This dissertation has been microfilmed exactly as received

FREI, Roland Walter, 1936–
THE DETERMINATION OF AMINO ACIDS
BY SPECTRAL REFLECTANCE.

University of Hawaii, Ph.D., 1965
Chemistry, analytical

University Microfilms, Inc., Ann Arbor, Michigan
THE DETERMINATION OF AMINO ACIDS
BY SPECTRAL REFLECTANCE

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN CHEMISTRY
January, 1965

By
Roland Walter Frei

Thesis Committee:
Michael M. Frodyma, Chairman
John J. Naughton
Richard G. Inskeep
Harry Zeitlin
John W. Hylin
to Eva, Urs and Christina
TABLE OF CONTENTS

Introduction

The paper chromatography of amino acids 1
Thin-layer chromatography and its application to amino acid analysis 5
Reflectance spectroscopy and some of its applications 9
The rationale of the research 15

Experimental

A. Factors affecting the reflectance of some dyes adsorbed on alumina 20
B. A simple semi-micro cell for the measurement of spectral reflectance 22
C. The application of spectral reflectance to thin-layer chromatography 23
D. Use of spectral reflectance in determining color stabilities of ninhydrin complexes of adsorbed amino acids 27
E. The determination by reflectance spectrophotometry of amino acids resolved on thin-layer plates 28
F. An improved method for the determination of amino acids by spectral reflectance 30

Results and Discussion

A. Factors affecting the reflectance of some dyes adsorbed on alumina 32
B. A simple semi-micro cell for the measurement of spectral reflectance 44
C. The application of spectral reflectance to thin-layer chromatography 53
D. Use of spectral reflectance in determining color stabilities of ninhydrin complexes of adsorbed amino acids
E. The determination by reflectance spectrophotometry of amino acids resolved on thin-layer plates
F. An improved method for the determination of amino acids by spectral reflectance

Summary
Appendix I
Derivation of the Kubelka-Munk function
References
Acknowledgements
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Dimensions of cell elements and sketch of assembled cell</td>
<td>24</td>
</tr>
<tr>
<td>2)</td>
<td>Transmittance spectra of fuchsin, eosine B and rhodamine B in ethanolic solution</td>
<td>34</td>
</tr>
<tr>
<td>3)</td>
<td>Reflectance spectra of eosine B adsorbed on alumina (regenerated at various temperatures)</td>
<td>35</td>
</tr>
<tr>
<td>4)</td>
<td>Reflectance spectra of fuchsin adsorbed on alumina</td>
<td>36</td>
</tr>
<tr>
<td>5)</td>
<td>Reflectance spectra of rhodamine B adsorbed on alumina</td>
<td>37</td>
</tr>
<tr>
<td>6)</td>
<td>Reflectance spectra of o-nitrophenol adsorbed on alumina</td>
<td>38</td>
</tr>
<tr>
<td>7)</td>
<td>Reflectance spectra of o-nitrophenol adsorbed on acidic, neutral and basic alumina</td>
<td>39</td>
</tr>
<tr>
<td>8)</td>
<td>Kubelka-Munk plots for a dilution series of rhodamine B adsorbed on alumina</td>
<td>40</td>
</tr>
<tr>
<td>9)</td>
<td>Reflectance spectra of various background materials relative to MgO</td>
<td>46</td>
</tr>
<tr>
<td>10)</td>
<td>Reflectance as a function of the number of filter paper introduced into the cell</td>
<td>47</td>
</tr>
<tr>
<td>11)</td>
<td>Reflectance spectra of various concentrations of McCormick blue adsorbed on silica gel</td>
<td>49</td>
</tr>
<tr>
<td>12)</td>
<td>Reflectance data obtained at 630 μν. for McCormick blue adsorbed on silica gel</td>
<td>50</td>
</tr>
<tr>
<td>13)</td>
<td>Reflectance spectra of eosine B adsorbed on filter paper, alumina and silica gel compared with the transmittance spectrum of an aqueous solution of the dye</td>
<td>55</td>
</tr>
<tr>
<td>14)</td>
<td>Reflectance spectra of dyes adsorbed on alumina</td>
<td>58</td>
</tr>
</tbody>
</table>
15) Reflectance spectra of various concentrations of eosine B adsorbed on alumina

16) 2-log % reflectance at 530 µm of eosine B adsorbed on alumina as a function of concentration

17) Kubelka-Munk values at 530 µm for eosine B adsorbed on alumina as a function of concentration and log C

18) Leucine series with spray "A". Per cent reflectance at 530 µm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time

19) Leucine series with spray "A". Per cent reflectance at 530 µm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time for samples stored below 10° C

20) Leucine series with spray "B". Per cent reflectance at 530 µm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time

21) Leucine series with spray "C". Per cent reflectance at 520 µm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time

22) Per cent reflectance and adsorbance at 520 µm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration

23) Reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent

24) Per cent reflectance at 515 µm of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of concentration

25) Absorbance at 515 µm of ninhydrin complex of various amino acids adsorbed on silica gel as a function of the square root of concentration

26) Two typical chromatograms obtained by two-dimensional chromatography

27) Typical chromatogram obtained by using an ammonia containing solvent mixture for the initial development
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Absorption maxima of transmittance and reflectance spectra of dyes</td>
<td>56</td>
</tr>
<tr>
<td>2)</td>
<td>Reproducibility of reflectance readings obtained for different spots of the same concentration of eosine B adsorbed on alumina</td>
<td>61</td>
</tr>
<tr>
<td>3)</td>
<td>Reproducibility obtained for different samples of identical concentrations of eosine B adsorbed on alumina at various wave lengths</td>
<td>66</td>
</tr>
<tr>
<td>4)</td>
<td>Test of reproducibility of packing reflectance cell</td>
<td>64</td>
</tr>
<tr>
<td>5)</td>
<td>Accuracy and precision of student determinations by spectral reflectance of dyes resolved on thin-layer plates</td>
<td>69</td>
</tr>
<tr>
<td>6)</td>
<td>Leucine series with spray &quot;B&quot;. Per cent reflectance at 530 m(\mu) of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time for samples stored below 10°C</td>
<td>76</td>
</tr>
<tr>
<td>7)</td>
<td>Leucine series with spray &quot;C&quot;. Per cent reflectance at 520 m(\mu) of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time for samples stored below 10°C</td>
<td>79</td>
</tr>
<tr>
<td>8)</td>
<td>Per cent reflectance at 530 m(\mu) of ninhydrin complex of leucine adsorbed on silica gel as a function of temperature and time of color development and of post-development storage time</td>
<td>83</td>
</tr>
<tr>
<td>9)</td>
<td>Reproducibility of reflectance readings obtained at 515 m(\mu) for different spots of the same concentration of amino acids adsorbed on silica gel and sprayed with ninhydrin reagent</td>
<td>86</td>
</tr>
</tbody>
</table>
10) Reproducibility of reflectance readings obtained at 515 µm for different spots of the same concentration of amino acids chromatographed in one dimension using a solvent mixture with 0.2% ninhydrin

11) Reproducibility of reflectance readings obtained at 515 µm for different spots of the same concentration of amino acids chromatographed in one dimension using a solvent mixture with 0.4% ninhydrin

12) Reproducibility of reflectance readings obtained at 515 µm for different spots of the same concentration of amino acids chromatographed in two dimensions

13) Reproducibility of reflectance measurement as a function of slit width

14) Probable relative error in the measurement of the concentrations of some amino acids

15) Probable relative error in the measurement of the concentration of glycine as a function of concentration
INTRODUCTION

One of the many difficult analytical problems which faces those working with biochemical systems is the separation, identification and determination of amino acids. Significant contributions in this area have resulted from the utilization of the thin-layer chromatographic technique to effect the resolution and identification of amino acid mixtures. This research represents an attempt to enhance the utility of the technique by devising a procedure for the determination of the resolved amino acids by means of diffuse reflectance and, in the process, to acquire more information about the analytical potential of reflectance spectroscopy.

The Paper Chromatography of Amino Acids

The employment of one- and two-dimensional paper chromatograms for the separation of amino acids was first described by Consden et al (1). Although the original paper dealt principally with the qualitative aspects of the problem, it did suggest the possibility of the method being used as a quick, convenient and accurate means of determining amino acids. Subsequently Boissonas (2), Keston et al (3), Woiwod (4) and Naftalin (5), among others, succeeded in determining amino acids and their radio-active derivatives after they had been separated by paper chromatography. In each case the acid or its derivative was removed from the paper by extraction and then determined by conventional procedures such as polarography, iodometry, colorimetry or micro Kjeldahl analysis. Hanes et al (6) improved the chromatographic technique by exercising a more rigorous control of such experimental variables as the temperature of the chromatographic
chamber and the composition of the solvent system, and by devising a new ninhydrin hydrantin spray reagent. All of these procedures suffered, however, from the disadvantage that they either required specialized equipment or were relatively tedious and time consuming.

A different approach to the quantitative evaluation of a paper chromatogram was suggested by Fisher et al. (7) who described several methods based on the observation that the area occupied by an amino acid or any other substance is a function of its own concentration. Unfortunately these methods have met with only moderate success because the control of experimental conditions that they require is difficult to maintain. Since the color intensity as well as the spot size varies with the quality of the substance being chromatographed, it is also possible to arrive at an estimate of the quantity by developing on the same chromatogram a dilution series of the substance of interest. Both procedures involve, as a final step, the visual comparison with respect to area or color density of the unknown spot with a matching standard spot, and so are subject to errors in judgment. Consequently a large number of replicate analyses must be carried out to give reasonably satisfactory results. Polson et al. (8), Berry and Cain (9) and others have employed such procedures in the analysis of a number of proteins, reporting an accuracy of between ±10% to ±15%.

More convenient, more rapid and usually more accurate than indirect methods based on the elution of spots has been the direct photometric estimation of amino acids resolved on paper chromatograms. Typical of this approach has been the procedures that depend on the
measurement of maximum spot density. Such procedure can not only be employed with one-dimensional paper chromatograms, but, unlike the techniques involving the mechanical scanning of spots, they are well suited for use with two-dimensional chromatograms.

Using the maximum density method in conjunction with two-dimensional paper chromatograms, Block (10), reported average errors of ± 15% and ± 10% when aqueous phenol and BuOH - AcOH - H₂O respectively, were used as the first solvent system. McFarren and Mills (11), carried out a complete amino acid analysis of β lactoglobulin with an approximate accuracy of 5% by employing a photovolt densitometer to determine the maximum density of the spots on one-dimensional paper chromatograms. Patton and Chism (12, 13), Salander et al (14) and others have also effected satisfactory amino acid analyses by this method. It was claimed by Roland and Gross (15), to be less tedious, more rapid and more reasonably accurate than any other procedure hitherto available for the analysis of protein hydrolyzates.

The limitations of the maximum density procedure have been discussed in several publications (13, 16, 17, 18, 19). One such limitation derives from the fact that, whereas round or elliptical spots are essential for the most reliable results, asymmetric spots are frequently obtained with standard solutions and particularly with extracts of natural products. This introduces uncertainties in the analytical results in that it is difficult to ascertain the point of maximum density. Lugg and McEvoy-Bowe (17, 18) achieved increased precision by employing a specially designed densitometer having a small aperture and by taking account of textural irregularities in the
chromatographic paper. Somewhat greater reliability appears to have been achieved by combining the maximum color density and the spot area methods (11, 15, 20). This combination, however, increases the number of operations required and introduces errors associated with the determination of the spot area.

Methods depending on the measurement of total spot density by scanning followed by integration of the resultant curves were introduced by Block (20), Bull et al (21), and Fosdick and Blackwell (22). Following these procedures, the concentration of amino acids resolved on one-dimensional paper chromatograms was determined by reference to standard curves consisting of plots of cartesian coordinates of concentration versus the area under the curve. For maximum accuracy, Block recommended that the standard curves include no fewer than four points. Redfield et al (16) attained an accuracy of ± 5% when he plotted % transmission versus the logarithm of the distance of the scan in centimeters and then determined the areas under the curve using a planimeter. Both he and Block (23) also described the use of the total spot density method in the determination of incompletely separated amino acids. All of these procedures, however, are much more involved than the maximum spot density method described earlier.

To meet this objection, Rockland et al (24, 25) proposed a technique which made it possible to determine the total spot density in a single, rapid operation. This procedure varied from those mentioned above in that the entire spot was read with a densitometer or with a colorimeter that had been adapted for this purpose. The spots themselves had to be relatively small and could not overlap one another.
It was also necessary to employ a series of masks which could just accommodate the size of spots that could be obtained with the concentrations being investigated. Various attempts were also made to eliminate the exceedingly tedious procedure of manual scanning by employing an automatic recording galvanometer with a logarithmic output. Müller and Clegg (26) proposed the use of a rotating sample drum and a chart to record the fluctuations in the photocurrent resulting from variations in the transmitted or reflected light. Rockland and Underwood (27) analyzed the amino acids in orange juice in less than twenty-four hours by employing an automatic recording instrumental setup. Automatic recording densitometers capable of being employed with irregularly shaped chromatographic spots were described by Wieme (28), and Johnstone and Briner (29). The latter reported obtaining increased sensitivity and an accuracy of ± 3% in the analysis of sugars. Recently Jellinek and Fridman (30), using an all-purpose recorder-integrator devised by Orr (31), carried out a thorough error analysis of the direct densitometric determinations of glycine.

Although most of these procedures have concerned themselves with amino acid analysis, direct photometric methods have also been worked out for such compounds of biological interest as sugars (29, 32, 33, 34) and for inorganic cations (35, 36).

**Thin-layer Chromatography and its Application to Amino Acid Analysis**

Although thin-layer chromatography had been employed as long ago as 1938 by Izmailov and Shraiber (37) as a means of separating organic mixtures, it did not gain general acceptance until 1949 when Meinhard
and Hall (38) introduced the use of starch as a binding agent to give the layer greater mechanical stability. Kirchner et al (39) refined the procedure and applied it to the separation of terpenes, and Reitsema (40) devised larger plates for the development of two-dimensional chromatograms. Stahl (41, 42) pointed out the importance of standardizing the adsorbents with respect to particle size and suggested the use of approximately 15% plaster of paris as a binder. This particular preparation is now available as "Silica gel G" from Merck, Darmstadt. Stahl also invented an ingenious application for the preparation of the plates which is commercially available from Desaga, Heidelberg. Even though thin-layer chromatography is still a relatively recent development, several reviews and books dealing with the technique are already available (43, 46).

It has been known for some time that thin-layer chromatography possesses several advantages over paper chromatography in the separation of lipophilic substances. More recently the method has also been applied successfully to the separation of hydrophilic compounds. In the case of amino acids it has been demonstrated that the thin-layer technique is superior to paper chromatography and excellent results have been obtained with both buffered and unbuffered silica gel films. Mutschler and Rochelmeyer (47) have obtained $R_f$ values for thirteen common amino acids on phosphate buffered silica gel films in three different solvent systems. Teichert et al (48) obtained $R_f$ values for fourteen amino acids on cellulose powder using a 4:1:5 amyl alcohol-acetic acid-water mixture as the solvent. Nürnberg (49) determined the $R_f$ values for twenty amino acids on silica gel G in two
solvents suitable for two-dimensional work. Brenner and Niederwieser
(50) undertook a systematic study of the conditions necessary for the
resolution of amino acid mixtures and concluded that very satisfactory
two-dimensional analyses can be carried out on silica gel G films.
In the course of this study, they obtained Rf values for twenty-five
amino acids on silica gel G with six different solvent systems. In
general, the most important advantages offered by the thin-layer
technique in the resolution of amino acid mixtures are the sharpness
of the separations, the high degree of sensitivity, and the great
speed. Separations that require many hours on paper can be accompl­
ished in minutes on a suitable film.

The methods employed in the quantitative evaluation of the chro­
matoplates have tended to parallel those used with paper chromatograms.
Accuracies reported ranged from 5% to 10% with the most accurate data
being obtained by eluting the species of interest from the adsorbent
and then determining it by conventional means (51 - 56). The spots
themselves are usually removed from the plates by scraping the adsor­
bent off with a razor blade or spatula, although Schilcher (55) and
Millet et al (56) have devised an apparatus which transfers the adsor­
bent quantitatively into a test tube by the application of a suction.
By using nozzles having the appropriate diameter, the time required
for an analysis can be reduced considerably. Regardless of how the
spot is removed from the plate, however, the elution techniques have
several built-in shortcomings. In addition to being very time consum­
ing and laborious, they are liable to methodic errors associated with
the use of the adsorbent. If the substance of interest is strongly
adsorbed, it may not be possible to achieve a quantitative recovery. On the other hand, it is conceivable that the adsorbent may contain extractable impurities which will complicate matters. Finally the location of the spots is often difficult to ascertain since chromogenic reagents may not be used.

While investigating the quantitative aspects of paper chromatography, Fisher et al. (7) found the spot area to be proportional to the logarithm of the weight of the material being chromatographed. A similar relationship was observed with thin-layer chromatographs over moderate concentration ranges (57). For larger concentrations extending up to 80 μg Purdy and Truter (58) noted that a linear relationship existed between the square root of the spot area and the logarithm of the weight. Since the absolute spot areas are dependent on the thickness of the film and the activity of the adsorbent, acceptable precision is achieved only if both samples and standards are developed on the same plate.

As might have been expected from prior experience with paper chromatograms, Hefendehl (59) found an integrated function of the optical density of a spot to be proportional to the concentration of the substance comprising the spot. He reported an accuracy of 3 to 5% for a procedure based on the preparation of photoprint copies of the chromatoplate by the phototrasing technique and their subsequent evaluation with a recording densitometer. Privet et al. (60) measured the optical density of the spots directly by scanning the plate at 1 mm. intervals with a fixed slit photodensitometer originally designed for use with paper chromatograms. Since it was transmitted light that was being
measured, the thickness of the film was most critical. More recently, Jacobsen (61) developed an interesting photogrammetric procedure for the analysis of estrone, estradiol and estriol mixtures separated by thin-layer plates. After the plates had been illuminated with ultraviolet light to make the spots fluorescence, photographs of the emitted light were enlarged and the dark areas evaluated densitometrically.

A most sensitive means of evaluating chromatoplates results from the application of radioactive isotopes to thin-layer chromatography. The methods employed for the detection and estimation of substances resolved on plates are essentially those used with paper chromatograms. A summary of the techniques and instruments available for these purposes, as well as a survey of the literature in the field, may be obtained from Nuclear-Chicago Corporation (62).

Reflectance Spectroscopy and Some of its Applications

Diffuse reflectance and the transparency of light-scattering and light-absorbing layers have been investigated by numerous workers because of their great practical significance in such areas as the paint, paper and ceramics industries. The most generally accepted theory concerning these phenomena, and one which has gained within the field of reflectance spectroscopy an importance akin to the Bouger-Beer law employed in transmittance spectroscopy, was developed by Kubelka and Munk (63, 64). As a rule, equations derived by other investigators have proved to be special cases or adaptations of the Kubelka-Munk equation (65 - 68). Kortüm and Vogel (65) 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 have to be those having a depth of approximately one millimeter. Judd and Wyszecki (68) compiled most of the different forms of the Kubelka-Munk function and pointed out some of their specific uses and applications.

Kortüm et al. (69 - 71) demonstrated that the Kubelka-Munk function for diffuse reflection on light-scattering layers, \( F(R_{oo}) = \frac{k}{s} \), holds only when the absorption coefficient, or \( k \), values are sufficiently small, as is the case with low concentrations of an adsorbed species. The same workers also pointed out the importance of adsorbent particle size, recommending particles having a diameter of 1 \( \mu \) or less for ideal diffuse reflectance. Zeitlin and Niimoto (72) also found that the particle size of the adsorbent or diluent can affect reflectance spectra, noting that the absorption bands tended to broaden as the particle size increased. Various attempts have been made to account for the deviations from the Kubelka-Munk law that are observed with increased concentration and particle size. Kortüm (65) ascribed them to the interference of regular, or Fresnel, reflection and verified his explanation experimentally (70) by measuring the reflectance spectra of powders between crossed polarization foils. In this manner he was able to separate diffuse from direct reflection.

The technique of carrying out reflectance measurements on powders is ideally suited for investigating color changes undergone by various compounds adsorbed on active surfaces (71 - 83). The polymorphic conversion of adsorbed mercuric iodide has been the subject of several investigations (71, 75, 77, 78, 82), and Kortüm and Vogel (81) have studied the effect of varying the adsorbent upon the reflectance
spectrum of malachite green o-carboxylic acid lactone. Zeitlin et al. (79) noted that larger bathochromic shifts resulted when the size of the cation of the alkaline earth metal carbonate being used as the adsorbent for nitrophenols was increased. Schwab and Schneck (82) used reflectance spectroscopy to investigate the behavior of several basic dyes adsorbed on substances exhibiting catalytic activity.

Numerous attempts have been made to explain the adsorption phenomenon and to postulate a mechanism for the adsorbate - adsorbent interaction. One generally accepted model is that suggested by Kortum (80, 81) who proposes that two types of adsorption are actually involved. The first of these is chemisorption in the first monomolecular layer due to polarization. It has also been suggested (79, 80, 81) that this bonding arises from the sharing of electrons or Lewis acid-base interactions. The second type is physical adsorption in subsequent layers due mainly to van de Waal's forces. Kortum used this model and the Kubelka-Munk law to determine the dissociation constants for various organic compounds adsorbed on different adsorbents (84), and to determine the surface areas of powdered adsorbents from adsorption isotherms (85).

The reflectance technique has also been applied to the study of both reversible and irreversible photochemical reactions undergone by substances adsorbed on active surfaces (86 - 88). It seems to be particularly useful with systems which undergo extremely rapid reactions in solution. High temperature diffuse reflectance spectroscopy has been made possible by the invention by Wendlandt et al. (89) of a sample holder which can be used for the visible portion of the spectrum.
at temperatures ranging from ambient to $500^\circ$ C.

The use of spectral reflectance for analytical purposes has been suggested by various authors. The advantages afforded by this technique for studies of biochemical systems have been pointed out by Naughton et al (90, 91). While investigating the heme pigments in tuna meat, these workers obtained reflectance spectra which were identical with the absorption curves found for transmittance measurements made on the same pigments in solution. Another obvious application of the technique was its use in obtaining analytically useful spectra of organic and inorganic compounds adsorbed on paper. Despite the fact that the textural irregularities of the paper make the reflectance technique superior to transmittance measurements for this purpose, published studies of this aspect of spectral reflectance are not numerous. In the earlier papers the spectral data were plotted in various ways. Winslow and Liebhafsky (92) employed plots of extinction, or $E_r$ ($E_r = -\log R$), versus concentration in their study of spot tests for copper and silver. Bevenue and Williams (93) determined reducing sugars separated on paper chromatograms by using calibration curves in which reflection density was graphed against concentration. Similar plots were employed by Goodban and Stark (94) in their analysis of amino acids in sugar beet processing juices. Regardless of the method used, linear calibration curves were obtained only at relatively low concentrations. Reasonably straight-line plots also resulted if data were graphed in the form percent reflectance versus the logarithm of concentration (95), reflectance versus the logarithm of concentration (96), or $2 - \log$ of reflectance versus the square root of con-
centration (98, 99). In all cases, however, the functions were
strictly empirical and held only for the particular concentration
range of the system under investigation.

The first application of the Kubelka-Munk law to the problem of
evaluating paper chromatograms was made by Vaeck (100, 101) who, in
determining divalent nickel on paper, found a linear relationship be-
tween \( f(R_{\infty}) \) and concentration over a range of 30 to 100 milligrams of
nickel per liter. Korte and Weitkamp (102) employed a modification of
the Kubelka-Munk equation in carrying out the determination of 2, 3,
6, trimethyl-fluorenone on paper chromatograms by means of reflectance
spectrophotometry, and Kortum and Vogel (103) undertook similar stud-
ies with malachite green. It was found in both cases that the Kubelka-
Munk function had only approximate validity for the systems investi-
gated, particularly at higher concentrations. The derivations obser-
ved by Korte and Weitcamp could be attributed, at least in part, to
the fact that they used only one thickness of paper, and so did not
fulfill the condition of infinite layer thickness which was assumed in
the derivation of the Kubelka-Munk equation. Kortum and Vogel employ-
ed ten thicknesses of filter paper of the same grade as backing mate-
rial to achieve infinite layer thickness but still observed deviations
at higher concentrations. These they attributed to the non-homo-
genous distribution of the dye and the textural irregularities and
varying moisture content of the paper. A critical comparison of the
reflectance and transmission techniques for the analysis of spots on
paper chromatograms was carried out by Ingle and Minshall (104), who
found that the paper appears optically more uniform in reflected light.
A precision of ± 0.43 in the percent reflectance measurements was reported for four replicate analyses of copper rubeanate on paper.

It has also been shown that the reflectance spectra of substances concentrated on particulate adsorbents can be used for their identification (105), and that the spectral reflectance can be employed to determine the concentration of dyes scavanged from solution by the batchwise addition of starch (106). A critical evaluation of the application of reflectance measurements to the direct analysis of solid mixtures of pigments, was made by Lermond and Rogers (107). Fisher and Vratny (95) developed a method for the determination of dyes, such as congo red or malachite green, adsorbed on powders, and observed that the preparation of the sample for analysis was the accuracy limiting operation. Kortum and Herzog (108) employed diffuse reflectance in the ultraviolet region of the spectrum for the analysis of rutile-anatase powder mixtures. Published data dealing with the quantitative analysis of powders by reflectance spectroscopy are still relatively scarce, however, because of the difficulties involved in the preparation of reproducible analytical samples having uniformly flat surfaces and identical densities. For powders whose particles have large or irregular dimensions, Kortum et al (109) recommended standardized grinding of the samples in a ball mill for several hours, a relatively inefficient and tedious process. This same article also includes an excellent summary of the principles and techniques of reflectance spectroscopy.
The Rationale of the Research

The extensive literature dealing with the direct estimation of amino acids of paper chromatograms reveals that there is a reproducible relationship between the color density of the ninhydrin complexes of the acids and their concentration on the chromatograms. Furthermore, a number of investigators have shown that reflection measurements can be carried out on powders with an acceptable degree of precision if certain experimental conditions are met. In view of these data, the application of spectral reflectance to the identification and determination of substances, such as amino acids, resolved on thin-layer plates seemed feasible.

For the purpose at hand probably the most appropriate theory treating diffuse reflection and the transmission of light scattering layers also happens to be the most general theory developed by Kubelka and Munk (63, 64). When applied to an infinitely thick opaque layer, the Kubelka-Munk equation may be written as

\[
\frac{(1-R_{\infty})^2}{2R_{\infty}} = \frac{k}{s}
\]

Where \( R_{\infty} \) is the absolute reflectance of the layer, \( k \) is its molar absorption coefficient, and \( s \) is the scattering coefficient (for the derivation of the equation see appendix, page 111). Instead of determining \( R_{\infty} \), however, it is customary in practice to work with the more convenient relative diffuse reflectance, \( R_{\infty} \), 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. Since the absolute reflectance of the standards
exhibiting the highest $R_{\infty}^1$ values never exceeds 0.98 to 0.99, however, one is actually dealing in such instances with the relationship

$$\frac{R_{\infty}^1 \text{ sample}}{R_{\infty}^1 \text{ standard}} = R_{\infty}$$

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 wave length by employing scattering particles whose size is large relative to the wave length being used. During the research described herein, the constancy of $s$ was ensured by making use of powders consisting of particles having an approximate diameter of 5 $\mu$.

A straight-line relationship between $F(R_{\infty})$ and $k$ is observed, however, only when dealing with weakly absorbing substances and only when the grain size of the powders employed is less than 1 $\mu$ in diameter. 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 absorbents having a large grain size or when large concentrations of the absorbing species are used, plots of $F(R_{\infty})$ versus $k$ or concentration deviate from straight lines in that there is a decrease in slope at higher concentrations.

In his explanation of this phenomenon, G. Kortum (65, 69, 70, 71) 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, that is to say, with no defined angle of emergence. Regular reflectance for cases involving normal incidence is described by the Fresnel equation

\[
R_{\text{reg}} = \frac{I_{\text{refl}}}{I_0} = \frac{(n-1)^2 + n^2 k^2}{(n+1)^2 + n^2 k^2}
\]

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

Although Kortum et al. (109) suggest the grinding of samples for twelve to fourteen hours in a ball mill as a means of diminishing the interference caused by regular reflectance, it was felt that there was no need to resort to such a procedure in this instance. Commercial grade adsorbents for thin-layer chromatography, since they consist of particles having an average diameter of 5 \(\mu\), seemed to be suited for reflectance measurements as received from the manufacturers. In addition, the grinding operation recommended by Kortum is not only too
inefficient and tedious for a routine analytical procedure, but also could result in the contamination of the samples.
Experimental

The first phase of the research concerned itself with the development of an experimental technique for the preparation of substances separated on thin-layer plates for reflectance measurements. Water-soluble dyes and aluminium oxide plates prepared according to a method devised by Mottier (110) constituted the system used in this pilot study. This system, in that it is stable and absorbs in the visible portion of the spectrum, lent itself most conveniently to the purpose at hand.

Once a satisfactory procedure had been devised, a systematic spectra of the dyes to determine the extent to which they could be depended upon for purposes of identification. The quantitative aspect of the study concerned itself with an evaluation of the factors affecting the precision and accuracy of the technique.

Finally, the experience gained during the pilot study was applied, first to systems including single amino acids, and then to amino acid mixtures of increasing complexity, in an effort to develop a method for their identification and determination. The thin-layer chromatographic technique employed to resolve the amino acid mixtures was one developed by Brenner and Niederwieser (50). It was necessary, at this point, to do some additional work on the nature and mode of application of the spray reagent, since no chromogenic agents were employed during the pilot study.
A. Factors affecting the reflectance spectra of some dyes adsorbed on alumina (Ref. 121).

All reagents used were of analytical grade. The o-nitrophenol was recrystallized repeatedly until its melting point range indicated satisfactory purity. Weighed amounts of eosine B, fuchsin, and rhodamine B dyes were dissolved in absolute ethanol and the solutions maintained in a moisture-free state by storage above anhydrous MgSO₄ for use as stock solutions. Thin-layer chromatographic grade alumina from "Merck" (Darmstadt) with a particle size of approximately 5 μ was employed as an adsorbent. Woelm alumina (basic and acid washed, activity grade 1) and the neutral Merck alumina were used in conjunction with o-nitrophenol in study of pH dependence.

The diffuse reflectance spectra were measured with a DK-2 Beckman spectrophotometer fitted with the standard reflectance attachment. A MgO plate served as the reference standard. A Beckman DU spectrophotometer equipped with a reflectance attachment was employed to carry out the photometric studies. Transmittance spectra were recorded with the DK-2 spectrophotometer employing 1-cm. matched quartz cells, and pH measurements were made with a Beckman pH meter, Model G, standardized against certified standard buffer solutions.

In order to evaluate the effect of moisture, samples were first prepared in an "air-dry" state. This was done by grinding 3-gram quantities of Merck alumina with 10 mg. of o-nitrophenol in an agate mortar for about one minute to achieve homogeneity. The sample was then "air-dried" by exposure to ambient temperature and humidity for approximately one hour, by which time an equilibrium condition with
respect to adsorption, color change, and humidity had been attained. Other samples of the same adsorbent were preheated carefully at temperatures of 200°, 800°, and 1100° C; transferred quickly to a vacuum dessicator containing P₂O₅ and evacuated to ca. 1 mm. Hg; and allowed to cool to room temperature. The mixing and grinding of the adsorbate and this preheated adsorbent in the proportions given above were carried out in a dry box which had been dried by dynamic adsorption through activated silica gel for 12 hours. The interaction was completed in one hour. The mixture was packed into a special cell devised by Barnes et al (111), the edge of the aluminum planchet was coated with silicone grease, and the diffuse reflectance spectra measured. The effect of pH upon the reflectance spectrum of o-nitrophenol was studied by preparing samples as described above with acid washed, neutral, and basic alumina. Slurries prepared with distilled water were employed to determine the pH of the different grades of alumina.

In the dye studies 1 ml. of stock solutions containing 0.3 mg. of fuchsin, eosine B, and rhodamine B, respectively was treated with 3 grams of adsorbent that had been preheated. The ethanol was removed in vacuo and the dry mixtures that resulted were prepared for measurement according to the procedure given earlier. A sample was also prepared in the "air dry" state and measured. During the minute required to carry out a measurement little or no moisture was adsorbed by the sample.

The photometric analysis of rhodamine B was carried out on dilution series of the dye adsorbed on alumina. 1 ml. aliquots of stan-
standard solutions of varying concentrations of dye were mixed with the adsorbent, following which the solvent was removed in vacuo. In this manner three six-membered series of samples were prepared, one in the "air-dry" state and two with adsorbents preheated to 200° and 1100° C. All measurements were carried out in duplicate. The reflectance of each sample at 562 nm was measured by means of a Beckman DU spectrophotometer equipped with a reflectance attachment.

B. A simple semimicrocell for the measurement of spectral reflectance (Ref. 122).

The cell employed consisted of white paperboard to which a 3.7 x 2.5 x 0.1 cm. microscope cover glass had been affixed with two pieces of masking tape. The white backing paper was cut to a size - 4.0 x 3.0 x 0.1 cm. - which permitted its introduction into the sample holder of the reflectance attachment of the Beckman model DU spectrophotometer. These data are presented schematically in Figure 1, as is a sketch of the assembled cell. The 40 mg. analytical sample, consisting of silica gel plus varying amounts of adsorbed dye, was carefully compressed between the cover glass and the paper until a thin layer having an approximate thickness of 0.4 mm. and an approximate diameter of 1.8 cm. was obtained. This last was necessary, since the impinging beam of light had an approximate diameter of 1.4 cm.

Merck silica gel G and McCormick blue, a food dye marketed by McCormick & Co., Inc., Baltimore, Md., constituted the system used to study the relationship between concentration of the dye and reflectance. It was prepared from a stock solution containing 1500 mg. of the dye in 100 ml. of distilled water. Solutions were applied to chroma-
toplates as spots by means of a 10 μl Hamilton microsyringe in 5 μl increments. After the plates had been dried for 15 min. at 110°C, each spot was removed from its plate and diluted with silica gel from the same plate until the aggregate totaled 40.0 ± 0.2 mg. This constituted the analytical sample which was then ground in a small agate mortar for two periods of 15 seconds each to ensure homogeneity and uniform particle size.

Cells employing white, gray and black backing paper were used to investigate background reflectance and interference. A Beckman model DU spectrophotometer fitted with a standard reflectance attachment was employed to determine the dye concentration - reflectance relationship. All other spectral data presented were obtained with the use of a similarly outfitted Beckman model DK-2 spectrophotometer.

C. The application of spectral reflectance to thin-layer chromatography. (Ref. 123).

Stock solutions containing 50 mg. of the dyes studied -- aniline blue, eosine B, basic fuchsin, malachite green, naphthol yellow S, and rhodamine B--per 100 ml. of solvent were applied as spots by means of a 10 μl Hamilton microsyringe. Except for the aqueous eosine B, the solvent used was 95% ethanol. The 10 x 7 x 0.15 cm. plates were cut from ordinary window glass and were coated with adsorbent by distributing the adsorbent-water mixture with a glass rod which rested on one thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.2 - 0.3 mm. thick. The plates were dried at 180°C for two hours and stored in a desiccator. Merck aluminum oxide and silica gel G were used as adsorbents.
Fig. 1. Dimensions of cell elements and sketch of assembled cell.
P - backing paper
C - microscope cover glass
S - sample
The dyes were chromatographed in n-butanol-ethanol-water (80:20:10 by volume) by the ascending technique according to Mottier (110), and the plates were then dried at 110°C for fifteen minutes. Direct spectral examination of these plates was accomplished by covering them with a clean glass plate of identical dimensions, fixing the ends together with masking tape, and then introducing them into the reflectance attachment of the Beckman model DK-2 spectrophotometer employed for this purpose. A sheet of paper, resembling in color the adsorbent material being used as a reference standard, was inserted behind the plate to serve as a reflecting background. The reference standard was prepared by grinding some of the adsorbent from the plate under examination and packing it into the cell described by Barnes et al (111).

A Beckman model DU spectrophotometer outfitted with a standard attachment for the measurement of diffuse reflectance was employed to examine spots scraped off chromatographic plates. The cells used to hold both sample and reference material were those described in the previous section. Fifty milligrams of material were carefully compressed between the cover glass and the paper until a thin layer having an approximate thickness of 0.4 mm. and an approximate diameter of 1.8 cm. was obtained. As before, the reference standard consisted of adsorbent from the plate under examination.

A student exercise was devised for a class in quantitative analysis to determine the reliability of the technique in the hands of an unexperienced analyst. Solutions containing several of thirteen commercially available dyes -- brilliant cresyl blue, brilliant green, brilliant yellow, crystal violet, eosine B, erythrosine B, fuchsin,
orange G, orange II, rhodamine B, safranine, spirit blue, and yellow AB were chromatographed in one dimension on silica gel plates that had been prepared according to the procedure given above and regenerated at 120° C. The solvent systems used to effect the separations were (50:50:50) and (90:10:10) butanol - acetic acid - water mixtures, with the choice being dependent on the nature of the sample being analyzed.

Each student identified the components of two mixtures, each consisting of at least three dyes, by the direct spectral examination of the chromatoplates with a Beckman DK-2 spectrophotometer. Identification was accomplished by comparing the spectra obtained in this manner with standard spectra. In addition, either crystal violet, fuchsin or rhodamine B, whichever had been included as a component of the mixture being analyzed, was determined by the spot-removal technique employing a Beckman DU spectrophotometer. Forty milligram samples were used for the analyses and the data obtained were evaluated according to one of two procedures. In the graphic method the concentration of the dye associated with a measured reflectance was read off on a standard F(R) versus concentration plot. A six-membered dilution series was employed in the construction of the plot and all assays of unknowns were done in triplicate. Since the concentrations of the unknowns all fell on the straight line portion of the calibration curve, it was also possible to use an alternative method. This involved the simultaneous analysis, in triplicate, of the unknown solution and a standard solution of approximately the same concentration. The concentration of the unknown was then determined by making use of the relationship
where \( C_u \) and \( C_s \) represent the concentration of the unknown and standard solutions, and \( F(R)_u \) and \( F(R)_s \) stand for the Kubelka-Munk functions of the unknown and standard, respectively.

D. Use of spectral reflectance in determining color stabilities of ninhydrin complexes of adsorbed amino acids. (Ref. 124).

The L-leucine used during this part of the investigation was of Calbiochem A Grade purity. A stock solution containing 500 mg. of the acid per 50 ml. of solution was employed in making up the dilution series used. Distilled water served as the solvent throughout and the solutions were applied as spots by means of a Hamilton microsyringe in 5 \( \mu l \) increments. The 20 x 5 x 0.35 cm. plates were coated with adsorbent by distributing a 4:10 Merck silica gel G-water mixture with a glass rod which rested on one thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.2 - 0.3 mm. thick. The plates were dried at 180\(^\circ\) C for 2 hours and stored in a desiccator. The acid was chromatographed in n-propanol-water (64:30) by the one-dimensional ascending technique described by Brenner and Niederwiesser (50) and the plates were then dried at 60\(^\circ\) C for 30 minutes in a mechanical convection oven. These drying conditions were selected after considering the results of various investigators (112, 113) which indicated that drying temperatures exceeding 60\(^\circ\) C substantially reduced recoveries of amino acids from paper chromatograms.
The various spray reagents used were applied by employing an atomizer in conjunction with compressed air at a distance of 30 to 40 cm. from the plate. After the gas pressure was adjusted to forestall impairment of the adsorbent surfaces, the plates were sprayed until they first appeared translucent. An excess of spray was avoided to preclude leaching out of the amino acids. The plates were next exposed to a stream of cold air for 5 minutes, dried in a mechanical convection oven at 60°C for 15 minutes, and then stored in the dark until required for study.

A Beckman model DU spectrophotometer fitted with a standard attachment for the measurement of diffuse reflectance was employed to examine spots scraped off the chromatographic plates. The cells used to hold both sample and reference material have already been described in Section B. Forty milligrams of material were carefully compressed between the cover glass and the paper until a thin layer having an approximate thickness of 0.4 mm. and an approximate diameter of 1.8 cm. was obtained. The reference standards consisted of adsorbent from the plate under examination. The 40 mg. comprising the sample were weighed to ± 0.3 mg. and then ground in a small agate mortar for two periods of 1 minute each to insure homogeneity and uniform particle size.

E. The determination by reflectance spectrophotometry of amino acids resolved on thin-layer plates. (Ref. 125).

The amino acids studied (DL - alanine, L - arginine, L - glutamic acid, glycine, L - isoleucine, L - leucine, L - lysine, DL - methionine, DL - norleucine, DL - norvaline, DL - phenylalanine, DL - serine, DL - threonine and DL - valine) were of Calbiochem A Grade purity.
Stock solutions containing 500 mg. of the acids per 50 ml. of solution were used in making up the dilution series employed in this research. Distilled water served as the solvent throughout. The solutions were applied as spots by means of a Hamilton microsyringe in 5 µl increments to 20 x 5 x 0.35 cm. plates which were coated with Merck silica gel G according to the procedure given in the preceding section. The amino acids were chromatographed in n-propanol - water (64:30) by the one-dimensional ascending technique described by Brenner and Niederwieser (50), and the plates were then dried at 60°C for 30 minutes in a mechanical convection oven.

The spray reagent, consisting of 90 g. of n-butanol, 10 g. of phenol and 0.4 g. of ninhydrin, was one described by Bull et al. (21). An even dispersion of the spray was achieved by using the technique outlined in the previous section. The plates were next exposed to a stream of cold air for 5 min., dried in a mechanical convection oven at 60°C for 15 min., and then stored in the dark at about 10°C in a refrigerator until required for the determination.

Direct spectral examination of these plates was accomplished with a Beckman model DK-2 spectrophotometer fitted with a standard reflectance attachment. A Beckman model DU spectrophotometer, likewise equipped for the measurement of diffuse reflectance, was employed to examine spots scraped off the chromatoplates. The cells used to hold the sample and reference material for examination have been described earlier. The reference standard in all cases consisted of adsorbent from the plate under investigation.

For the analysis of spots removed from chromatoplates, the 40 mg.
comprising the sample were weighed to ± 0.3 mg. and then ground in a small agate mortar for two periods of 1 min. each to insure homo-
geney and uniform particle size. Samples were weighed and worked up in a low humidity, air-conditioned room to avoid the condensation of moisture. With the exception of this period, the samples were stored in a large desiccator from the time they were removed from the refri-
gerator until they were introduced into the reflectance attachments of the spectrophotometers. In addition to the calcium chloride desiccant, the desiccator contained dry ice as a coolant to insure color stabili-
ity.

F. An improved method for the determination of amino acids by spec-
tral reflectance. (Ref. 126).

The amino acids used for this study (DL- alanine, L- arginine, L- glutamic acid, glycine, L- leucine, L- lysine, DL- methionine, DL- phenylalanine, DL- serine and DL- valine) were of Calbiochem A Grade purity. Stock solutions of the acids containing 500 mg. in enough distilled water to make 50 ml. of solution were applied as spots by means of a Hamilton microsyringe in 5 μl increments. The 20 x 5 x 0.35 cm. plates used for one-dimensional resolutions were coated with Merck silica gel G according to the procedure given in Section D. After resolutions had been achieved, the plates were heated in a mechanical convection oven at 60°C for 30 min. to dry them and to develop the colors.

Both one- and two-dimensional chromatograms were used in investi-
gating the applicability of four solvent mixtures: (1) n-propyl alcohol - water - acetic acid (64:36:20); (2) n-butyl alcohol - water -
acetic acid (60:20:20); (3) phenol - water (75:25); and (4) n-propyl alcohol - 34\% ammonia (67:33). The first three solvent systems were employed in conjunction with one-dimensional analyses carried out by the ascending technique. Systems three and two, and four and one were paired off during the two-dimensional analyses with the first of each pair being used for the initial development. Chromatograms were dried at 60\degree \text{C} for 30 min. prior to development in the second dimension. Successful resolutions of mixtures of the ten amino acids were achieved in 10 hours or less when the solvent fronts were permitted to move 18 cm. in each dimension by the ascending technique.

The acids were identified by using $R_f$ values (50) or, in ambiguous situations, by simultaneously running standards for comparison purposes. A Beckman model DU spectrophotometer fitted with a standard attachment for the measurement of diffuse reflectance was employed for the quantitative evaluation of the spots, which were scraped off the chromatographic plates and worked up into 40 mg. analytical samples. The cells used to hold the samples and reference material as well as the procedure followed in preparing material for examination have been described in Section B.
RESULTS AND DISCUSSION

A. The Factors Affecting the Reflectance Spectra of Some Dyes Adsorbed on Alumina. (Ref. 121).

Observations made by Weitz et al. (114, 115), and DeBoer and Houben (116) concerning the color changes undergone by various compounds following their adsorption on active surfaces have stimulated the investigation of these phenomena with the aid of spectral reflectance. This technique is particularly suited for the measurement and examination of the diffuse reflectance spectra of species in the solid state. More recent studies (80, 82, 84), have endeavored to interpret similar observations made with various two-component solid systems on the basis of Lewis acid-base interactions and the polarization theory. In attempts to explore the analytic potential of spectral reflectance, Kortum et al. (69, 71, 103, 109) have carried out a critical evaluation of the Kubelka-Munk function with respect to its use for photometric purposes. For a number of adsorbate-adsorbent systems the function was found to be proportional within limiting concentrations to the molar concentration of the adsorbate, since straight lines passing through the origin were obtained when \( F(R) \), the Kubelka-Munk function, was plotted against molar concentration. Kortum concluded that this relationship may be used for quantitative analysis much in the same manner as the Beer-Lambert law. Careful control of experimental conditions must be exercised in any analytical application, however, since variables such as grain size, packing density, moisture, and pH can influence and modify spectra. The findings of Kortum have been confirmed by Zeitlin and co-workers (79, 117) in studies of the
behavior of mononitrophenols adsorbed on alkali metal carbonates and alkaline earth oxides.

The present study was undertaken with the objective of examining the effect of such variables as humidity, regeneration temperatures, and pH on the reflectance spectra of a number of dyes adsorbed on grades of alumina commonly used in thin-layer chromatography to gain information concerning the experimental conditions necessary for the attainment of reproducible and useful data. The dyes studied were eosine B, rhodamine B, and fuchsin. Q-nitrophenol was included since its behavior following adsorption on various metal carbonates and oxides had been the subject of previous studies (79, 117).

Figure 2 presents transmittance spectra of ethanolic solutions of the three food dyes. Typical reflectance spectra for the three dyes as well as for q-nitrophenol are given in Figures 3 - 7. Graphs in which the Kubelka-Munk function is plotted against concentration of rhodamine B in milligrams per millimeter of ethanolic solution are shown in Figure 8.

As is customary in quantitative spectrophotometry, the reflectance spectra were examined in order to determine the wave length of maximum adsorption, since the sensitivity of the determination is greatest at this wave length. A comparison of the transmittance spectra of the three food dyes with corresponding reflectance spectra of the dyes adsorbed on alumina showed in all cases, in going from transmittance to reflectance, that the absorption maximum was retained in reflectance. The peaks in the latter were broader and displaced bathochromically, a phenomenon which has been observed by several
Fig. 2. Transmittance spectra of (1) fuchsin, (2) eosin B, and (3) rhodamine B, in ethanolic solution.
Fig. 3. Reflectance spectra of eosin B adsorbed on
(1) Alumina ("air dry" state)
(2) Alumina (preheated to 200°)
(3) γ-Alumina (preheated to 800°)
(4) α-Alumina (preheated to 1100°)
Fig. 4. Reflectance spectra of fuchsin adsorbed on
(1) Alumina ("air dry" state)
(2) Alumina (preheated to 200°)
(3) $\delta$-Alumina (preheated to 800°)
(4) $\alpha$-Alumina (preheated to 1100°)
Fig. 5.
Reflectance spectra
of rhodamine B adsorbed on
(1) Alumina ("air dry" state)
(2) Alumina (preheated to 200°)
(3) 𝛾-Alumina (preheated to 800°)
(4) 𝛼-Alumina (preheated to 1100°)
Fig. 6.
Reflectance spectra of o-nitrophenol adsorbed on
(1) Alumina ("air dry" state)
(2) Alumina (preheated to 200°)
(3) γ-Alumina (preheated to 800°)
(4) α-Alumina (preheated to 1100°)
Fig. 7. Reflectance spectra of o-nitrophenol adsorbed on
(1) Alumina (acidic)
(2) Alumina (neutral)
(3) Alumina (basic)
Fig. 8. Kubelka-Munk plots for a dilution series of rhodamine B adsorbed on
- Alumina ("air dry" state)
- Alumina (preheated to 200°)
- Alumina (preheated to 1100°)
workers for a variety of systems (72, 74, 105). For all four compounds, the peaks in the reflectance spectra appear to be usable for photometric purposes. A key factor, in this particular situation is the elucidation of the influence of moisture, pretreatment, and pH on the reflectance absorption maxima.

The spectral data show clearly the effect of the pretreatment of the adsorbent on the reflectance spectra. This treatment, which included heating of separate samples of alumina at 200°C, 800°C, and 1100°C prior to cooling and mixing with the adsorbate as well as the preparation of an "air dry" system in which the adsorbent-adsorbate mixture was equilibrated with atmospheric moisture at room temperature, yielded reflectance spectra in which there were definite displacements of the absorption maximum. The spectra showed, in all cases, a bathochromic shift in the following order: Al₂O₃ (1100°C) > Al₂O₃ (800°C) > Al₂O₃ (200°C) > Al₂O₃ ("air dry"). These results are in accord with the findings of Schwab et al (82), for a series of adsorbed dyes. A similar trend was reported by Zeitlin et al (117) for o-nitrophenol adsorbed on alkaline earth oxides.

These displacements are explainable in terms of Lewis acid-base interaction between adsorbate and adsorbent, the extent of the shift being governed by the amount of water co-adsorbed with the adsorbate. This interpretation, originally suggested by Kortum (80, 81, 109), for a different system, is one which stresses the importance of the moisture content which in turn is directly dependent on the preheat treatment of the adsorbent. In short, an equilibrium condition which is moisture-dependent exists between unadsorbed and adsorbed species.
relative to the active adsorbent. The competition for active adsorbent sites between molecules of each of the four compounds studied and water favors the latter. The $200^\circ C$ heat treatment accorded to the adsorbent prior to mixing with the adsorbate, eliminates some of the adsorbed water. As a result, the organic adsorbate, is more effectively and directly exposed to the polarization action of the adsorbent, and the adsorption peak is shifted to a longer wave-length than that observed with the "air dry" system containing a higher concentration of water. This is particularly noticeable for o-nitrophosphol (Fig. 6). At $800^\circ C$ further dehydration of the alumina enhances the bathochromic shift with the largest shifts being observed after the alumina is subjected to a pretreatment temperature of $1100^\circ C$. It is noteworthy that at this temperature there is a transition from the $\gamma$-to the $\alpha$-modification of alumina. The reversibility of the phenomena can be demonstrated by exposing the dry systems to atmospheric humidity and measuring the reflectance spectra over a period of 24 hours. Hypsochromic shifts of the absorption maxima are observed until the spectra are identical with those obtained for the "air dry" mixtures.

Since commercial grades of chromatographic alumina are prepared according to various specifications, pH-dependence tests were performed with three commonly used grades to ascertain whether their use would result in changes in the reflectance spectrum of a given species. pH determinations carried out on aqueous slurries of acid-washed, neutral, and basic alumina yielded values of 4.2, 7.6, and 10.0, respectively. As may be seen in Figure 7 the reflectance
spectra of o-nitrophenol adsorbed on the three grades of alumina and measured under similar conditions show the marked effect of the type of alumina used, particularly acid-washed, on the spectrum of the adsorbed species. Of interest is the bathochromic shift observed with increasing pH of the adsorbent.

Quantitative measurements were carried out on three six-membered dilution series of rhodamine B to ascertain the analytic potential of applying spectral reflectance to thin-layer chromatography as well as to examine the effect of moisture from a slightly different standpoint. The three sets of data—one for "air dry" system and two for systems in which the adsorbent had been preheated to 200°C and 1100°C—were plotted in the form Kubelka-Munk function, versus milligrams of dye per ml. of solution. As may be seen in Figure 8, calibration plots passing through the origin were obtained in each case for the concentration range employed. Such plots can be utilized in the same manner as ones derived from transmittance measurements to determine the concentration of an adsorbed dye species. In order to emphasize the effect of moisture, the reflectance measurements for all three curves were taken at the absorption peak wavelength (562 μm) of the dye-alumina system in which the adsorbent had been preheated to 1100°C. When this is done, the slope of the calibration curve is observed to decrease with increasing humidity. These observations are in close agreement with those reported by Kortum and Vogel (103) for malachite green spotted on paper chromatograms. Although the change in slope is undoubtedly related to the displacement of the absorption maxima, an additional factor which may be involved is the varying intensity of
rhodamine B associated with the variation in moisture. Figure 5 depicts the increase in color intensity observed with the decrease in moisture content of a constant amount of dye (0.3 mg. of dye per 3 g. "air dry" adsorbent). Similar observations have been made with the other dyes (Figures 3, 4, 5). The interpretation given above for the bathochromic shifts of the absorption maxima can also be invoked to explain the relation between color intensity and humidity.

In conclusion it appears that spectral reflectance can be employed successfully for the quantitative analysis of dyes adsorbed on alumina. It is essential for reproducible results, however, to use the same grade of adsorbent and to prepare samples for measurement under uniform conditions of regulated humidity so as to control effectively the important moisture factor.


Early in the investigation the need for a cell capable of accommodating 40 to 100 mg. samples became apparent. After such a cell had been devised, the components of dye mixtures resolved on thin-layer plates could be determined by carrying out reflectance measurements on spots removed from the plates and packed in the cell. The present study represents an attempt to correlate the performance of the cell with current theories of reflectance spectroscopy.

The reflectance of three empty cells employing white, gray and black backing paper, respectively, was measured relative to MgO to determine the extent of background interference that might be anticipated with the use of the cell. As expected and as from a considera-
tion of Figure 9, which presents the spectra obtained, the values observed differed considerably. At 640 μm, for example, the reflectance ranged from 90%R to 12%R. Based on MgO having an absolute reflectance of 98%, the absolute reflectance values, R_a, found at this wave length for the white, gray and black cells were 88%, 40% and 10% respectively. At 400 μm, the other end of the visible spectrum, the corresponding R_a values were 68%, 28% and 10%. When 40 mg. of silica gel were introduced into each of the three cells and their reflectance was measured relative to silica gel of the same quality, however, the three spectra were almost identical, differing by no more than one reflectance unit between 350 and 700 μm. In all respects the three closely resembled the MgO curve shown in Figure 9. Since the 1% difference in reflectance observed among the spectra is coincident with the degree of precision inherent in the reflectance technique (see Section C), one can conclude that when the cell is used with finely powdered samples compressed to an approximate thickness of 0.4 mm, the background interference encountered is too small to be of practical significance and the sample layer may be assumed to have an infinite thickness.

To check the suitability of the cell for paper chromatography, the reflectance of the cell employing the black backing paper was measured relative to Whatman No. 1 filter paper. As indicated in Figure 10, which shows cell reflectance as a function of the number of thicknesses of filter paper introduced into the cell, five layers of paper were required to eliminate background interference. These results are in agreement with those obtained by Ingle and Minshall.
Fig. 9. Reflectance spectra of various background materials relative to MgO.
(1) MgO. (2) White backing paper. (3) Gray backing paper. (4) Black backing paper.
Fig. 10. Reflectance as a function of the number of thicknesses of filter paper introduced into the cell.
Varying concentrations of McCormick blue absorbed on silica gel were employed to verify the relationship between dye concentration and reflectance. In that it absorbs in the visible portion of the spectrum and is stable in the absence of moisture and light, the system proved to be most convenient for this purpose. The spectra obtained for different concentrations of absorbed dye are presented in Figure 11. As may be seen there, variation of the dye concentration had no significant effect upon the positions of the absorption maxima when the measurements were carried out under controlled experimental conditions. When the reflectance at 630 μm is plotted against the concentration of absorbed dye, as shown in Figure 12, the result is the typical smooth curve that is capable of being employed for calibration purposes. Alternatively these data may be graphed as concentration versus \((1-R)^2/2R\), the form of the Kubelka-Munk expression that is most often used. A consideration of this curve, which is also presented in Figure 12, supports the validity of applying the Kubelka-Munk law to the system being investigated when concentrations less than 300 mg. of dye/100 ml. of solvent are involved, or, in other words, when conditions of low absorbance and ideal diffuse reflectance obtain. In this region the dye has been so diluted with silica gel, which acts as a neutral diluent, that the contribution to the reflected radiation of regular reflectance has become negligible and one is dealing principally with diffuse reflectance. Accordingly within this concentration range it is appropriate to employ the relationship \(F(R_{\text{diffuse}}) = kcd_{oo}\), which is valid for reflectance from layers having an infinite
Fig. 11.
Reflectance spectra of various concentrations of McCormick blue adsorbed on silica gel.
Concentrations in mg/100ml:
(1) 10; (2) 20; (3) 40; (4) 60;
(5) 120; (6) 180; (7) 240; (8) 300;
(9) 600; (10) 900; (11) 1200;
(12) 1500.
Fig. 12. Reflectance data obtained at 630 μm for Mc Cormick blue adsorbed on silica gel.

- % reflectance as a function of concentration.
- ▼ Kubelka-Munk values as a function of concentration.
- ○ Kubelka-Munk values as a function of the logarithm of concentration.
thickness, \( d_{oo} \), that precludes background interference. At higher concentrations increased absorptivity is associated with increased deviation from ideal behavior as manifested by the gradual increase in slope which eventually results in the curve becoming asymptotic to the horizontal axis. This last was ascribed by Kortum (80) to a saturation effect in the first absorption monolayer marking the shift from chemical to physical absorption.

Some of the interference encountered was no doubt due to Fresnel reflectance resulting from the use of thin-layer chromatographic grade silica gel which has an approximate grain size of 5 \( \mu \). Although it is possible to eliminate this effect by milling the particles down to a 1 \( \mu \) grain size as recommended by Kortum (59), the employment of such a step in an analytical procedure would not only result in an inordinate expenditure of time and effort but would also introduce more problems than it would solve. If it is desired to work with higher concentrations of an absorbing species, one can make use of the relationship \( (1-R)^2/2R = k \log C \), which was found to be applicable to more highly concentrated dye systems. When the data obtained during the present study were plotted in the form \( (1-R)^2/2R \) versus the logarithm of concentration, as is done in Figure 12, the result was a straight line which extended from a concentration of 300 mg./100 ml. of solvent to one of 1400 mg./100 ml. of solvent.

The difficulties associated with the use of the microscope cover glass were circumvented by employing matched cells for the sample and the reference standard. In this way it was possible to cancel out the effects due to radiation being absorbed by the glass, which can
amount to a decrease in reflectance as large as 6.0 reflectance units, and to radiation being reflected from its surface. When reflectance measurements for a dilution series were carried out employing cells with and without cover glasses, identical slopes were obtained for Kubelka-Munk function - concentration plots of the two sets of data.

The packing of the sample in the cell presented no problems. Because the background interference was negligible, the sample layer thickness was not overly critical and a packing reproducibility corresponding to 0.7 reflectance units for three replicate packings of the same sample was observed without any difficulty (see Section C).

The cell described herein has proved to be most useful for the measurement of the spectral reflectance of semimicro samples such as those which are obtained from thin-layer chromatographic plates. Indeed, since there is no significant background interference with sample layers having an approximate thickness of 0.4 mm., it is capable of being employed with any finely powdered samples. The cells are inexpensive and simple to construct and can, therefore, be produced in large numbers to expedite serial analyses. Its size can be varied to adjust to the requirements of the reflectance attachment and it can be mounted vertically as well as horizontally if the proper care is exercised. For measurement in the near UV and IR regions, the cell may be employed with a quartz cover or without any cover glass at all provided that the surface of the sample is smooth.

The situation that would exist if the cell were employed with sample layers that were thinner and more transparent would be analogous to one that would result from the use in reflectance photometry
of a flat, highly reflective backing paper in conjunction with a more or less transparent sample distant from the light, as suggested by J. W. H. Lugg (18). Such a procedure might provide advantages over transmission methods when dealing with paper chromatograms characterized by low transmission. In these instances, however, the thickness of the sample layer becomes more critical and it would be necessary to employ a high quality grade of chromatographic paper. Furthermore the amount of radiation reflected from the backing sheet would reach such proportions that the Kubelka-Munk relationship would no longer apply (103).

C. The Application of Spectral Reflectance to Thin-layer Chromatography. (Ref. 123).

Although the thin-layer chromatographic technique offers many distinct advantages, its analytical utility is restricted by two shortcomings. In the first instance, the difficulty experienced in obtaining reproducible R_f values with thin plates usually makes it necessary to run standards alongside the samples for comparison purposes. Secondly, the quantitative removal and extraction of individual spots from plates is a tedious process which often cannot be accomplished without decomposition occurring. Both of these operations would become superfluous if it were possible to effect the in situ identification and determination of chemical species separated on thin plates.

The use of spectral reflectance for these purposes was suggested by various studies (92 - 104) which demonstrated its utility with respect to paper chromatography. Furthermore, it has been shown that
the reflectance spectra of substances concentrated on particular adsorbents can be used for their identification (105), and that spectral reflectance can be employed to determine the concentration of dyes scavenged from solution by the batchwise addition of starch (106). A critical evaluation of the application of reflectance measurements to the direct analysis of solid mixtures has established the fact that analytically useful data can usually be obtained with samples in powdered form (107). In view of these results, it was decided to investigate the analytical applications of spectral reflectance to thin-layer chromatography using, as a pilot system, water-soluble dyes and aluminum oxide plates prepared according to a method devised by Mottier (110). This system, in that it is stable and absorbs in the visible portion of the spectrum, lent itself most conveniently to the purpose at hand.

**Direct examination of chromatographic plates**

As indicated in Figure 13 which contrasts the transmittance spectrum of an aqueous solution of eosine B with the reflectance spectra of $2.5 \times 10^{-3}$ mg. of the dye adsorbed on filter paper, alumina and silica gel, the spectra obtained for the different dyes were influenced by the nature of the adsorbent employed. The positions of the absorption maxima obtained for the dyes under various experimental conditions are summarized in Table 1. In all cases the absorption maxima obtained for transmittance shifted to higher wave-lengths when the reflectance spectra of the dyes adsorbed on Whatman #42 filter paper were determined. These results agree substantially with those
Fig. 13 Reflectance spectra of eosine B adsorbed on filter paper, alumina, and silica gel compared with the transmittance spectrum of an aqueous solution of the dye. (1) Silica gel (Merck thin-layer chromatography grade). (2) Filter paper (Whatman No. 42). (3) Alumina (Merck thin-layer chromatography grade). (4) Transmittance spectrum.
<table>
<thead>
<tr>
<th>Dye</th>
<th>H₂O</th>
<th>EtOH</th>
<th>Transmittance</th>
<th>Reflectance</th>
<th>Filter Paper</th>
<th>Alumina</th>
<th>Silica Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline Blue</td>
<td>---</td>
<td>600</td>
<td>615</td>
<td>594</td>
<td>592</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosine B</td>
<td>516*</td>
<td>524</td>
<td>530</td>
<td>528</td>
<td>520</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basic Fuchsin</td>
<td>---</td>
<td>549</td>
<td>553</td>
<td>550</td>
<td>540</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malachite Green</td>
<td>---</td>
<td>620</td>
<td>628</td>
<td>615</td>
<td>615</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthol Yellow S</td>
<td>---</td>
<td>435/389</td>
<td>443/394</td>
<td>436/391</td>
<td>431/390</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodamine B</td>
<td>---</td>
<td>546</td>
<td>550</td>
<td>550</td>
<td>547</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Readings are given as mu
of Yamaguchi et al. (97). That no such general trend is observed in the case of the alumina or the silica gel is attributable to the larger number of experimental variables introduced by their employment (see Section A). For these reasons, the standardized procedure described earlier was employed for the preparation and development of the plates. There was no discernible change in the reflectance spectra of dyes separated on plates which had been stored in a desiccator over silica gel for periods up to three days following their development. It might also be noted that a complete inhibition of the fluorescence exhibited by eosine B and rhodamine B in transmittance measurements occurs when the dyes are adsorbed on filter paper, alumina or silica gel.

With proper precautions it is possible by direct examination of chromatographic plates to obtain spectra suitable for identification purposes as shown in Figure 14, which depict the reflectance spectra for the various dyes adsorbed on alumina. Spots having diameters as small as 5 mm. could be centered by using the red portion of the visible spectrum and measured with ease. In the resolution of complex dye mixtures, a spot separation of one centimeter sufficed to permit spectral identification of the component dyes.

The quantitative potential of the technique was demonstrated by examining plates spotted with dilution series of the dyes. Figure 15, which shows the reflectance spectra of various concentrations of eosine B adsorbed on alumina, typifies the data obtained during this study. The solutions were added in 5 µl. increments to give spots whose diameters approximated 1 cm. As indicated earlier, the thick-
Fig. 14  (a) Reflectance spectra of dyes adsorbed on alumina. (A) Eosine B. (B) Rhodamine B. (C) Fuchsin. (b) Reflectance spectra of dyes adsorbed on alumina. (D) Naphthol yellow S. (E) Malachite green. (F) Aniline blue.
Fig. 15 Reflectance spectra of various concentrations of eosine B adsorbed on alumina. Concentrations in mg/100 ml: (1) 0.78; (2) 1.56; (3) 3.12; (4) 6.25; (5) 12.5; (6) 25.0; (7) 50.0; (8) 75.0; (9) 100.0
ness and particle size of the adsorbent and the drying temperatures of
the plates were kept fairly constant. It was found that excessive
tailing decreased the precision of measurements, and an effort was
made to keep it to a minimum. Precision was likewise affected by the
improper positioning of the spot in the impinging light beam, and care
was taken to center it in the manner described previously. No signi-
ficant changes in reflectance were observed over a period of a week
when the plates were stored in a desiccator in the dark. When these
precautions were taken, the largest difference in reflectance found
between any two members of thirteen sets of triplicate samples of
eosine B was 4.0 units on the 100-unit reflectance scale. These sam-
ples represented a concentration range of $5 \times 10^{-3}$ to $4 \times 10^{-5}$ mg. of
added dye. The data for this study are presented in Table 2, which
indicates the reproducibility that can be expected for reflectance
readings obtained for different spots of the same concentration. A
consideration of Figure 16, in which these data are plotted in the
form $2-\log \% R$ versus concentration, reveals that Beer's law holds only
as a limiting law for reflectance and that a linear relationship
obtains only for concentrations less than 5 mg. of dye/100 ml. of
solution. As may also be seen in Figure 16, plotting the same data
in the form $2-\log \% R$ versus the square root of the concentration
extends this linear relationship ten-fold to an upper limit of 50 mg./
100 ml. The same relation between reflectance and concentration was
found to exist for rhodamine B. These results are in agreement with
those obtained by Yamaguchi et al. (98) for different food dyes adsor-
bed on filter paper.
TABLE 2

REPRODUCIBILITY OF REFLECTANCE READINGS
OBTAINED FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION
OF EOSINE B ADSORBED ON ALUMINA
(Readings taken at 530 nm)

<table>
<thead>
<tr>
<th>Concentrations of Dilution Series (mg. dye/100 ml. sol'n.)</th>
<th>Trail 1</th>
<th>Trail 2</th>
<th>Trail 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.78</td>
<td>98.0%  R</td>
<td>98.5%  R</td>
<td>98.4%  R</td>
</tr>
<tr>
<td>1.56</td>
<td>94.5</td>
<td>93.8</td>
<td>93.5</td>
</tr>
<tr>
<td>3.12</td>
<td>89.5</td>
<td>91.0</td>
<td>91.0</td>
</tr>
<tr>
<td>4.30</td>
<td>87.0</td>
<td>88.0</td>
<td>87.3</td>
</tr>
<tr>
<td>6.25</td>
<td>83.0</td>
<td>82.0</td>
<td>81.5</td>
</tr>
<tr>
<td>8.50</td>
<td>79.0</td>
<td>77.5</td>
<td>78.5</td>
</tr>
<tr>
<td>12.50</td>
<td>76.5</td>
<td>74.0</td>
<td>73.5</td>
</tr>
<tr>
<td>18.00</td>
<td>71.0</td>
<td>71.5</td>
<td>69.0</td>
</tr>
<tr>
<td>25.00</td>
<td>66.5</td>
<td>62.5</td>
<td>63.5</td>
</tr>
<tr>
<td>37.50</td>
<td>60.0</td>
<td>59.3</td>
<td>59.5</td>
</tr>
<tr>
<td>50.00</td>
<td>55.0</td>
<td>55.5</td>
<td>54.0</td>
</tr>
<tr>
<td>75.00</td>
<td>49.0</td>
<td>50.0</td>
<td>49.0</td>
</tr>
<tr>
<td>100.00</td>
<td>44.5</td>
<td>47.0</td>
<td>45.0</td>
</tr>
</tbody>
</table>
Fig. 16 2—log % reflectance at 530 m\(\mu\) of eosine B adsorbed on alumina as a function of concentration. ●—● \(C\); ○—○ \(C^{\dagger}\).
Examination of spots removed from the chromatographic plates

The precision attained in the determination of the adsorbed dyes by the direct examination of the chromatographic plates was improved by scraping the spots off the plates and measuring the reflectance of this material with the use of the cell described in Section B. Such a device proved to be necessary, as the amount of material removed from the plates was insufficient to fill the cells available for this purpose. The addition of more alumina was undesirable, since it introduced a considerable dilution factor that decreased the sensitivity of the method. Using this improved cell and taking precautions to insure a homogeneous sample of relatively uniform particle size, it was possible to get reflectance readings obtained at various wave lengths for three different samples of eosine B of identical concentration.

The procedure employed was identical with that followed in the determination of the dyes by direct examination up to the point the sample material was removed from the plate. The fifty milligrams comprising the sample were weighed to ± 0.2 mg. and then ground in a small agate mortar for two periods of fifteen seconds each to insure homogeneity and uniform particle size. The grinding procedure was standardized, as it was found that a measurable difference in reflectance was produced by varying the operation. Lermond and Rogers (107) reported similar results for the screening of sample materials. The greatest difficulty encountered was the attainment of reproducible packing of the sample in the cell. To assure the degree of precision achieved in the test of reproducibility which is summarized in Table 4, it is essential that the samples have approximately the same dia-
**TABLE 4**

**TEST OF REPRODUCIBILITY OF PACKING REFLECTANCE CELL.**

**EOSINE B ADSORBED ON ALUMINA**

<table>
<thead>
<tr>
<th>Wave Length, mμ</th>
<th>1st Packing</th>
<th>2nd Packing</th>
<th>3rd Packing</th>
</tr>
</thead>
<tbody>
<tr>
<td>620</td>
<td>98.4% R</td>
<td>98.4% R</td>
<td>99.0% R</td>
</tr>
<tr>
<td>600</td>
<td>97.2</td>
<td>96.8</td>
<td>97.2</td>
</tr>
<tr>
<td>580</td>
<td>93.3</td>
<td>93.7</td>
<td>93.8</td>
</tr>
<tr>
<td>560</td>
<td>89.0</td>
<td>89.1</td>
<td>89.5</td>
</tr>
<tr>
<td>540</td>
<td>86.9</td>
<td>87.2</td>
<td>86.5</td>
</tr>
<tr>
<td>530</td>
<td>86.7</td>
<td>86.8</td>
<td>86.3</td>
</tr>
<tr>
<td>520</td>
<td>87.2</td>
<td>87.2</td>
<td>86.9</td>
</tr>
<tr>
<td>500</td>
<td>89.6</td>
<td>89.6</td>
<td>89.6</td>
</tr>
<tr>
<td>480</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td>460</td>
<td>93.4</td>
<td>93.7</td>
<td>93.8</td>
</tr>
</tbody>
</table>
meter and thickness and possess a uniformly smooth surface. The largest observed difference for any pair of readings obtained for the sample repacked in the same holder was 0.7 reflectance units. When one considers that these differences are of the same order as the ones obtained for the replicate samples listed in Table 3, it becomes apparent that the precision of the technique is limited by the reproducibility of packing the sample.

The relationship between reflectance and the concentration of eosine B adsorbed on alumina was investigated again, this time by means of the spot removal technique. A plot of the data obtained in the form \(2 \log \%R\) versus concentration gave the same type of smooth curve as was obtained by direct examination and depicted in Figure 16. The only notable difference was the upward extension of the linear relationship to a dye concentration of 20 mg./100 ml. of solution. It is possible to extend this upward even further to 40 mg./100 ml. if, as is done in Figure 17, the concentration is plotted against \((1-R)^2/2R\), the form of the Kubelka-Munk expression most often used (68). Beyond this point, the curve is so smooth that it can serve a quantitative function. By plotting the logarithm of the concentration versus \((1-R)^2/2R\), as shown in Figure 17, it is possible, if this is desired, to expand the linear relationship to the highest concentration studied, 350 mg./100 ml.

Finally some measurements were made on a dilution series using the cell without the glass cover, which increased readings by as much as 6.0 reflectance units at 530 nm. For samples having a smooth surface, the precision was unaffected by the removal of the glass.
TABLE 3

REPRODUCIBILITY OBTAINED FOR DIFFERENT SAMPLES OF
IDENTICAL CONCENTRATIONS OF EOSINE B ADSORBED ON ALUMINA

<table>
<thead>
<tr>
<th>Wave Length, μm</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
</tr>
</thead>
<tbody>
<tr>
<td>540</td>
<td>85.7%</td>
<td>86.1%</td>
<td>86.3%</td>
</tr>
<tr>
<td>530</td>
<td>85.2%</td>
<td>85.4%</td>
<td>85.7%</td>
</tr>
<tr>
<td>520</td>
<td>85.9%</td>
<td>86.3%</td>
<td>86.4%</td>
</tr>
</tbody>
</table>
Fig. 17 Kubelka-Munk values at 530 m\(\mu\) for cosine B adsorbed on alumina as a function of concentration. \(\circ\) --- \(C'\); \(\bullet\) --- \(\log C'\).
Utilization of the cell without the glass cover might be desirable when dealing with samples of low reflectance or with spectral regions requiring a quartz plate.

The check of the reliability of the technique in the hands of inexperienced analysts yielded encouraging results. A class of twenty-eight beginning students in analytical chemistry went through the exercise outlined in the experimental section and obtained the quantitative data presented in Table 5. The results of four students were rejected because they had committed obvious operative errors. Other than this, no effort was made to restrict the participation of students on the basis of ability. Furthermore, none of the students had had any prior experience with spectrophotometry or chromatographic separations. Despite this, about 90% of the dyes were identified correctly, and the determinations were carried out with an accuracy of approximately ± 5% using the graphic method and of approximately ± 7% using the algebraic method. Although the accuracy of the algebraic method is less than that of the graphic method, its use approximately doubles the speed of the analysis. The lower degree of accuracy observed with crystal violet and fuchsin can be explained by the inability to achieve clean-cut separations with these dyes because of their tendency to tail.

The above results show that the components of complex mixtures of dyes separated on thin-layer plates can be identified rapidly, without recourse to $R_f$ values, by direct examination of the plates by spectral reflectance. The same operation is capable of providing quantitative data having a precision of approximately ± 5%. A degree
### TABLE 5

**ACCURACY AND PRECISION OF STUDENT DETERMINATIONS BY SPECTRAL REFLECTANCE OF DYES RESOLVED ON THIN-LAYER PLATES.**

<table>
<thead>
<tr>
<th>METHOD</th>
<th>RANGE</th>
<th>% DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GRAPHIC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CRYSTAL VIOLET</strong></td>
<td>3.5 - 9.5</td>
<td>0.3 - 9.6</td>
</tr>
<tr>
<td><strong>FUCHSIN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RHODAMINE B</strong></td>
<td>1.3 - 8.0</td>
<td></td>
</tr>
<tr>
<td><strong>ALGEBRAIC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CRYSTAL VIOLET</strong></td>
<td>0.0 - 15.0</td>
<td>0.6 - 15.0</td>
</tr>
<tr>
<td><strong>FUCHSIN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RHODAMINE B</strong></td>
<td>2.0 - 10.8</td>
<td></td>
</tr>
</tbody>
</table>
of precision identical to that afforded by transmittance is attained if the reflectance measurements are carried out on spots removed from the chromatographic plates and packed in an appropriate cell. By applying the Kubelka-Munk function, linear reflectance-concentration relationships can be obtained for concentration ranges of interest to the analytical chemist. The data provided by the student also demonstrated that the technique can be employed by people without specific skills or experience and still provide results of reasonable validity.

Although the present study was restricted to the visible portion of the spectrum, other regions can be used as well if the cell is provided with a quartz plate or employed without a cover. In general, one can say that the application of spectral reflectance to thin-layer chromatography would enhance its utility greatly by simplifying and expediting the analysis of complex mixtures.

D. Use of Spectral Reflectance in Determining Color Stabilities of Ninhydrin Complexes of Adsorbed Amino Acids. (Ref. 124).

Although spectral reflectance has found application in the solution of a wide range of problems, surprisingly little use has been made of this technique in analytical work. Some of the potentialities of the method, particularly in biochemical analysis, have been pointed out by Naughton et al (90). More recently the analysis of amino acids by means of thin-layer chromatography has provided another example of a situation where spectral reflectance can be used to obtain analytical data simply and with a minimum of preparation and extractive operations.
Several direct photometric techniques have been devised for the in situ estimation of amino acids separated by paper chromatography (10 - 27). Although some of these procedures could be adapted for use in such investigations as the study of factors affecting recoveries from paper chromatograms and the stabilization of colors developed on paper, they are not applicable to chromatoplates. The possibility of using reflectance measurements to accomplish this in separations involving thin plates was suggested by the study of the application of spectral reflectance to thin-layer chromatography described on the previous section. As a result of this study, it was decided to employ the technique to investigate some of the factors affecting the color stability of the ninhydrin complex of leucine adsorbed on silica gel.

Various sprays were studied from the standpoint of the color stability of the ninhydrin complex resulting from their employment. Dilution series of leucine ranging from 5.0 to 50 μg. were used for these studies with readings being taken at 530 μm. Spots were removed from chromatoplates and readied for analysis according to the procedure outlined in the experimental section. Spray "A" was prepared by mixing 50 parts by volume of a 0.2% solution of ninhydrin in absolute alcohol with 3 parts by volume of a 1% solution of Cu(NO₃)₂·3H₂O in absolute alcohol shortly before use. As may be seen in Figure 18, the color intensity of the ninhydrin complex obtained with this spray decreased by as much as six reflectance units within one hour when the samples were kept at room temperature (28° C). The effect of temperature in determining color stability was pointed up by repeating the study with plates which had been maintained below 10° C from the
Fig. 18. Leucine series with spray "A." Per cent reflectance at 530 mg of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time: (●) after 3 hr; (○) after 4 hr; (□) after 5 hr; (△) after 8 hr at room temperature (28°C).
time they were dried until the analytical samples were prepared. This expedient succeeded in reducing the observed decrease in intensity to a maximum value of one reflectance unit per hour over a five-hour period, as depicted in Figure 19. Even these results, however, would be unsatisfactory as the basis for any precise determination of amino acids.

The employment of Spray "B", which was prepared by adding 20 ml. of glacial acetic acid to 100 ml. of Spray "A" as suggested by Brenner and Niederwieser (50), afforded a readily apparent increase in color stability. Figure 20, which shows the % reflectance of the ninhydrin complex of leucine as a function of concentration and time at room temperature (28°C), clearly indicates the stabilizing influence of the acetic acid. The color stability attained was of the same order as that achieved with Spray "A" at temperature below 10°C. As indicated in Table 6, the average decrease in stability noted within the concentration range studied when Spray "B" was used in conjunction with plates maintained below 10°C amounted to 2.7 reflectance units over a 24-hour period. In no case was a decrease in excess of 3.7 units observed for this time span. Within the specified limitations, the technique provided data having a standard deviation of ± 2 reflectance units.

Spray "C", of the three studied, not only afforded the best results as regards color stability but also provided greater sensitivity. This reagent, consisting of 90 g. of n-butanol, 10 g. of phenol and 0.4 g. of ninhydrin, was one described by Bull et al (21). As is shown in Figure 21 the color intensity of the ninhydrin complex
Fig. 19. Leucine series with spray "A." Per cent reflectance at 530 nm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time: (●) after 2 hr; (○) after 7 hr; (□) after 24 hr; (Δ) after 3 days below 10°C.
Percent reflectance at 530 μm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time:

- •-• after 2 hours
- o-o after 6 hours
- v-v after 24 hours
at room temperature (28°C)

Fig. 20 Leucine series with spray "B"
TABLE 6

LEUCINE SERIES WITH SPRAY "B"

% REFLECTANCE AT 530 M\textmu{} OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF CONCENTRATION AND TIME FOR SAMPLES STORED BELOW 10° C.

<table>
<thead>
<tr>
<th>Conc. (µg)</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
<th>%R range</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>89.1</td>
<td>90.0</td>
<td>92.7</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>80.0</td>
<td>80.2</td>
<td>83.4</td>
<td>3.4</td>
</tr>
<tr>
<td>12</td>
<td>76.2</td>
<td>77.2</td>
<td>79.9</td>
<td>3.7</td>
</tr>
<tr>
<td>14</td>
<td>74.0</td>
<td>74.8</td>
<td>77.3</td>
<td>3.3</td>
</tr>
<tr>
<td>16</td>
<td>70.0</td>
<td>70.4</td>
<td>73.2</td>
<td>3.2</td>
</tr>
<tr>
<td>18</td>
<td>67.8</td>
<td>68.4</td>
<td>70.8</td>
<td>3.0</td>
</tr>
<tr>
<td>20</td>
<td>66.9</td>
<td>67.7</td>
<td>69.2</td>
<td>2.3</td>
</tr>
<tr>
<td>22</td>
<td>64.7</td>
<td>65.1</td>
<td>67.3</td>
<td>2.5</td>
</tr>
<tr>
<td>24</td>
<td>62.2</td>
<td>63.7</td>
<td>66.0</td>
<td>3.8</td>
</tr>
<tr>
<td>28</td>
<td>60.3</td>
<td>60.6</td>
<td>62.5</td>
<td>2.2</td>
</tr>
<tr>
<td>32</td>
<td>58.3</td>
<td>58.1</td>
<td>59.9</td>
<td>1.8</td>
</tr>
<tr>
<td>34</td>
<td>57.8</td>
<td>57.7</td>
<td>59.3</td>
<td>1.6</td>
</tr>
<tr>
<td>36</td>
<td>57.0</td>
<td>56.0</td>
<td>57.6</td>
<td>1.6</td>
</tr>
<tr>
<td>40</td>
<td>55.9</td>
<td>55.2</td>
<td>56.9</td>
<td>1.7</td>
</tr>
<tr>
<td>50</td>
<td>54.2</td>
<td>54.2</td>
<td>56.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Average %R range 2.7
Fig. 21. % reflectance at 540 nm of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time. ●● after 1 h; ○○ after 24 h; △△ after 3 days at room temperature (28°).
of leucine decreased by no more than five reflectance units within 24
hours at room temperature (28°C) when Spray "C" was employed. When
the storage temperature of the chromatoplates was maintained below 10°C,
the average change over a 24-hour period was found to be 1.0 reflectance unit and the greatest decrease noted was of the order of 2.0
reflectance units. These data, which are presented in Table 7, indi-
cate that it should be possible to determine amino acids with the
degree of precision inherent in the reflectance technique by using
Spray "C" at reduced temperatures. Sensitivities found for leucine
were 0.5 μg. for Spray "C" as opposed to 2 μg. for Spray "B".

Similar results were obtained when Spray "C" was used with DL -
alanine, L - argine, glycine, L - isoleucine, L - lysine, DL -
methionine, DL - norleucine, DL - norvaline, DL - serine, DL -
Thrreonine and DL - valine. This would seem to indicate that the
technique is applicable to most, if not all, amino acids.

E. The Determination by Reflectance Spectrophotometry of Amino Acids
Resolved on Thin-layer Plates. (Ref. 125).

The promise of reduced tailing, increased sensitivity, and greater
speed and resolution has induced many investigators to resort to the
thin-layer technique in the analysis of amino acids. Unfortunately,
since methods developed for the estimation of acids separated on paper
are not applicable to chromatoplates, there is need for a procedure
whereby this can be accomplished in separations involving thin plates.
The possibility of using reflectance measurements for this purpose was
suggested by the results presented in the two preceding sections.
These data demonstrated that the components of dye mixtures resolved
### TABLE 7

**LEUCINE SERIES WITH SPRAY "C"**

% REFLECTANCE AT 520 μM OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF CONCENTRATION AND TIME FOR SAMPLES STORED BELOW 10°C.

<table>
<thead>
<tr>
<th>Conc. (μg)</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
<th>%R range</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>71.7</td>
<td>71.9</td>
<td>70.9</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>59.3</td>
<td>59.3</td>
<td>58.7</td>
<td>0.6</td>
</tr>
<tr>
<td>12</td>
<td>56.0</td>
<td>55.9</td>
<td>55.2</td>
<td>0.8</td>
</tr>
<tr>
<td>14</td>
<td>52.4</td>
<td>51.4</td>
<td>51.2</td>
<td>1.2</td>
</tr>
<tr>
<td>16</td>
<td>50.0</td>
<td>49.2</td>
<td>48.5</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td>46.4</td>
<td>45.8</td>
<td>44.4</td>
<td>2.0</td>
</tr>
<tr>
<td>22</td>
<td>45.0</td>
<td>44.6</td>
<td>44.0</td>
<td>1.0</td>
</tr>
<tr>
<td>24</td>
<td>43.1</td>
<td>42.2</td>
<td>41.7</td>
<td>1.4</td>
</tr>
<tr>
<td>26</td>
<td>41.8</td>
<td>41.8</td>
<td>42.2</td>
<td>0.4</td>
</tr>
<tr>
<td>28</td>
<td>40.1</td>
<td>39.5</td>
<td>38.9</td>
<td>1.2</td>
</tr>
<tr>
<td>30</td>
<td>39.2</td>
<td>38.9</td>
<td>37.8</td>
<td>1.4</td>
</tr>
<tr>
<td>32</td>
<td>38.5</td>
<td>38.5</td>
<td>38.1</td>
<td>0.4</td>
</tr>
<tr>
<td>36</td>
<td>37.8</td>
<td>37.2</td>
<td>37.2</td>
<td>0.6</td>
</tr>
<tr>
<td>40</td>
<td>37.2</td>
<td>36.3</td>
<td>36.0</td>
<td>1.2</td>
</tr>
<tr>
<td>50</td>
<td>36.8</td>
<td>36.3</td>
<td>36.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Average %R range 1.0
on thin-layer plates can be determined with a precision of approximately ± 5% by direct examination of the plates. When the reflectance measurements were carried out on spots removed from the plates and packed in an appropriate cell, the degree of precision attained was identical to that afforded by transmittance. Finally, it was shown that it should be possible to employ both techniques successfully with mixtures of amino acids.

**Color development**

The ninhydrin spray used initially was a modification of the one which was described by Brenner and Niederwieser (50) and which is the Spray "B" referred to in Section D. The spray described by Bull et al (21), which is the spray "C" of Section D, was found to provide greater sensitivity, however, and was employed in the acquisition of the data presented in this Section. Figure 22 which depicts % reflectance and absorbance at 520 μm of the ninhydrin complex of leucine adsorbed on silica gel as a function of concentration, shows clearly the increased color intensity resulting from the use of the latter spray. Both sprays yield analytically useful data which take the form of a straight line up to concentrations of 30 μg. when absorbance is plotted versus the square root of the concentration. Sensitivities found for leucine -- 0.5 μg. for the Bull spray and 2 μg. for the modified Brenner-Niederwieser spray -- were consistent with the 0.5 μg. value reported by Pratt and Auclair (118) for a 0.1% solution of ninhydrin in n-butanol used in conjunction with paper chromatograms. The difference can be ascribed to the three or four-fold dilution of
Fig. 22. % reflectance and absorbance at 320 μg of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration. Modified Brenner-Niederwieser spray: O - O % reflectance vs. C, ● - ● absorbance vs. C, Bull et al. spray: △ - △ % reflectance vs. C, ▲ - ▲ absorbance vs. C.

the ninhydrin complex with silica gel adsorbent that occurs during the preparation of the sample for analysis.

The procedure outlined in the experimental section for the development and measurement of the color due to the ninhydrin complex was suggested by the results of a study of the effects of temperature and time of development, and of post-development storage time upon color intensity. Chromatoplates, spotted with identical amounts of leucine (30 μg.) and sprayed with the Bull reagent, were developed at different temperatures (27, 60 and 90°C) for varying periods of time (10, 15 and 20 minutes) and then read at 1, 24, and 72 hour intervals following their storage in the dark at room temperature. The results of the study are summarized in Table 8 as mean values of % reflectance readings obtained from three replicates for each set of experimental conditions. Although plates read an hour after the ninhydrin color had been developed yielded the lowest reflectance readings regardless of the other variables, a longer interval was utilized since it lends itself more to serial analyses. Because it afforded greater precision, development at 60°C for 15 minutes was preferred to development at room temperature despite the fact that the latter method produced a more intense color. Indeed, of the readings taken at the twenty four-hour interval, only those obtained at room temperature exhibited a range in excess of 1.5 reflectance units. There the ranges found correspond to 3.0 reflectance units. These results agree substantially with those obtained by McFarren et al (11) with paper chromatograms. Such was not the case with an attempt to enhance color intensity by treating the chromatoplates with steam for 5 minutes following
TABLE 8

% REFLECTANCE AT 530 μM OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF TEMPERATURE AND TIME OF COLOR DEVELOPMENT, AND OF POST-DEVELOPMENT STORAGE TIME (AT 27°).

<table>
<thead>
<tr>
<th>Development Temperature</th>
<th>90° ± 2</th>
<th>60° ± 1</th>
<th>27° (Room Temperature)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development Time (Min)</td>
<td>(RaR)</td>
<td>(RaR)</td>
<td>(RaR)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Storage Time (Hrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>47.6</td>
<td>46.6</td>
<td>46.9</td>
</tr>
<tr>
<td>24</td>
<td>54.2</td>
<td>52.7</td>
<td>53.2</td>
</tr>
<tr>
<td>72</td>
<td>81.6</td>
<td>79.0</td>
<td>80.2</td>
</tr>
</tbody>
</table>
development at 60°C for 10 minutes. Whereas Bull et al (21) employed this technique successfully with paper chromatograms, similar treatment of thin-layer plates had the reverse effect. In one case the % reflectance was found to increase from 43.9 to 55.2.

Direct examination of chromatoplates

The quantitative potential of the direct examination technique is indicated by Figure 23, which shows the reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent. Since it has already been shown that the precision provided by this procedure is of the same order as that afforded by direct transmission methods applied to paper chromatography, no further work was done on this aspect of the research.

Examination of spots removed from chromatoplates

A considerable increase in precision was achieved by analyzing spots removed from chromatoplates. The reproducibility one can anticipate for readings obtained for different spots of the same concentration of various amino acids adsorbed on silica gel and sprayed with ninhydrin reagent is indicated in Table 9. In the case of each acid three 30 µg. replicates were chromatographed over a distance of 15 cm. in one dimension and prepared for analysis according to the procedure outlined in the experimental section. For this study the size of the analytical samples was increased to 60 mg. and the reflectance at 515 µm was determined 12 hours after the ninhydrin color had been developed. An average standard deviation of 1.42% was obtained for the fourteen sets; the largest standard deviation found for any one set
Fig. 23. Reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent. Concentrations in mg: (1) 2.00; (2) 5.00; (3) 10.0; (4) 15.0; (5) 20.0; (6) 25.0; (7) 30.0; (8) 35.0; (9) 40.0.
TABLE 9

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515

NJ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO

ACIDS ADSORBED ON SILICA GEL AND SPRAYED WITH NINHYDRIN REAGENT.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Range (%R)</th>
<th>Mean (%R)</th>
<th>Std. Dev. (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>45.4 - 47.0</td>
<td>45.8</td>
<td>2.32</td>
</tr>
<tr>
<td>Arginine · HCl</td>
<td>60.4 - 62.2</td>
<td>61.2</td>
<td>1.37</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>60.9 - 63.0</td>
<td>62.3</td>
<td>1.94</td>
</tr>
<tr>
<td>Glycine</td>
<td>68.5 - 71.0</td>
<td>69.6</td>
<td>1.83</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>47.4 - 49.0</td>
<td>48.0</td>
<td>1.80</td>
</tr>
<tr>
<td>Leucine</td>
<td>48.8 - 49.6</td>
<td>49.3</td>
<td>0.88</td>
</tr>
<tr>
<td>Lysine · HCl</td>
<td>59.8 - 60.2</td>
<td>60.0</td>
<td>0.33</td>
</tr>
<tr>
<td>Methionine</td>
<td>54.2 - 55.7</td>
<td>55.0</td>
<td>1.38</td>
</tr>
<tr>
<td>Norleucine</td>
<td>48.4 - 49.2</td>
<td>49.0</td>
<td>0.34</td>
</tr>
<tr>
<td>Norvaline</td>
<td>46.8 - 47.9</td>
<td>47.6</td>
<td>1.47</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>69.5 - 71.2</td>
<td>70.5</td>
<td>1.26</td>
</tr>
<tr>
<td>Serine</td>
<td>53.2 - 54.9</td>
<td>54.3</td>
<td>1.76</td>
</tr>
<tr>
<td>Threonine</td>
<td>60.0 - 61.8</td>
<td>61.3</td>
<td>1.85</td>
</tr>
<tr>
<td>Valine</td>
<td>48.4 - 49.8</td>
<td>49.2</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Av. Std. Dev. 1.42
was the 2.32% value observed with alanine. This degree of precision was attainable, however, only with sets subjected to the procedure at one and the same time. Particularly large deviations resulted when plates were sprayed at times differing by an hour or more. In fact the precision of the determinations was limited by elements associated with the generation of the ninhydrin color and not by deviations arising from the packing of the sample cell, which were of secondary importance. This was in contrast to results obtained for a stable system whose analyses involved no chromogenesis. On the other hand, it was possible to achieve this order of precision with spots containing as little as 2 µg. of an amino acid. The best possible results were obtained when sets containing 3 to 4 replicates were chromographed in the same chamber.

The possibility of using a single standard curve in the determination of several amino acids was suggested by a consideration of the color densities and standard deviations recorded in Table 9. Accordingly the relationship between reflectance and the concentration of the ninhydrin complexes of various amino acids adsorbed on silica gel was determined by means of the procedure employed for the precision study. Reflectance readings were taken 6 hours after the ninhydrin color had been developed for dilution series covering the 0.5 to 50 µg. range. Some of the standard curves obtained during the course of this study are shown in Figure 24. It was found that one standard curve could indeed be used for the determination of norvaline, valine, norleucine, isoleucine and leucine without affecting the precision significantly. As may be seen in Figure 24, the largest spread found
Fig. 24. % reflectance at 515 m\(\mu\) of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of concentration. (A) Glycine. (B) Serine. (C) Threonine. (D) Leucine. (E) Norvaline. (F) Alanine.
between the curves for norvaline and leucine, which bracketed those for the other group members, only amounted to two reflectance units. Even this small dispersion is lessened by plotting the data in the form percent reflectance versus concentration in \( \mu \)-moles. When this is done, the calibration curve for alanine as well as for the five members of the group become identical up to concentrations of 30 \( \mu \)g. Although standard curves for the other nine acids in general resembled those shown in Figure 24, no other grouping analogous to the norvaline-leucine set was found. This was not surprising since the acids differed not only in their color development characteristics but also in their sensitivities. Sensitivities (in \( \mu \)g.) found for the acids were as follows: alanine, glutamic acid, isoleucine, leucine, norleucine, norvaline, serine and valine -0.5; glycine -0.8; methionine -2.5; threonine -5; lysine -8; arginine -10; and phenylalanine -12. The interchange that occurred in the relative positions of the curves for glycine and threonine, which are included in Figure 24, with increased concentration is indicative of some of the complexities encountered. A more useful form of these data is obtained when absorbance is plotted versus the square root of the concentration, as is done in Figure 25, since this results in a linear relationship in the concentration range of analytical interest.

In conclusion, when specified precautions are observed in the generation of the ninhydrin color, amino acids resolved on chromatoplates can be determined by reflectance spectrophotometry. Direct examination of the plates yields results having the same range of precision, 5 to 12\%, as is reported for direct photometric methods.
Fig. 25. Absorbance at 515 m\(\mu\) of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of the square root of concentration. (A) Glycine. (B) Serine. (C) Threonine. (D) Leucine. (E) Norvaline. (F) Alanine.
developed for paper chromatograms. A degree of precision approaching
that afforded by transmittance is achieved if the reflectance measure-
ments are carried out on spots removed from the chromatoplates.
Unlike analyses dealing with stable systems and involving no chromo-
genesis, the precision attained is limited by elements associated
with the color development process and not by deviations arising from
the preparation of the analytical sample. The dilution of the nin-
hydrin complex with silica gel adsorbent that occurs during the sample
preparation reduces the sensitivity of the reflectance method to
approximately half that reported for transmission measurements carried
out with paper chromatograms, but the advantages afforded by the thin-
plate technique more than compensate for this reduced sensitivity.
These include more rapid resolution, increased precision and no re-
quirement that the substrate be treated to compensate for textural
irregularities. Over the concentration range best suited for analysis,
2 to 30 μg., a linear relationship is observed with most of the acids
studied when absorbance is plotted against the square root of the con-
centration, or when the Kubelka-Munk function is plotted against con-
centration. Although the present study was restricted to one-dimen-
sional thin film chromatography, there appears to be no reason why
spectral reflectance cannot be applied to the two-dimensional process
with equal success.

F. An Improved Method for the Determination of Amino Acids by Spec-
tral Reflectance. (Ref. 126).

The precision attained with the procedure discussed in the pre-
ceding Section was found to be limited by elements associated with the
color development process, such as the incomplete reaction of the acids with the ninhydrin reagent and the leaching out of the acids during the spraying operation.

Since the degree of precision thus achieved was less than that provided when the same procedure without the chromogenic step was applied to a stable system, it was felt that substantial improvement in the method would result if the color were developed without sprays. Accordingly it was decided to investigate the possibility of adapting a nonspray method suggested by El Khadem et al. (119) for the identification of amino acids and sugars separated on paper chromatograms to the problem at hand. By adding the detecting reagents to the solvent mixtures, these investigators succeeded in eliminating not only the spraying operation but also the drying step preceding it.

Identification of amino acids

When solvent mixtures containing 0.3% ninhydrin were used to develop one-dimensional chromatograms, it was not possible to observe the coloration noted by El Khadem et al. (119) with paper chromatograms when the solvent front reached the amino acids. The spots that appeared after drying, however, were sharply defined and exhibited no tailing. An increase in the ninhydrin concentration to 0.4% did succeed in producing a faint pink coloration during the development of the plates, which made it possible to follow the movement of the spots, as well as a greater sensitivity. This last was offset somewhat by the appearance of some tailing.

A clean separation of a mixture consisting of 3 μg. of each of
the ten amino acids was achieved in 8 to 10 hours when solvent three
without ninhydrin was used for the initial development and solvent two
which was 0.2% with respect to ninhydrin was employed in the second
dimension. Some idea of the degree of resolution attained as well as
the feasibility of using $R_f$ values for the identification of the acids
can be gained from a consideration of Figure 26, which shows one chro­
matogram obtained by means of this procedure superimposed on a second.
It should be remembered that the thickness of the adsorbent layer was
somewhat greater than usual so as to provide enough material for the
determination of the acids, and that the adsorbent was applied not
with a precision applicator but manually with masking tape and a glass
rod.

Similar success, insofar as resolution is concerned, was realized
when solvent mixture four was employed for development in the first
dimension and 0.2% ninhydrin in solvent mixture one for development in
the second. As may be seen in Figure 27, which depicts a typical
chromatogram obtained with these solvent systems, it is possible to
make use of ammoniacal solvents in these separations provided they are
employed for the first development only and provided that the ammonia
is removed by a drying preceding the second development. Enough
adsorbed ammonia remains, however, to impart a brownish or purplish
tinge to the entire plate. This results in a lack of contrast between
the color of the adsorbent and that of the ninhydrin complexes of the
amino acids which renders ammoniacal solvents unsuitable for use in
conjunction with the method described herein.
Fig 26 Two typical chromatograms obtained by using solvent mixture three for the initial development and 0.2% Ninhydrin in solvent mixture two for the second development. Trial one, dotted line; trial two, solid line.
Fig 27 Typical chromatogram obtained by using solvent mixture four for the initial development and 0.2% ninhydrin in solvent mixture one for the second development.
Determination of amino acids

The reproducibility that can be expected of the method was determined by chromatographing four 5 μg. replicates of each acid over a distance of 15 cm. in one dimension by the ascending technique and preparing them for analysis according to the procedure outlined in the experimental section. When solvent mixture one to which 0.2% ninhydrin had been added was employed to develop the plates, an average standard deviation of 0.49% R was obtained for the ten sets. As may be seen in Table 10, which summarizes the results of this experiment, the largest standard deviation found for any one set was the 0.84% R value observed with lysine. Similar data were obtained using solvent mixture two that was 0.2% with respect to ninhydrin.

These results represent a considerable increase in precision over that provided by spray methods. An average standard deviation of 1.45% R and a maximum standard deviation for a single set of 2.32% R were found for the previous study conducted with three 30 μg. replicates of the same ten acids. The two studies differed principally in the solvent systems used and in that the ninhydrin was applied as a spray. Since the results of this research indicate that precision changes of the magnitude being discussed were not observed when the solvent systems were varied, one must ascribe the increase in reproducibility to the elimination of the spraying operation. This conclusion is in accord with the findings reported by Jellinek and Fridman (30), who carried out a critical evaluation of the errors in a densitometric analysis of glycine on paper chromatograms.

The effect produced by increasing the ninhydrin concentration was
TABLE 10

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 µM FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN ONE DIMENSION USING SOLVENT MIXTURE #1. (0.2% NINHYDRIN).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Range (%)</th>
<th>Mean (%)</th>
<th>Std. Dev. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>72.8 - 73.6</td>
<td>73.0</td>
<td>0.39</td>
</tr>
<tr>
<td>Arginine · HCl</td>
<td>80.3 - 81.6</td>
<td>80.8</td>
<td>0.59</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>80.5 - 81.7</td>
<td>81.2</td>
<td>0.51</td>
</tr>
<tr>
<td>Glycine</td>
<td>77.3 - 77.9</td>
<td>77.5</td>
<td>0.27</td>
</tr>
<tr>
<td>Leucine</td>
<td>73.8 - 74.5</td>
<td>74.2</td>
<td>0.30</td>
</tr>
<tr>
<td>Lysine · HCl</td>
<td>79.0 - 80.9</td>
<td>79.8</td>
<td>0.84</td>
</tr>
<tr>
<td>Methionine</td>
<td>78.3 - 79.4</td>
<td>78.8</td>
<td>0.58</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>81.3 - 82.6</td>
<td>82.3</td>
<td>0.71</td>
</tr>
<tr>
<td>Serine</td>
<td>75.5 - 76.4</td>
<td>76.1</td>
<td>0.41</td>
</tr>
<tr>
<td>Valine</td>
<td>74.2 - 74.7</td>
<td>74.4</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Av. Std. Dev. 0.49
ascertained by repeating the preceding experiment with solvent three to which 0.4% ninhydrin had been added. As indicated in Table 11, the reproducibility attained was only slightly less than that observed with 0.2% ninhydrin solutions. An average standard deviation of 0.53% R was obtained for the ten sets; the largest standard deviation found for any one set was 0.87% R. There was, however, an increase in sensitivity from the 1.0 µg. value found for most of the acids with solvents one and two that had been made 0.2% with respect to ninhydrin to the 0.5 µg. value observed with the 0.4% ninhydrin solution of solvent three. These last results are essentially the same as those obtained when the ninhydrin was applied as a spray.

As expected there was some decrease in reproducibility where the ten acids were chromatographed in two dimensions, though the precision was still considerably better than that achieved in one dimension with the use of sprays. The results obtained when four 5 µg. replicates were chromatographed in the first dimension with solvent mixture three and in the second dimension with solvent mixture two to which 0.2% ninhydrin had been added are presented in Table 12. The average standard deviation for these data was 0.77% R, with no standard deviation in excess of 1.18% R being found for any of the acids.

Despite the increased reproducibility resulting from the elimination of the spraying operation, elements associated with the ninhydrin reaction continued to be the chief factors limiting the precision of the method. Among the most important of these was the close dependence of the color stability of the ninhydrin complexes of the adsorbed amino acids upon the nature of the solvent system used to develop the
**TABLE 11**

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 M\(\mu\) FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN ONE DIMENSION USING SOLVENT MIXTURE #3 (0.4% NINHYDRIN).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Range (%R)</th>
<th>Mean (%R)</th>
<th>Std. Dev. (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>50.2 - 51.1</td>
<td>50.8</td>
<td>0.60</td>
</tr>
<tr>
<td>Arginine · HCl</td>
<td>58.3 - 59.8</td>
<td>59.0</td>
<td>0.66</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>57.7 - 58.9</td>
<td>58.3</td>
<td>0.57</td>
</tr>
<tr>
<td>Glycine</td>
<td>55.8 - 56.9</td>
<td>56.3</td>
<td>0.56</td>
</tr>
<tr>
<td>Leucine</td>
<td>51.0 - 51.6</td>
<td>51.2</td>
<td>0.30</td>
</tr>
<tr>
<td>Lysine · HCl</td>
<td>56.3 - 57.6</td>
<td>56.9</td>
<td>0.61</td>
</tr>
<tr>
<td>Methionine</td>
<td>55.9 - 57.0</td>
<td>56.7</td>
<td>0.57</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>59.0 - 60.9</td>
<td>60.1</td>
<td>0.87</td>
</tr>
<tr>
<td>Serine</td>
<td>54.2 - 54.8</td>
<td>54.4</td>
<td>0.30</td>
</tr>
<tr>
<td>Valine</td>
<td>52.7 - 53.5</td>
<td>53.2</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Av. Std. Dev. 0.53
TABLE 12

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 M\(\mu\) FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS CHROMATOGRAPHED IN THE FIRST DIMENSION WITH SOLVENT MIXTURE #3 AND IN THE SECOND DIMENSION WITH SOLVENT MIXTURE #2 (0.2% NINHYDRIN).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Range (l%R)</th>
<th>Mean (l%R)</th>
<th>Std. Dev. (l%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>67.2 - 68.1</td>
<td>67.8</td>
<td>0.41</td>
</tr>
<tr>
<td>Arginine · HCl</td>
<td>78.3 - 80.4</td>
<td>79.3</td>
<td>0.90</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>78.7 - 80.3</td>
<td>79.5</td>
<td>0.78</td>
</tr>
<tr>
<td>Glycine</td>
<td>74.3 - 75.3</td>
<td>74.8</td>
<td>0.41</td>
</tr>
<tr>
<td>Leucine</td>
<td>68.8 - 70.8</td>
<td>69.8</td>
<td>0.74</td>
</tr>
<tr>
<td>Lysine · HCl</td>
<td>77.9 - 80.4</td>
<td>79.3</td>
<td>1.15</td>
</tr>
<tr>
<td>Methionine</td>
<td>75.8 - 77.2</td>
<td>76.6</td>
<td>0.65</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>80.3 - 82.4</td>
<td>81.1</td>
<td>0.90</td>
</tr>
<tr>
<td>Serine</td>
<td>70.6 - 73.0</td>
<td>72.3</td>
<td>1.18</td>
</tr>
<tr>
<td>Valine</td>
<td>70.2 - 71.5</td>
<td>70.9</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Av. Std. Dev. 0.77
chromatoplates and on the temperature at which the developed plates were kept. The effect of temperature was such that developed plates which were stored any length of time had to be maintained below $10^0 \text{C}$ to reduce color density changes to a level consistent with precision requirements of the order discussed above. Under these temperature conditions the solvent mixture containing phenol was found to be preferable to those containing acetic acid from the standpoint of color stability. Over a 24 hour period no variation in excess of 1% R was observed in the case of plates developed with solvent mixture three while plates developed with mixtures one and two exhibited variations as large as 3% R. This increased stability associated with the phenolic solvent was apparent even when it was employed as the first solvent in the development of a two-dimensional chromatogram. To insure maximum precision, therefore, it is essential that plates on which unknowns and reference standards are being resolved be processed at the same time and under identical conditions. By proceeding in this manner, it is possible to cancel out not only variations related to the ninhydrin reaction but also those which occur during the drying of the chromatograms because of oxidation and volatilization of the amino acids.

Errors associated with the other operations constituting the procedure were of secondary importance. Variations attributable to the packing of the sample cell were found to amount to an average standard deviation of 0.2% R for the stable system whose analysis involved no chromagenesis. Although excessive tailing and poor resolutions can curtail the accuracy considerably, especially if large concentrations
of acids are involved, such errors can be avoided to a large extent by choosing suitable separation procedures; by increasing the thickness of the adsorbent layer; and by extending the development time. When working with more than 20 to 30 µg. of acid, it was necessary to make the analytical sample larger than 40 mg. to accommodate the increased amount of test material as the areas of the spots were approximately proportional to the concentration. To determine the loss of precision that might be attributed to tailing, plates on which were paired developed and undeveloped spots consisting of 30 µg. of the same acid were sprayed, dried at approximately 55°C, and subjected to analysis by spectral reflectance. In the case of no acid were differences greater than those ascribable to the ninhydrin reaction found between paired spots. This observation is in keeping with the results reported for paper chromatography (112, 113).

The effect upon reproducibility of varying the slit width of the spectrophotometer was ascertained by measuring the reflectance at 515 µm of a 10 µg. sample of glycine complexed with ninhydrin relative to a standard consisting of adsorbent removed from the same plate. Four consecutive measurements were made at each of several slit openings using the blue-sensitive phototube at load resistor setting 2 and at sensitivity setting 3. A consideration of the results obtained, which are set forth in Table 13, revealed that standard deviations of 0.05% R or less can be expected for slit widths in the range 0.25 - 0.4 mm. There is a decrease in precision with widths greater than this while smaller widths are not suited for the measurement of diffuse reflectance. The band widths isolated at the various slit settings were
### TABLE 13

**REPRODUCIBILITY OF REFLECTANCE MEASUREMENTS AS A FUNCTION OF SLIT WIDTH.**

**READINGS OBTAINED AT 515 μM WITH A SAMPLE OF GLYCINE COMPLEXED WITH NINHYDRIN.**

<table>
<thead>
<tr>
<th>Slit Width (mm)</th>
<th>0.25</th>
<th>0.30</th>
<th>0.40</th>
<th>0.50</th>
<th>0.60</th>
<th>0.70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band Width (μm)</td>
<td>5.00</td>
<td>6.00</td>
<td>8.00</td>
<td>10.00</td>
<td>12.00</td>
<td>14.00</td>
</tr>
<tr>
<td>Mean of Four Readings (%R)</td>
<td>67.50</td>
<td>67.50</td>
<td>67.60</td>
<td>67.70</td>
<td>67.50</td>
<td>69.10</td>
</tr>
<tr>
<td>Standard Deviation (%R)</td>
<td>0.05</td>
<td>0.00</td>
<td>0.05</td>
<td>0.08</td>
<td>0.14</td>
<td>0.22</td>
</tr>
</tbody>
</table>
obtained from dispersion data provided for the Beckman DU spectrophotometer.

Finally the probable relative error in the measurement of the concentrations of alanine, leucine, serine and valine was determined by making use of the precision data obtained with four 5 μg. replicates of the acids and listed in Table 10, and of the calibration curves for these same acids which were presented in Section E. A similar investigation was carried out to ascertain the relationship between the probable relative error and the concentration of glycine. In this instance solvent one which was 0.2% with respect to ninhydrin was employed in conjunction with four replicates of acid at each concentration investigated. Data relative to these two studies are presented in Tables 14 and 15, respectively. The change in measured concentration equivalent to the deviations observed for the various acids was obtained from the appropriate calibration curves and expressed as a probable % relative error in concentration. For the five acids at 5 μg. concentration this figure ranged from a low value of 2.8% for valine to a high of 5.0% for serine. In the case of glycine minimal values were obtained in the intermediate concentration range. The relatively large 9.0% value observed at the 2 μg. concentration may be attributed to the fact that this concentration approached the 1 μg. sensitivity limit for glycine as well as to the increased contribution of volumetric and gravimetric errors associated with such operations as the preparation of the standard solutions. At the opposite end of the scale, the 7.0% figure found for 20 μg. concentrations can be ascribed to the flattening of the calibration curve that occurs at
<table>
<thead>
<tr>
<th></th>
<th>Alanine (%R)</th>
<th>Leucine (%R)</th>
<th>Serine (%R)</th>
<th>Valine (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>72.8 - 73.6</td>
<td>73.8 - 74.5</td>
<td>75.5 - 76.4</td>
<td>74.2 - 74.7</td>
</tr>
<tr>
<td>Mean</td>
<td>73.0</td>
<td>74.2</td>
<td>76.1</td>
<td>74.4</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.39</td>
<td>0.30</td>
<td>0.41</td>
<td>0.27</td>
</tr>
<tr>
<td>Equivalent Change in Measured Concentration of Acid (\mu g)</td>
<td>0.18</td>
<td>0.15</td>
<td>0.25</td>
<td>0.14</td>
</tr>
<tr>
<td>Probable % Relative Error</td>
<td>3.60</td>
<td>3.80</td>
<td>5.00</td>
<td>2.80</td>
</tr>
</tbody>
</table>
**TABLE 15**

PROBABLE RELATIVE ERROR IN THE MEASUREMENT OF THE CONCENTRATION OF GLYCINE AS A FUNCTION OF CONCENTRATION.

<table>
<thead>
<tr>
<th>Concentration of Glycine (µg per spot)</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (%R)</td>
<td>84.3 - 86.0</td>
<td>77.3 - 77.9</td>
<td>63.6 - 65.1</td>
<td>55.3 - 57.1</td>
</tr>
<tr>
<td>Mean (%R)</td>
<td>85.1</td>
<td>77.5</td>
<td>64.4</td>
<td>56.3</td>
</tr>
<tr>
<td>Standard Deviation (%R)</td>
<td>0.92</td>
<td>0.27</td>
<td>0.66</td>
<td>0.76</td>
</tr>
<tr>
<td>Equivalent Change in Measured Concentration of Glycine (µg)</td>
<td>0.18</td>
<td>0.20</td>
<td>0.50</td>
<td>1.40</td>
</tr>
<tr>
<td>Probable % Relative Error</td>
<td>9.00</td>
<td>4.00</td>
<td>5.00</td>
<td>7.00</td>
</tr>
</tbody>
</table>
high concentrations. Of the acids investigated, this effect is parti-
cularly noticeable in the case of glycine.

The application of a nonspray method devised by El Khadem et al
(119) for use in paper chromatography to the determination by reflec-
tance spectrophotometry of amino acids resolved on thin-layer plates
has enhanced the utility of the latter technique considerably. By
dissolving ninhydrin in the solvent mixtures employed to develop the
chromatograms, one drying and the subsequent spraying of the plates
are eliminated. Despite this modification, the Rf values of the amino
acids are not altered with the result that values obtained by means of
the conventional spray method can still be utilized for purposes of
identification. The nonspray procedure can be applied successfully to
two-dimensional chromatography by adding the ninhydrin to the second
solvent system. When no quantitative work is contemplated, ammoniacal
solvents can be employed for the first development provided that the
ammonia is removed by drying prior to the development in the second
direction.

Depending on the concentration of ninhydrin used, the sensitivity
of the method is between $5 \times 10^{-9}$ and $1 \times 10^{-8}$ moles for most amino
acids. Although this is somewhat less than that afforded by the in
situ estimation of amino acids separated on paper, this slight
decrease in sensitivity is more than compensated for by an increase in
precision and accuracy. The elimination of the spraying operation
with its attendant irregularities, such as leaching out of the amino
acids, has resulted in a precision which is comparable to that
reported for transmission measurements made of these same spots
through the paper (104). The comparison becomes all the more favora-
ble when one considers that the latter data were obtained with a
stable system - copper rubeanate - and with undeveloped spots. The
overall accuracy attained in the measurement of the concentrations
of amino acids equals or exceeds that achieved when such techniques
as total scanning and the electronic integration of curves are
employed in conjunction with paper chromatograms (28, 29, 30).

Although the present study was restricted to amino acids, there
appears to be no reason why an appropriate modification of the pro-
cedure described herein cannot be applied to other systems. For
example, El Khadem et al (119) have also employed the nonspray tech-
nique for the separation of sugars on paper while Bevenue and Williams
(96) have devised a method for the direct estimation by reflectance
spectrometry of sugars separated by paper chromatography. It should
be possible to make use of these results in conjunction with such
procedures for the resolution of sugars on thin-layer plates as those
proposed by Stahl and Kaltenbach (120), and by Patuska (51).
SUMMARY

The various factors affecting the diffuse reflectance spectra of eosine B, rhodamine B, fuchsin, and o-nitrophenol adsorbed on chromatographic grade alumina were studied to determine the analytic potential of employing reflectance spectroscopy in conjunction with thin-layer chromatography. The results obtained indicated that spectral reflectance could be utilized for the photometric analysis of food dyes adsorbed on alumina, since straight lines passing through the origin were obtained when the Kubelka-Munk function was plotted versus a suitable concentration range of rhodamine B. As a next step, components of dye mixtures resolved on thin-layer plates were identified by direct examination of the plates by spectral reflectance. The amounts of adsorbed dye were determined at the same time with a precision of approximately ± 5%. Reflectance measurements carried out on spots removed from the plates and packed in a simple cell suited for the measurement of the spectral reflectance of semimicro samples, such as those obtained from chromatoplates, afforded a degree of precision identical to that attained with transmittance. An attempt was made to correlate the performance of the cell with current theories of reflectance spectroscopy using a system consisting of Merck silica gel G and McCormick blue, a food dye.

The techniques developed with the stable dye systems were then employed, first to investigate some of the factors affecting the color stabilities of the ninhydrin complexes of adsorbed amino acids, and second in an attempt to determine amino acids separated on thin-layer
chromatograms. Direct examination of the plates yielded a degree of precision comparable to that afforded by direct transmission methods applied to paper chromatography. Precision approaching that afforded by transmittance was attained if the reflectance measurements were carried out on spots removed from the plates and specified precautions were observed in the generation of the ninhydrin color. Since it was found that the spraying procedure is the limiting factor for the last method, a nonspray method was developed to circumvent this limitation. In addition to facilitating the determination, the elimination of the spraying operation with its attendant irregularities resulted in a substantial increase in precision and accuracy. In this respect the method is competitive with paper chromatography used in conjunction with such techniques as total scanning and the electronic integration of curves.
APPENDIX I

Derivation of the Kubelka-Munk function:

When a plane parallel layer of thickness \(d\) is irradiated diffusely and monochromatically with a beam of intensity \(I_0\), the radiation flow in the positive \(x\)-direction can be represented by \(I\) and the radiation flow in the negative \(x\)-direction (caused by scattering) can be represented by \(J\). An infinitesimally thin layer \(dx\) parallel to the surface is penetrated by the radiation in all possible directions \(\rho\) with respect to the normal. The average path of the radiation is, therefore, not \(dx\) but

\[
\frac{dJ}{I} = dx \int_0^{\frac{\pi}{2}} \frac{dI}{I \sigma \cos \rho} = u dx \quad \text{or} \quad \frac{dI}{J} = dx \int_0^{\frac{\pi}{2}} \frac{dJ}{J \sigma \cos \rho} = v dx \quad (1)
\]

where \(\frac{dI}{I}\) and \(\frac{dJ}{J}\) stand for the angular distribution of the radiation.

Assuming conditions for ideal diffuse radiation,

\[
\frac{dI}{I} = I \sin 2 \rho \quad \text{and} \quad \frac{dJ}{J} = J \sin 2 \rho \quad (2)
\]

where \(u = v = 2\). This factor is included in \(s\) (scattering coefficient) and \(k\) (absorption coefficient). The component \(k I dx\) of \(I\) is absorbed in the layer \(dx\) while the component \(s I dx\) is scattered backward. The radiation \(J\) in the negative \(x\)-direction contributes radiation \(s J dx\) by scattering in the positive \(x\)-direction. The change in intensity of \(I\) in the layer \(dx\) is, therefore, composed of the following elements:

\[
dI = -(k+s) I \ dx + s J dx \quad . \quad \text{By analogy, the decrease in intensity of } J \text{ is: } \frac{dJ}{J} = -(k+s) J \ dx + s J dx \quad (3) \quad (4)
\]
These are the basic differential equations which describe the absorption and scattering processes. The indefinite integrals are:

\[ I = A(1 - \beta) e^{\sigma x} + B(1 - \beta) e^{-\sigma x} \quad (5) \]

\[ J = A(1 + \beta) e^{\sigma x} + B(1 + \beta) e^{-\sigma x} \quad (6) \]

with \( \sigma' = \sqrt{K(K + 2s)} \quad (7) \)

and \( \beta = \frac{\sigma'}{K + 2s} = \sqrt{K(K + 2s)} \quad (8) \)

The constants \( A \) and \( B \) are determined by the limiting conditions. If one integrates for the entire thickness, \( d \), of the layer the conditions

for \( x = 0 \) : \( I = I_o \)

for \( x = d \) : \( I = I(x=d); J = 0 \)

are valid and one obtains

\[ A = -\frac{(1 - \beta) e^{-\sigma d}}{(1 + \beta)^2 e^{\sigma d} - (1 - \beta)^2 e^{-\sigma d}} I_o \quad (9) \]

\[ B = \frac{(1 + \beta) e^{\sigma d}}{(1 + \beta)^2 e^{\sigma d} - (1 - \beta)^2 e^{-\sigma d}} I_o \quad (10) \]

The transmission of the layer is therefore given by

\[ T = \frac{I(x=d)}{I_o} = \frac{4\beta}{(1+\beta)^2 e^{\sigma d} - (1-\beta)^2 e^{-\sigma d}} = \frac{2\beta}{(1-\beta)^2 \sin h\sigma d + 2\beta \cos h\sigma d} \]
the diffuse reflectance by

\[ R = \frac{\mathcal{I}(x = 0)}{I_0} = \frac{(1 - \beta)^2 (e^{d} - e^{-d})}{(1 + \beta)^2 e^d - (1 - \beta)^2 e^{-d}} = \frac{(1 - \beta)^2 \sin h\delta d}{(1 + \beta)^2 \sin h\delta d + 2 \beta \cos h\delta d} \]

For \( s = 0 \) (non-scattering layer) and \( \beta = 1 \), eq. (11) becomes the Bouguer-Lambert's law \( T = e^{-kd} \) and \( R' \) becomes zero.

For infinite layer thickness \( d \) approaches 0 and one obtains

\[ R'_\infty = \frac{1 - \beta}{1 + \beta} = \frac{s + K - \sqrt{K(K + 2s)}}{s} \]  

These conditions are achieved experimentally with 1 mm. layers of fine powders and \( R'_\infty \) can therefore be measured. Equation (13) can be transformed to a more convenient form

\[ \frac{(1 - R'_\infty)^2}{2 R'_\infty} = \frac{K}{s} \]
REFERENCE LIST


Polarization and colorchange with the adsorption of surface active substances. E. Weitz, F. Schmidt and J. Singer. Z. Elektrochem., 46, 222 (1940).

Colorchange and catalytic action as consequences of the polarization due to adsorption on surface-active substances. E. Weitz, F. Schmidt and J. Singer. Z. Elektrochem., 47, 47 (1941).


Acknowledgements

Grateful acknowledgement is made to

Dr. Michael M. Frodyma, who suggested the problem and gave his enthusiastic support in many organizational matters.

Dr. Richard G. Inskeep, the Graduate Research Committee and the Chemistry Department for their interest and encouragement.

Finally my wife Eva Maria Frei, a tireless and understanding companion during my years of study.