas-filled incandescent lamp, B is representative of sunlight, and C represents daylight from an overcast sky. A specification is also possible in the form of color temperatures resulting from a comparison of the energy distribution in the visible range of the spectrum to that of a black-body radiator.[125] The corresponding color temperature would then be 2854°K for illuminant A. Illuminant B would make use of the same source in conjunction with a suitable filter to give a color temperature of about 5000°K, and illuminant C would essentially correspond to A and B, but it uses a filter which brings its color temperature up to 6800 °K. These concepts then form the basis for instrumental measurement of color and the representation of color values in numerical form within the CIE system.

Three types of instrumental approaches to such measurements can be chosen: the equi-contrast method, the trichromatic method, and the spectrophotometric method.

2. *The Equi-contrast Method*

In this method the eye is used as a detector, which means that the measurements still depend on certain characteristics of the eye, such as normal color vision, limited field of vision, etc. Additive mixing of three primaries (e.g., red, green, and blue) and matching of instrumentally measurable quantities of these three sources vs. a reference standard (white light) and then vs. the color to be measured, are used as the principle of operation.

It is now possible to eliminate the restrictions of the human eye detector simply by replacing the eye with a suitable photodetector. This modification would then lead to the second approach, known as the trichromatic method.

3. *The Trichromatic Method*

Light filters (tristimulus filters) are positioned in front of the phototubes to simulate standard observer conditions. This adaptation is generally applied in the red, green, and blue ranges of the spectrum and should give the phototube an approximation of normal color vision.

Direct measurements of tristimulus values can be carried out with this experimental arrangement,[126, 127] provided the photocell response is approximately proportional to some linear combination of the distribution curves of these tristimulus filters in the visible spectral region. According to Grassmann's law[124] it would then be possible to test whether any two light beams have the same color. The accuracy of this approach depends largely on how closely the photocell-tristimulus filter combination can match the characteristics of the CIE standard observer.

For the interpretation of colorimeter readings the measurements (usually made vs. an MgO or $BaSO_4$ standard and illuminated with a CIE illuminant) are taken through each of the filters and the corresponding readings R_x, R_y, and R_z are multiplied with factors characteristic of the set of filters used.

4. *The Spectrophotometric Method*

For higher accuracy requirements in color measurements the investigator would probably choose a spectrophotometric approach. In this case tristimulus values are determined ideally from each individual spectral color. In practice, the method consists of measuring the reflectance spectrum of the compound under observation. This could be done with any of the existing commercial spectrophotometers with reflectance attachment (section III C). Judd[128] has given a detailed discussion on the computation of tristimulus values from reflectance spectra. The first step consists of the use of suitable multiplying factors M_n ($n = x$, y, or z) by which a spectral curve between 380 and 780 nm can be represented with color coordinates. Mathematically this could be represented as

$$X = \int_{380}^{780} R\,M_x\,d\lambda \qquad (30)$$

R stands for percent reflectance measured on the transmission scale of the regular spectrophotometer; λ is used for wavelength, and M is the multiplying factor. Equations for Y and Z would be similar. A combination of Equations 29 and 30 results in the following equations:

$$x = \frac{\int_{380}^{780} R\,M_x\,d\lambda}{\sum_{n=x}^{z} \int_{380}^{780} R\,M_n\,d\lambda} \qquad (31)$$

$$y = \frac{\int_{380}^{780} R\,M_y\,d\lambda}{\sum_{n=y}^{z} \int_{380}^{780} R\,M_n\,d\lambda} \qquad (32)$$

The factor M is dependent on the type of illuminant (A, B, or D) used.

For actual computation of the chromaticity coordinates it is obvious that the above equations would be rather tedious to use. One could therefore adopt approximation methods which would involve the integration at certain preselected wavelength intervals. The color points can then be determined by means of trichromatic coefficient computing forms usually provided by the instrument manufacturers. However, there are limitations for these approximation methods. One is that the spectra should not exhibit any sharp peaks (which is usually the case with reflectance spectra) and fluorescence should be completely absent. It is obvious that these computing functions could be done very well with a small digital computer; this would eliminate the need for approximation methods and would enable computation of large amounts of samples. Indeed, there are instruments on the market which use built-in computers for instant readout of color coordinates (see section III B).

B. The Measurement of Pigments

Professional color matchers still do a large proportion of the color mixing and matching in the pigment industry. Spectral reflectance techniques, however, are becoming increasingly important in this segment of application, particularly in cases where the pigments are mixed with a large proportion of yellow or white materials. In such an event, the Kubelka-Munk theory is fairly applicable and the coefficients of absorption at any given wavelength are additive for the individual components in the mixture (see Section II D). If one deals with samples with an opaque surface, such as powder, which adheres to the rule of infinite layer thickness, then the Kubelka-Munk function can be used in the usual form (Equation 3). For paints, linoleums, plastics, and similar materials, in which the pigment particles are held together by means of a continuous medium, a correction factor has to be introduced which takes into account specular and internal reflections occurring at the air/medium interface.[129,130] Other exceptional cases exist when the colored pigments are present in high concentrations, which again results in deviation from the Kubelka-Munk law. In such instances the sample can occasionally be diluted or semi-empirical functions have to be introduced, which extend the linear range of the concentration-reflectance relationship.

Duncan[131-133] and others[134,135] have discussed a number of practical examples of pigment analyses based on such an approach. Often the analysis time can be reduced by using computers, such as designed specifically for this purpose by Davidson and Hemmendinger.[136-138]

The use of spectral reflectance for the identification of pigments is another interesting aspect which has been elaborated on in much detail by Duncan.[131] If only binary mixtures of pigments are used, simple inspection of the spectra can yield sufficient information for positive identification. For more complex systems, absorption coefficients K obtained at certain wavelength intervals can be tabulated and used for identification purposes.

Investigations such as discussed in this section usually precede final color-matching procedures. They constitute still relatively crude approximations and the final step then involves the measurement and computation of color coordinates as discussed in section IV A.

C. The Measurement of Miscellaneous Samples

1. Biological Systems

Brinkman and Zijlstra[139] have reported on a reflectance method for the measurement of blood saturation for oxygen. The name for this method was proposed as "Reflection Oximetry." An equation for the relationship of oxygen saturation (OS) and corresponding reflectance was suggested by Rodrigo[140] and can assume the following form:

$$OS = A + \left[\frac{B}{I_r(\lambda)}\right] \qquad (33)$$

where A and B are constants which depend on instrumental factors such as optical geometry, wavelength, and intensity of illumination, and I_r (λ) stands for the diffuse radiation at wavelength λ. In order to have infinite layer thickness of the sample, Rodrigo chose samples of a thickness ≥ 3 mm. The obvious dependence of this equation on instrumental variables prompted Polanyi and Hehir[141] to construct a reflection oximeter which permitted the measurement of absolute OS values.

Some applications of a rather unusual nature have been reported. Lubnow[142] studied several strains of birds with respect to the melanine in their plumage. On the basis of this investigation he was able to distinguish two different strains of lark. Others[143,144] have reported on the reflectance spectroscopic study of hummingbird feathers and found amazingly good agreement between certain types of feathers taken from various subjects.

Derksen and Monahan[145] measured human skin in the visible and near-infrared regions with a homemade reflectometer and used a semisphere for the collection of the diffusely reflected radiation. The tanning of human skin as a result of UV light irradiation from several artificial UV sources for various lengths of time was studied by spectral reflectance by Luckiesh et al.[146] Measurements were carried out on a brunette, and intermediate, and fair blonde subject. Ardent racial segregationists may find this method useful for the establishment of a skin-scale, which would help them to establish whether a person belongs to a white or non white race. Obviously, fluctuations due to tanning during the summer could cause considerable consternation and confusion.

Some workers have reported on reflectance studies of foliage and leaves.[147,148] Factors such as chlorophyll production in sea plants or the influence of chemicals on the appearance of foliage can be studied conveniently by this approach.

Despite this rather sketchy account of biological applications, it can easily be seen that the possibilities of use for problems of this nature are endless, and it remains up to the ingenuity of the individual investigator to devise suitable procedures for his particular problems.

2. *Building Materials*

Spectral reflectance techniques have found wide acceptance in the construction industry for quality comparison and matching of building materials. They have also been found suitable for the investigation of lighting conditions, thus enabling proper choice of interior design and assisting the illumination engineers. Moon[149-153] has published a large number of such studies. All his work was carried out with the Hardy Reflectance Spectrophotometer. The samples investigated included floor coverings, such as cork, linoleum, and oak flooring, asphalt tiles, and carpets. Similar measurements were made on materials used in schoolrooms, such as chalk boards, wall paints, and window shades, as well as certain types of metal finishes.

For lighting and decoration studies of bathrooms and kitchens, tables of reflection factors for ceramic tiles were found useful.[151] Moon has further used this technique for the investigation of acoustic materials for ceilings and walls,[152] for wall paper and latex paints, and many other media. For ceramic tiles and glossy paints, gloss properties may be of importance since a large portion of the reflected light may be given off as specular components. Usually illumination engineering makes use of the total reflection properties, including both reflection modes.

3. *Foodstuff*

In recent years, diffuse reflectance spectroscopy has been adopted in a number of food laboratories and processing industries in an attempt to eliminate personal factors in visual comparison of color changes in foods. Only a few examples for the application of this technique to studies of foods are discussed here.

Guerrant[154] studied changes in reflectance as a result of variations of ascorbic-acid content in foods during storage at deep-freeze conditions. Naughton et al.[155,156] have investigated the "greening" process in tuna fish flesh upon precooking. Various heme pigments were investigated and the greening phenomenon was traced to an anomalous heme protein oxidation process. Similar studies on heme pigments in fresh beef were carried out by Kraft and Ayres[157] and by Pirko and Ayres.[158] The relative proportions of myoglobin, oxymyoglobin, and metmyoglobin could be indicated from the corresponding reflectance spectra.

The irradiation effect on meat samples treated with γ rays was studied by Ginger et al.[159] A group of instruments was tested and rated as to their suitability for the measurement of canned tomato juice.[160] A reflectance scale for different grades of tomato puree was proposed by Yeatman et al.[161] and measurements were made with a tomato reflectance colorimeter disigned by Hunter[162] in collaboration with Yeatman and his group.

Many food dyes were investigated by spectral reflectance techniques. Yamaguchi et al.[163] measured food colorants adsorbed on filter paper. Food dyes were also quantized by this technique following separation on thin-layer chromatograms.[164] Some of the factors influencing the reflectance of food dyes adsorbed on alumina were reported by Frei and Zeitlin.[165]

In general it appears again from all the work reported in this area that reflectance spectroscopy can solve many of the problems pertinent to the food-processing industries. Much of the reflectance work done up to now by tedious measuring procedures, however, could be largely automated, resulting in a significant saving of time and labor.

4. *Geological Systems*

An excellent discussion on the measurement of color during microscopic ore examinations has been written by Piller.[166] The classification and identification of ores and minerals color is considered very important, and it was desirable to eliminate the inherent drawbacks of visual observation. The lack of suitable microscope reflectometers and the unfamiliarity of geologists with this technique and with color-measuring systems can be given as a reason for the relatively late adoption of instrumental color-measuring methods to geological problems of this nature. Commercial microscope reflectometers are now available. The work by Piller, for example, was carried out with Zeiss instrumentation designed by the author himself. Joyce, Loebl & Co. Ltd. is another company manufacturing such equipment. Piller has discussed the use of the CIE color system in connection with these systems, but the specification of mineral colors according to Helmholtz is often considered more desirable since a closer correspondence to hue and saturation can be achieved.[166] With regard to errors encountered in microscopic reflectance measurements systematic errors were found not to exceed 1%. Experimental errors due to equipment or sample parameters can be eliminated to some degree by the proper choice of technical procedures.

An interesting investigation of desert surfaces has been carried out by diffuse reflectance spectroscopy.[167] For this purpose a specific instrument had to be constructed which permitted measurements of an extended surface. The measurements were made from heights between 1.5 and 2 m above the surface and in some cases from a helicopter at an altitude of 200 m. The data revealed that for all types of desert surfaces investigated, the reflectance in the blue portion of the spectrum was significantly less than in the red. The range investigated was from 390 to 650 nm. Varying the height of observation between 2 and 200 m did not result in significant variations of reflectance data.

5. *Paper and Pulp Material*

The literature on reflectance spectroscopy pertaining to this field is highly developed since the measurement of color, whiteness, brightness, and gloss properties are important qualities in paper manufacturing. A few examples dealing more specifically with diffuse reflectance spectroscopy will be treated in this section.

One of the earlier investigations of this nature was reported by Taylor,[168] who studied reflectance factors of a number of commercial newspaper grades. Anacreon and Noble[89] and Stenius[72] studied different grades of colored papers. Investigations of cellulose layers and paper in connection with chromatographic processes will be discussed in section IV G.

The major use of spectral reflectance techniques has been for the measurement of whiteness and brightness. Luner and Chen[169] discussed the control of these factors in wood pulp during the paper manufacturing process.

For the final product itself the white color is an important indicator of its quality. Differences in whiteness are not solely attributable to differences in lightness but are also due to real color differences. As revealed by a detailed study[170,171] by Vaeck, a touch of blue suggests a higher quality of white and any other hue would be taken as a lower quality. Recently a system was proposed[172] which permits arranging color coordinates of white colors according to a specific scale. The system is so designed that individual differences in color perception between different observers can be taken into account.

Stenius[173,174] critically discussed the parameters in brightness and whiteness measurements of papers. The Zeiss Elrepho Colormeter received particular attention in these discussions. Many of the principles involved in the investigations of paper are also valid in the field of textiles where optical brighteners are used widely.

The proper choice of reference standards is important for the measurement of samples with such a low color saturation. Budde and Chapman[76] recently discussed some of the problems related to the calibration of standards for "absolute brightness" measurements. Further discussions on reference standards have been given earlier (section III A). The validity of the Kubelka-Munk function in reflectance measurements of papers has been assessed in a number of publications.[175-177]

6. *Pharmaceutical Products*

Diffuse reflectance spectroscopy is a well-established technique in pharmaceutical industries. Aging, illumination, temperature, and humidity effects on tablets, powders, creams, and emulsions are best studied by this technique and it also has an important place in production control.

A sample holder has been described which permits the reproducible measurement of tablets on the Beckman DU equipped with the reflectance attachment.[178] The precision obtained with this holder was reported to be better than 2%. The same holder was used to investigate the light fastness of water-soluble dyes officially permitted for the coloring of tablets.[179-181] Figure 20 shows the influence of excessive and normal illumination over a maximum period of 84 days in the form of kinetic plots for FD&C Blue No. 1. Rate constants for the fading process can then be calculated from these plots.

The method for investigating fading processes has been further improved by Everhard and Goodhart.[182] A first-order rate equation has been proposed by these authors which permits the calculation of fading processes as they occur under normal illumination conditions, based on data obtained with a high-intensity irradiation. The major advantage of this approach is, as expected, the substantial time-saving factor. For the previously discussed method[179,180] each intensity had to be considered as a separate case to which a different kinetic constant was associated. Everhard et al.[183] have also discussed spectrophotometric reflectance methods for matching the color of solid dosage forms. Even though techniques similar to those discussed in section IV B can be used, Everhard has pointed out the particular difficulties associated with pharmaceuticals, e.g., the limited number of permitted dyes available and the limited choice of tabletting materials. The use of a computer, such as described by Davidson and Hemmendinger,[137] again proves to be of great help in this type of investigation.

7. *Textiles*

One of the earlier applications of reflectance spectroscopy to textiles was reported by Hardy[4] who measured a piece of green silk to illustrate the performance of an instrument which he designed and built and which was a forerunner of the Hardy Reflectometer produced commercially by General Electric Co. Since then a large number of investigators have reported on color measurements of dyed fabrics by this technique.

Moon and Cettei[184] analyzed a large number of samples of men's wear fabrics in order to study contrasts that can be obtained with pedestrian clothing and their surrounding background. Conventional street illuminants were used by the investigators for illumination of the samples.

A custom-made reflectometer with a semi-

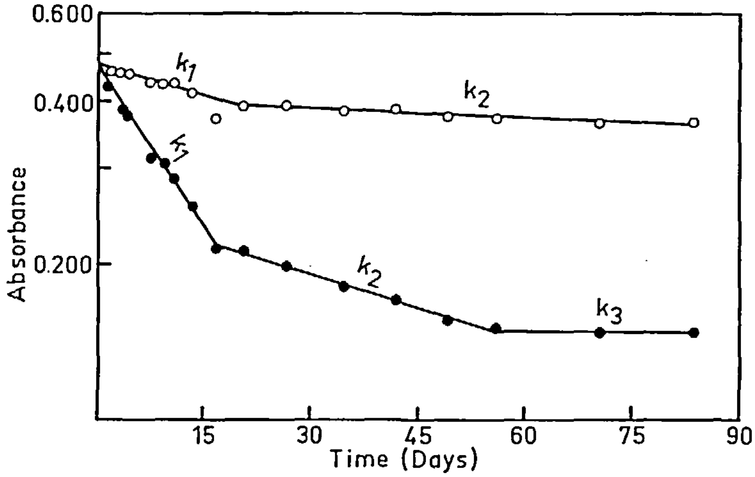


FIGURE 20. Influence of light intensity on the fading of the surface of tablets colored with FD&C Blue No. 1. o, ordinary light; •, accelerated light.[179]

sphere design was used by Derksen and Monahan[145] for fabrics and other samples in the visible and UV regions of the spectrum. The utility of such techniques to the investigation of dyeing mechanisms was shown by a number of workers.[185] Another area is the investigation of phenomena such as bleaching as a result of intense illumination[186] and the influence of elevated temperatures, humidity, and aging on numerous fabrics.[187]

Fourt and Sookne[188] investigated the influence of angle of incident light on measured reflectance on some cotton yarn samples. The optical geometry as well as the orientation of the yarn with regard to the plane of light was found to be critical (see also Table 5). For strongly structured samples such as this, a diffuse illumination via an integrating sphere or ellipsoidal mirror is often preferable. Laundering processes which enable the objective evaluation of the effectiveness of soaps and detergents have also been studied with this technique.[189,190] The evaluation of optical brighteners often used in modern detergents has been discussed by Vaeck.[170] The determination of whiteness as a result of such chemicals can be determined by the new reflectance spectroscopic method proposed by Vaeck[170] without interference of fluorescent components present in the system. This can be achieved with suitable filters discussed by the same author.

Müller-Gerber[187] has presented a comprehensive treatment of the use of diffuse reflectance spectroscopy in the textile field. The investigation of dyed fabrics by differential reflectance spectroscopy was first advocated by Lermond and Rogers.[26] It has proven to be advantageous in cases of strongly dyed samples with low reflectance, where dilution of the sample is not possible (see section II D). Instead of using a regular reference standard such as $BaSO_4$ or MgO the instrument scale is set to 100% R with the least concentrated sample (absolute reflectance as low as 2% R) positioned in the reference port. The resulting scale expansion permits differentiation of a series of highly concentrated samples which otherwise would show approximately the same reflectance value.

Numerous methods were suggested for the presentation of dye concentration on fabrics vs. the corresponding reflectance. Using the Kubelka-Munk function or percent R vs. concentration resulted in smooth curves suitable for analytical purposes, but obviously the Kubelka-Munk theory is not strictly applicable for such systems since linearity in the Kubelka-Munk plot is only observed for very low concentrations. Plotting the same function vs. log of concentration resulted in an extension of the linear concentration range.

The reproducibility of measurements one can observe with this type of investigation as well as

TABLE 5

Studies on Yarns Dyed with Ponsol Brown, Showing Reproducibility of Measurements and Effects on Reflectance of Pressing Surface of Mounted Sample and of Changing Direction of Yarn. Samples were Measured against Magnesium Carbonate[26]

Wavelength (nm)	Removal and replacement of given sample, % R ± S.D.* (4 measurements)	Pressed surface, (Av. % R of 2 measurements)	Direction of threads in sample changed 90°, % R ± S.D. (4 measurements)
400	7.67 ± 0.06	8.25	7.20 ± 0.10
420	5.93 ± 0.10	6.27	5.63 ± 0.07
440	4.79 ± 0.03	5.13	4.63 ± 0.04
460	4.68 ± 0.01	5.10	4.50 ± 0.06
480	5.62 ± 0.03	6.19	5.39 ± 0.15
500	7.76 ± 0.07	8.44	7.55 ± 0.10
540	11.50 ± 0.10	12.20	10.90 ± 0.10
580	14.90 ± 0.10	15.80	13.90 ± 0.10
620	32.50 ± 0.20	33.80	31.40 ± 0.40
660	54.70 ± 0.10	55.80	53.00 ± 0.20
700	62.10 ± 0.20	62.60	51.00 ± 0.30

S.D. (standard deviation)= $\frac{\sqrt{\Sigma(x - \bar{x})^2}}{n - 1}$

the effects of sample preparations on the resultant reflectance are presented in Table 5.

D. Surface Phenomena

The use of reflectance techniques for the investigation of surface phenomena is one of the most appropriate fields where this technique can be applied. The only alternate approach for the investigation of adsorbed species, for example on powder adsorbents, would be transmission of light through thin layers of the material, which in the infrared region of the spectrum has met with good success due to a substantial decrease of light scattering. At lower wavelengths, however, this scattering process becomes a serious interference, and the difficulty of reproducing the layer thickness of the samples is another handicap of this technique.

1. Sample Preparation

The preparation of samples for problems of this nature is extremely critical. Reflectance spectra have been found to vary considerably with differences in particle size of the adsorbent. It is therefore recommended to standardize grinding and sifting procedures. Grinding in ball mills or mortars has been used by Kortüm and co-workers. For this purpose porcelain ball-mill containers with two to six balls of varying sizes, made of the same material, were recommended. For harder materials, agate containers and balls are preferred. The same type of grinding equipment should be used within one dilution series. At least six hours grinding was suggested. The errors occurring due to abrasion of the grinding equipment were found to be negligible in comparison to the overall reliability of the reflectance technique.

The simpler sample preparation procedures reported by Zeitlin and co-workers consisted of 15-min grinding in agate mortars, followed by sifting through fine mesh screens (e.g., 200 mesh), and they were usually satisfactory. When packing the samples, the surfaces should be smooth and free from gloss, particularly in the case when directional illumination, e.g. at 45°, is used. With the use of sample cells such as the one described by Barnes et al.[92] these surface conditions are fulfilled quite satisfactorily. The gloss from the cover glass is usually negligible for a zero-degree angle of incidence. Without the use of a cover glass, a glass or metal tamp can serve conveniently to press the sample into a smooth surface. The sample itself can be placed in a small planchet. In cases where gloss becomes a significant problem, a thin layer of the same powder can be sifted over the surface. The pressure of packing should be standardized as far as feasible. Powder layers between 2 and 5 mm thickness will usually give an infinite layer thickness.

The use of reference standards has been discussed in section III A. Often the adsorbent or neutral diluent used in a particular experiment is also chosen as the reference standard.

Air humidity is generally considered an undesirable factor for investigations of this nature since the highly polar water molecules can compete for the available adsorption sites on the sample surfaces. Sample preparation is therefore often carried out in controlled atmospheric conditions, e.g., weighing, mixing, grinding, and sample packing are preferably done in a glovebox with dynamic air-drying through a bed of silica gel or molecular sieves. The transfer of samples from the dry atmosphere to other locations is conveniently done in a small dessicator containing phosphorus pentoxide or another suitable drying agent. The cell mentioned earlier[92] can be sealed with silicon grease at the edges of the planchet to keep the moisture out during the measuring process.

To obtain a homogeneously mixed sample, the mixing and grinding procedures described above are quite satisfactory for solid-solid adsorbent-adsorbate interactions. Adsorption from the gas phase can be done in a closed system which is heated in order to obtain a high vapor pressure for the adsorbate. Temperature, atmospheric, and concentration conditions can be controlled conveniently with such an approach. The disadvantages are slow equilibration of the system and the restriction to compounds with relatively high vapor pressure.

For adsorption from solutions control of the various parameters poses problems and the influence of the solvent has to be taken into account. On the other hand, no tedious grinding procedures are needed for a homogeneous mixing of the two phases. The solvent, which should be completely neutral, can be withdrawn by a vacuum stripping procedure while the sample is kept in a controlled environment, or if volatile enough, it can be left to evaporate into the atmosphere of the drybox. Obviously, before exposing the adsorbent to the adsorbate, it has to be treated so as to have a uniform and defined particle size and a controlled state of regeneration.

Sample preparation for analytical purposes need not be as rigorous as described in this section.

2. *Adsorbent-adsorbate Interaction*

Weitz et al.[191, 192] and de Boer and Houben[193] have reported color changes for many organic systems after adsorption on active surfaces. Polarization, Lewis acid-base interactions and formation of electron donor-acceptor complexes are but a few interpretations given to the occurrence of these phenomena. They can all be classified under the expression chemisorption, and there is a considerable amount of controversy on the subject.

Polarization was taken as a basis to explain shifts of absorption maxima in reflectance spectra of Hg (II) compounds adsorbed on a number of active substances.[194] Table 6 shows the wavelengths of the initial and final absorption maxima for the Hg compounds with three different adsorbents. After periods of 60 to 90 days it was assumed that reactions were complete. From these data it is evident that silica gel and alumina exert a stronger polarizing action on the mercury compounds (larger shifts are observed on these two adsorbents). The data also confirm the different polarizabilities of the anions, which–in adherence to the theoretical predictions–result in bathochromic shifts in the order Br > Cl and S > O. The data for the HgI_2-alumina system did not follow the general pattern. The anomalous behavior of this system has been discussed elsewhere.[195,196] Later, the polarization theory was extended to organic adsorbates on adsorbents such as starch, talcum, and alkaline carbonates. Monoitrophenols were chosen as the first systems to be investigated.[197-199] Large bathochromic shifts of different magnitude were observed for the reflectance maxima of the organic compound, depending on the adsorbent. The order observed was $K_2CO_3 > Na_2CO_3 > Li_2CO_3$. This corresponds to the order of effective nuclear charges of the adsorbent cations involved, and it is assumed that as a result of the decrease in polarization power in going from Li to K, the excitation energy of the nitrophenols is lowered, hence causing the observed bathochromic shift.

An extension of this investigation was carried out by Zeitlin et al.[198] Only *o*-nitrophenol was examined, and a group of alkaline earth oxides served as adsorbents. Again, as expected, bathochromic shifts in the order BaO>SrO>CaO>NgO> (BeO) were observed. BeO behaved rather anomalously, due to its different crystal structure (zincblend rather than rock-salt structure). Table 7 summarizes some of the results obtained in this study and demonstrates also the influence of the regeneration temperatures. It is assumed that the heat treatment of the adsorbent results in a gradual removal of co-adsorbed water molecules and the phenol gets more exposed to the polarization action of the adsorbent (first monomolecular layer). As would be expected, this

TABLE 6

Wavelength (nm) of the Initial and Final λ_{max} of Hg(II) Compounds Mixed with Alumina, Silica Gel, and Sodium Fluoride[194]

	λ_{max}(initial)		λ_{max}(final)	
Alumina				
HgI_2	575	290		290
$HgBr_2$		248		255
$HgCl_2$		249-230		233-234
HgS	570		570	
HgO	501		501	
Silica gel				
HgI_2	568	269-272		317-320
$HgBr_2$		240-243		263-264
$HgCl_2$				220-225
HgS	563-565		568-569	
HgO	518		517-520	
Sodium fluoride				
HgI_2	573	293	573	293
$HgBr_2$		248-249		248-249
$HgCl_2$		233		233
HgS	560-566		560-566	
HgO	520-528		520-528	

TABLE 7

Wavelength Maxima (nm) of the Reflectance Spectra of *o*-Nitrophenol Adsorbed on Alkaline Earth Oxides[198]

Oxide	Air-dry	Regeneration temperature		
		200°C	600°C	1000°C
BeO	416 ± 2	434 ± 3	440 ± 3	444 ± 3
MgO	420 ± 2	420 ± 2	427 ± 2	428 ± 3
CaO	424 ± 2	426 ± 2	428 ± 3	430 ± 3
SrO	434 ± 2	440 ± 2	452 ± 2	ca. 454
BaO	440 ± 2	447 ± 2	458 ± 3	

phenomenon is reversible when the samples are exposed to humid air. Zeitlin and Lieu,[199] in an attempt to shed more light on the nature of the adsorbed phenolic species, studied the sodium salts of a number of nitrophenols as well as di- and trinitrophenols. No shifts were observed for the phenolic salts, and it was believed that the spectra of the salts were those of the phenoxide anions in the adsorbed state. With the mononitrophenols, which are relatively weak acids, an equilibrium, which is in favor of the non-ionized species, is assumed to exist. This equilibrium would be strongly influenced by the polarizing power of the cations in the adsorbents, resulting in a significant shift of the spectrum (as has been observed[197, 198]). Once the phenoxide ion is the only species present, however, polarization could not affect the position of the spectrum any longer.

Similar shifts were observed with nitroanilines[200] as with the previously investigated nitrophenols (see Figure 21), except that the shifts were somewhat less pronounced. In this study it was also attempted to ascertain whether a correlation existed between the intensity of absorption maxima and the adsorbent used. Figure 21 shows the relative peak heights for equal mole ratios of *o*-nitroaniline on alkaline earth oxides of 200 mesh particle size, regenerated at 300°C, and measured vs. MgO as a standard. A decrease in color intensity in the order Mgo>CaO>SrO>BaO was observed. BeO again behaved differently. An explanation for this phenomenon was given on the basis of differences in active surface areas of the adsorbents investigated. According to this, MgO would have the largest and BaO the smallest active surface area or, better, the largest amount and smallest amount of adsorption sites per unit area, respectively. The same order was observed with increasing size of anions, resulting in decreasing peak heights in the order $MgO>MgCO_3>MgSO_4$. Although the conclusions reached above are quite interesting and might stimulate further research in this area, the author believes that actual surface area measurements by the BET method or by reflectance spectroscopy (see section IV-D3) would be required to further substantiate this postulation.

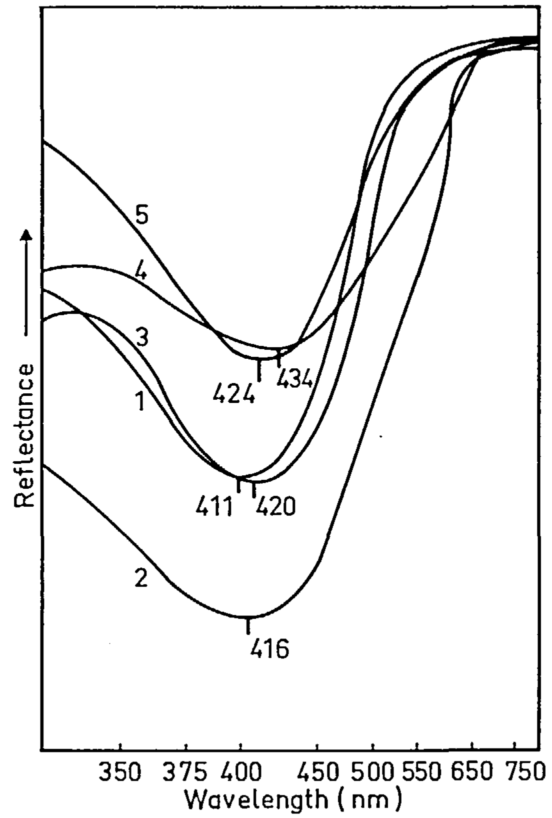


FIGURE 21. Reflectance spectra of 0.04 g *o*-nitroaniline adsorbed on 0.05 mole of 1, BeO; 2, MgO; 3, CaO; 4, BaO; 5, SrO of 200-mesh particle size and regenerated at 300°C.[200]

Schwab et al.[201, 202] investigated a group of basic dyes of the benzene-derivative type. For each dye bathochromic shifts were observed with increasing acidity of the adsorbent in the order quartz powder < Al_2O_3 air-dried < SiO_2 air-dried < Al_2O_3 dried at 200°C < SiO_2 dried at 200°C < Al_2O_3 dried at 900°C < SiO_2 washed with HCl < pure solid adsorbent < Al_2O_3 washed with HCl < *a* Al_2O_3 < bentonite. In these examples the acidity could be attributed to the presence of active hydroxy groups or to a Lewis-type acidity as a function of incomplete coordination. It should also be possible, however, to find a satisfactory explanation of the phenomena observed in this study on the basis of the polarization theory as discussed by Zeitlin et al.[197-199]

Griffiths et al.[203] have demonstrated some of the possible uses of the reflectance technique in the UV region; specifically for inorganic systems. Lithium fluoride was chosen as a relatively neutral diluent, but surface phenomena still were found to interfere seriously with the investigations of strictly inorganic problems.

Kortüm[7] has undoubtedly made the major contribution to bringing diffuse reflectance spectroscopy to its present prominence in this field.

The system p-dimethylaminoazobenzene (DMAB) adsorbed on regenerated $BaSO_4$ and $CaSO_4$ was chosen by Kortüm et al.[32] to study transition from chemisorption to physical adsorption. The adsorbents were carefully heated to eliminate co-adsorbed water. It was then possible to observe a color change of the DMAB from yellow to red upon adsorption on the surface of the salts. The red band between 18000 and 21000 cm^{-1} (Figure 22) can be attributed to the interaction (chemisorption) between adsorbate and adsorbent in the first monomolecular layer. With an increase of the DMAB concentration, some of the material is progressively adsorbed in the second layer by physical adsorption, and as a result one observes the gradual formation of a yellow band at about 24000 cm^{-1} (Figure 22). The corresponding Kubelka-Munk plot of this system is depicted in Figure 23. If one plots the $F(R_\infty)$ values as computed from the reflectance values of the red band as a function of the mole fractions of adsorbent and adsorbate, one can easily determine the saturation mole fraction for chemisorption by extrapolating the two linear branches of the plot. The horizontal branch would correspond to the condition of complete saturation in the first monomolecular layer. The chemisorption process itself was explained by Kortüm on the basis of Lewis acid-base interaction, with the adsorbent acting as an acid.

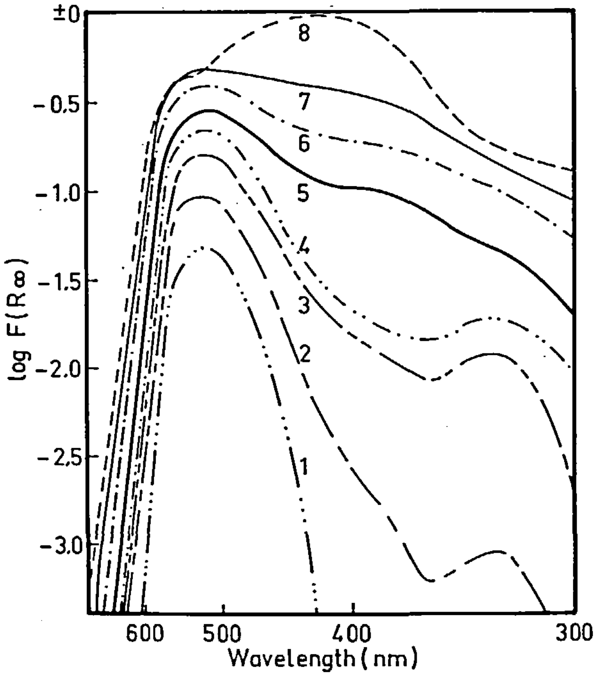


FIGURE 22. Reflectance spectra of p-dimethylaminoazobenzene adsorbed on dry $BaSO_4$ as a function of concentration. (1) $x = 1.40 \times 10^{-4}$; (2) $x = 2.42 \times 10^{-4}$; (3) $x = 4.07 \times 10^{-4}$; (4) $x = 6.69 \times 10^{4}$; (5) $x = 1.035 \times 10^{-3}$; (6) $x = 1.572 \times 10^{-3}$; (7) $x = 2.531 \times 10^{-3}$; (8) $x = 4.412 \times 10^{-3}$ [32]

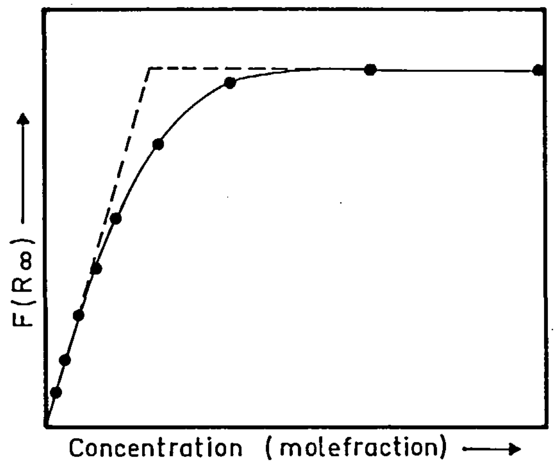


FIGURE 23. Adsorption isotherm as determined by reflectance measurements.

The adsorption of mercury halides such as investigated by Zeitlin et al.[194] could, according to Kortüm,[204] also be classified as a Lewis acid-base type interaction, except that in this case the adsorbent would act as a base. Probably the most widely investigated mercury salt is HgI_2.[13, 195, 196, 205] Visual color changes have been reported to occur upon adsorption of the HgI_2 in the red modification[191, 192] and have been attributed to the formation of individual Hg^{2+} and I^- ions as a result of bond cleavage. This phenomenon was later studied by reflectance spectroscopy with the mercuric iodide alumina system.[196] On the basis of their results the authors assumed that a change from the stable red to a metastable yellow modification occurred. On lithium fluoride, which is a rather inactive medium, no such color change was observed.[196, 205]

Kortüm[13] later investigated a mercuric iodide system (HgI_2-MgO) and concluded from this experimental evidence that neither of the two postulated reactions is valid. He suggested the existence of a tetrahedral mercury complex adsorbed on the surface of the adsorbent. This complex could be of a similar nature to that known in solution, and it is possible, according to his postulation, that active sites on the adsorbent could partially displace the I^- ligands on the mercury complex. The evidence compiled by

Kortüm seems most convincing and lends strong support to the mechanism proposed by him.

The possibility of participation of active adsorption sites in actual complexing processes has also been suggested by Frei and Zeitlin[206] in a reflectance study of some adsorbed metal chelates of pyridine-2-aldehyde-2-quinolylhydrazone (PAQH). If the 2:1 chelates of Cu or Ni were applied to starch or cellulose a change from the brown bis chelate to the red 1:1 species could be observed to occur within a few seconds. These observations led to the conclusion that the cellulose or starch-water complex formed in air humidity may actually be blocking some coordination sites on the metal, hence permitting only the formation of a 1:1 chelate. As a result the existence of a mixed ligand complex of the formula

$$[\mathrm{Ni(PAQH)\text{-}cellulose{:}(H_2O)_x}]$$

with unknown charge was suggested.

Kortüm has also described some systems which he classified as electron donor-acceptor complexes. Of particular interest were the relatively simple systems involving adsorption of iodine or bromine on adsorbents such as alkaline halides,[204,207] alkaline earth, and aluminum oxides.[208] In the adsorption processes occurring with these systems, the adsorbent is believed to act as an electron donor by virtue of its negative ions, and adsorbed halogen, as expected, acts as an electron acceptor. Complexes of the general structure X^-I_2 and X^-Sr_2 ($X^-=I^-$, Br^-, Cl^-, OH^-) have been suggested; reflectance spectra were found to be in reasonably good agreement with equivalent spectra in solution.

Redox reactions, ring cleavage, and photochemical reactions have occasionally been observed to occur on surfaces.[209-215] The coexistence of two benzene species, attributable to the redox equilibrium $C_6H_6H^+ + \text{Catalyst} \rightleftarrows C_6H_6{}^+ + \text{Catalyst}^- + H^+$, were reported to exist on a mixed catalyst surface (Al_2O_3-SiO_2).[211] Many other hydrocarbons were observed to behave similarly. Triphenylchloromethane has been studied on SiO_2, CaF_2, and $MgSO_4$; triphenylmethylcation was formed as a result of the adsorbent-adsorbate redox interaction.[212] The redox process is believed to proceed even further when stronger adsorbents such as alumina or alkaline earth oxides are used.

Reversible ring cleavage of ring-shaped aromatic compounds also has been occasionally reported on active surfaces. Kortüm and Vogel[209] have investigated such a process involving the adsorption of malachite green-*o*-carboxylic acid lactone on alkaline halides When the colorless lactone was adsorbed on preheated and waterfree active surfaces a blue color developed as a result of lactone ring cleavage. The blue form, according to an infrared study of the COO^- group[213] is believed to exist as a zwitterion in the adsorbed state. Desorption occurs at a relatively slow rate upon subjection of the sample to air humidity. To make the reaction possible, the adsorbent probably must exert a catalytic influence which lowers the activation energy substantially, hence permitting this reaction to occur at room temperature. Other reversible ring cleavage occurs with spiropyrans when adsorbed at room temperature at suitable surfaces, such as MgO and NaCl.[214] Changes of activation energies as evidenced in the above discussions can also influence photochemical reactions on solid surfaces, and again these were easily studied by spectral reflectance.[210,215]

Kortüm et al.[215] have investigated the formation of a blue quinoid-type structure as a result of illuminating 2-(2′,4′-dinitrobenzyl)-pyridine. In the dark the reaction is reversible to the colorless form. The kinetics of this reversible reaction have been studied in detail by the same group (see section IV D4). A similar investigation deals with the reversible photochemical oxidation of anthracene on alumina and other adsorbents in the presence of oxygen.[208] Usually anthrachinone is formed, but if adsorbed on alumina the reaction proceeds further to give compounds such as chinizarine, alizarine, or chryazine, depending on the specific properties of the alumina. The pronounced influence of the adsorbent is evidenced by the fact that on KCl no further reaction of the anthraquinone occurs and on silica gel the rate is much slower.

Reflectance methods as applied to surface studies and investigations of surface phenomena also can have great use to the analytical chemist. Studies of stability, position, and intensity of spectra as a function of temperature of the environment,[216] regeneration temperature of the adsorbent,[165, 206] drying temperature of the adsorbent—adsorbate system,[217] time of storage,[216] influence of light, etc., are but a few examples and can prove useful to elucidate optimum conditions for the analysis of certain compounds. Other examples are studies of pH dependence and influence of spray-reagent composition[217] in connection with chromatographic techniques followed by in situ evaluation of the separated compounds; these are discussed in detail in section IV G.

3. *Determination of Surface Area*

From experimental evidence (Figure 22) it becomes clear that chemisorption can usually only take place in the first monomolecular layer. In the case of the DMAB this change from chemisorption to physical adsorption is easily followed by the pronounced color change that is associated with it. Another even more drastic example is the previously discussed adsorption of malachite green lactone (MGL) on NaCl,[209] where upon chemisorption a change from colorless to blue occurs. If one then plots the Kubelka-Munk function of the green MGL bond vs. concentration of MGL in mole fractions over a sufficiently wide concentration range, one obtains essentially a Langmuir-type isomer similar to the one in Figure 23. The curve eventually reaches a limiting value, at which point the first monomolecular layer is believed to be covered completely and extrapolation of the two straight branches of the plot enables reading off the saturation concentration. This in turn provides a relative measure of the active surface area of the adsorbent. The MGL:NaCl system has been used by Kortüm and Oelkrug[218] for investigations to this end. The active surface area of NaCl of different particle sizes was studied by this approach, and the results were found to be in fairly good agreement (slightly lower) with those of surface areas determined by the BET method. Obviously the determination of absolute values is impossible since the way in which this complicated organic molecule adheres to the adsorbent surface is unknown. The method, however, should have good potential for problems such as the determination of relative surface areas of chromatographic adsorbents. The values that could be expected are likely to be more realistic than if obtained by the BET method since nitrogen gas tends to penetrate into cavities of the adsorbent which are not available in chromatographic processes. The resulting BET values therefore tend to be too large. Compilations of relative surface areas of certain groups of chromatographic adsorbents in turn permit prediction of loading capacity and facilitate the proper choice of adsorbent.

4. *Kinetic Studies on Surfaces*

There is hardly an alternate method to diffuse reflectance spectroscopy available for the study of kinetic processes in the solid state. Such studies can be of great significance in investigations of catalytic processes, and many reactions which occur at extremely high rates in solution can be studied at a convenient slower rate if the system of interest is adsorbed on a suitable matrix. The reversible photochemical reaction of 2-(2′,4′-dinitrobenzyl)-pyridine mentioned earlier[215] was chosen by Kortüm et al. as a test case to demonstrate the use of reflectance techniques to problems of this nature. The procedure used involved the sealing of the sample into quartz cells which were kept in thermostated condition after irradiation. The reverse reaction from the blue to the colorless form was monitored at 600 nm at certain time intervals and the measurements were made against pure adsorbent as reference standard and converted to absolute values. The kinetic plots were obtained by plotting log $F(R)_{abs.}$ vs. time for various temperatures. First-order reactions were observed and the kinetic constants were computed in the usual manner by taking the slope of the kinetic plots.

Corresponding activation energies can also be determined from the slopes of log K vs. 1/T (T, absolute temperature) plots. For the reaction discussed here, the activation energies on silica gel were about three times higher in comparison to the same system in solution.

Although the experiment described above is of little practical value to the analytical chemist, the author believes that this area could be expanded into kinetic methods of analysis in solid systems (e.g., chromatographic systems) similar to the already well-known catalytic methods in solution.[219]

5. *Determination of Equilibrium Constants*

The knowledge of equilibrium constants for dissociation or association equilibria of adsorbed systems could again be useful for general analytical problems related to such systems or to problems of catalysis or kinetics. Several studies were reported by Kortüm and Braun[220-223] on the feasibility of reflectance techniques of the measurement of equilibrium constants in the solid state. Deviations from the Kubelka-Munk law were used to compute equilibria of systems in the adsorbed state, in analogy to procedures in solution where deviations from the Bouger-Lambert law can be used for certain systems. The constants were found to be several orders of magnitude smaller than corresponding constants determined in chloroform.

In all investigations the dissociation phenomenon was believed to be strongly dependent on the amount of co-adsorbed moisture which apparently is a condition necessary for the establishment of a true equilibrium in the boundary phase. If the molecules are not able to move freely, e.g., when they are rigidly adsorbed on the crystal lattice of a dry adsorbent, no such dissociation can occur.

In practical analytical procedures adsorbents are often kept at room conditions and may contain a significant amount of co-adsorbed water from the atmosphere. Conditions for the establishment of a true equilibrium therefore exist for many systems and may be of interest to the analytical chemist. Even though it would be desirable to know these equilibrium constants, say, for example, for metal chelates adsorbed on chromatographic adsorbents, the method for the determination of such constants as proposed by Kortüm and Braun is too involved to be of practical significance, and one might prefer to use data gathered from solutions, inaccurate as they may be for the system at hand. The development of alternative and simpler reflectance spectroscopic methods for the measurement of equilibrium constants remains a tough but rewarding and useful problem to be solved.

E. Spectral Reflectance at Low and Elevated Temperatures

Most diffuse reflectance work has been carried out at room temperature. Occasionally samples are heated before measurement in order to investigate temperature effects on the kinetics of systems or to remove co-adsorbed water, but by the time the measurement is carried out the samples have cooled to ambient temperatures. This section deals mainly with reflectance work carried out at temperatures below or above ambient conditions, using suitable cells, heating blocks, and cooling devices.

1. High-temperature and Dynamic Reflectance Spectroscopy

The investigators responsible for having brought high-temperature reflectance methods into the limelight are Wendlandt and co-workers. Several reviews are now available from the same author on this topic.[8,97,100,224] Wendlandt distinguishes between two different modes of operation. One mode deals with the measurement of reflectance spectra at isothermal conditions and has generally been referred to as "high-temperature reflectance spectroscopy" (HTRS).[98] The other approach is a dynamic method of continuous reflectance measurements at a fixed wavelength over certain temperature ranges. It has been named "dynamic reflectance spectroscopy" (DRS).[225]

Figures 24 and 25 demonstrate the working principle of these two modes of operation. The HTRS curves for a hypothetical system heated to increasing temperatures T_1, T_2, T_3, T_4, etc. are shown in Figure 24. An increase of the maximum at wavelength λ_1 can be observed with increasing temperature at the expense of the maximum at wavelength λ_2. The corresponding DRS curves are depicted in Figure 25a and b, as measured at fixed wavelengths λ_1 and λ_2, respectively. The temperatures for the start and end of the thermal transitions can be seen clearly from these isolambdic curves. Although DRS does not yield any new data in comparison to other thermal analysis techniques, such as differential thermal analysis DTA or thermogravimetric analysis TGA, it can be rated as a valuable complementary method for the study of thermal stability and structural changes. Its major advantage is the absence of interference with thermodynamic and thermogravimetric processes. Applications of the

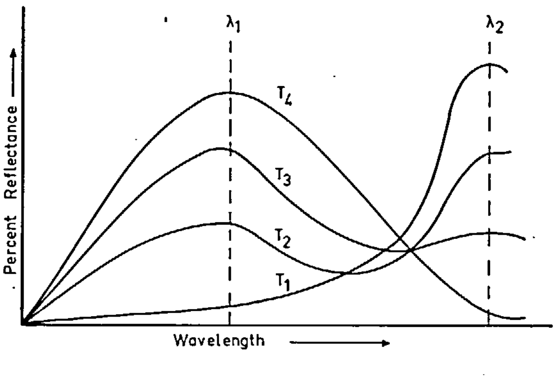
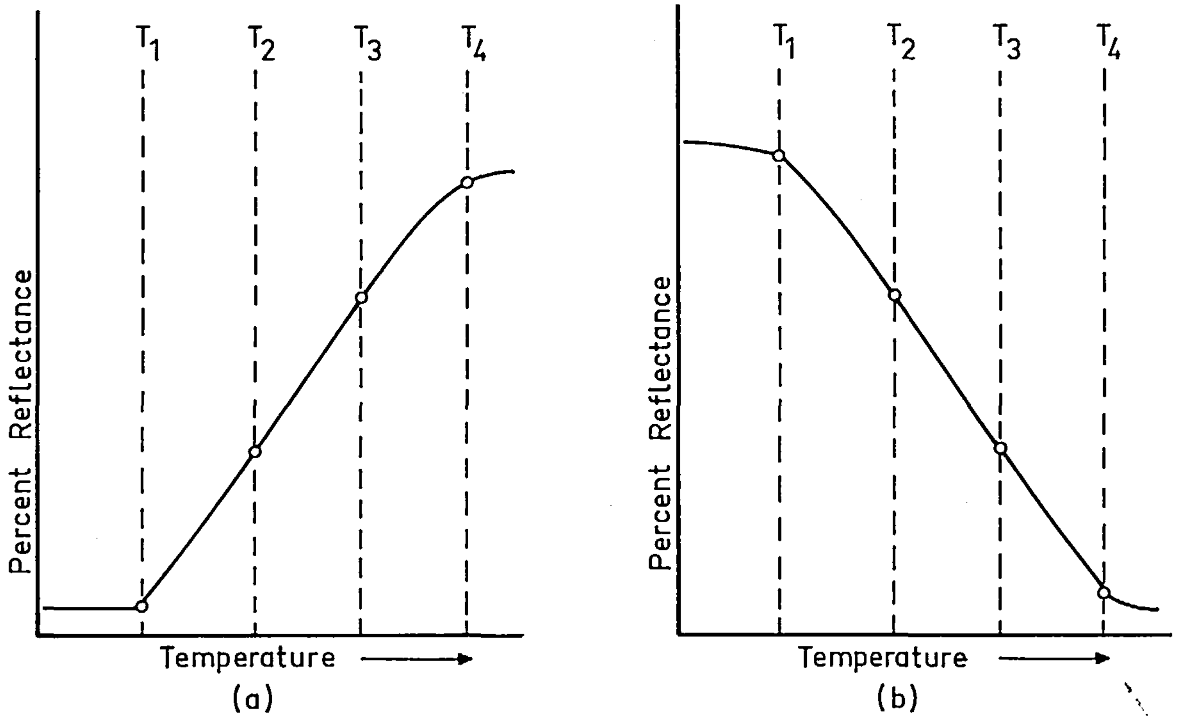


FIGURE 24. Hypothetical high-temperature reflectance spectroscopic curves.



(a) (b)

FIGURE 25. a, b. Hypothetical dynamic reflectance spectroscopic curves, at λ_1 and λ_2, respectively.

technique have been primarily restricted to inorganic systems, even though this need not be so, and can be attributed to the general research interest of the major contributor to the field. Studies have been carried out in the UV, visible and near-infrared regions of the spectrum. A typical example, dealing with the deaquation of a coordination compound,[224,226] is given in Figure 26 for [Cu (en) $(H_2O)_2$] SO_4. Two distinct sets of absorption maxima are observed: one at 25° (identical at 75°) located approximately at 625 nm and the other with a hypsochromic shift to 575 nm for a temperature of 150° (identical at 180°). From these data Wendlandt concluded that the deaquation process in the reaction [Cu(en) $(H_2O)_2$] $SO_4 \rightarrow$ Cu(en) SO_4 + $2H_2O$ had to occur between 75° and 150°. Dynamic reflectance spectroscopy was then used to obtain a more precise value for the transition temperature.

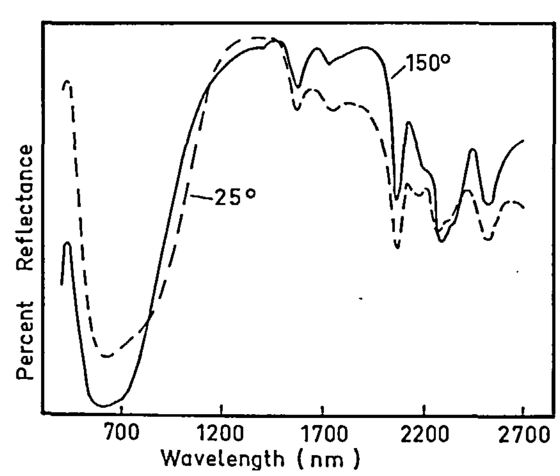


FIGURE 26. Visible and near-infrared reflectance spectra of [Cu(en) $(H_2O)_2$] SO_4.[224,226]

The usefulness of the near-infrared region for diffuse reflectance spectroscopic investigations of such systems is also demonstrated in Figure 26. When comparing the spectrum at room temperature to the high-temperature spectrum recorded at 150°, one observes the disappearance of a band at 2150 nm and a bathochromic shift of two bands from 2260 nm to 2280 nm, and from 2510 nm to 2525 nm, respectively, at the elevated temperature. The intensity of the bands also changes.

Another possible area of application is the study of thermochromic transition temperatures.[112] With Cu_2HgI_4, for example, a reversible color change from a red to a black modification has been observed at elevated temperatures. The DRS curve of this system showed a continuous decrease of reflectance from room temperature to about 80°C, with the most drastic color change taking place at 70°C. Thermochromic reactions have also been investigated by other workers, but only in an isothermal mode. Hatfield et al.[95] studied thermochromism of bis (N,N-diethylethylenediamine) copper (II) perchlorate which at about 50°C undergoes a color change from red to blue. The polymorphic conversion of red HgI_2 has been scrutinized by Kortüm[13] at elevated temperatures. The results have been discussed in section IV D2. An isothermal study of the kinetics of interaction between Fe_2O_3 and CaO has been reported by Baistrocchi.[227] The project was carried out in the HTRS mode at temperatures between 500 and 900°C and represents an interesting variation to Kortüm's work.[215]

2. *Measurement at Low Temperatures*

Diffuse reflectance spectroscopy at low temperatures has been reported by a number of investigators.[228-232] The studies were primarily in the field of inorganic chemistry as an aid to interpretation of complex spectra in terms of ligand field theory. The resulting spectra, which are often recorded at liquid nitrogen temperatures, have sharper structural features compared with similar spectra recorded at ambient temperatures. This is attributed to some degree to the elimination of "hot bands" which originate from vibrationally excited ground state transitions. No unusual problems are encountered in the technique of low-temperature reflectance spectroscopy. A low-temperature cell designed for work with liquid nitrogen has been described in section III E3.

F. Inorganic Systems

A comprehensive review on the use of reflectance spectroscopy in inorganic chemistry was written by Clark.[233] The use of this technique, especially for problems in structure analysis of metal complexes and other metal compounds, has been emphasized. The use of this approach is evident in cases where transmission spectra cannot be measured easily because of solubility or stability reasons, or where additional valuable data can be gained in the solid state. Interpretation of the data has been attempted in terms of crystal field or ligand field theories.

Applications of more interest to the analytical chemist include an investigation of rare earth metal oxides and other rare earth compounds by White[234] and Ropp.[235] The latter used a Cary Model 15 high-resolution Spectrophotometer equipped with a double-beam reflectance sphere attachment coated with $BaSO_4$. The estimated resolution (taken directly from the charts) he was able to obtain for rare earth compounds measured in the UV and visible regions of the spectrum was about 1 Å. The author recommends flushing of the reflectance spheres, photomultiplier housing, and monochromator compartment with nitrogen or another inert gas to improve the resolution. Standards and samples were carefully compressed with a Bausch & Lomb powder press. A high-intensity xenon 150 W lamp served as the energy source. No mention was made, however, about particle size of samples and related problems in sample preparation, which from previous work is known to be critical for the resolution that one can obtain. With high-resolution reflectance spectroscopy one has the possibility to identify optically narrow bands of the rare earth which result from upper energy states and unperturbed 4f transitions. Schatz[56,236] has reported on the effect of pressure on the reflectance spectra of compacted powders (see section III A). The effect of particle size with samples under high compression has been studied by the same investigator,[253] and little dependence on particle size was found under such high-pressure conditions (up to 35300 psi) as long as the particles remained large in comparison to the wavelength used for the investigation. Several workers[237,238] have investigated bulk uranium oxide by spectral reflectance over the UV and visible regions of the spectrum. The reflectance values were observed to change with varying $UO^{2.66}$ to $UO^{2.00}$ ratios.

A group of inorganic matrices have been scrutinized by Griffiths et al.[203,205] by UV reflectance spectroscopy. LiF was chosen as a relatively neutral diluent for the highly absorbing specimens such as metal iodides, iodates, periodates, chromates, and various sulfur salts, as well as commercial sodium peroxide. The activity of the lithium fluoride surface, however, could not be eliminated entirely and was considered a serious drawback. He found the method quite useful, however, for the investigation of relatively unstable compounds such as sodium ozonide, super oxides, and peroxides which are hard to prepare as single crystals. The analytical use of the method was also judged to be satisfactory, and linear calibration plots were reported for absorbance plotted vs. concentration of KIO_3 (see Figure 27) over a relatively large concentration range. This is quite astonishing and rather unexpected, particularly at these high concentrations. On closer inspection of the curves one in fact wonders whether presentation of the data in the form of linear calibration curves is really justified. Inorganic powder materials that are difficult to dissolve have been explored by this technique by Lermond and Rogers.[26] The reproducibilities obtained for repeated measurements of a single sample were between ± 0.1 and 0.2% and for five samples measured in a series ± 2% maximum on the 100-unit reflectance scale. With the present knowhow and instrumentation one should be able to improve this considerably. The use of differential reflectance measurements, particularly for samples with low reflectance, was also explored by these workers in connection with copper (II) and iron (II) oxides. Linear relationships were reported over concentration ranges 0.05 to 14.3% Fe_2O_3 in $BaSO_4$ with plots $(R\text{-}R_m)$ vs. C^{-1}. R is the reflectance of the sample and R_m the reflectance of the sample with the highest concentration of colored compound. Other relationships such as the Kubelka-Munk function, or $(R\text{-}R_m)$ vs. $C/(1\text{-}C)$, were also discussed.

Two or multicomponent mixtures of inorganic compounds have been analyzed by reflectance techniques.[236, 239, 240] The principle has essentially been discussed in section II D. Kortüm and Herzog[239] studied two titanium modifications (rutile and anatase) in the UV region since there is a considerable interest for a method which enables distinction between these two pigments (anatase possesses a much better covering power). The UV absorption maxima are sufficiently wide apart to enable the quantitative determination of the two modifications, each with an accuracy of about ± 2%. The Kubelka-Munk function was used to achieve additivity of the individual reflectances and to construct linear calibration curves.

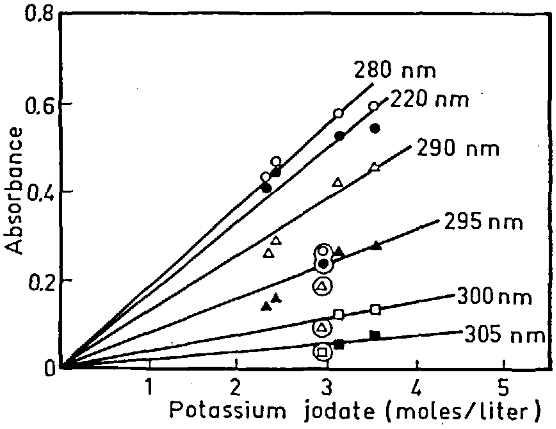


FIGURE 27. Absorbance of KIO_3 vs. concentration, LiF as diluent.[205]

Doyle and Forbes[240] used a Unicam SP500 Spectrophotometer equipped with the reflectance attachment to carry out similar studies with lead monoxide, silver oxide, and zinc oxide. The diluents used were silica gel and magnesium oxide, and the wavelengths of analysis were chosen at 515, 425, and 368 nm, respectively. These workers preferred an empirical function $(A/R)^{1.383}$ to the Kubelka-Munk function for the construction of calibration curves. The standard deviation from the actual percentage of known samples was found to be 6.6% for zinc oxide and 5.2% for the lead monoxide. This is certainly satisfactory for an analysis of this kind and comes close in accuracy to single-component analysis. The use of the empirical function, while feasible, seems a poor choice if one has a theoretically sound and easily available function, such as the Kubelka-Munk function, at hand.

Fisher and Vratny[241] investigated inorganic powders such as Fe_2O_3 diluted in magnesium carbonate and reported a linear relationship for plots of 100% R vs. log weight % iron oxide in magnesium carbonate. Other systems included copper amine and iron thiocyanate on filter strips. Reflectance techniques proved useful for the examination of spot tests carried out on filter papers. As an example, the spot test for copper, using α-benzoin oxime, was studied by Winslow and Liebhafsky.[242] These workers concluded, however, that transmission measurements through the paper gave a better signal to concentration relationship, which at the time when this work was carried out may have been attributable to the lack of suitable instrumentation for reflectance work, but this would not be true any longer.[254]

In some cases diffuse reflectance techniques have been applied following concentration steps by ion exchange procedures. The concentration of nickel and manganese on cation exchange paper consisting of oxidized cellulose has been carried out by Ermolenko et al.[243] The reflectance spectra were recorded following suitable reaction with chelating agents. Interference up to a

100-fold excess was negligible from other metal ions and the detection limits for Ni and Mn were reported to be 0.01 μg. Fujimoto and Kortüm[244] used the same evaluation technique to measure heavy metal chelates after applying suitable scavenging techniques with the use of ion exchange resins.

Again, these few examples should merely serve to illuminate the vast application possibilities for this technique. The last two applications seem particularly promising in this age of trace analysis of pollutants, for example.

G. Chromatography

1. General Experimental Procedure

a. Detection of Spots

For quantitative reflectance work the reactions generated by the chromogenic sprays have to be instantaneous and reproducible, as well as stable over the time required for a complete analysis. Reflectance techniques are very suitable to study such parameters, as can be seen from an investigation of color stabilities of ninhydrin complexes of amino acids.[216] Simple heating of the plates will occasionally result in the formation of a colored product[93] and if a fluorescent compound is available, observation under UV light enables location of the spot.[245] For many UV-active compounds detection of the spots as dark areas is possible on a fluorescent background. This fluorescent background can be produced by incorporating a luminous pigment into the adsorbent,[94,246,247] or by just taking advantage of the natural fluorescence of adsorbents such as silica gel or cellulose. Another approach to the detection of UV-active substances was described by Frodyma and Lieu.[94] A direct scanning approach is used in connection with the regular reflectance attachment for the Beckman DK-2, by holding the chromatoplate, which is taped to a protective shield made of plastic, against the sample port of the integration sphere. In this way the adsorbent along the chromatographic path is exposed to the impinging light beam and a sudden increase of reflectance occurs when an area containing the spot material is reached. After location of the spot, either under UV light or by the technique described above, the position is marked with a grease pencil on the backside of the plate. A similar approach has been used successfully on the Bausch & Lomb Spectronic 505 reflectance attachment. In both instances a black cloth was used to cover the measuring assembly in order to keep out stray light. With the development of mechanical scanning devices the above method of detection has become somewhat obsolete. Nevertheless, the advantage of not requiring any specialized equipment would make it useful in certain cases.

With the new line of specialized recording instruments now available for the evaluation of chromatograms (section III E) mechanical scanning has become the major approach to detection problems. Some direct scanning procedures have been discussed, particularly in connection with the Zeiss PMQ II Spectrophotometer, by Frei et al.,[248,249] Stahl and Jork (see review by Gänshirt[250]), Klaus,[106,107] and de Galan et al.[108] On two-demensional chromatograms, however, visual or UV lamp detection techniques still have to be used to detect the spots.

b. Recording of Reflectance Spectra

The spectra of spots can be recorded directly on the plate after location of the spot area. The chromatoplate can be positioned against the sample port of a reflectance attachment and the light beam is centered on the spot of interest. A glass plate of identical size can be taped on top of the thin-layer plate for protection of the adsorbent. For UV reflectance work paper masks with a size identical to the plate and with holes punched out where the spots are located, or the use of the previously mentioned protective plastic plate, are recommended. Since in direct measurements one rarely has an infinite layer thickness of the sample surface, it is recommended to use a reflecting background mounted behind the plate, consisting of a sheet of nontransparent material with good reflection properties at the wavelength of interest. The glass or quartz window reflectance cells discussed in section III D can also be used conveniently for the recording of spectra.[251] In this procedure spots are excised and placed on top of 30 to 50 mg of adsorbent removed from the same chromatogram and packed in the appropriate cell. For reference standards the same procedures can be applied and the reference material usually consists of adsorbent from the same plate. Procedures for recording of spectra with the Zeiss Chromatogram-Spectrophotometer have been described elsewhere.[248,252,253] Automatic recording of spectra can now be carried out directly on the chromatograms in a matter of minutes with the Zeiss chromatogram scanner equipped with the mechanical wavelength drive or with any of the double-beam instruments discussed in section III.

c. Quantitative Measurement of Reflectance

Semiquantitative measurements have been made by carefully adjusting the light beam to the center of the spot. An accuracy of about 10% was obtained with instruments such as the Spectronic 505 or the Beckman DK-2 Spectrophotometers,[164] provided that no excessive tailing of the spots occurred. Spots no more than 0.75 cm in diameter and separated on Eastman chromatogram sheets can be cut out and placed in the Beckman DU or Spectronic 20 single-beam instrument. The area of the impinging light beam is adjusted to a size large enough to cover the entire spot and the measurement is made in a single reading.[254] Light-scattering phenomena and nonhomogeneity of the material contained in the spot pose a problem, and corrections have to be used with the Kubelka-Munk function. This rapid technique, however, is still superior to the previously discussed method. Braun and Kortüm[266] have previously investigated this problem in conjunction with paper chromatography and have shown that the measured reflectance depends on the irregularity of the spot shape and the empty space around it which is also exposed to the light beam and measured. Simulation of infinite layer thickness was achieved in their experiment by using several clean sheets of chromatogram paper as a background for the spot. The Kubelka-Munk equation (Equation 3) has been modified by the same workers for such a single beam approach to the following form:

$$F'(R_\infty) = \frac{b^2}{2n(1-b)} - \frac{F(R_{\infty'})}{n} \tag{34}$$

which is proportional to the absorbing material on the spot. From the mean observed diffuse reflectance $\overline{R}_\infty$ of the complete area under the light beam (spot and surrounding matrix) and the diffuse reflectance of the paper itself R'_∞ one can compute the term b as follows:

$$b = 1 - n\overline{R}_\infty + (n-1)\,R'_\infty \tag{35}$$

n is the ratio of the area under observation to the actual spot area. With this relationship a reasonable adherence to the Kubelka-Munk theory was achieved even though the spread of values was such that Kortüm et al.[7] concluded that it would be very difficult to render this technique truly quantitative. With the instrumentation and knowhow available on in situ measurement of chromatograms at the time of this investigation, this may well have been the case. With today's sophisticated chromatogram scanners on the market, however, one can state with confidence that direct evaluation of chromatograms by reflectance spectroscopy can be of a quantitative nature. Relative percent errors of 3 and 4% were reported by Jork[255] and Pataki[253] for quantitative work in the UV range of the spectrum, using the mechanical scanning approach with the Zeiss instrument. As a rule, a greater precision can be achieved in the quantitative evaluation of chromatograms when the measurements are carried out on spot material removed from the plate.[164] The procedure works as follows: the substance of interest is removed with sufficient adsorbent to make up a sample of exact predetermined weight (20 to 80 mg, depending on the sample). This mixture is ground in a small mortar for a given period of time (30 to 60 sec) and packed in the aforementioned cells (see section III D). The reference material from the same plate is treated in the same way. Further expediting of sample preparation has been suggested by Lieu et al.[256] by removing the sample with a circular planchet glued to a cork stopper. The thickness of the layers and the spot areas to be excised will dictate the size of the planchet to be used. Once the sample is cut from the adsorbent layer it is moved on a direct path to the nearest edge of the plate and deposited in an agate mortar for further handling. This method enables the spot removal for quantitative analysis in less than one minute. The accuracy of this technique depends strongly on the evenness of the layers, but in the same study it was found that with commercial applicators the quality of chromatoplates is sufficiently good to approach the precision of the more time-consuming gravimetric removal technique.

The reproducibility of coating procedures is also of importance if direct scanning techniques are used. Reflectance spectroscopy, however, is somewhat less sensitive to fluctuations in layer thickness than corresponding transmission techniques.[254] An efficient means of eliminating such fluctuations is the use of double-beam scanning instruments. Klaus[257] and Huber[258] recently conducted critical investigations of the effects of fluctuations in layer thickness, layer quality, and other experimental parameters on the reproducibility of reflectance measurements. The somewhat longer time of analysis involved in the spot-removal technique is offset by its precision. Its major advantage, however, is its simplicity and the possibility to better control sample conditions such as humidity and homogeneity, as well as layer thickness. The spot-removal technique can doubtless be used in connection with practically every conventional spectrophotometer equipped with a reflectance attachment. Disadvantages of this approach include the inability to measure paper chromatograms or prefabricated chromatogram sheets, and automation possibilities are practically nil. For handling large numbers of samples in routine analyses the author believes that direct scanning techniques will become predominantly used, particularly with the advent of more and more sophisticated commercial chromatogram scanners, some of which are now available with electronic readout equipment. For nonroutine problems, however, where adsorption properties of systems have to be investigated before the actual design of an anlytical method, the spot-removal method will retain its importance.

2. *Visible Reflectance Spectroscopy of Organic Systems*

Much of the work in the category of organic compounds which possess their own color, or which can be made visible with suitable sprays, has so far been evaluated by in situ transmission techniques. The majority of these methods[103, 259, 260] should be easily adaptable to diffuse reflectance spectroscopy with its inherent advantages[254] such as higher sensitivity, less noise when operated in single beam mode, reasonable adherance to the Kubelka-Munk theory (with proper precautions), etc. Spectral reflectance has been used for the in situ identification and determination of many organic substances in the visible region following resolution on thin-layer plates[73, 261] and occasionally on paper and electrophoresis strips.[262, 263]

a. Dyes and Pigments

Yamaguchi et al.[163, 264] were the first to report on some reflectance spectroscopic studies of food dyes on filter paper. The dye malachite green was investigated on chromatographic paper by Kortüm et al.,[177, 265, 266] with the purpose of evaluating the use of spectral reflectance techniques in the field of paper chromatography. The production of spots with a homogeneous distribution of the spot material was considered one of the major difficulties. Reproducible calibration curves of the Kubelka-Munk function plotted vs. concentration, however, could be obtained with careful sample preparation procedures. About ten layers of paper were needed to achieve infinite layer thickness.[265] The same workers showed that with appropriate correction factors for spot size and self-absorption of the paper a form of the Kubelka-Munk function could be developed, which results in linear calibration curves over a useful concentration range of the dye. The following equation was proposed:

$$F'(R_\infty) = \frac{b^2}{2n(1-b)} - \frac{F(R_\infty)}{n} \qquad (36)$$

For b the following relationship holds: $b = 1 - n\bar{R}_\infty + (n-1)R_{\infty 1}$. $\bar{R}_\infty$ is the average reflectance of the total measuring area, which includes the spot and the surrounding paper. $R_{\infty 1}$ is the reflectance of the paper only, measured over the same area. A simpler way of overcoming the inhomogeneity effects, however, is the method of scanning a spot with a narrow slit or by means of a "flying spot" technique, such as described by Goldman and Goodall.[34]

The first application of diffuse reflectance spectroscopy to TLC was carried out with dyes,[178] which, in being stable and not requiring detection by spraying, lent themselves readily to such investigations. Direct spectral examination of the plates was carried out with the Beckman DK-2 Spectrophotometer according to the previously discussed procedure. A precision similar to that obtained by transmittance was obtained by carrying out reflectance measurements on spots removed from the chromatoplate and packed in appropriate cells (section III D). The same procedure was later employed for student experiments.[267] Data were obtained either by using a calibration curve (average accuracy 3.8 to 5.7%) or an algebraic method (average accuracy 5.8 to 7.8%). For the calibration curves the Kubelka-Munk function of the measured reflectance was plotted vs. concentration of dyes in µg/spot. Since the unknown concentrations all fell on the linear portion of the curve, the following equation was used for computation purposes:

$$C_u = \frac{C_s F(R)_u}{F(R)_s} \qquad (37)$$

C_u and C_s are the concentrations of the unknown and standard, respectively, and $F(R)_u$ and $F(R)_s$ stand for the Kubelka-Munk functions. The students correctly identified 90% of the dyes on the basis of reflectance spectra of the dyes. From these results it can be concluded that the reflectance technique can yield useful data in the hands of an inexperienced analyst. The usefulness of this technique to the examination of humidity and regeneration temperature effects on the spectra of dyes adsorbed on alumina has been demonstrated by Frei and Zeitlin.[165]

Several workers have analyzed plant pigments by this technique. Yamaguchi et al.[268] studied the relationship between reflectance and the concentration of chlorophyll in paper partition chromatography. Calibration curves 2-log R vs. $\sqrt{C}$ were recommended. Garside and Riley[269] recently reported on a combined thin-layer chromatographic-reflectance spectroscopic procedure for the chlorophylls and carotenoids in marine particulate matters. In situ measurements of the pigment spots were made with a Joyce, Loebl Chromoscan with TLC attachment operated in the reflectance mode. The sensitivity of the method was reported to be from 0.03 μg for β-carotene to 0.14 μg for chlorophyll a, with an integrator reading of 3 being taken as the lowest significant level. For chlorophylls and major xanthophylls analyzed in a composite phytoplankton culture the coefficient of variation of the method did not exceed 5%, and good agreement was also observed between different methods of analysis. A complete analysis was performed in about one hour. The major advantage of the in situ reflectance method seemed to be the time factor.

b. *Amino Acids*

TLC is of great importance to amino-acid analysis and has surpassed paper chromatographic methods in speed, resolution, and sensitivity.[270] Discussions on in situ evaluation of amino acids separated by these techniques are therefore concentrated on TLC work in this section. Reviews on the application of reflectance spectroscopy to amino-acid analysis on thin-layer plates have appeared recently.[253,271] The colors obtained with conventional ninhydrin sprays for the resulting ninhydrin-amino acid complexes are not much differentiated. With the use of the modified spray reagent recommended by Moffat and Little,[272] however, the resulting colors were fairly specific (see Table 8), hence permitting the identification of complex amino-acid mixtures by making use of a combination of reflectance spectra, visual observation and R_F values.[251]

Reflectance spectroscopic studies served also to investigate the stability of ninhydrin complexes.[216] The factors limiting the precision of the method were associated with the spraying procedure. Incomplete reaction of the amino acids with the reagent, leaching out of color spots, etc. were hard to control, and it was felt that a substantial improvement of the method could be achieved if the colors were developed without sprays, merely by adding the detecting agent to the solvent system.[273] With this approach an average standard deviation of 0.49% R was reported for a set of ten samples, which represents a significant improvement in the precision over that attained by the spray method where an average standard deviation of 1.45% R was obtained. As is to be expected, a decrease in reproducibility results after two-dimensional chromatography, and an average standard deviation of 0.77% R was reported. This is still much better than with the spray method.

Probable relative errors as determined from the reproducibility data and the corresponding calibration curve have been reported for various concentrations of glycine. Minimum errors were obtained for an intermediate concentration range of 5 to 10 μg/spot (Table 9).

Pataki[253] has reported on amino-acid analysis by way of ninhydrin complexes, using the Zeiss Chromatogram-Spectrophotometer. The spray reagent was similar to the one used by Frodyma and Frei.[273] Calibration curves peak area *vs.* $\sqrt{C}$ were linear over a reasonable concentration range. The reproducibility reported by Pataki was considerably poorer than with the above-discussed technique. With direct scanning, background irregularities and tailing of the spots can result in serious errors. Heathcote and Haworth[274] recently reported similar work carried out with the Joyce, Loebl Chromoscan. No new aspects were introduced by their method, and they seemed unaware of the above-mentioned work.

Ninhydrin complexes on paper chromatograms have been evaluated by reflectance techniques as

TABLE 8

Color, Absorption Maxima, and R_F-Values for Some Amino Acids[251]

Amino acid	Concentration (µg/spot)	Color	Absorption maxima (nm)	(µg/spot)		
				DK-2	Visual	R_F-value
Alanine	5	Blue-violet, fades to red-violet after 24 hr	540,480,412			0.26
	2	As above	532,486,416	2		
	1	As above	528,473		0.2	
Arginine-HCl	10	Light violet, fades pink	547,483,419			0.08
	5	As above	535,484	5	3	
Cystine	8	Pink-gray	447			0.16
	6	Pink-gray	455			
	5	Pink-gray		5	3	0.06
Glutamic acid	5	Dark violet with pale orange ring, brown after 24 hr	532,482,409			0.27
	3	As above	536,481,416	3		
	2	As above			1	
Histidine-HCl	5	Gray with yellow ring	455			
	3	Yellow, brown-gray after 24 hr	455			
	0.5	As above	455	0.5	0.5	
Methionine	10	Violet with yellow ring, pink after 24 hr	535,478.421			0.40
	8	As above	538,483,410			
	5	As above	537,480	5		
	2	As above			1	
Serine	5	Dark violet with yellow ring, fades to pink	540,478,415			0.47
	3	As above	540,478,415			
	2	As above	537,479,429.	2		
	1	As above			0.3	
Tyrosine	5	Orange with yellow ring, light yellow after 24 hr			2	0.47

TABLE 9

Probable Relative Error in the Measurement of the Concentration of Glycine as a Function of Concentration (Number of determinations n = 4)[273]

	Concentration of glycine (µg/spot)			
	2	5	10	20
Range (% R)	84.3-86.0	77.3-77.9	63.6-65.1	55.3-57.1
Mean (% R)	85.1	77.5	64.4	56.3
S.D.* (% R)	0.92	0.27	0.66	0.76
Equivalent change in measured concentration of glycine (µg)	0.18	0.20	0.50	1.40
Probable % relative error	9.0	4.0	5.0	7.0

*S.D. (standard deviation)= $\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$

far back as 1953[275] when amino acids were determined in sugar-beet processing juices. Only semiquantitative results were obtained, and it is believed that transmission techniques through paper were superior at that time, mostly due to better instrumentation available for in situ transmission densitometry.

c. Sugars

Mixtures of sugars were successfully determined by spectral reflectance following resolution on thin-layer chromatograms.[276,277] For Aldopentoses chromatographic resolution of sugars on cellulose layers by a procedure set forth by Vomhof and Tucker[278] was chosen. A more uniform distribution of the chromogenic reagent was brought about by using the dipping technique, which in the case of the highly stable cellulose layers posed no particular problems. An accuracy of about 5% and a sensitivity limit of 5 μg/spot were reported for this technique. The stability of the sugar-aniline derivatives is good for about one week. A naphtharesorcinol-phosphoric acid mixture, such as described by McCready and McComb,[279] was tried with good success for hexoses as well as pentoses.[277] Stable red spots were observed for fructose, and blue-green and blue spots for rhamnose, arabinose, and glucose. Bevenue and Williams[280] used similar visualization methods before quantitatively analyzing raffinose and mellibiose on paper chromatograms by spectral reflectance. Plots log 1/R vs. concentration resulted in limited linearity over relatively low concentrations. The quantization of reducing sugars such as fructose and glucose has been described by the same workers,[281] using this approach. Spectral reflectance was measured with a Photovolt reflectance unit Model 501-A (no longer available) equipped with a filter for 515 nm. The useful linear range for calibration curves as described above ranged from 2 to 20 μg/spot. Small amounts of galactose were determined by Owens et al.[282] on paper chromatograms, using the same procedure. The overall accuracy obtained with the Photovolt instrument and experimental procedures similar to the ones used by Thaller[276] was reported to be ± 5% after one-dimensional chromatography. This is about the same as observed with the TLC procedure.

When one compares these methods for sugars to the ones developed for amino acids or pigments, one can readily see the need for further improvement in the methodology of reflectance spectroscopic sugar analysis. Since few alternative methods are available, however, there is still great demand for such an improved method.

3. Ultraviolet Reflectance Spectroscopy of Organic Systems

The use of diffuse reflectance spectroscopy in the UV region of the spectrum was demonstrated as early as 1958, when Zeitlin and Niimoto[283,284] reported UV reflectance spectra of a number of ketones on filter paper. A modified form of the Kubelka-Munk equation was used by Korte and Weitkamp[285] to quantitatively determine 2,3,6-trimethyl-fluorenone on paper chromatograms. In the UV region of the spectrum the advantage of in situ reflectance spectroscopy over in situ transmission through the layers becomes particularly obvious due to the high absorption of supports and adsorbents used in thin-layer chromatography. It was not unitl 1965, however, that the first applications of this technique to TLC were published.[93]

a. Aspirin and Salicylic Acid

Mixtures of aspirin (acetylsalicylic acid) and salicylic acid were selected for a first investigation of the feasibility of spectral reflectance in this area.[93] This system had the advantage of forming brown spots upon heating the plates, hence facilitating the location of the spot areas before a spectral investigation. After separation the plates were dried for 2 hr at 90°C, during which period the aspirin converted quantitatively to salicylic acid. The resulting shift of absorption maxima from 278 to 302 nm can readily be observed. Samples were prepared according to the method discussed earlier (section IV Glc) and the spots were measured at 302 nm. Linear calibration curves for absorbance plotted vs. $\sqrt{C}$ were obtained for a concentration region of 0.2 to 1.7 μmole/spot. The precision for this method, in which the samples were packed in a cell covered with quartz glass, was reported as 0.37% and 0.47% standard deviation for salicylic acid and aspirin, respectively.

b. Amino-acid Derivatives

DNP amino acids are obtained by suitable dinitrophenylation procedures. The resulting derivatives are UV-active and can be analyzed suitably by the reflectance technique or by fluorescence quenching methods. The DNP amino acids can also be separated conveniently from interfering salts, and TLC separation techniques have been reported in detail.[270]

Another well-known derivatization procedure is the reaction of amino acids with phenylisothiocyanate to give 3-phenyl-2-thiohydantoins, also known as PTH amino acids. Most PTH amino acids are soluble in organic solvents and can be separated from interfering impurities by selective extraction processes. They too are UV-active, hence suited for quantization by the above-mentioned techniques. The preparation and chromatographic separation have been discussed by Pataki.[270] Pataki[253] and Zürcher et al.[286] have reported on the qualitative and quantitative evaluation of DNP and PTH amino acids by diffuse reflectance spectroscopy after separation of the derivatives on thin-layer plates. The reflectance spectra served conveniently for the identification of the amino acids; further differentiation of the spectra could be achieved by subsequent treatment of the spots with HCl or NaOH spray solutions.[253] The instrument used for these investigations was the Zeiss Chromatogram-Spectrophotometer and the reproducibilities that can be expected for such derivatives range between 94 and 96% (computed from relative standard deviations after one-dimensional chromatography). Detection limits are about 1 µg/spot. The methods described here for DNP and PTH amino acids do not necessarily offer any advantage over the nonspray visible reflectance method reported in section IV G2b; in fact, both the sensitivity and reproducibility of the ninhydrin method are slightly better. Nevertheless, it is useful to have these methods since the derivatization procedures mentioned above are widely used to isolate amino acids in complex biological systems.

c. Nucleo Derivatives

Several nucleotides have also been analyzed by the "spot-removal" technique.[245] The spots were located under a UV lamp (254 nm) by the fluorescence quenching method, removed from the plate, and packed in a windowless cell (section III A) for measurement. Accuracies were reported to range between 2 and 6%.

Pataki[253] and Frei et al.[248] reported on a much more extensive investigation of nucleo derivatives with the use of the Zeiss Chromatogram Scanner in the reflectance mode. The data agreed well with the results reported by Lieu et al.[245] The wavelength values for the maxima (Table 10) were found to fluctuate by no more than 2nm, even over extended periods of up to six days and with concentrations varying from 0.5 to 5.0 µg/spot. The spectra were recorded directly from the plate by focusing the light beam to the center of the spot. The excellent reproducibility of the spectra rendered them very suitable for identification purposes, as can clearly be seen from Table 10. For a quantitative study a group of five compounds (hypoxanthine, uridine, inosine, uracil, and thymine) was initially chosen for investigation.[249] Two-dimensional chromatography was carried out on cellulose layers. The chromatogram scanning peaks obtained with the Zeiss instrument were evaluated planimetrically. Fairly linear curves were obtained with peak areas plotted vs. the square root of concentration, which would correspond to a rough approximation of a Kubelka-Munk plot. The use of internal standards for the compensation of fluctuations inherent in the chromatographic procedure was introduced by Klaus[287] in connection with one-dimensional TLC of bands. For two dimensional procedures this was expected to be even more advantageous, as can indeed be seen from Table 11. Improvements in reproducibility between 3 and 4% relative standard deviation were observed with both uracil and adenine used as internal standards. Since uracil is itself a component of the biological samples of interest, it was decided to use adenine for further work since it does not interfere with any of the nucleo derivatives of interest on the chromatogram (see Figure 28). The resulting chromatogram as

TABLE 10

Reflectance Maxima of Nucleo Derivatives and Related Compounds Adsorbed on Cellulose[248]

Compound	λ_{max} (nm)	Compound	λ_{max} (nm)
Xanthine	270-271	Nicotinamide	265
Guanine	248/277-278	Cytidine	280-281
Uridine	265	Guanosine	255
Inosine	250	AMP-3'	262
Uric acid	288	AMP-2'	261-262
Nicotinic acid	263	AMP-5'	261-262
Hypoxanthine	251	CMP-3'	279
Uracil	261	GMP-3'	255-256

TABLE 11

A Comparison of the Reproducibility of Data with and without the Use of Internal Standards.[249]

	Hypoxanthine	Uracil	Adenine	Hypoxanthine/ uracil	Hypoxanthine/ adenine
Mean and standard deviation* (n = 6)	752 ± 69	790 ± 65	780 ± 75	0.96 ± 0.069	0.96 ± 0.05
S.D. (%)	9.1	8.3	9.6	6.3	5.1
Mean and standard deviation (n = 12)	700 ± 70	760 ± 81	740 ± 60	0.924 ± 0.066	0.948 ± 0.065
S.D. (%)	10.0	10.7	8.1	7.1	6.9

*S.D. (standard deviation) = $\sqrt{\frac{\Sigma(x-x)^2}{n-1}}$

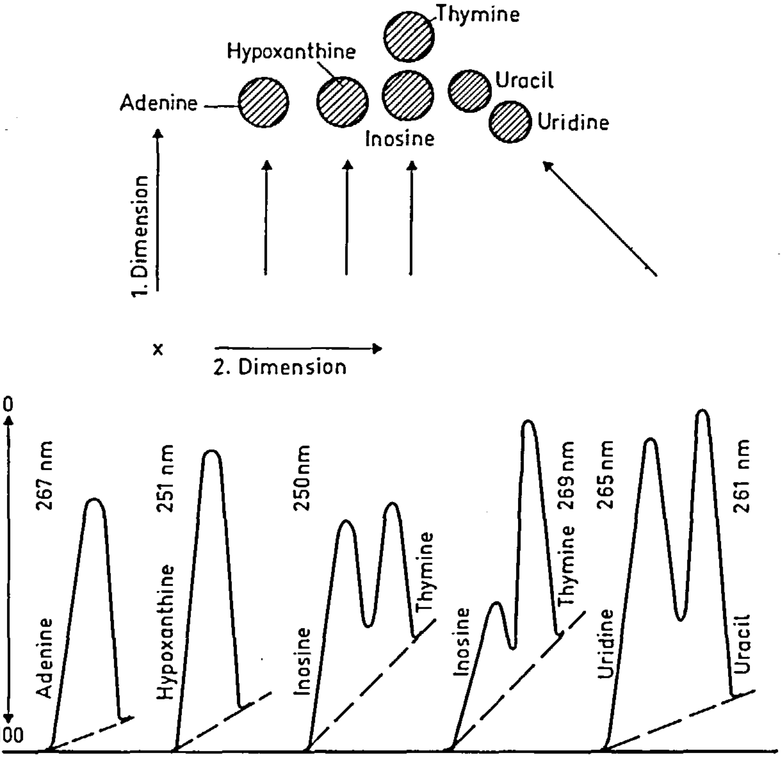


FIGURE 28. Thin-layer chromatogram and corresponding chromatographic peaks for nucleo derivatives separated on purified cellulose. Scanning speed, 7.5 cm/min; recorder speed, 8 cm/min. Arrows mark the scan direction.[249]

well as the corresponding chromatographic peaks is shown in Figure 28. The peak evaluation technique depicted in this figure has proven to be the best of a number of approaches studied in connection with this problem, particularly for poorly resolved peaks. Calibration curves of ratios (substance/adenine) plotted vs. the square root of concentration resulted again in a linear relationship useful over a reasonable analytical concentration range. About two days are required for a complete analysis of these components in complex biological samples. This includes complete chromatographic resolution (one day) and the evaluation of the data by this internal standard method. The actual working time for one technician, with planimetric evaluation of the peak areas, is 6 to 8 hr. The possibility of using one set of calibration curves for the determination of mixtures analyzed on different days was also studied. For such an approach an average percent error of around 16% was obtained. However, this method may have some merit due to the enormous time-saving factor.

Nicotinamide was selected in a later investigation[288] for a comparison study of calibration curves obtained by fluorimetry, reflectance spectroscopy, and fluorescence quenching. For UV reflectance and for quenching methods the same shape of calibration curves was obtained, thus supporting the assumption that both methods work on the basis of UV absorption of the compound of interest. In both cases linear calibration curves were obtained over about the same concentration range by plotting (peak area)^2, which corresponds essentially to reflectance (R)^2, vs. concentration. This corresponds to a rough approximation of the Kubelka-Munk function.[252] In the case of reflectance spectroscopy, a direct signal is obtained for this absorption phenomenon, whereas in the quenching method the compound acts as a UV filter and as a result a dark spot appears where the spot material is adsorbed. This darkening effect is an indirect measure of the concentration of the adsorbed UV-active compound. Under ideal instrumental conditions, the quenching method should be as sensitive as the reflectance technique, but usually reflectance is superior because optimum conditions can be chosen more easily. Fluorescence quenching therefore offers absolutely no advantage over UV reflectance spectroscopy, and it suffers from several drawbacks, such as lower reproducibility of data due to higher background fluctuations and lower specificity.

d. Vitamins

The spot-removal technique in conjunction with UV reflectance spectroscopy was successfully adapted to the nondestructive determination of several vitamins.[94] The spectra were recorded directly on the plate with the Beckman DK-2 Spectrophotometer. In some instances luminous pigments were added to facilitate the location of the spots; this, however, would not be necessary in view of the natural fluorescence of the silica gel. Neither R_F-values nor the position of the absorption maxima is greatly affected by the presence of luminous pigment (Table 12), but the sensitivity is better on pure silica gel G. Table 12 reveals also that with the exception of nicotinic acid and nicotinamide a unique reflectance spectrum has been obtained for the vitamins tested, hence permitting their identification.

For a quantitative analysis the samples were prepared in the windowless cell described before (section III D). Calibration curves (% R vs. log C) were not quite linear, and the use of the modified Kubelka-Munk function suggested in other work[249, 288] may be recommended instead. Values of ± 0.33% R to ± 0.39% R mean standard

TABLE 12

Absorption Maxima, R_F-Values, and Sensitivities of Vitamins Absorbed on Silica Gel G and Silica Gel G–Luminous Pigment Mixture[94]

	Absorption maximum (nm)		R_F-value		Sensitivity (μmole)	
Vitamin	Silica gel G–lum.pigm. mixture	Silica gel G	Silica gel G–lum.pigm. mixture	Silica gel G	Silica gel G–lum.pigm. mixture	Silica gel G
Thiamine hydrochloride	278	278	0.00	0.00	0.01	0.01
Pyridoxine hydrochloride	298	298	0.17	0.18	0.05	0.01
Nicotinamide	263-268	264	0.51	0.51	0.02	0.01
Nicotinic acid	262-268	264	0.66	0.65	0.02	0.01
*p*Aminobenzoic acid	295	295	0.85	0.86	0.01	0.005

deviation were reported in a study of reproducibility. The accuracy of this method (2.3%) is superior to the one reported by Pataki[253] for similar compounds.

e. Hormones

Struck et al.[289] have described an in situ reflectance procedure for the determination of Δ^4-androstene-3,17-dione and testosterone. This is one of the earlier studies carried out with a prototype of the Zeiss Chromatogram-Spectrophotometer. Quantities of 0.2 to 0.5 μg of the studied compounds were estimated with an accuracy of ± 10% after two-dimensional chromatographic separation on silica gel GF (with fluorescence indicator). The detection limits were reported to be 1 μg/spot. It is reasonable to assume that the use of internal standards such as reported earlier might considerably improve the accuracy of this method.

f. Herbicides and Pesticides

The possibility of nondestructive detection of *s*-triazines by fluorescence quenching at about 254 nm[262] prompted the use of UV reflectance spectroscopy for the quantitative evaluation of these compounds after resolution on silica gel plates.[247] Camag silica gel DF-5 with organic fluorescence indicator, suitable for low UV work, was used. The spectroscopic study was carried out with a Spectronic 505 Spectrophotometer equipped with a reflectance attachment suitable for work in the lower UV range. Some spectra recorded directly on the plate are shown in Figure 29. Atraton has a maximum as low as 230 nm and for simazine, which is not shown here, a maximum at 218 nm was reported and still used with good success for quantitative determination. This work therefore constitutes the lowest UV in situ reflectance study, carried out in a spectral region where self-absorption of the adsorbent becomes a real problem. As can be seen in Figure 29, the spectra are sufficiently characteristic to be suitable for identification purposes. Comparing the reflectance spectra to the corresponding transmission spectra in ethanol revealed relatively good resemblance, but broader maxima were observed for reflectance spectra due to light-scattering phenomena, and bathochromic shifts of a few nm resulted on the adsorbent.

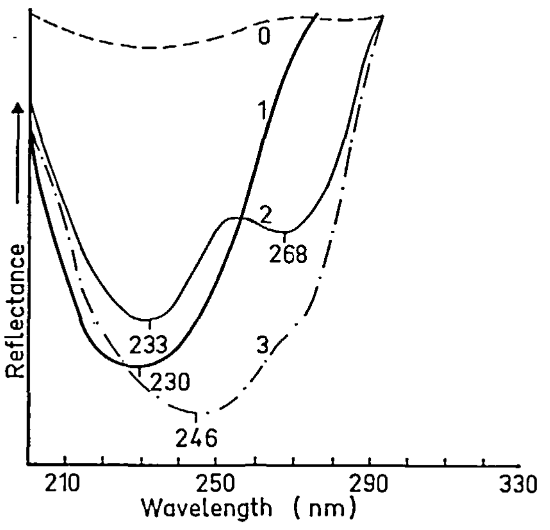


FIGURE 29. Reflectance spectra of 1, atraton; 2, atrazine; 3, amethryne, adsorbed on silica gel (0, base line).[247]

Atrazine was chosen for a detailed quantitative investigation. The spectrum (Figure 29) reveals two maxima at 230 and 270 nm, respectively, which can both be used for analytical procedures. The precision of this technique was reported as 0.50% R for 270 nm and 0.84% R for 230 nm (standard deviation for six 10-μg samples). The decrease in precision at lower wavelength can be attributed to increased self-absorption of the silica gel and a loss in radiation energy output; this in turn requires more amplification of the signals and results in an increase of the noise level. As expected from the spectrum (Figure 29) the sensitivity is somewhat better at wavelength 230 nm. Sensitivities between 1 and 3 μg have been reported for all triazines (given at a 50% accuracy level), but these would have been considerably better if direct scanning procedures had been used. The accuracy of the method is ± 3% for a 10-μg sample.

In a later study dealing with the in situ determination of *s*-triazines,[290] the UV reflectance technique was compared to other methods of in situ evaluation (Table 13). The accuracy is best for the spectral reflectance technique. The loss in accuracy for the fluorescence quenching method can be attributed to background fluctuations. Methods III and IV are strictly semiquantitative, and IV may have the advantage of a shorter analysis time. Since no electronic equipment is needed for methods III and IV, they may have some potential as field techniques.

TABLE 13

Quantitative Analysis of Atrazine
by Four Different Methods[290]

I: Ultraviolet reflectance spectroscopy[247]
II: Fluorescence quenching: fluorimetric[290]
III: Fluorescence quenching: spot-area measurement[291]
IV: Flourescence quenching: visual comparison of spots[246]

Amount of atrazine added (μg): x_1	Amount of atrazine found (μg): x_2			
	I	II	III	IV
10.0	9.7	9.2	11.2	12.1
6.0	5.9	5.8	6.7	5.6
4.0	3.8	3.8	4.6	4.5
Relative % Error				
$\frac{x_1 - x_2}{x_1}$ 100%	3.3	5.6	12.9	13.4

Chlorinated pesticides such as aldrin, dieldrin, endrin, lindane, heptachlor, methoxychlor, toxaphene, and DDT and some of its derivatives were studied by Beroza et al.[111] with the use of a fiber optics instrument constructed in their laboratory (section III E). The same measuring device was also used for the reflectance spectroscopic determination of some organophosphorus pesticides, which were separated on thin-layer plates coated with fluorisil. For the chlorinated pesticides silver-nitrate-impregnated alumina plates were used. The relative merits of Beroza's instrumental approach have been discussed in section III E.

g. *Pharmaceutical Compounds and Drugs*

Stahl and Jork have investigated a number of systems with the Zeiss Chromatogram-Spectrophotometer. Reflectance spectra of caffeine,[292] phenazetine,[293] and the *Strychnos* alkaloids, strychnine and brucine,[294] were measured directly on the chromatograms. In an investigation of drug samples spectra and quantitative analyses of five opium alkaloids were reported.[295] Characterization of classes of drugs was attempted in a study of band positions of individual phenol ethers and phenylpropanes.[296,297] Selective identification of zones was possible with this approach in a chromatographic study of oils from certain plants. Other applications include an investigation of aromatic hydroxyaldehyde derivatives, such as vanillin, anisaldehyde, and asarylaldehyde,[293] and a study of the pyrethrines of pyrethrum and peony flowers.[298]

Kraus et al.[299] have reported the measurement of scylla glycosides by spectral reflectance with the Zeiss instrument following the separation of these components on silica gel thin-layer plates. Schunack and co-workers[300] have discussed similar procedures for the quantitative determination of some xanthine derivatives in blood. Accuracies ranging between ± 4% and ± 18% were reported for the various compounds, but again the author believes that many of the methods were carried out under far from possible optimum conditions inherent in the technique. Lack of fundamental knowledge of the field of diffuse reflectance spectroscopy may be but one of the reasons. The relative popularity of in situ reflectance methods in the pharmaceutical field without doubt can be attributed to the many contributions of Stahl and Jork, who are among the earlier users of this technique.

In general, the author believes that in situ UV reflectance techniques will gain wide acceptance in practically all branches of organic analysis, particularly since they make use of some of the major advantages of reflectance over densitometric or fluorescence quenching techniques, such as more efficient recording of signals, elimination of mirror effect in the TLC support, less interference from self-absorption of adsorbent and support, better specificity, etc.[254] It also seems quite obvious that the instruments now available for this work are far better than the corresponding chromatographic techniques and that further improvements of quantitative methods will have to be made primarily through improvements of the separation and, if necessary, chromogenic steps.

4. *Reflectance Spectroscopy of Inorganic Systems*

The first application of spectral reflectance to the evaluation of chromatographically separated inorganic systems was reported by Vaeck,[301,302] who determined Ni^{2+} in microgram quantities on paper chromatograms. Ingle and Minshall[303] presented a critical comparison of reflectance and transmission techniques for the analysis of copper spots on paper chromatograms. They reported that the paper appeared optically more uniform in the reflected light and therefore recommended reflectance spectroscopy for in situ work. They determined copper as the rubeanic-acid complex and reported a precision of ± 0.43% R for four replicate determinations.

The first paper on the combination of reflectance spectroscopy and TLC for the determination of trace metals appeared in 1967.[304] The following sections give an account of this and other work in this area·that has been done since.

a. Rubeanic-acid Complexes

The first study[304] dealt with the investigation of rubeanic complexes, which had been used with good success in a combination of paper chromatography and spectral reflectance.[303] Fluctuations of the stability and intensity of the spots as a function of varying temperature, humidity, and pH conditions were investigated by reflectance spectroscopy. Instrumental detection limits with samples prepared in the spot-removal mode with 20 mg material per sample were reported to be 0.05 μg for nickel and copper and 0.1 μg for cobalt. The Kubelka-Munk theory was valid up to about 10 μg/spot. The reproducibility of the method ranged between 0.55 and 0.77% R standard deviation when the spraying technique was used. With cellulose layers it was possible to use a dipping technique, and as a result improved reproducibilities ranging between 0.39 and 0.44% R were obtained for all three metals in a medium concentration of 1.6 μg/spot. The accuracy that can be expected with varying concentrations of the metal is depicted in Table 14. The accuracy decreases at low concentration to 20% relative error for 0.1-μg samples, which is already close to the detection limit (0.05 μg/spot). The best concentration range is around 10 μg/spot and then a loss of accuracy is observed, again due to the flattening of the calibration curve. A similar trend has been reported in a systematic error analysis carried out by Lieu and Frodyma[23] and has been represented graphically (Figure 2). If one were to plot the probable percent error of Table 14 as a function of concentration or percent reflectance, a similar error curve would be obtained.

b. Pyridine-2-aldehyde-2-quinolylhydrazone Complexes

This relatively new chelating agent was developed by Heit and Ryan[305] and extensively investigated in connection with TLC work and in situ reflectance spectroscopy.[217,306,307] A procedure similar to the rubeanic-acid method[304] has been discussed for the determination of nickel, copper, cobalt, and iron. A solution of PAQH[217] (0.03% w/v) stabilized in 0.01N ethanolic HCl was used. The reflectance spectroscopic study of pH dependence for the nickel complex is demonstrated in Figure 30, which shows the nickel complexes adsorbed on silica gel. A red complex (maximum 520 nm) corresponding to the 1:1 species is predominant when the plate is sprayed with a 0.025N NaOH solution. With increasing basicity a shift from the red modification to the brown 2:1 (reagent to metal) complex with absorption maximum at 480 to 490 nm is observed, until at 1.0N NaOH strength a complete conversion to the brown species has occurred. The same shift from a 1:1 to a 2:1 complex has been noticed for the copper complex. For analytical purposes the 2:1 species is of interest, and in both cases spraying with a 1.0N NaOH solution has been recommended for optimum results. This pH study serves as an elegant example to demonstrate

TABLE 14

Probable Relative Error in the Measurement of the Concentration of Nickel as a Function of Concentration[304]

	Concentration of Ni (μg/spot)							
	0.1	0.5	1.0	2.0	4.0	6.0	10	20
S.D.*	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Equivalent change in measured concentration of nickel (μg/spot)	0.02	0.04	0.05	0.07	0.10	0.14	0.22	1.0
Probable % error	20	8.0	5.0	3.5	2.5	2.3	2.2	5.0

*S.D. (standard deviation) = $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n-1}}$

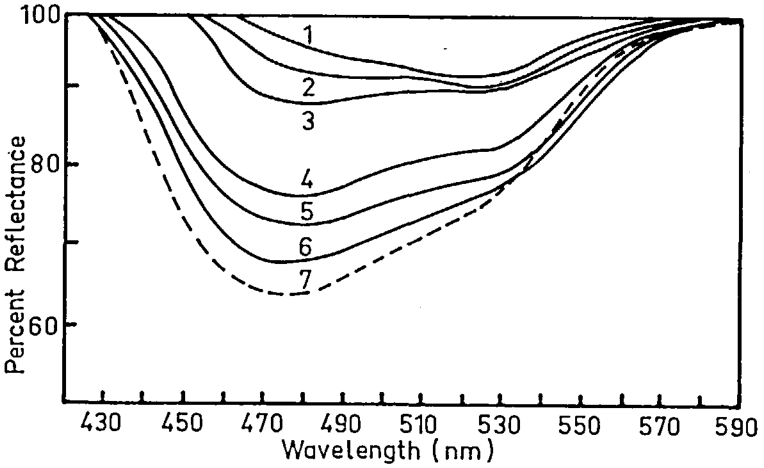


FIGURE 30. Reflectance spectra of nickel complex adsorbed on silica gel and sprayed with NaOH solutions. (1) 0.025N NaOH; (2) 0.05N NaOH; (3) 0.10N NaOH; (4) 0.25N NaOH; (5) 0.50N NaOH; (6) 0.75N NaOH; (7) 1.0N NaOH.[217]

the use of spectral reflectance for investigations of this nature. A temperature study carried out similarly revealed the best conditions to be a one-hour waiting period after spraying, to allow the reaction to come to equilibrium conditions and then heating for 5 min at 110°C, which serves as a final drying step and results in a further increase of color intensity. The previously mentioned competition between a 1:1 and a 2:1 complex was found to be also temperature-dependent. For best results on cellulose the samples were just air-dried for 1 hr. In the case of the copper and nickel species only the 1:1 complexes were observed on cellulose layers (section IV D2). The detection limits for this improved method were reported to be about 0.01 µg/spot at the 50% accuracy level and were obtained by the spot-removal technique. The reproducibility was similar to that of the rubeanic-acid method discussed earlier.[304] Iron, zinc, lead, cadmium, and manganese reacted with PAQH at suitable conditions, but did not interfere in this method in up to tenfold excess if the pH and temperature conditions recommended were followed. Trace metals in cereals have been determined by a combination of these two methods.[306] Nickel and copper were determined as the rubeanic-acid complexes and cobalt as the PAQH chelate. The concentration levels of cobalt in oats came close to the detection limit, which resulted in a high relative standard deviation of 20%.

In another study[307] PAQH was used as a semiselective scavenger and the complexes were separated on alumina layers with chloroform as an eluting agent. This approach required no spraying procedure, hence rendering spectral reflectance methods more precise. Interference from impurities in the chromatographic adsorbent was also eliminated, and the separation with the less polar fast-flowing solvent was achieved in a small fraction of time. The scavenging step entailed an additional concentrating effect, hence rendering this approach particularly suitable for trace analysis in natural water sources.

c. *Dithizone and Oxine Complexes*

Dithizone and oxine complexes are among some other systems investigated by reflectance spectroscopy.[308] The aim in this work was to find a qualitative analysis scheme which enabled the identification of a wide number of cations by a combination of spectra and R_F-values analogous to the method proposed for amino acids.[251] The plates were sprayed with a dithizone-oxine reagent mixture and the spectra were recorded with the Beckman DK-2 Spectrophotometer. After recording of the first set of spectra the chromatograms were exposed to ammonia fumes and a second set of spectra was recorded. A complete list of absorption maxima obtained before and after treatment for all the cations investigated is depicted in Table 15. A rather specific pattern of change was observed due to the ammonia treat-

TABLE 15

Spectral Data for Cations Absorbed on MN-Cellulose[308]

	After spraying with dithizone-oxine reagent		After spraying with dithizone-oxine reagent followed by ammonia treatment	
Cation	Absorption maximum (nm)	Sensitivity (μg)	Absorption maximum (nm)	Sensitivity (μg)
Aluminum	–	–	380	2.3
Bismuth	505	2.2	515	2.2
Cadmium	498	1.6	519	1.6
Chromium	–	–	414	6.5
Cobalt	512	0.5	564	0.5
Copper	502	0.6	398	0.6
Iron	380	2.7	597	0.9
Lead	498	0.5	556	2.2
Manganese	510	2.2	507	2.6
Mercury	492	0.5	–	–
Nickel	503	1.2	514	0.6
Silver	492	2.0	574	0.6
Tin	510	0.9	–	–
Zinc	513	0.7	526	0.7

ment, which renders the method proposed by Zaye et al.[308] suitable as a fingerprint device for a large number of metals, which, if sprayed under just neutral conditions, would show little differentiation in color.

d. Complexes of 1-(2-pyridylazo)-2-naphthol

Galik and Vincourova[309] have investigated complexes of 1-(2-pyridylazo)-2-naphthol (PAN) of cobalt, copper, nickel, and iron in a manner similar to the approach mentioned by Frei et al.[307] in connection with PAQH chelates. A Zeiss Jena (DDR) Recording Reflectance Filterphotometer ERI-10 served for the direct recording of the chromatogram spots. The peak areas were integrated manually and calibration plots peak area vs. μg/spot, which were slightly bent, were used for quantitative work. The following instrumental detection limits were observed: Co, 0.016 μg; Ni, 0.007 μg; and Fe, 0.012 μg. Detection limits were defined as readings of 10 mm^2 peak areas. Interfering metal ions were eliminated in the separation process. The stability of the chelates of interest was excellent over several days.

Other workers[310, 311] used PAN as a chromogenic spray reagent for a number of metal ions. The separation process was carried out on cellulose layers impregnated with liquid ion exchanger Primene-JM-T hydrochloride. In the first portion of this study parameters involved in the in situ reflectance spectroscopic investigation of the Zn-PAN complex were investigated.[310] The measurements were made with the Joyce, Loebl Chromoscan with TLC attachment in the reflectance mode. As in previous studies, the spraying procedure and the inhomogeneity of spot-material distribution were found to be the limiting factors on the reproducibility of the technique. Despite the spraying error, an accuracy of 5% was deemed possible for this method at the 1-μg level of metal concentration. Later, a similar method was discussed for Co, Cu, Zn, Cd, Pb, Bi (III), and UO$_2^{2+}$, [311] and compared to a spot-removal technique followed by conventional transmission spectroscopic measurement of the samples. Both methods were found to be comparable in accuracy (about ± 4%) but the in situ technique was more favorable with regard to time of analysis and the range of metals that could be investigated.

PAN and closely related reagents such as 4-(2-thiazolylazo) resorcinol (TAR) have been investigated as detecting agents following the separation of a large number of heavy metals on various adsorbent layers.[312, 313] Diffuse reflectance studies carried out on these systems[314] suggested the feasibility of this technique to similar quantitative evaluation methods to be used after separation and visualization of the metal ions.

e. Miscellaneous

A method for the quantitative determination of copper, nickel, and zinc has been developed by Frodyma et al.[315] Copper and nickel were analyzed in the presence of 11 other cations without interference, by employing neocuproine and dimethylglyoxime as spray reagents. 3,3-dimethylnaphthidine served as chromogenic reagent for zinc, with only tin, cadmium, and iron interfering. Accuracies of 2.1% and 2.8% were reported for nickel and copper, and 5.6% for zinc. All three were evaluated by the spot-removal technique and measured on the Beckman DU Spectrophotometer. The method seems to have no distinct advantages or disadvantages over the other procedures discussed in which spray reagents were used for the detection of metals. PAQH and PAN tend to give better sensitivity but may be subject to more serious interferences than rubeanic acid or the reagents used in this technique. The proper choice obviously depends on the type of sample to be analyzed.

V. CONCLUSIONS

The major use and therefore development of diffuse reflectance spectroscopy will be in the field of color comparison and control in the various industries mentioned previously. These operations will be still further automated and more and more instruments along the line of the Zeiss Model DMC-25 Color Spectrophotometer will become available. With the availability of new and sophisticated instruments new industries and also new sciences will be able to benefit from the technique. Synthetic building materials, plastics and food-processing industries, health sciences, geological and space sciences are but a few examples.

In other areas utilization and progress are more limited. Physical chemists and physicists will continue to use this technique for studies of adsorption phenomena and insoluble systems, but progress and advancement will depend largely on instrumental improvements and advancement in sampling techniques, e.g., the development of temperature-and humidity-controlled cells, improvement of resolution and efficiency of collecting diffuse radiation, and improvement of standard reference materials and electronic design of measuring circuits which give better stability. In analytical chemistry, on the other hand, the instruments available are adequate and the success of diffuse reflectance spectroscopy will depend more on the ingenuity of the analyst to devise new methods to which this technique can be applied. Particular popularity for spectral reflectance is foreseen in the field of chromatography, where in situ reflectance together with fluorescence techniques will be the major tools to make paper and thin-layer chromatography quantitative methods of analysis. In conjunction with recent developments in thin-film chromatography and other micro TLC techniques,[316] microscope reflectance methods such as described in section IV C 5 will become important. Development of better-controllable chromatographic techniques will be necessary to make full use of the excellent quality of chromatogram spectrophotometers that have been developed during the past two to three years. The design of cheaper instruments (for example those that use fiber optics) which can be used for routine monitoring of pollution sources in field laboratories may be a promising area of expansion. Designs which permit automated readout of data after scanning of chromatograms, and computer interfacing of chromatogram spectrophotometers in order to control data acquisition, are also of interest, and the field is still wide open.

Utilization of diffuse reflectance techniques in the infrared region of the spectrum will probably never become important due to the inherent difficulties such as light-scattering phenomena, small penetration of radiation into the sample, etc. Direct reflection techniques; e.g., attentuated total reflectance and multiple internal reflectance, will be likely to receive increased attention in this area and gain importance in the characterization of complex organic systems (natural-products chemistry, polymer chemistry, etc.).

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