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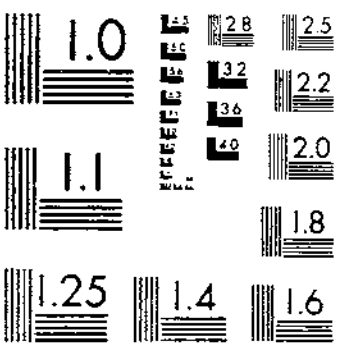

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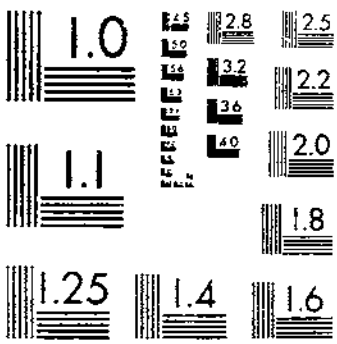

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THE VISUAL SPECTROPHOTOMETRY OF DYES

By W. C. HOLMES, *Senior Chemist*, J. T. SCANLAN, *Associate Chemist*, and A. R. PETERSON,[1] *Color and Farm Waste Division, Chemical and Technological Research, Bureau of Chemistry and Soils*

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INTRODUCTION

COLOR AND ABSORPTION

The phenomenon of color arises from the selective absorption of light in the visible spectrum. The visible spectrum is that relatively restricted portion of the entire spectrum of radiant energy to which the eye is sensitive. The practical working range includes, roughly, the light of all wave lengths between 400 mμ and 720 mμ. (The millimicron, denoted by the symbol mμ, is the millionth part of a millimeter, and is equivalent to 10 Ångström units.)

The color of substances, whether in the solid form or in solution, is determined by the location of their dominant absorption of light within the visible spectrum. Their color is complementary to the color of the light they absorb. A violet dye, for example, appears violet because of its predominant absorption of yellow light. A red dye absorbs principally green light and a blue dye, red light.

The unaided eye perceives but can not analyze color. The color of substances may be of simple origin. It is more commonly of very complex origin, depending upon the varying degree of absorption of light over the extensive spectral ranges. The eye merely registers a composite effect, which may be the resultant of an endless variation of factors. It is possible, however, to resolve this effect into its component factors with the spectrophotometer.

[1] Research fellow supported by the Commission on the Standardization of Biological Stains.

The spectrophotometer may reveal that two violet dyes which are indistinguishable to the unaided eye may differ very decidedly in the details of their absorption of light. A third dye, which may appear identical in color, may be shown by the spectrophotometer to be a mixture of blue and red dyes, with an absorption spectrum radically different from that of a true violet dye.

The spectrophotometer enables the analyst to measure the degree in which a colored substance absorbs light in all portions of the spectrum. This absorption, in the instance of dyes, usually occurs in well-defined bands. If the measurements are expressed in terms of light absorbed and are plotted against wave length, the analyst obtains an absorption curve or graphical representation of the dye spectrum, which will show the precise manner in which the dye absorbs light throughout the spectrum.

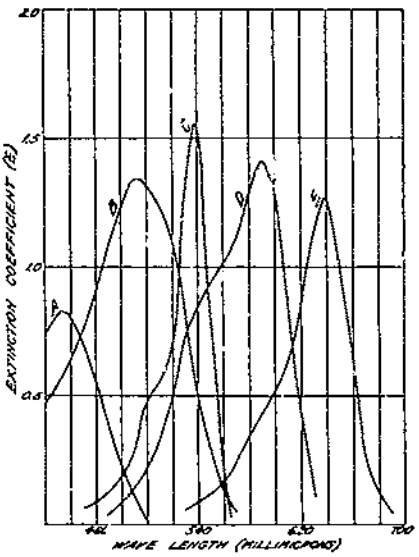

FIGURE 1.—Typical dye spectra: A, Tartrazine; B, orange G; C, phloxine; D, crystal violet; E, Neptune blue

Typical absorption curves of dyes are illustrated in Figure 1.

When measured under suitable conditions, the spectral positions and general forms of absorption spectra are characteristic of individual dyes, and the magnitude of absorptive indices (the height of the bands) is a direct measure of the concentration of dye present.

The advantages of the spectrophotometer over the colorimeter are obvious. It enables the analyst to dispense with comparative standards and express measurements in absolute values. It provides photometric fields for matching which are completely uniform in hue. Whereas the colorimeter merely provides relatively favorable conditions for the visual comparison of total color intensity, the spectrophotometer affords an analysis of color which is not only frequently illuminating in regard to the nature of the origin of the color, but is also of great value in respect to the applications in which it may be utilized.

The term, "depth of color," as employed by the colorist carries no implication with respect to color intensity, but refers entirely to dominant hue. In any series of colored compounds the color of the simplest member is usually yellow. The color of such a compound may be deepened by weighting the molecule with suitable substituent groups, passing from yellow to orange and then from orange to red. In like manner violet is a deeper color than red, and blue is a deeper color than violet. By referring to Figure 1 it will be observed that the phenomenon of deepening color is simply one of the progressive passage of the absorption band through the visible spectrum. When the band first enters the spectrum, the color is yellow. As it passes through the spectrum in the direction of longer wave length, the color

changes successively to orange, red, violet, and blue. Green is a still deeper color than blue. The spectra of dyes are more complex than is apparent from their absorption of visible light. They absorb light in the ultra-violet region of the spectrum as well as in the visible spectrum. The passage of the absorption bands through the visible spectrum with deepening color is accompanied by a corresponding progress of other absorption bands in the ultra-violet. In passing from blue to green color, the second band passes from the ultra-violet into the far-violet region of the visible spectrum. The spectra of green dyes show absorption in the far-violet as well as in the region of red light.

In some instances the absorption bands may progress even farther. The primary band may leave the visible spectrum and enter the infra-red region, in which, of course, it will cease to influence color. The secondary band will then give rise to what is termed "color of the second order."

COLOR AND CHEMICAL CONSTITUTION

It is obvious that the manner in which a substance absorbs light, whether within the visible spectrum or elsewhere, must be related, primarily, to its chemical constitution. Chemists make this assumption in establishing the structure of substances on the basis of the analogy of their spectroscopic characteristics and behavior with that of other substances of known structure.

Countless investigations during the past 50 years have revealed many facts regarding the manner and degree in which minor constitutional variations in dyes influence the spectral locations of band maxima. It has even been found possible to calculate the positions of maxima on empirical principles with considerable success (*48*, *49*).[2] Recent studies of the quantitative aspects of absorption carried on in the Color and Farm Waste Division indicate clearly that an analogous correlation exists between the constitution of dyes and their intensity of color.

Although much has been learned respecting the modification of color by means of constitutional variations, the ultimate cause of color has remained obscure. Simple theories as to the relation between color and constitution have been repeatedly shown to be inadequate for universal application, and no explanation of known facts has replaced them which has met with universal acceptance (*58*).

It is probably the general belief that the vibrations which are responsible for color are electronic. It appears certain, furthermore, that color is conditioned upon factors which can not be expressed by conventional methods of defining chemical structure. There is considerable evidence which indicates that the arrangement of residual affinities of molecules may have a decided effect upon color.

Any protracted discussion of theoretical aspects of color and absorption would be out of place in this bulletin. It is advisable, however, to emphasize one fact. Color is not completely dependent upon constitution in the ordinary sense. The absorption of dyes is affected in appreciable degrees by factors which do not modify structure

[2] Italic numbers in parentheses refer to Literature Cited, p. 39.

as expressed in conventional terms. It is never safe for the color analyst to assume that the absorption of a dye will be identical under different conditions. He should determine by experiment the factors which may affect the absorption of any given dye and base his technic with that dye upon his findings.

EQUIPMENT AND TECHNIC FOR VISUAL SPECTROPHOTOMETRY

The essential principle of the visual spectrophotometer is simple. Two parallel beams of light of equal intensity are passed through a photometer and thence through a spectrometer to reach the eye in contiguous fields, enabling the observer to compare the two spectra accurately. The photometric arrangement provides for the uninterrupted passage of one beam and for the reduction of the intensity of the second beam in any proportion desired. A glass cell containing the solution of the dye which is under examination is interposed

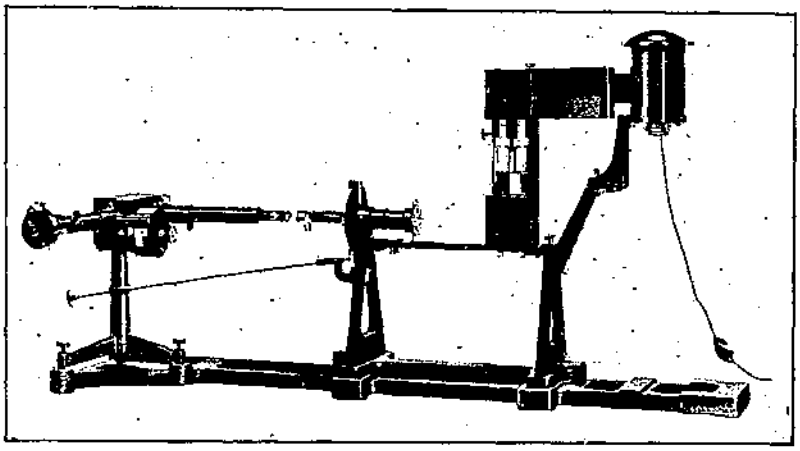

FIGURE 2.—Modern spectrophotometer

in the path of the first beam, and a similar cell containing solvent alone in the path of the second beam. The spectrum of the beam which has passed through the dye solution will be deficient of such light as has been absorbed by the dye, and the light thus absorbed may be measured by reducing the intensity of the second beam until it matches that of the first beam.

Figure 2 shows a spectrophotometer of recent design which is exceptionally convenient for investigating the absorption of solutions of dyes in the visible spectrum. Figure 3 is a schematic diagram of the same instrument.

A 250-watt Mazda projection lamp is employed as the light source. This may be disconnected from the carriage readily if any occasion arises in which it may be desired to employ a light source of a different character.

The absorption cells are removable vertical cups of optical glass, which may be raised and lowered about plungers of optical glass, thus varying at will the depth of the layer of solution through which the light passes. The cells rest upon a platform provided with a

graduated scale and vernier which enables the analyst to measure to one-tenth of a millimeter the depth of the solution layer over the range from zero to 6 centimeters.

This feature of the instrument is a very valuable aid to convenience. It is necessary to restrict absorption within somewhat narrow limits in order to measure it to best advantage. If the analyst employs absorption cells of fixed depth he can only vary and adjust the degree of absorption by the relatively laborious (and sometimes expensive) means of preparing solutions of different dye concentrations. With the calibrated-cup and plunger arrangement, readjustment is possible within wide limits by merely turning an adjusting screw.

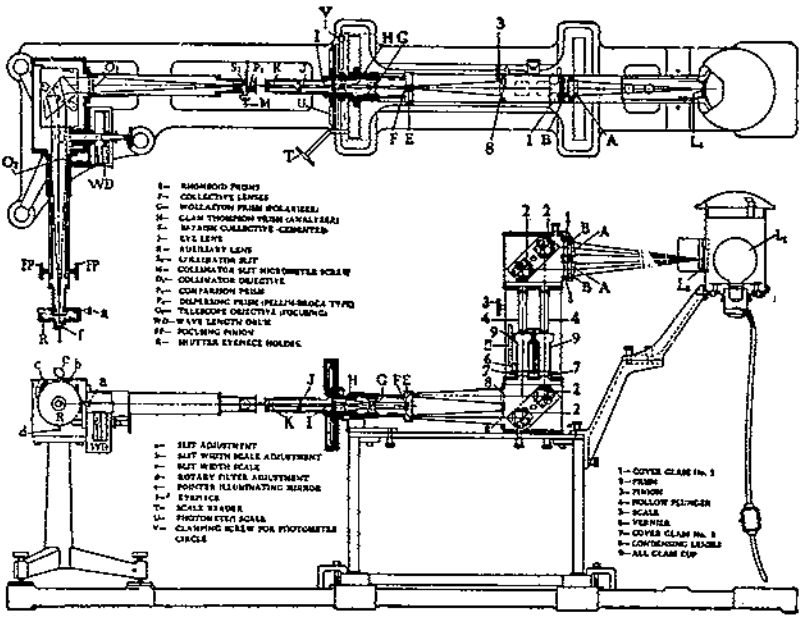

FIGURE 3.—Diagram of modern spectrophotometer

The photometer is of the improved Martens type, depending upon polarization for varying light intensity. The analyzer is rotated by a large circle which is so graduated that the analyst may read from it the angle of rotation or, more conveniently, read directly the transmissive or absorptive values derived therefrom. The photometer field may be reversed to compensate for possible errors arising from polarization within solutions and from minor optical imperfections in the instrument, making the reversal of absorption cells unnecessary.

The spectrometer is provided with a screw drum, calibrated in wave lengths, by means of which the prism may be rotated in such a manner as to bring light of any desired wave length into the center of the field of vision. The eyepiece shutter may be opened wide enough to permit a view of nearly one-third of the visible spectrum or reduced to a narrow slit through which only a narrow

spectral range is visible to enable the eye to concentrate upon obtaining an exact match within that range.

The entire equipment is mounted on a single substantial base, which renders the accidental displacement of the separate units impossible or, at least, improbable.

The same instrument is provided with accessories which enable the analyst to utilize absorption cells in a horizontal position. An illuminating sphere may also be supplied with which the color of colored solid substances may be investigated by comparing the light reflected from their surfaces with identical light reflected from a standard white surface of magnesium carbonate. (The investigation of dyed materials will not be discussed in this bulletin.)

In measuring the absorption spectrum of a dye the wave-length drum is set at a definite wave length, the photometer circle rotated until the light in both visual fields is equal in intensity, and the extinction coefficient read from the circle. Further measurements are then carried out throughout the visible spectrum (or as long as appreciable absorption continues) at intervals of 10 mμ, with intermediate measurements in the region of the dye maximum or in any other portion of the spectrum wherein it may be desirable to bring out detail. If extinction coefficients are then plotted against wave length, a graphic representation of the absorption of the dye, or its absorption curve, is obtained.

In dealing with the absorption of dyes it is very convenient to express spectrophotometric values in terms of extinction coefficients (E). (The terms Bunsen extinction coefficient and absorptive index are somewhat more specific, but are less frequently employed.) The extinction coefficient (E) is the logarithm (expressed as a negative number) of the transmittancy (T). The latter (T) is merely the ratio of the intensity of the light emerging from the solution to the intensity of the light entering it. Such a ratio must necessarily fall between zero and unity. Its logarithm, accordingly, will be negative and will range between zero in the instance of complete transmission (T=1) and infinity when extinction is complete (T=O). The extinction coefficient is therefore a direct measure of the absorptive power of the solution.

In accordance with Lambert's law (*46*), values of E should increase in direct proportion with the thickness of the solution layer, and in accordance with Beer's law (*1*), they should also increase in direct proportion with solute concentration. Lambert's law is found valid whenever the photometric provisions and technic are satisfactory. Beer's law (*1*) also proves valid in instances in which the conditions are such as will exclude alteration in either the physical or chemical solute molecule.

The use of the spectrophotometer makes but little demand upon technical experience or skill on the part of the analyst. In the matching of the photometric field, in operating the instrument, the most common error on the part of the novice is that of too great deliberation. The average observer will find that he can obtain the most reliable data by making his matches rapidly. (It is assumed, of course, that such readings will be checked repeatedly before final acceptance.) The eye tires rapidly, and its sensitivity decreases greatly with fatigue. It is well for the analyst to match fields as

rapidly as possible and allow the eye a brief period in which to recover its normal sensitivity before attempting to duplicate the first match.

There are two factors which influence visual sensitivity in a decided degree which the analyst should bear in mind at all times. The ability of the eye to recognize differences in light intensity varies considerably both with the wave length of the light and with the degree of illumination. Visual sensitivity is greatest in the yellow-green portion of the spectrum, at about 560 mμ, and decreases somewhat rapidly as either end of the spectrum is approached. Data are available on the relative sensitivity of the average eye in different spectral regions, but it is questionable if they are entirely adequate to serve as a criterion in spectrophotometric practice. It is well for the analyst to determine for himself the spectral range within which his readings may be relied upon, and to determine also, the general order of variation in measurements which he may expect in other spectral regions.

In various practical applications of spectrophotometric data, which will be referred to subsequently, the analyst may have considerable latitude in respect to the wave lengths at which his measurements are carried out. In making his selection the factor of relative sensitivity should be given serious consideration. It may frequently prove advisable to select wave lengths which may involve some measure of disadvantage in other respects in order to effect improvement in the precision of the measurements.

The same consideration may influence the proper choice of solvents and conditions employed. The bands of dyes which may lie in spectral regions in which the eye is relatively insensitive, in such solvents and under such conditions as are ordinarily employed in their examination, may frequently be transposed into more favorable regions for examination by resorting to other solvents or other conditions.

The second factor which influences visual sensitivity is illumination. The eye is most sensitive to changes in light intensity over a limited photometric range, and accuracy suffers with both excess and deficiency of illumination. The analyst should determine for himself the photometric range within which his measurements are most trustworthy and confine his work to it. He may find, for example, that he reads most accurately when the extinction coefficients are approximately 1.00 and that his readings become somewhat less certain when values fall below 0.70 or exceed 1.30. He should, then, modify his technic in such a manner as will enable him to carry out all critical measurements within the photometric zone referred to. With the equipment which has been described, this may be effected readily by the adjustment of layer thickness.

In the outer portions of the visible spectrum, in which sensitivity is relatively poor, better results are obtained with more intense illumination. For this, and other reasons, it may occasionally prove advantageous to replace the heterogeneous light source with a mercury or helium vacuum-tube lamp for work in these regions. It is hardly possible to obtain reliable absorption values with certain yellow dyes unless this is done.

The analyst should know his dyes. He may obtain much information respecting their chemical constitution and general properties

from textbooks and color indices (*52*, *53*). Kayser's review of early spectroscopic investigation (*42*) and Formánek's tables of technical synthetic dyes (*6*) supply considerable detail on qualitative aspects of the absorption spectra of natural coloring matters and commercial dyes. Holmes's tables (*31*) are intended to supplement these sources and enable the reader to obtain whatever information may be available in the literature on both qualitative and quantitative aspects of the absorption of dyes in general. It will be seldom, however, that the analyst will be able to obtain definite information on dyes which will be thoroughly adequate for his purposes. He should know how the spectra of individual dyes are modified by various conditions; on the one hand, in order to avoid inadvertent modifications in absorption which may invalidate his results, and, on the other hand, in order to utilize such modifications in various applications. He will find it necessary, in general, to obtain such information for himself.

The visual method compares favorably in accuracy and reliability with photoelectric and thermoelectric methods over the greater portion of the visual spectrum (*9*). Photoelectric methods are somewhat more satisfactory in the extreme violet and thermoelectric methods in the extreme red.

In the practical applications of spectrophotometric data described subsequently it will be noted that the critical values employed for the most part are ratios. Their validity is conditioned upon relative accuracy in two measurements, rather than upon absolute accuracy of measurements in general. It is believed that this factor will render the employment of the methods less influenced by the type of equipment employed and by the personal equation than would otherwise be the case.

THE INFLUENCE OF CONDITIONS UPON ABSORPTION

It is advisable to discuss the influence which various conditions exert upon the absorption spectra of dyes before taking up any of the specific practical applications which may be made of such spectra. The principal factors which require consideration are (1) solvent, (2) dye concentration, (3) temperature, (4) colloidal state, and (5) hydrogen-ion concentration.

SOLVENT

The earliest investigators recognized that the absorption spectra of colored solutes were affected by solvents even when the solvents themselves were colorless. It was found that absorption bands of dyes occupied somewhat different spectral locations, in general, with each solvent employed in their examination. The rule was formulated by Kundt (*45*) that bands shift toward the region of longer wave length with increasing refractive indices in solvents. The controversy which ensued over the validity of this generalization and its possible interpretation is reviewed and discussed by Kayser (*42*).

Kundt's rule has subsequently been thoroughly discredited. The recorded instances in which the bands of dyes undergo displacement in the opposite direction to that postulated equal or outnumber those of conformity to the rule. Even among the instances in which the

behavior of dyes is in qualitative accordance with Kundt's rule, the degree of displacement with changes in solvent varies greatly with different dyes. The uniformity which would result if purely physical factors were operative is lacking in a conspicuous degree.

The influence of chemical factors upon the phenomenon in question, on the other hand, is readily apparent. Thus, Meek (*47*) has shown that the spectral position of the band of alizarin-cyanine in organic solvents is influenced by the chemical nature of the solvents, and Holmes (*16*) has pointed out that a definite correlation exists between the displacement of dye bands with change in solvents and the constitutional variations in dyes.

The solvents considered in the last-mentioned instance were water and alcohol. Dyes undergoing band displacement in the direction of longer wave length in passing from aqueous to alcoholic solutions were termed "alcopositive"; those exhibiting the reverse behavior, "alconegative." It was shown that a variety of types of substitutions in dyes exert a definite influence upon their behavior in the respect in question, with the effect depending, in some measure, upon the structure of the parent substance and upon the position in which substitution occurs. The effect of substitution within the amino groups in particular is clearly defined with dyes of a variety of constitutional types. Alkylamino substitution exerts an alconegative effect in diphenylmethane, triphenylmethane, rhodamine, azine, oxazine, and thiazine derivatives, whereas arylamino substitution operates in the contrary manner.

It was suggested that these phenomena may find explanation through the hypothesis that the absorption of dyes depends primarily upon the distribution of their residual affinities, and that with solutions of dyes the interplay of residual affinities of solute and solvent results in rearrangements of affinity within the dye molecule whereby their absorption is modified.

It is a logical deduction from this constitutive hypothesis that the behavior of dyes with change in solvent should prove highly individual. This is found to hold true. It appears very probable that the absorption of two dyes is never modified in precisely the same manner and degree in passing from one solvent to another. In instances in which this may appear to occur it seems probable that actual differences exist which are too slight for recognition by available means. In general, appreciable differences are observed. Advantage is taken of that circumstance in differentiating between dyes. When dyes have practically identical absorption maxima in one solvent it is often found that a resort to other solvents will develop sufficient differences in maxima locations to make it possible to distinguish between them.

The normal displacements of dye bands with changes in solvents are relatively small, in most instances amounting to 5 or 10 mμ. When extreme displacements of bands occur it appears probable that radical intramolecular changes have taken place involving definite valence rearrangements.

The displacements of absorption bands with change in solvent are usually accompanied by modifications in the intensity of absorption. In passing from dilute aqueous solutions to dilute alcoholic solutions of rosaniline chloride, for example, the dye maximum not only shifts

from approximately 542 to 548 mμ, but the extinction coefficients of the solutions at their maxima increase by approximately 25 per cent.

It appears probable that such modifications in intensity of absorption may be attributed, primarily, to the same rearrangement in residual affinities of the dyes which has been held responsible for the shifting of the band. In numerous instances, however, the operation of other factors may be recognized. Changes in solvents may modify tautomeric equilibria between dye forms or effect alterations in their state of molecular aggregation. In either instance the dye spectrum undergoes a corresponding modification.

In the specific instance cited, for example, a careful examination of absorption curves reveals the fact that in aqueous solutions of the dye a small proportion of the dye is present in an orange form and that in alcoholic solutions the dye exists entirely in the (normal) red form. It is evident that a part of the increase in absorption at the dye maximum, observed in passing from aqueous to alcoholic solution, is due to the effect of the change in solvent upon the equilibrium between the tautomeric forms of the dye. (P. 11.)

In this particular instance it appears improbable that colloidal change is a contributory factor. It is found that a variety of other salts of rosaniline, including the bromide, iodide, nitrate, sulphate, oxalate, citrate, and benzoate, behave in an analogous manner. They have a common molecular absorption in water, on the one hand, and a different common molecular absorption in alcohol, on the other hand. It appears improbable that such dissimilar compounds should all be equally colloidal in any given solvent, and the conclusion appears warranted that they all give true solutions in both water and alcohol, at all events at the great dilutions employed in spectrophotometric examinations. All the solutions in question, moreover, are spectrophotometrically stable. Their solutions give no indication of alteration in absorption on prolonged standing.

In the transition from solution in water to solution in chloroform, however, these same dye salts behave quite differently. Fresh solutions of rosaniline chloride, bromide, and iodide in chloroform absorb about 8 per cent more light than do their aqueous solutions, whereas fresh solutions of rosaniline acetate and benzoate absorb about 15 per cent less light than corresponding aqueous solutions. The chloroform solutions are unstable in each instance, and the absorption of the acetate and benzoate solutions decreases more rapidly than does that of the chloride, bromide, and iodide solutions. It seems evident that all these solutions are distinctly colloidal, and that the acetate and benzoate solutions are decidedly more colloidal than are the solutions of the other salts referred to.

Colloidal phenomena with dye solutions are discussed subsequently. (P. 14.) They frequently influence the changes in absorption which result from a change in solvent. In all quantitative applications of spectrophotometry, maximum molecular dispersion is favorable to accuracy, and consideration must be given to the selection of solvents which are favorable.

DYE CONCENTRATION

The fundamental basis of all colorimetry is formulated in Beer's law (*1*) which states in effect that the intensity of color (and absorption) in the solution of a colored solute is directly proportional to the

solute concentration. This law has been tested in innumerable investigations with many solutes and solvents and found relatively unreliable in a large proportion of instances, and the causes of its failure have been the subject of endless controversy. In the following discussion the consideration of its application will be confined to dye solutions.

Dyes are most commonly examined and analyzed in aqueous solution. It is found that comparatively few dyes conform rigidly to Beer's law over any very extensive range of variation in concentration in aqueous solutions, and the behavior of many dyes is decidedly anomalous over even limited ranges of dilution. Such abnormalities in behavior were formerly attributed to electrolytic or hydrolytic dissociation, solvation, or molecular aggregation of solute particles. It seems probable, however, that molecular tautomerism is more frequently the most important factor concerned.

One common type of dye in particular is susceptible of alteration of an extreme order. It has been shown by Holmes (*15*) that aminated dyes of a large variety of constitutional classes undergo radical modifications of absorption spectra with change in concentration in aqueous solution. The typical phenomenon is one of progressive transition between two definite bands of widely separated spectral location. It may be illustrated by a graph showing the absorption of Nile blue 2B at various dilutions. (Fig. 4.) Analogous data have been obtained with more than 100 other aminated dyes.

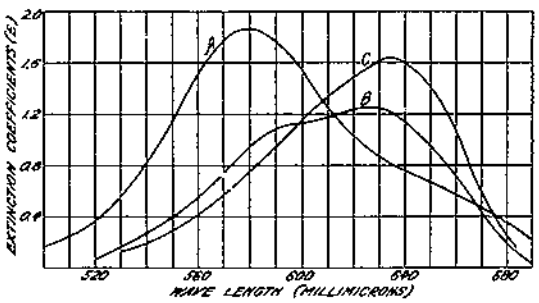

FIGURE 4.—Absorption spectra of Nile blue 2B at various aqueous dilutions: A, 665 mg of dye per liter (0.043-cm layer); B, 20 mg of dye per liter (1-cm layer); C, 0.8 mg of dye per liter (20-cm layer)

Such dyes apparently exist in two different structural forms. Of these forms one exists in the solid dye, and the second is found in alcoholic solutions irrespective of the dye concentration of the solution. In aqueous solutions both forms are found in a state of equilibrium, which alters as the solutions undergo dilution.

The equilibria appear practically unaffected by variation in temperature, hydrogen-ion concentration, or neutral salt concentration. They are decidedly affected, however, by the addition to the aqueous solutions of a variety of organic substances which have diverse chemical characteristics but have the common attribute of unsaturation (*27*). The influence of such substances appears to increase with the degree of their unsaturation. Thus, the effect of allyl alcohol is greater than that of isopropyl alcohol, and the effect of m-phenylenediamine greater than that of its hydrochloride. In a general way these substances exert the same influence upon aqueous solutions of the dyes as does simple aqueous dilution.

It seems probable that this phenomenon is another instance in which the interplay of residual affinity in solute and solvent results in affinity rearrangements within the solute molecule. The modification in absorption is so considerable, in general, that a definite readjustment in valence arrangement seems indicated. A hypothesis, which need not be discussed here, has been suggested respecting the nature of this readjustment (*23*).

It is obvious that these phenomena are of practical as well as of theoretical interest. The dilution ranges in which they occur often coincide with the working ranges in colorimetric practice. In such instances they invalidate Beer's law and give rise to anomalous results. In any application of spectrophotometry to aqueous dye solutions, the analyst should bear in mind the possible interference which may arise from this source. Practical means of obviating the difficulty are discussed on page 28.

The influence of constitutional factors in dyes upon the phenomenon of the modification of their spectra with the dilution of their aqueous solutions is clearly evident. Substitutional variations in dyes may decidedly influence their behavior in the respect in question. Crystal and benzyl violets supply an excellent illustration (*21*). The measurement of dilution effects, accordingly, may sometimes serve as a valuable means of differentiating between similar dyes.

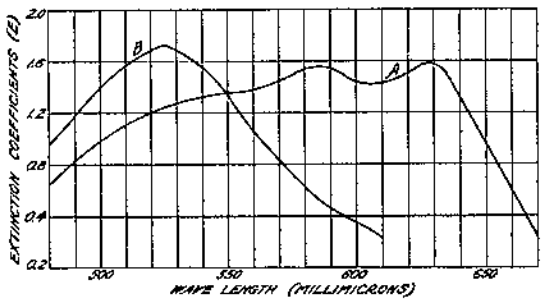

FIGURE 5.—Absorption spectra of Erie violet 3R in aqueous solution (80 parts per million): A, solution prepared with cold water; B, same solution after heating

TEMPERATURE

When Erie violet 3R (NAC) is dissolved in cold water the resulting solution is heterogeneous, containing preponderant proportions of blue and of violet components in addition to the dye proper, which is red. If the solution is then heated, however, or if alcohol is added, the blue and violet components are completely transformed into the dye proper and can not be regenerated subsequently (*12*). (Fig. 5.) It is believed that the blue and violet substances are intermediate compounds in the formation of the true dye. Analogous effects of a less extreme order have been observed with certain other azo dyes, but this type of irreversible alteration has not been reported with dyes of other classes.

It is probably very seldom that the heating of a dye solution results in actual decomposition of the dye. Dye manufacturers warn their customers to exercise caution in resorting to heating in the preparation of solutions of basic dyes, but the basic dyes of some classes appear to be very stable to heat. With basic dyes of other classes, however, the molecule is susceptible to attack at its substituted amino groups. Dealkylation may occur at these points, and amino groups may even, in some instances, be replaced by hydroxyl groups. These transformations proceed very slowly, except under distinctly alkaline

conditions, but are considerably accelerated by heating. Probably few basic dyes would undergo any appreciable degree of alteration of this type in the ordinary course of preparing their solutions by heating, but it is doubtless advisable to bear that possibility in mind.

Only one dye which finds any extensive application is notably unstable to heat. Auramine undergoes hydrolysis very readily in aqueous solutions, particularly under acid conditions (*33*). Solutions of auramine should never be warmed.

Sheppard has observed a very striking type of temperature effect with photosensitizing dyes of the cyanine group (*54*). Increase in temperature gave rise to a progressive transition between one dye band in one portion of the spectrum and a different band in a widely separated location. This alteration was accompanied by changes in the state of solute aggregation and was completely reversible, the original characteristics of the solution being restored when the solutions were cooled. This reversible type of behavior does not appear to have been noted with dyes of other classes.

Radical alterations of dye spectra, however, whether of irreversible or reversible type, are rarely observed in varying the temperature of dye solutions. In general, temperature exerts only very minor effects upon the absorption of dyes.

G. and H. Krüss (*44*) point out that decided changes in temperature may give rise to appreciable errors through their effect upon the optical constants of spectrophotometric equipment. With Bremer (*2*) they investigated the effect of considerable variations in temperature upon the absorption of some 20 dyes. They observed small displacements in the spectral location of bands, occurring in both directions and averaging less than 3 mμ over the extreme temperature range of 60° C., and they also noted appreciable effects upon absorption intensities.

Gibson, McNicholas, Tyndall, Frehafer, and Mathewson (*9*) obtained relatively uniform data with five food dyes which indicate a decrease in absorption over the range between 25° and 40° C. of approximately 2.1 per cent. (Aqueous expansion over this range would account for about 0.5 per cent of the decrease.) An opposite effect was noted with light green SFY, which may probably be attributed to other factors. Their data give little or no indication of band displacements. They concluded that such temperature effects as might be met with in ordinary practice would be insufficient to result in any appreciable inaccuracies in the spectrophotometric examination of the dyes in question.

This conclusion is supported by a limited number of data obtained at this laboratory, principally with azo dyes. It seems probable that the typical temperature effect is a small decrease in absorption unaccompanied by any decided band displacement. The minor variations in temperature incidental to ordinary practice may probably be disregarded, even when solvents are employed with higher coefficients of expansion than that of water.

In spectrophotometric applications with indicators, however, the influence of temperature change upon the dissociation constants of the indicators should be borne in mind, and variations in temperature minimized.

COLLOIDAL STATE

The hypothesis of Stenger (*55*) that radical alterations in the spectral position of absorption bands may be attributed to variations in the size of the physical molecule does not appear tenable in the instance of dyes.

Radical modifications of absorption spectra have, indeed, been found to accompany changes in the state of molecular aggregation in some instances. Apart from the phenomena observed with dyes of the cyanine group, already referred to (p. 13), the instance of indigotine monosulphonate (*14*) may be cited. Such radical modifications of absorption as those are seldom found associated with colloidal change. It is evident that the alteration in both absorption and in colloidal state in such instances must be attributed to molecular tautomerism or some other factor.

It was shown by Pihlblad (*51*) in a study of five selected dyes under a variety of conditions which influence colloidal state, in which spectrophotometric data were correlated with ultramicroscopic measurements, that the spectrophotometric criterion of increasing molecular aggregation is a symmetrical flattening of the absorption band, in which the maximum is depressed and the lower slopes broadened, without appreciable displacement of the band or of its maximum. The same conclusion was reached by Holmes (*15*) in the course of an investigation of dilution effects with aqueous solutions of many dyes. Typical curves in illustration of this effect are given in Figure 6.

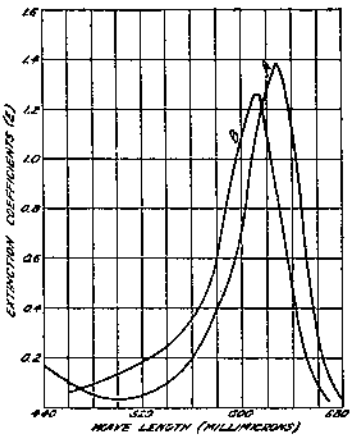

FIGURE 6.—Effect of dilution on the absorption spectra of aqueous solutions of pontacyl green S: A, Very dilute solutions; B, concentrated solutions

The influence which the solvent may have upon the colloidal state of dye solutions (p. 10) has been discussed.

Dye concentration may influence colloidal state in a decided degree. An extreme instance has been reported (*28*) in which the absorption of an aqueous solution of rosaniline phenolate increased by about 28 per cent on dilution with an equal volume of water, instead of decreasing by 50 per cent, as would, of course, be anticipated through the normal operation of Beer's law. It seems probable, however, that with dyes in general such variations in dye concentration as are incident to ordinary spectrophotometric technic will seldom exert any appreciable effect upon the state of solute aggregation.

Neutral salts may modify the spectra of dyes through their effect upon the colloidal state of the solutions. Typical effects are illustrated in Figure 7. The salt effects recorded are immediate ones. Much more decided effects are observed upon the prolonged standing of the solutions.

The effect of such quantities of inorganic salts as may be found associated with dyes in commercial products will be negligible, owing

to the great dilutions at which spectrophotometric examinations of dye solutions are carried out. When, however, the use of buffered solutions is resorted to for the purpose of regulating the hydrogen-ion concentration the dye spectra may sometimes be affected to an appreciable degree.

HYDROGEN-ION CONCENTRATION

Most dyes are stable within the zone of approximate neutrality. Nearly all dyes, however, are modified in constitution, color, and absorption by sufficient concentrations of either acids or alkalies. They are, accordingly, potential indicators of hydrogen-ion concentration, although few of them possess exceptional qualifications for practical application as indicators.

The behavior of dyes with acids and alkalies is influenced by their general structural features and their specific minor constitutional variations. Even minor variations in dyes frequently give rise to decided difference in their dissociation ranges. The measurement of the effect of suitable regulated variations in hydrogen-ion concentration upon the absorption of dyes, accordingly, is often of great service in enabling the analyst to distinguish between them. (P. 19.)

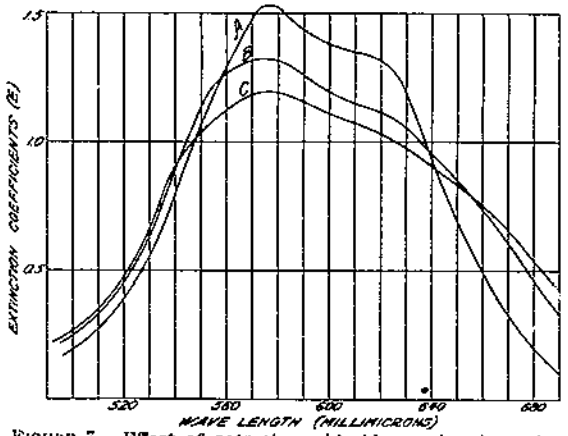

FIGURE 7.—Effect of potassium chloride on the absorption spectra of aqueous solutions of night blue (dye concentration=1 part in 50,000 parts water): A, In distilled water; B, with 0.5 per cent potassium chloride; C, with 1 per cent potassium chloride

The question of the advisability, or necessity, of buffering solutions of dyes intended for spectrophotometric examination, in order to insure a definite hydrogen-ion concentration, is one which may, perhaps, give rise to considerable difference of opinion. It is doubtless necessary to "regulate" the solutions of some dyes in that manner (*17*). In other instances it is unnecessary and entails unfavorable effects. The authors prefer to prepare dye solutions in general for spectrophotometric examination without the addition of buffer agents and to regulate solutions of only such dyes as have been found to be distinctly influenced by minor variations in hydrogen-ion concentration within the zone of approximate neutrality.

It may sometimes prove decidedly advantageous to resort to definite acid or alkaline conditions in the examination of dye solutions in order to transpose absorption bands in the spectrum. This may enable the analyst to carry out measurements in a spectral region in which vision is more sensitive than it is in the region in which he would have to make his measurements on neutral solutions of the same dye.

THE IDENTIFICATION OF DYES

The practical dye tester relies, in part, upon such systematic chemical schemes as that of Green (*10*) and in part upon such reactions and alterations in color with chemical reagents as are cited by Schultz (*53*) or the Colour Index (*52*) for the identification of dyes. He usually has a comprehensive collection of dye samples available for direct comparison, and may sometimes resort to comparative dyeings as a final step in his method. He often acquires great proficiency in the rapid identification of the majority of dyes of principal textile importance.

The differentiation obtained by such means, however, is approximate rather than precise. Even when color reactions are carried out under well-regulated conditions (*32*), they frequently yield results which resemble so closely, as far as unaided visual observation is concerned, those of closely related dyes, that they fail to accomplish the desired purpose. In customary usage they prove notably ineffective. The average dye tester finds it difficult or impossible to distinguish between methyl and crystal violets, for example, or between guinea green B and light green SFY. His rough identifications may prove sufficient for his immediate practical requirements. They are, however, inadequate for scientific purposes.

The resort to more elaborate chemical methods of identification is seldom entirely effective. Chemical methods usually enable the analyst to distinguish readily between dyes which differ radically in structure, but do not ordinarily afford any decisive differentiation between products which differ only in minor constitutional aspects. With dyes in general a more positive identification may be obtained readily by spectroscopic means than is possible by relatively laborious chemical investigation.

With azo dyes only may the superiority of spectroscopic methods of identification be open to question. The absorption spectra of azo dyes, as a class, are somewhat less distinctive than those of other dyes in general, while an exceptionally effective means of chemical investigation is applicable with azo dyes. They may be broken down into their component parts by reduction, yielding the intermediates employed in their manufacture or simple derivatives of those intermediates. The identification of these scission products will serve, in turn, to identify the dyes from which they were derived. Holmes (*20*) has supplied a digest of such data as are available for the purpose. The ability to identify a few hundred amino derivatives, accordingly, will qualify the analyst to identify positively a vast number of azo dyes.

Although this method is one of great possible utility, it appears somewhat doubtful whether it will find very extensive employment. Both the isolation of the reduction products in a suitable condition for identification, and their subsequent positive identification will frequently prove difficult. The labor involved and the technical ability required seem likely to restrict the general use of this method.

There are indications that this reduction method for azo dyes may eventually develop, in large measure, into one of indirect spectroscopic examination. One of the best general means of identifying the reduction products, and one which is increasing in favor, con-

sists of converting them into azo dyes by coupling them with suitable intermediates, and identifying the resulting dyes with the spectroscope. Peter, Mayer, Wegemann, and Marshall (*5*) and Brunner (*4*) supply considerable data suitable for that purpose in some of the most important recent contributions to the analysis of azo dyes.

The direct examination of azo dyes by spectroscopic methods is far less difficult and laborious than is the resort to reduction methods. It is probably nearly as effective and reliable in most instances. It would appear preferable, except in instances in which it has been found to be ineffectual or in which the requisite spectroscopic data are unavailable and unobtainable.

With the dyes of other groups, spectroscopic methods almost invariably prove much superior to chemical methods in respect both to convenience and decisiveness. They have the added advantage of being applicable to extremely minute quantities of material.

Spectroscopic methods may be either spectroscopic, in the restricted sense of the term, or spectrophotometric.

THE SPECTROSCOPIC METHOD

The spectroscopic method of dye identification was developed by Formánek (*6*) through comprehensive study of the light absorption of dye solutions extending over years.

The general characteristics of the spectra of dyes, together with the manner in which they underwent modification with changes in conditions, were found to depend upon the more fundamental structural aspects of dyes. Distinct minor variations in absorption were found with even minor constitutional variation, and usually they proved sufficiently definite to enable the analyst to distinguish between even closely related dyes.

In examining dyes the general type of spectrum and the influence upon it of solvent and dilution were noted. The locations of absorption maxima were determined with great care with a variety of solvents and conditions. Vat dyes were investigated in xylol, tetralin, sulphuric acid, and sulphuric-boric acid. Certain of these solvents were found useful in the examination of dyes of other classes, but with dyes in general the observations were usually carried out on neutral, slightly acid, and slightly alkaline solutions in water, ethyl alcohol, amyl alcohol, and 90 per cent acetic acid.

Part I of Formánek's work (in collaboration with E. Grandmougin and subsequently with J. Knop) is concerned primarily with descriptions of equipment and technic and discussions of such matters as the correlation of color and constitution. Subsequent sections consist largely of compilations of data and incidental information for hundreds of dyes of commercial grades. Sections I and II of Part II deal with soluble green, blue, and red dyes in general; Section III with yellow dyes, and Section IV with vat dyes. Within each general category the subtabulation is based upon the general type of the absorption spectrum and its behavior with changes in conditions.

In identifying a dye of unknown character with the aid of Formánek's tables, the spectroscopist determines the subtable in which it

should be found, and compares the absorption maxima of his unknown sample with those of the various known dyes recorded in it. If his values are in close agreement with those of a known dye, he is justified in concluding that the two dyes are identical beyond reasonable doubt.

The value of the recorded data for purposes of identification necessarily varies with different dyes. The spectra of the dyes of some classes are much less characteristic, in general, than are those of other classes. Individual dyes are encountered, irrespective of dye class, more particularly among yellow, brown, or black products, which absorb light in a manner which is practically devoid of distinctive features. In most instances, however, Formánek's data are reasonably satisfactory for the purpose in view.

The comprehensive character of the tables may be emphasized. It is inevitable that such compilations can not be completely exhaustive, as new dyes are constantly appearing on the market. It is comparatively seldom, however, that dyes are met with in practice which are not to be found in Formánek's tables.

The Formánek system of identification is both rapid and convenient in operation. The proportion of dyes with which it proves useless or seriously ineffective is relatively small, as is that of dyes with which the data necessary for its application have not been provided. It is unquestionably the most valuable means available at present for identifying the majority of dyes with comparative ease and certainty.

The principal objections which can be advanced against the Formánek spectroscopic method are that it places nearly complete reliance upon precise determinations of absorption maxima and that the differences in location between maxima in closely related dyes are often too small to serve as an entirely satisfactory means of differentiation. The precise determination of maxima, moreover, is a matter of considerable difficulty, in which the results obtained may be influenced by the type of equipment used, by the technic employed, and by the personal equation. Commercial products, finally, frequently contain varying proportions of subsidiary coloring matters which may modify their maxima in some degree.

The analyst will frequently find that his data on samples of known identity do not conform exactly with the data of Formánek's tables. He can not rely implicitly, accordingly, upon a comparison of his values with those of Formánek as a means of definitely establishing the identity of his samples of unknown character.

THE SPECTROPHOTOMETRIC METHOD

The spectrophotometric method of dye identification was developed in the Color and Farm Waste Division in the hope and expectation of reducing the uncertainties referred to.

Constitutional variation in dyes influences not only the position of their bands but also the manner in which their spectra undergo alteration with changes in solvent, hydrogen-ion concentration, dilution, and other conditions. The degree of such alteration in consequence of definite modification in conditions is characteristic for individual dyes. It may be determined by measuring extinction coefficients at suitable wave lengths, and may be expressed in terms

of the ratio of such coefficients. In the spectrophotometric method such ratios are employed to supplement data on absorption maxima, and are usually found to afford a much more decisive means of distinguishing between similar dyes than are maxima.

The method has been tested with a group of closely related dyes of the patent blue type (*13*) with one of basic fuchsines (*19*) and with one of basic triphenylmethane violets (*21*).

It may be illustrated by means of the following condensed tabulation of data (Table 1) obtained with certain of the more closely related acid dyes of the patent blue class.

TABLE 1.—*Spectrophotometric constants of dyes of the patent blue type*

Dye	λ [1] in—			Ratio of E at λ [1]—		
	91 per cent alcohol	Neutral aqueous solution	Alkaline aqueous solution	In concentrated aqueous solution to E at λ in dilute aqueous solution	With 0.1 per cent NaOH (after standing) to E at λ in neutral aqueous solution of same dye concentration	With 2 per cent H_2SO_4 to E at λ in neutral aqueous solution of same dye concentration
	mμ	mμ	mμ			
Patent blue A (C. I. No. 714)	630	637.5	627.5	0.76	0.58	0.30
Patent blue V (C. I. No. 712)	630	637.5	627.5	.98	.85	(2)
Alphazurine A [3]	630	637.5	637.5	.77	(2)	.24
Xylene blue AS (C. I. No. 763)	630	637.5	637.5	.79	(2)	.315
Xylene blue VS (C. I. No. 672)	632.5	640	640	.97	.21	(2)
Alphazurine 2G [3]	631	640	639	.90	.34	(2)
Alphazurine (C. I. No. 671)	628	630	630	.81	(2)	.37

[1] λ denotes the wave length of maximum absorption.
[2] Nil.
[3] Alphazurine A and alphazurine 2G have ethylbenzyl and diethyl substitutions, respectively, at the amine groups and a 2,5-disulpho substitution in their nonaminated nuclei.

The values of the second, third, and fourth columns are approximate absorption maxima of the dyes in (1) alcoholic, (2) neutral aqueous, and (3) alkaline aqueous solutions. Those of the last three columns are spectrophotometric ratios, or ratios of extinction coefficients at suitable wave lengths measured under definite variations in conditions. Their variations in value afford a quantitative index of alteration in the dyes under those conditions. The values in column 5 are affected by tautomeric alteration in the dyes resulting from measured variation in dye concentration. Those in column 6 measure resistance to decolorization by alkali, the recorded data indicating the proportion of the dye which withstands the effect of 0.1 per cent of sodium hydroxide over an extended period. Those in column 7 indicate the proportions of dye which withstand immediate conversion into yellow polyacidic derivatives with 2 per cent sulphuric acid. The original paper should be consulted for further detail.

The general principle of such ratio application may be clarified by a more detailed illustration of the significance of the values recorded in column 7. The curves of Figure 8 enable the reader to visualize the effect of acid upon pontacyl blue A (which is identical

with the xylene blue AS referred to in Table 1). The effect of 2 per cent sulphuric acid is measured by dividing the extinction coefficient of an acid (2 per cent) solution of the dye at 637.5 mμ by the extinction coefficient of a neutral solution containing the same quantity of dye at the same wave length. The other dyes of the group undergo analogous transformations, but their degree of transformation at any definite hydrogen-ion concentration is different.

It is of interest to note that such constitutional variation as is found in this group of dyes has a decidedly greater effect upon the general properties and behavior of the dyes than upon their color. The utility of the ratios for differentiation arises from that circumstance.

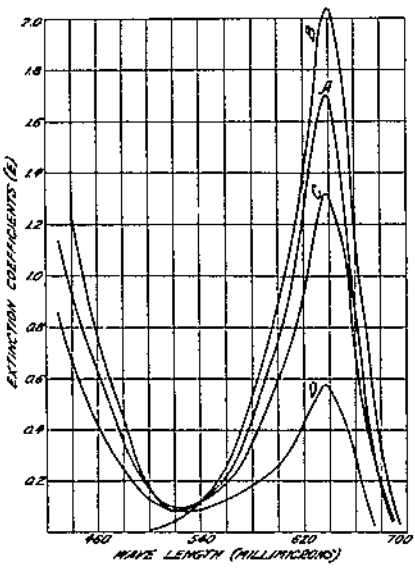

FIGURE 8.—Effect of sulphuric acid upon pontacyl brilliant blue A; A, 40 parts of dye per 1,000,000 parts of water; B, 100 parts of dye per 1,000,000 in 1 per cent sulphuric acid; C, 100 parts of dye per 1,000,000 in 2 per cent sulphuric acid; D, 100 parts of dye per 1,000,000 in 4 per cent sulphuric acid

Although any constitutional variation doubtless exerts some influence upon the position of the band in the spectrum, the effects in these instances are relatively small. Leaving out of consideration alphazurine FG, which differs from the remaining dyes in both the number and nuclear position of its sulphonic acid groups, it will be seen that the absorption maximum is but little affected by (1) variation in the relative position of sulphonic groups within the nonaminated nucleus, by (2) the introduction of a hydroxyl group therein, or even by (3) the type of amino substitution; that is, whether the diethyl or the ethylbenzyl substitution is involved.

On the other hand, the influence of these constitutional factors is plainly evident in the ratio values. Upon dilution the dyes with diethyl amino substitution behave very differently from those with ethylbenzyl substitution. Their ratios (four) range from 0.96 to 0.98 as against a range of 0.76 to 0.79. They are also decidedly more stable to alkali and less stable to acids. The introduction of a hydroxyl group into the nonaminated nucleus confers indicator properties. It has little effect upon stability to acids but increases stability to alkali materially. Within the nonaminated nucleus the 2,4-disulpho substitution is more stable to acidity and less so to alkalinity than is the 2,5-disulpho substitution.

With these dyes the ordinary chemical methods of differentiation are practically useless. Identification on the basis of absorption maxima alone is also unsatisfactory. It would probably prove both difficult and uncertain even for an expert provided with the most favorable equipment for the purpose.

The value of the recorded ratios for differentiating between the various dyes will be obvious. Xylene blue VS may be distinguished from alphazurine 2G by means of its behavior under alkaline conditions, alphazurine A from xylene AS through its behavior under acid conditions, and patent blue A and V behave very differently with both alkalies and acids as well as upon mere dilution. In each of these instances it is extremely difficult to distinguish with any certainty between the dye pairs referred to by means of absorption maxima alone.

Such ratios may be determined with relative ease and substantial accuracy with any type of spectrophotometer, and the results obtained are independent in large measure of the equipment employed, of the technic followed, and of the skill of the analyst. There can be no doubt that they will be found a very valuable aid in the identification of dyes in general, and will frequently afford a more reliable and precise differentiation between similar dyes than is possible by other means.

ABSORPTION RATIOS

There are definite limitations to the accuracy with which it is possible to determine the locations of absorption maxima on the basis of a mere direct comparison of extinction coefficients. The analyst determines that the maximum must lie between certain limits, say, between 540 and 550 mμ. It is seldom, however, that the spectrum is sufficiently well defined to enable him to state positively that the maximum falls at a definite wave length, say at 545 mμ. The heads of dye bands are usually somewhat flat. In most instances in which he reports a value of 545 he is by no means sure that the true value may not be, rather, 544 or 546 mμ. In some instances even less definite conclusions are possible.

In applying the spectrophotometric method of identification to basic violets of the triphenylmethane group (*21*), determinations of maxima were supplemented by determinations of a spectrophotometric ratio of a type different from those hitherto referred to. Wave lengths were selected on opposite slopes of the bands of the dyes and ratios of extinction coefficients of solutions at these points obtained. Although the conditions chosen in that particular instance were not ideal for the purpose, it was found that the ratios obtained provided a much better means of distinguishing between the dyes in question than did the dye maxima.

The term "absorption ratio" was adopted, subsequently, for a ratio of this type. It may be defined as the ratio of the extinction coefficients of a solution of a colored substance at two specific wave lengths. If the wave lengths in question are selected on opposite sides of the absorption maximum, the absorption ratio defines the spectral location of the absorption band. If they are selected on the same side of the maximum (as may be necessary in visual measurements on yellow dyes) the absorption ratio defines the gradient of the slope of the band within the region of measurements. The slope of an absorption band is usually less characteristic than its spectral location.

The application of the absorption ratio may be seen by reference to Figure 9.

The curves represented are related in the manner which is usual between curves of dyes of similar structure which differ only in minor constitutional aspects. Dyes A, B, and C may be distinguished by their maxima, which are, respectively, 590, 595, and 600 mμ. They may also be distinguished on the basis of absorption ratios. If the ratio adopted be that of extinction coefficients of their solutions at 580 mμ to those at 610 mμ, the ratio values of A, B, and C will be found to be, respectively, 0.88, 1.13, and 1.48. The range of variation in absorption ratio values is much greater than that in maxima values.

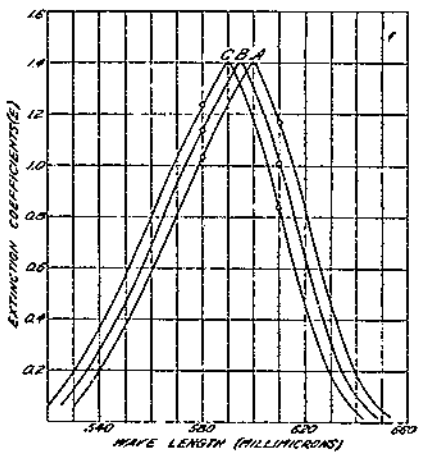

FIGURE 9.—Representative absorption spectra of closely related dyes

In the particular instance illustrated it would not be difficult to differentiate between the dyes on the basis of their maxima, but the heads of the bands of dyes are sometimes so poorly defined that it may be difficult to distinguish between maxima even when bands are as widely separated as those illustrated. Even when dealing with dyes which have clean-cut spectra, the maxima method has definite limitations. If the existence of a fourth dye, D, be assumed, the band of which lies 1 mμ farther toward the region of longer wave lengths than does that of dye B, it is evident that its maximum will fall at 596 mμ and that it will be difficult or impossible to distinguish between it and that of dye B with any certainty. Its absorption ratio value, however, will be 1.20, which can be distinguished readily from 1.13, the ratio value of dye B.

Holmes and Peterson (*34*) have tested the value of absorption ratios as a means of identification in an investigation of 90 dyes employed in biological staining. (Table 2.)

TABLE 2.—*Absorption ratio of various dyes* (*33*)

Dye	Color Index No.	Solvent [1]	Wave lengths	Absorption ratio [2]
			mμ	
Fluorescein (sodium salt)	766	+5 drops 1 per cent Na_2CO_3	500, 530	36.5
Auranin	655			30.0
Martius yellow	0			21.0
Orange G	27			5.46
Resorcin yellow	148			3.61
Chrysoidin Y	20			2.83
Chrysoidin R	21			2.63
Sudan I	24	95 per cent alcohol		2.49
Bismark brown Y	331			2.20
Orange II	151			1.95
Orange IV	143	+10 drops 6N.HCl		1.75
Bismark brown R	332			1.58
Congo red	370			1.22
Purpurin 4B	448			1.19
Vital red	456			1.17

See footnotes end of table.

TABLE 2.—*Absorption ratio of various dyes* (*33*)—Continued

Dye	Color Index No.	Solvent [1]	Wave lengths	Absorption ratio [2]
			mμ	
Sudan III	248	95 per cent alcohol		1.10
Crystal ponceau	89			1.04
Biebrich scarlet	280			1.00
Orange I	150	1 per cent Na_2CO_3		1.00
Fast red A	176		500, 530	.97
Methyl orange	142	+10 drops 6N.HCl		.97
Sudan IV	258	95 per cent alcohol		.94
Erio garnet B	375			.94
Bordeaux B	88			.88
Dibromofluorescein		+5 drops 1 per cent Na_2CO_3		3.84
Mercurochrome		do	505, 535	2.83
Eosin	768	do		.03
Janus red	266		510, 540	1.12
Amaranth	184			1.07
Eosin B	771	+5 drops 1 per cent Na_2CO_3		1.41
Phenosafranin	810			1.13
Erythrosin	773	+5 drops 1 per cent Na_2CO_3		1.01
Safranin	841			1.02
Phloxin B	778	+5 drops 1 per cent Na_2CO_3	515, 545	.97
Methyl eosin	769	do		.96
Ethyl eosin	770	do		.86
Neutral red	825	+5 drops acetic acid		.75
Methylene violet 2R	842			.61
Rhodamin S	743			1.51
Pararosanilin	676			1.13
Neutral violet	826	+5 drops acetic acid		1.05
Magenta II				.90
Rosanilin				.89
Acid fuchsin	692	95 per cent alcohol	530, 560	.81
Pyronin G	739			.80
Rhodamin G	750			.78
New fuchsin	678			.76
Rhodamin B	749			.68
Pyronin B	741			.52
Rose Bengal 3B	779	+5 drops 1 per cent Na_2CO_3	540, 570	.92
Alizarin red S	1034	N. NaOH		1.21
Alizarin	1027	do	545, 575	.81
Chrome black F	200			.94
Magdala red	857			.90
Meldola's blue	909		560, 590	1.13
Methyl violet	680			1.405
Iris violet	847			1.29
Benzyl violet	683			1.14
Aniline blue (spirit soluble)	689		575, 605	1.08
Crystal violet	681			1.065
Nigrosin (water soluble)	865			1.01
Ethyl violet	682			.91
Thionin	920			1.44
Cresyl violet (Grübler)				1.20
Aniline blue (water soluble)	707			1.08
Trypan blue	477			1.08
Niagara sky blue	520		590, 620	.99
Methyl blue	706			.97
Methylene violet (Bernthsen)				.835
Indigotin	1180			.79
Victoria blue B	729			1.18
Night blue	731			1.04
Cresyl blue (NAC)	877		605, 635	.90
Malachite green	657			.83
Fast green FCF				.76
Guinea green B	666			.72
Brilliant green	662			1.51
New methylene blue N	927			1.21
Cresyl violet (NAC)				1.16
Toluidin blue O	925		620, 650	1.04
Light green SFY	670			.98
Methyl green	684			.91
Nile blue A	913			.90
Methylene green	924			1.12
Janus green	133			.97
Nile blue 2B	914		635, 665	.93
Methylene blue	922			.59
Thionin blue	920			.51
Capri blue GON	876		650, 680	1.27

[1] Unless otherwise specified, the solvent used was 50 per cent water and 50 per cent of 95 per cent alcohol. Any addition to the standard solvent is indicated by +.

[2] The absorption ratio was calculated by dividing the extinction coefficient obtained at the shorter wave length specified by that obtained at the longer wave length.

In general, the solvent employed was 50 per cent water and 50 per cent of 95 per cent alcohol. (P. 29.) (As was to be anticipated, the ratios proved less satisfactory when dealing with yellow, orange, or brown dyes than with dyes of other colors, and less satisfactory with azo dyes than with the dyes of other classes. The accuracy with which they could be determined depended, naturally, upon relative visual sensitivity within the region in which it was necessary to undertake the measurements. Under the least favorable conditions encountered results varied by as much as 1 or 2 per cent, but an excellent agreement in values was generally obtained.

It was found that absorption ratios alone were ample for the purpose of differentiating between the dyes investigated, and unquestionably they provide an exceptionally convenient and valuable means for identifying dyes in general, although it may prove advisable to resort to other methods for corroborative evidence.

With some of the dyes examined, different samples from different sources proved substantially uniform. With numerous dyes, however, different samples gave some diversity in values. Obviously the nature of the absorption ratio is such that its values with any given dye will usually be affected by the presence of other dyes. Most commercial dyes contain varying proportions of subsidiary coloring matters which have ratio values decidedly different from that of the dye with which they are associated. Such color impurities modify the ratio values of the dyes with which they occur in a degree corresponding with the proportion in which they are present.

It might appear that this circumstance would invalidate the use of absorption ratios, or would at least detract seriously from their practical utility for purposes of identification. It seems probable, however, that the proportion of subsidiary dyes will seldom be large enough to obscure the identity of the principal dye. The differences in ratio values between different dyes are so considerable, in general, that the danger of erroneous conclusions is small. The analyst will usually recognize the dye with which he is dealing and realize that he has to do with an abnormal sample of that dye.

The same circumstance, on the other hand, is of great service to the discriminating analyst who is interested in the nature of his samples, apart from the mere identity of the principal dye present. The absorption ratio is an excellent criterion of dye purity, in the restricted sense of purity from contamination with other coloring matters. The deviation of values may frequently enable the analyst to estimate the proportion of subsidiary dyes present within somewhat narrow limits. (P. 34.)

A simple absorption ratio, accordingly, which may be determined easily within a few minutes, is not only an effective means for establishing the identity of dyes, but at the same time it may also furnish considerable information respecting their purity, which could be obtained by other means only with difficulty, if at all.

Absorption ratios have also been found of value as a criterion of dye purity in the synthesis and purification of dyes in the Color and Farm Waste Division. They may be determined readily with material at any point in the process, and they enable the chemist to follow the course of his operations intelligently. If recrystallization effects the removal of a subsidiary dye, for example, the absorption ratio

will indicate that fact and will also inform the chemist when the elimination of the impurity in question has been made as complete as is possible by the means employed. No other criteria are as easy to apply for the purpose or have as wide a range of application.

It will be obvious that the methods of dye identification outlined are applicable not only with dyes but also with a great variety of colorless substances which are of such a character as will enable the analyst to convert them readily into colored substances. The phenols, as a class, supply an excellent illustration. They were converted into phthalein dyes by Gsell (*11*) and by Formánek and Knop (*7*); into azo dyes by Palkin and Wales (*50*, *57*), and into indophenols by Gibbs.[3] In each instance spectroscopic means were employed to identify the resulting dyes. Their identification serves, in turn, to identify the phenols used in their preparation.

THE SPECTROPHOTOMETRIC EVALUATION OF DYES

INDIVIDUAL DYES

The employment of the spectrophotometer for the quantitative estimation of dyes is very simple in theory. The Bunsen extinction coefficients of any dye solution vary in direct proportion with the concentration of dye. When standard absorption data have once been obtained on solutions containing a known concentration of a dye the analyst can determine readily the concentration of other solutions of that dye (in the same solvent) on the basis of comparison of its absorption data.

This statement is completely valid, of course, only in instances in which the normal operation of Beer's law is the sole determining factor. It has already been pointed out that a variety of other factors may be encountered in general practice which influence the absorption of dyes. It is obvious that these may give rise to appreciable errors in the evaluation of dyes unless the technic followed and the conditions provided are such as will preclude such a possibility.

The first requirement for spectrophotometric analysis is that of standard data. In some instances, perhaps, the analyst may be concerned merely with relative values, as is the practical dye tester. In general, however, he will require absolute data. It is impossible to calculate the extinction coefficients which solutions of a given dye will have. They have to be determined experimentally.

Although some data are available in the literature on the quantitative absorption of certain dyes, they seldom can be relied upon, unfortunately, for purposes of quantitative analysis. Even when otherwise adapted for analytical needs, they are usually supplied with so little information respecting purity of material and precise technic that the analyst can not feel assured of their dependability or of the exact conditions which their use demands. It is always advisable, and it is usually necessary, for him to obtain his own standard data in order to insure reliable results.

The first requisites for that purpose are samples of known dye. Absolute purity is seldom attainable, nor is it necessary. The presence of moisture, inorganic salts, or colorless organic substances is

[3] Unpublished investigations.

immaterial. The essential requirements are that the sample shall be substantially free from foreign coloring matters and that its actual dye content shall have been determined by reliable methods.

Purification from color impurities is obtained by recrystallization, separation with immiscible solvents, or other treatments adapted to the requirements of specific instances. The results of such treatments may be followed with the spectrophotometer. Substantial freedom from color impurities may be assumed when further treatment ceases to modify the precise form of the spectrum. More simply, it is indicated by a constant absorption ratio. (P. 21.)

The dye content of samples should usually be determined by means of titration with a standard titanous chloride solution (*43*). This reduction method is applicable with most dyes and is exceptionally convenient. The presence of such foreign substances as are ordinarily found associated with dyes is immaterial. The reduction method is direct, requiring no preliminary standardization in operation. It is seldom that other chemical methods of dye analysis are equally suitable or satisfactory, although it may prove advisable or necessary to resort to them in specific instances.

If resort is made to other chemical methods of analysis it is well to make certain that the dye samples with which they are employed are free from interfering substances. The experience of Holmes and Peterson (*35*) with neutral red and the pyronines may be cited. With these dyes, which can not be evaluated by the reduction method, it was found that all available samples contained appreciable proportions of organic dye intermediates, which invalidated results obtained by other chemical methods. In order to obtain material of known dye content it was necessary to eliminate these organic impurities. This was effected by repeated crystallization. The material was analyzed and examined with the spectrophotometer after each crystallization, and the results of chemical analysis were accepted as reliable only when a constant ratio had been obtained between extinction coefficients and apparent dye content as indicated by chemical means.

It is obvious that the method employed in obtaining standard data should be the same as that which is used subsequently in the evaluation of the same dye. It is improbable that any analyst would deliberately employ different solvents in the two instances. It is, however, quite possible that he might minimize the necessity for uniformity in seemingly immaterial details of practice. Minor details of manipulation are of little consequence when dyes give true solutions in the solvents employed. When dyes have decided colloidal tendencies in the solvents used, however, minor variations in manipulation may frequently lead to appreciable variations in results.

Let us suppose, for example, that it is intended to evaluate a dye on the basis of its extinction coefficients in 50 per cent alcohol. With colloidal dyes it will be found that the absorption values of their solutions may differ decidedly depending upon whether (1) a stock solution is prepared in 50 per cent alcohol and diluted with the same solvent, or (2) an aqueous solution is made up and diluted with alcohol, or (3) an alcoholic solution is made up and diluted with water. It may also be found that different values are obtained, depending upon whether or not a resort is made to heating in order to insure

complete solution of dye in the stock solution. It will be found, finally, that it may matter decidedly whether the absorption readings are taken immediately after the preparation of the solutions or somewhat later, for example, over the week-end.

In such instances the physical state of the freshly prepared solution may vary considerably in accordance with the method of its preparation. Physical readjustment ensues, but may often require a period of days or weeks for completion. It is not feasible for the analyst to wait until the final state of physical equilibrium has been reached. The only practicable course is that of carrying out the absorption measurements upon freshly prepared solutions, and depending upon a strict adherence to a rigid technic in their preparation to insure uniformity in results.

The indirect method of preparing solutions for examination (that is, by dilution of a stock solution) should be followed unless only minute quantities of material are available. With many technical dye products it is necessary to start with several tenths of a gram of dye in order to insure obtaining a representative sample.

The choice of solvent is of the greatest importance. No single solvent, unfortunately, is suitable for employment with all dyes.

A digression seems advisable at this point in order to consider the bearing which the general character of the analyst's work may have upon his choice of solvent and technic.

If the analyst is concerned merely with the evaluation of solutions of individual dyes, it will be advisable for him to investigate the general question of solvent and conditions with some care. The solvent selected should, if possible, be one in which the dye will be completely dispersed. If any regulation of hydrogen-ion concentration is necessary or advisable the provisions adopted should be such as will allow of minor inadvertent variation in conditions with safety; that is, the point selected should lie within a pH range in which the dye is chemically stable. The technic followed should be one which has been tested and found to give reliable results with the particular dye in question.

If the analyst is concerned with the analysis of dye mixtures as well as with that of individual dyes, this course, unfortunately, is not sufficient. Standard absorption data are valid only when the exact conditions under which they were obtained are duplicated. Mixtures of dyes can be analyzed only when standard data are available which have been obtained with the component dyes of the mixture under the same conditions as are utilized in examining the mixture.

It is well, accordingly, for the analyst to obtain standard data on dyes by two methods—a specific and a general procedure. The specific method may then be employed to best advantage when the dye is alone. The general method will probably give somewhat less reliable results, but will enable the analyst to evaluate the dye when it is in dye mixtures.

Aqueous solutions have been employed somewhat generally in the spectrophotometric evaluation of dyes, but their use has unquestionably resulted in many inaccuracies. The authors have pointed out that many dyes undergo tautomeric alteration with variation in concentration in aqueous solution. These alterations frequently occur over the range of concentration within which measurements are usually carried out in analytical practice. The data of French (*8*)

on aqueous methylene blue solutions illustrate the magnitude of errors which may arise from this source.

It has been customary in spectrophotometric analysis to depend upon a comparison of extinction coefficients at or near the dye maximum in solutions which may differ decidedly in actual dye concentration. The analyst, for example, has determined that a 1-centimeter layer of an aqueous solution of methylene blue containing 10 milligrams of actual dye per liter has a certain extinction coefficient at 673 mμ. He measures the extinction coefficient of a 1-centimeter layer of a second aqueous solution of the dye at 673 mμ and finds its ratio to his standard data to be 70:100. He concludes that the concentration of actual dye in the second solution is 7 milligrams per liter. In reality, however, it is appreciably less.

In this instance the analyst is dealing with a mixture of two dye forms, the relative proportions of which are altered with every change in actual dye concentration, rather than with a single dye form. His measurements are carried out in a region in which one dye form absorbs light much more strongly than does the other. The relative proportion of the strongly absorbent dye form increases upon dilution. His extinction coefficients have no fixed relation to actual dye content. If the solution he is evaluating is more dilute than that with which he obtained his standard data, his results (indicated dye contents) will be too high. If he employs more concentrated solutions, his results will be too low.

There are a variety of ways in which this difficulty may be obviated. The analyst may adopt the principle of carrying out all critical measurements at nearly identical concentrations of actual dye. This, however, would necessitate means whereby solution layers could be varied and measured with precision, or would involve the repeated preparation of dye solution. The latter course is inapplicable, moreover, when dealing with dye mixtures. The analyst may employ his usual technic and standardize his extinction values against dye content experimentally over such a working range as might be encountered in general practice; but this course would prove decidedly laborious. He may employ his usual technic without standardization by determining the particular wave length at which both dye forms have the same absorption and at which, accordingly, the relation of the extinction coefficient is unaffected by variation in dye concentration. (P. 30.) Although data obtained at this wave length would prove adequate for the evaluation of dye, it is desirable to have reliable data at other wave lengths for employment with dye mixtures.

The only thoroughly adequate and satisfactory course appears to be to avoid the use of aqueous solutions in general practice.

The phenomenon of tautomeric alteration in dyes with changing concentration is seldom encountered in alcohol, and alcohol has other advantages as a solvent. With aqueous solutions of basic dyes, adsorption of dye base occurs on the surface of containing vessels and absorption cells in varying degrees, depending upon the nature of the dye, the composition of the glass, and other factors. With aqueous solutions of safranines, for example, a 10 per cent decrease in absorption values, arising from this cause, has been observed repeatedly when solutions were left in the absorption cells for about

45 minutes. This type of adsorption is not encountered, or is negligible in proportion, in alcoholic solutions.

The use of 90 per cent alcohol has been recommended for employment with dyes which undergo tautomeric alterations in aqueous solution and with basic dyes in general (*25*). It will be found, however, that 50 per cent alcohol is nearly as effective in stabilizing dye solution to variations in dye concentration and in minimizing adsorption. It is decidedly more economical in use, and for many dyes it is probably even preferable to a solvent with a higher content of alcohol.

The use of 50 per cent alcohol has proved very satisfactory with dyes in general, and the writers have little hesitation in advocating its employment in a general technic, intended for application in particular with dye mixtures. It is suggested that it be used both for the preparation of the stock solution and for its dilution. Although it is an effective solvent for most dyes, it may be advisable to warm the stock solutions of some dyes to promote solution. It is suggested, accordingly, that all stock solutions be warmed in their preparation to insure uniformity in that respect. The addition of buffering agents to insure a definite hydrogen-ion concentration is not advocated. Dyes dissolve less readily in buffered solutions, and their colloidal tendencies are accentuated in them. Definite regulation of hydrogen-ion concentration is essential only with a few dyes. It is suggested, finally, that in all instances absorption measurements be made on the freshly prepared solutions.

It will be understood that this practice will not prove suitable in the examination of all dyes. It is not advocated for use with any individual dye, although it will usually prove reasonably suitable. It is recommended only for general use in the field of the analysis of dye mixtures in which the use of a single technic is requisite which will prove comparatively reliable with dyes of many different classes and types.

In obtaining standard absorption data it is well to carry out measurements throughout the visible spectrum. The standard spectrum obtained will enable the analyst to detect the presence of appreciable quantities of foreign coloring matters when found in future samples, and will also provide him with all the data he may require in the evaluation of dye mixtures. For the analysis of individual dyes he will require values obtained at one, two, or three wave lengths. These should be selected at or near the wave length of the dye maximum rather than upon the slopes of the absorption curves, since the disturbing influence of foreign dyes, which may be encountered in practice, may prove more serious in the latter instance. With yellow dyes, however, it usually will be necessary to deal with band slopes. This course may also prove very advisable whenever dye maxima lie near the extremes of the visible spectrum. In such instances the advantage of improving photometric sensitivity would outweigh all other considerations.

Although the spectrophotometric method of dye analysis is somewhat less accurate with some dyes, particularly with most yellow dyes, than is the reduction method with titanous chloride, it compares favorably with the latter method with dyes in general. When the provisions of the technic are judicious it gives reliable results with most dyes. It may be employed with some dyes to which the

reduction method of evaluation is inapplicable. It may, if necessary, be applied to extremely small quantities of material.

It is, perhaps, unnecessary to add that the spectrophotometer may be utilized to considerable advantage in connection with many of the color reactions which the analytical chemist employs. A single illustration will be cited. It was recently shown that the familiar turmeric test for boron could be modified in such manner as would render it possible to follow its operation with the spectrophotometer, and the results obtained indicated that it would prove possible to detect thereby the presence of 1 part of boron in 25,000,000 parts of aqueous solution (*29*).

DYE MIXTURES

Spectrophotometric methods have exceptional utility in the analysis of dye mixtures. It has been found possible to analyze certain simple mixtures of dyes on principles of differential reduction (*22*), but the possibilities along such lines are definitely limited. In general, dye mixtures can be analyzed by chemical means only after the separation of their individual components has been effected, and such separations are usually very laborious and frequently relatively ineffective. Spectrophotometric methods, on the other hand, are very convenient and have an extensive range of application.

The extinction coefficients of a mixture of dyes (when chemical action between such dyes is excluded) is the simple sum of those of its component dyes. When standard data have been obtained on the individual dyes in a mixture the analyst is equipped for the analysis of that mixture.

It is obvious that all standard data must be obtained with the same solvent and technic, and the dye mixture examined under precisely the same circumstances to obtain reliable results.

It has already been pointed out that water is unsuitable as a solvent in the analysis of dye mixtures, and a provisional technic has been outlined, employing 50 per cent alcohol, which is considered advisable. (P. 29.)

In obtaining data on individual dyes it is advisable to carry out measurements throughout the visible spectrum at intervals which are small enough to define the dye spectrum in detail. It is advantageous to adopt a standard dye concentration for such measurements or, at all events, to calculate the results to terms of a common dye concentration. Standard absorption curves can then be constructed on the basis of a standard concentration. These will be referred to as specific absorption curves. The utility of such specific curves is illustrated in Figure 10.

These curves illustrate the fact that there is a certain wave length at which the absorption of a mixture of two dyes is unaffected by variations in their relative proportions. At the point of intersection of specific absorption curves of dyes the only factor which influences the absorption of a mixture of those dyes is that of the total dye concentration (*24*).

In order to determine the total dye content of a mixture of two dyes, accordingly, it is only necessary to prepare a solution of appropriate concentration and determine its extinction coefficient at the wave length of intersection of specific curves of the component dyes. The ratio of the value obtained to the corresponding value on the

specific curves will be that of the concentration of total dye in the solution under examination to the standard concentration of dye employed in obtaining the specific data. The total dye content of the mixture may be calculated on that simple basis.

In some instances this single measurement may be all that the analyst may require. Nothing further is needed, for example, in determining whether mixtures of two food colors comply with the regulation of the Food and Drug Administration that they shall be labeled with a true statement of total dye content. More frequently, however, the analyst will also wish to determine the dye content of the individual components of the mixture. There are a variety of ways in which this may be accomplished.

When one of the components of a dye mixture absorbs light in a region of the spectrum in which the second component does not (at such dye concentrations as are employed in the examination of the mixture), it is obvious that its concentration may be determined directly by means of absorption measurements in that region. In the instance illustrated in Figure 10, for example, it would be possible to determine the concentration of dye B by means of measurements carried out at 610 mμ.

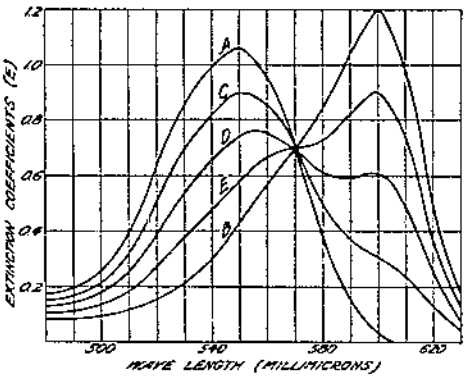

FIGURE 10.—Absorption spectra of two dyes and of mixtures of these dyes in which the total dye concentration is the same as that of the individual dye solutions: A, Dye A, 100 per cent; B, dye B, 100 per cent; C, 75 per cent dye A and 25 per cent dye B; D, 50 per cent each dyes A and B; E, 25 per cent dye A and 75 per cent dye B

Frequently, however, the analyst may find it necessary to depend upon measurements in regions in which the absorption of the dye components overlap. In such instances he may measure the extinction coefficient of the solution of his mixture at the maximum of one of its components. The value so obtained is reduced to specific conditions. In other words, he calculates what it would be if the total dye concentration of the solution under examination were the standard dye concentration employed in obtaining his specific data. (It is assumed, of course, that he has already determined the total dye concentration of his solution in the manner previously outlined and has, accordingly, the data necessary for the calculation in question.) The percentage of dye in the mixture may then be calculated by means of the formula

$$X=\frac{(a'-b)\,100}{a-b}$$

Where X=the percentage of dye A in the mixture.

a=the extinction coefficient of the specific curve of dye A at its maximum.

b=the extinction coefficient of the specific curve of dye B at the maximum of dye A.

a'=the extinction coefficient of the dye mixture solution at the maximum of dye A, reduced to specific value.

Referring again to Figure 10 it is assumed that a solution of a mixture of dyes A and B is found to have extinction coefficients of 0.56 and 0.72, respectively, at 570 mμ and 550 mμ. Then

$$a'=\frac{0.72\times 0.70}{0.56}=0.90$$

$$\text{and } X=\frac{(0.90-0.42)\,100}{1.06-0.42}=75 \text{ (per cent)}$$

Then 75 per cent of the dye mixture is dye A and 25 per cent dye B.

These values may be checked by means of further measurements at other wave lengths. In the absence of appreciable quantities of other dyes, it should be possible to obtain consistent results throughout the spectrum.

If the analyst prefers, he may establish the relative proportions of dyes in simple mixtures in a different manner. He may employ the ratio of extinction coefficients at, or near, the respective maxima of the dyes with which he is dealing. It will be evident that the value of this ratio will be unaffected by variation in total dye content but will be affected in a decided degree by any variation in the relative proportions of the components. Having specific curves of component dyes available, the analyst can calculate readily what values this ratio will have over the entire range of variation in percentage composition of mixtures of those dyes.

There is probably little choice between these two methods of determining percentage composition of mixtures in general practice. The analyst should make his selection between them on the basis of their relative advantages and disadvantages under the particular conditions with which he may be concerned.

These methods have certain practical limitations. They are of little value if one of the dyes is present in only very small proportion. Maximum accuracy is obtained only when the dyes involved have bands which are well separated in spectral location. They are very convenient to apply, however, and yield results, under favorable conditions, which are accurate within about 1 per cent.

With dye mixtures containing three components the proportion of instances in which accurate results may be obtained is smaller. When the forms of the specific curves are such as preclude reliable measurements of the concentration of any one or any two of the dyes, it becomes necessary to resort to complex calculations, and appreciable errors may result. Under favorable circumstances, however, it may be possible to obtain satisfactory results.

Having obtained standard specific absorption curves on three such dyes the analyst may evaluate a mixture of them by measuring E_1, E_2, and E_3 at $m\mu_1$, $m\mu_2$, and $m\mu_3$, where these wave lengths represent the maximum absorption of the dyes in question, A, B, and C, respectively.

Then it is obvious that—

$$E_1=X_1+Y_1+Z_1$$
$$E_2=X_2+Y_2+Z_2$$
$$E_3=X_3+Y_3+Z_3$$

where X_1, X_2, and X_3 represent the extinction coefficients of dye A at $m\mu_1$, $m\mu_2$, and $m\mu_3$, and Y_1, Y_2, and Y_3 and Z_1, Z_2, and Z_3 represent

the extinction coefficients of dyes B and C, respectively, at the same respective wave lengths. Inasmuch as the ratios of the values of the extinction coefficients of each individual dye at $m\mu_1$, $m\mu_2$, and $m\mu_3$ may be calculated from the basic data previously obtained, and are constant under suitable working conditions, it is possible to solve these equations, obtaining values for X_1, X_2, X_3, Y_1, Y_2, Y_3, Z_1, Z_2, and Z_3. The percentage of the component dyes in the mixture may then be calculated by dividing these values by the specific extinction coefficients of the dyes at those wave lengths.

Applying this method in the evaluation of three mixtures of the dyes erythrosine, phloxine B, and rose Bengal B the authors obtained the results shown in Table 3.

TABLE 3.—*Percentage of the component dyes in mixtures of erythrosine, phloxine B, and rose Bengal B*

Dye mixture No.	Erythrosine		Phloxine B		Rose Bengal B	
	Present	Found	Present	Found	Present	Found
1	43.15	42.3	25.3	27.3	13.9	12.7
2	26.5	26.5	42.2	41.55	14.0	14.6
3	17.3	16.9	25.3	25.0	34.9	36.0

When independent measurements may be obtained upon one of their component dyes, mixtures of three dyes may be analyzed more simply and with greater accuracy. An example is given in Figure 11.

In evaluating such a mixture the concentration of dye C could be obtained directly by measuring extinction coefficients within the region between 575 $m\mu$ and 600 $m\mu$. Further measurements within the region between 540 $m\mu$ and 560 $m\mu$ would give values for B and C from which the concentration of dye B could be obtained, since the values for dye C alone in the region could be calculated from the data already available. With the concentrations of dyes B and C established, it would be possible to make appropriate allowance for the absorption of those dyes in the region of shorter wave length, and obtain values for the concentration of dye A from absorption measurements therein.

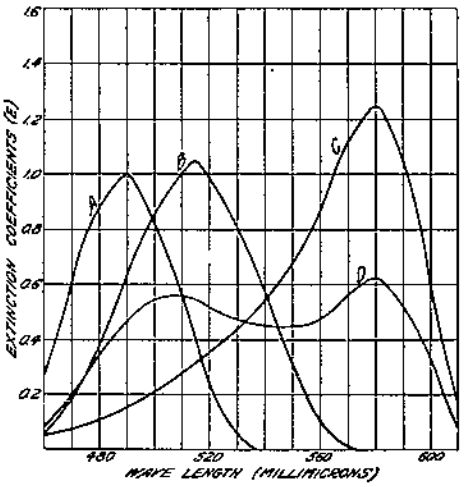

FIGURE 11.—Absorption spectra of aqueous solutions of three dyes and of a mixture of the same dyes: A, B, and C, Individual dyes; D, a mixture consisting of 20 per cent A, 30 per cent B, and 50 per cent C

Such methods as have been outlined hitherto for dye mixtures prove ineffective when the dye components differ but slightly in their hue and in the spectral locations of their absorption bands. This condition is seldom encountered in deliberate mixtures, but may frequently be met with in general practice. The analyst may wish to investigate the character of dyes which are commonly marketed as mixtures of varying proportions of similar individual components, or study the decomposition of dyes into products of similar color. In these, and in similar instances, the employment of absorption ratios may prove extremely useful.

Referring to Figure 9 it will be evident that if dye B contained as an impurity any appreciable proportion of dye A its absorption ratio would be increased, and that the increase would be proportionate to the percentage of dye A which was present. It can be calculated that it would require only approximately 3 per cent of dye A to raise the absorption ratio of dye B from 1.13 to 1.14. A careful determination of absorption ratios, accordingly, would enable the analyst to calculate the approximate percentage of dye A in mixtures of dyes A and B.

The accuracy of such determinations will vary considerably with conditions, depending in large measure upon visual sensitivity in the region in which the measurements are carried out and upon the spectral interval between the bands of the dyes in question. Under favorable conditions it is probable that results may be obtained which are accurate within about 2 per cent. In most instances, however, the method will prove decidedly less satisfactory. Even when conditions are such, however, that results obtained are only roughly quantitative, the method affords a very convenient means of obtaining information which is usually more definite than that obtainable by laborious chemical methods.

The absorption ratio method has been utilized in evaluating mixtures of methylene blue and trimethyl thionine (*30*), and in investigating the atmospheric dealkylation of methylene blue (*41*) and of cresyl blue (*36*).

SPECTROPHOTOMETRIC EVALUATION AND PRACTICAL DYE TESTING

The practical dye tester does not concern himself with absolute dye strengths. He adopts a convenient dye strength arbitrarily to serve as his standard, or type, and evaluates dye samples in terms of that standard, basing his estimate upon the results which he obtains in small-scale comparative dyeings. His operations are unquestionably more cumbersome and crude than are spectrophotometric or chemical methods of dye analysis. It might appear probable that the latter methods might advantageously replace those now in use for practical dye testing.

This possibility has been investigated at this laboratory and elsewhere, and the conclusions reached have been in substantial agreement. Spectrophotometric values can not be interpreted (as yet, at all events) in precise terms of brilliance and hue. Neither spectrophotometric evaluation nor chemical methods of dye analysis, moreover, can be relied upon to afford a reliable index of dye strength, with dyes in general, in the practical sense in which dye strength is judged in the industries. With some dyes analytical

results may agree closely with the dye tester's verdict, but with other dyes appreciable discrepancies may be encountered.

It is necessary to admit that analytical methods are not sufficiently developed, and that our knowledge of dyeing processes is not sufficiently complete, to enable one to define the essential dyeing properties of technical dyes from their analytical examination. Analytical methods, in short, can not replace practical dye testing.

INDICATOR METHODS

The application of spectrophotometric methods to indicator practice was made practically simultaneously by Holmes (*18*, *37*, *38*, *39*, *40*), by Brode (*3*), and by Thiel, Dassler, and Wulfken (*56*).

Indicators commonly change between two colors, although some indicators, like phenolphthalein, may exist in only one colored form. Certain indicators, such as the sulphonphthaleins, exist in three forms and have two useful transition ranges in color. A limited number of indicators exist in more than three forms.

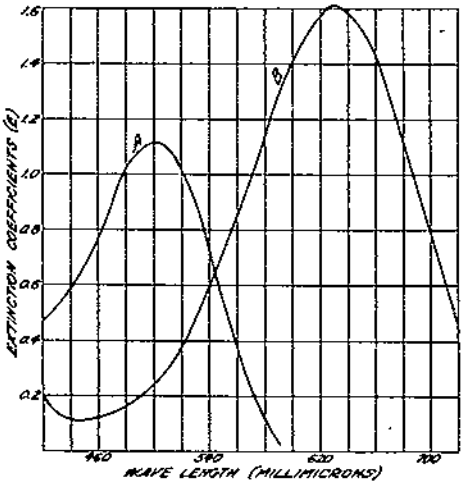

FIGURE 12.—Absorption spectra of aqueous solutions of 1-naphthol-2-sodium sulphonate indophenol at different hydrogen-ion concentrations: A, 75 mg per liter at pH 5.82; B, 37.5 mg per liter at pH 10.19

Indicator changes are invariably accompanied by decided alterations in absorption spectra, and the degree of transformation of any indicator at any definite hydrogen-ion concentration within the range, or ranges, of its utility may be measured with the aid of the spectrophotometer and given numerical expression in terms of spectrophotometric ratios. The empirical calibration of such ratios against known hydrogen electrode values yields data which may be utilized subsequently in the determination of the Sörensen exponents of solutions of unknown hydrogen-ion concentrations.

In point of fact the necessary spectrophotometric data for indicator practice can be calculated on theoretical assumptions. All that is required for the purpose is a knowledge of the apparent dissociation constant of the indicator and its absorption curves obtained under conditions which insure practically complete conversion, respectively, into its two color forms. The only assumptions required are that the indicator shall exhibit the essential behavior of a monobasic substance and that it shall conform to Beer's law. It is never entirely safe, however, to rely implicitly upon these assumptions, and an experimental verification of ratios is always advisable. Typical indicator transformations are illustrated in Figures 12, 13, and 14.

Within the region of its transition an indicator is essentially a mixture of two dyes, and the methods by which it may be investigated are those which have already been outlined for the analysis of dye mixtures. The courses which may be followed are illustrated by the graph of 1-naphthol-2-sodium sulphonate indophenol. (Fig. 12.)

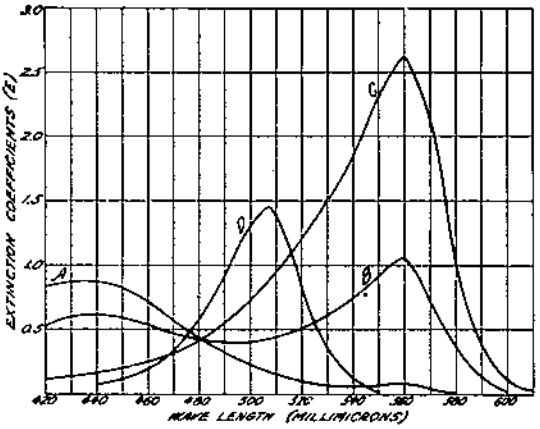

FIGURE 13.—Absorption spectra of aqueous solutions of phenolsulphonphthalein at different hydrogen-ion concentrations: A, At pH 6.3; B, at pH 7.5; C, at pH 10.2; D, in concentrated sulphuric acid

With increasing alkalinity, the indicator changes from a red form with maximum absorption at approximately 500 mμ to a blue form with maximum absorption at about 630 mμ. This transformation is practically complete in passing from pH 5.7 to pH 11.7, but 80 per cent of the alteration occurs between pH 7.7 and pH 9.7, and this smaller interval constitutes the useful working range of the indicator. Within the latter range any appreciable change in hydrogen-ion concentration is accompanied by a corresponding alteration in the relative proportions of the red and blue forms of the indicator and, consequently, by a corresponding alteration in the relative intensities of their absorption bands.

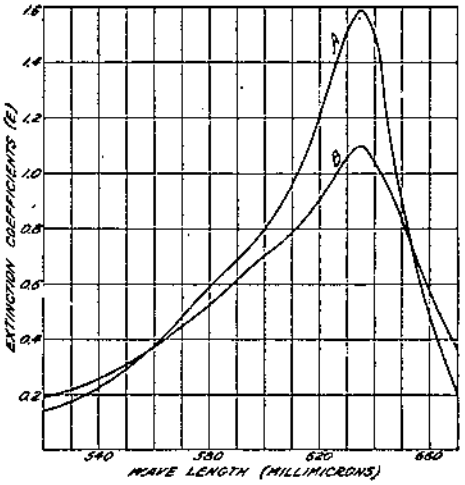

FIGURE 14.—Absorption spectra of aqueous solutions of fast green FCF at different hydrogen-ion concentrations: A, At pH 5.0; B, at pH 10.0

The degree of transformation of the dye at any given hydrogen-ion concentration in this range may be determined conveniently by means of absorption measurements carried out (1) at, or near, 630 mμ or (2) at, or near, 500 mμ, or (3) at both wave lengths.

In the first instance, the blue form of the indicator would be measured alone, since the red form does not have appreciable absorption at that wave length in such concentrations as would be employed. The spectrophotometric ratio employed would be that of the extinction coefficient at 630 mμ under the conditions of the test to the extinction coefficient

at the same wave length of an equal quantity of indicator under conditions which insured practically complete conversion into the blue form. This type of ratio will be referred to by the term R_1. It will be evident that an R_1 ratio can be employed only with solutions in which the precise concentration of indicator is known.

In the second instance the red form of the indicator would be measured, primarily. In actual practice with this particular indicator, it is improbable that the analyst would employ an R_1 ratio at 500 mμ. Not only is the red form of the dye less intense than the blue form, but the alteration in extinction coefficients at 500 mμ per unit change in indicator transformation would be further reduced by the fact that the blue form of the indicator absorbs some light at that wave length, and that its absorption there would increase as that of the red form diminished. The analyst, accordingly, would prefer to measure the absorption of the blue form of the dye.

In the third instance the ratio employed would be that of the extinction coefficient of the solution under examination at 500 mμ to the extinction coefficient of the same solution at 630 mμ, or vice versa. This type of ratio, which will be termed an R_2 ratio, affords a maximum degree of alteration in value per unit change in indicator transformation, since both the increase in one absorption band and the simultaneous decrease in the other contribute to it. It will be evident that values of R_2 ratios will not be affected by changes in indicator concentration and that the R_2 ratios, accordingly, may be employed with solutions in which the concentration of indicator is not known.

In actual practice the analyst would probably prefer to employ some other wave lengths, for instance 520 and 600 mμ, rather than the wave lengths referred to in the previous discussion. The visual sensitivity of the analyst is appreciably greater at 520 mμ than at 500 mμ and at 600 than at 630 mμ. If such changes were made, the differentiating value of the ratios would decrease ostensibly, but it is probable that the actual accuracy of the methods would be distinctly improved, owing to the improved accuracy with which measurements could be carried out.

It is advisable, in general, to obtain both R_1 and R_2 values in calibrating spectrophotometric methods for use with indicators. Although R_2 values afford greater differentiation than do R_1 values, they do not necessarily afford greater accuracy. If the absorption bands of both color forms are in such spectral locations as will enable the analyst to obtain reliable measurements on both bands, R_2 values will prove preferable. If, however, the band of one color form is so situated that accurate measurements on it are difficult, as is the case with the sulphonphthaleins and many other indicators, it will usually be found that R_1 values prove distinctly more reliable.

Even in instances, however, in which the analyst may find that R_1 values prove somewhat more accurate than R_2 values, he may find the latter values very useful, and at times nearly indispensable. The fact that they are unaffected by variations in indicator concentration renders their use of great value in examining solutions of small volume or in other instances in which it may be difficult or impossible to insure a precise indicator concentration.

The accuracy of spectrophotometric methods with indicators is excellent. With certain samples of bromcresol green it was found

that their apparent dissociation constants (4.68, 4.65, and 4.69, respectively) could be checked so consistently in any extensive series of careful measurements that it was possible to distinguish between the samples in question with fair certainty (*18*, *37*, *38*, *39*, *40*). The methods may be relied upon to check electrometric methods nearly to the second decimal point in Sörensen (pH) units.

A table of solubilities of representative dyes at 26° C. in water and in 95 per cent alcohol is included for the benefit of any investigators who may have occasion to prepare dye solutions. (Table 4.)

TABLE 4.—*Dye solubilities at 26° C.*

[Data are expressed as grams of anhydrous dye per 100 cubic centimeters of saturated solution. Data in parentheses are expressed as grams of anhydrous dye per 100 grams of saturated solution]

Dye	Color Index No.	In water	In 95 per cent alcohol
Pieric acid	7	1.18	8.96
Victoria yellow	8	1.66	1.18
Martius yellow (sodium salt)	9	4.57	.16
Martius yellow (calcium salt)		.05	1.90
Naphthol yellow S	10	8.96	.03
Aurantia	12	(1)	.33
Wool yellow G	16	18.40	.24
Chrysoidin Y	20	.86	2.21
Chrysoidin G	21	.23	.99
Sudan I	24	(1)	.37
Orange G	27	10.86	.22
Ponceau 2G	28	1.75	.21
Chromotrope 2R	29	19.30	.17
Alizarole yellow GW	36	25.84	.04
Alizarole orange G	40	.40	.57
Sudan II	73	(1)	.30
Azo Bordeaux	88	3.83	.19
Crystal ponceau	89	.80	.06
Erika B	130	.64	.17
Janus green	133	5.18	1.12
Metanil yellow	138	5.36	1.45
Methyl orange	142	.52	.08
Helianthin (color acid of methyl orange)		.015	.015
Orange IV	143	.16	.20
Azo acid yellow	146	2.17	.81
Resorcin yellow	148	.37	.19
Orange I	150	5.17	.64
Orange II	151	11.37	.15
Narceine	152	10.02	.06
Fast red A	176	1.67	.42
Amaranth	184	7.20	.01
Ponceau 6R	186	12.98	.01
Sudan III	248	(1)	.15
Brilliant croceine	252	5.04	.00
Erythrin X	254	6.41	.06
Sudan IV	258	(1)	.09
Biebrich scarlet	280		.05
Bismark brown Y	331	1.36	1.08
Bismark brown R	332	1.10	.98
Congo red	370		.19
Purpurin 4B	448		.13
Sky blue	520	13.51	(1)
Alizarin O	655	.74	4.49
Victoria green (oxalate)	657	7.60	7.52
Victoria green 3B	659	.01	2.24
Guinea green B	666	(28.40)	7.30
Light green SFY	670	20.35	.82
Fast green FCF		10.04	.35
Pararosanilin (chloride)	678	.26	5.93
Pararosanilin (acetate)		4.15	13.63
Rosanilin (chloride)		.39	8.16
New fuchsin (chloride)	678	1.13	3.20
Methyl violet	680	2.93	(15.21)
Crystal violet (chloride)	681	1.681	3.87
Crystal violet (iodide)		.035	1.78
Aniline blue (spirit soluble)	689	(1)	1.10
Victoria blue 4R	690	3.23	20.49
Patent blue A	714	8.40	5.23
Cyanole extra	715	1.38	.44
New Victoria blue R	728	.54	3.98
Pyronin G	739	8.96	.60
Pyronin B (iodide)	741	.07	1.08
Rhodamin B	749	.78	1.47
Rhodamin G	750	1.34	6.31
Fluorescein (color acid)	766	.03	2.21
Fluorescein (sodium salt)		50.20	7.10
Fluorescein (magnesium salt)		4.51	.35
Fluorescein (calcium salt)		1.13	.41
Fluorescein (barium salt)		6.54	.56
Eosin (sodium salt)	768	44.20	2.18
Eosin (magnesium salt)		1.43	.28
Eosin (calcium salt)		.24	.09
Eosin (barium salt)		.18	.06
Ethyl eosine	770	.03	1.13
Eosin B	771	39.11	.75
Erythrosin (sodium salt)	773	11.10	1.87
Erythrosin (magnesium salt)		.38	.52
Erythrosin (calcium salt)		.15	.35
Erythrosin (barium salt)		.17	.04
Phloxin (sodium salt)	774	(50.00)	9.02
Phloxin (magnesium salt)		20.84	29.10
Phloxin (calcium salt)		3.57	.45
Phloxin (barium salt)		6.01	1.17
Rose Bengal (sodium salt)	779	36.25	7.53
Rose Bengal (magnesium salt)		.48	1.59
Rose Bengal (calcium salt)		.30	.07
Rose Bengal (barium salt)		.17	.05
Thioflavine T	815	2.11	3.17
Neutral red (chloride)	825	5.64	2.45
Neutral red (iodide)		.15	.16
Neutral violet	826	3.27	2.22
Safranin	841	5.45	3.41
Methylene violet 2RA	842	.69	3.18
Iris violet	847	3.12	3.66
Cresyl violet (NAC)		.38	.25
Nile blue 2B	914	.16	.62
Thionin	920	.25	.25
Methylene blue (chloride)	922	3.55	1.48
Methylene blue ($ZnCl_2$ double salt)		2.75	.05
Methylene blue (iodide)		.09	.13
Methylene green	924	1.46	.12
Toluidine blue O	925	3.82	.57
New methylene blue N	927	(13.32)	1.65
Alizarin	1027	(1)	.125
Alizarin WC	1034	7.69	.15
Indigo carmine	1180	1.68	.01

[1] Nil.

LITERATURE CITED

(1) BEER, A.
1852. BESTIMMUNG DER ABSORPTION DER ROTHEN LICHTS IN FABRIGEN FLÜSSIGKEITEN. Ann. Phys. [Leipzig] 86: 78–88.

(2) BREMER, H.
1892. ÜBER DEN EINFLUSS DER TEMPERATURE GEFÄRBTER LOSUNGEN AUF DIE ABSORPTIONSPEKTREN DERSELBEN. Ztschr. Anorgan. Chem. 1: 112.

(3) BRODE, W. R.
1924. THE DETERMINATION OF HYDROGEN-ION CONCENTRATION BY A SPECTROPHOTOMETRIC METHOD AND THE ABSORPTION SPECTRA OF CERTAIN INDICATORS. Jour. Amer. Chem. Soc. 46: 581–596, illus.

(4) BRUNNER, A.
1929. ANALYSE DER AZOFARBSTOFFE. 124 p., illus. Berlin.

(5) FIERZ-DAVID, H. E
1926. KÜNSTLICHE ORGANISCHE FARBSTOFFE. 719 p. illus. Berlin.

(6) FORMÁNEK, J., GRANDMOUGIN, E., and KNOP, J.
1908–27. UNTERSUCHUNG UND NACHWEIS ORGANISCHER FARBSTOFFE AUF SPEKTROSKOPISCHEM WEGE. t. 1–2, illus. Berlin.

(7) ——— and KNOP, J.
1917. ÜBER DEN NACHWEIS DER PHENOLE AUF SPEKTROSKOPISCHEM WEGE. Ztschr. Analyt. Chem. 56: [273]–298, illus.

(8) FRENCH, R. W.
1926. EFFECT OF VARIATIONS IN CONCENTRATION OF DYES IN SOLUTION UPON THEIR QUANTITATIVE DETERMINATION SPECTROPHOTOMETRICALLY. METHYLENE BLUE. Indus. and Engin. Chem. 18: 298–299, illus.

(9) GIBSON, K. S., MCNICHOLAS, H. J., TYNDALL, E. P. T., and FREHAFER, M. K., with the cooperation of MATHEWSON, W. E.
1922. THE SPECTRAL TRANSMISSIVE PROPERTIES OF DYES: I. SEVEN PERMITTED FOOD DYES, IN THE VISIBLE, ULTRA-VIOLET, AND NEAR INFRARED. U. S. Dept. Com., Bur. Standards Sci. Paper 440, p. 121–184, illus.

(10) GREEN, A. G.
1920. THE ANALYSIS OF DYESTUFFS AND THEIR IDENTIFICATION IN DYED AND COLOURED MATERIALS, LAKE-PIGMENTS, FOODSTUFFS, ETC. Ed. 3. 150 p., illus. London.

(11) GSELL, H.
1916. DIE SPEKTRALANALYTISCHE IDENTIFIKATION DER PHENOLE. Ztschr. Analyt. Chem. 55: [417]–426.

(12) HOLMES, W. C.
1923. INFLUENCE OF THE TEMPERATURE AT WHICH THE SOLUTION OF DYES IS AFFECTED UPON THE NATURE OF THE SOLUTION. Color Trade Jour. 13: 54.

(13) ———
1923. THE SPECTROPHOTOMETRIC IDENTIFICATION OF DYES. I—ACID DYES OF THE PATENT BLUE TYPE. Indus. and Engin. Chem. 15: 833–836, illus.

(14) ———
1924. THE SPECTROSCOPY OF THE SULFONATED INDIGOTINS. Jour. Amer. Chem. Soc. 46: 208–214, illus.

(15) ———
1924. THE INFLUENCE OF VARIATION IN CONCENTRATION ON THE ABSORPTION SPECTRA OF DYE SOLUTIONS. PRELIMINARY PAPER. Indus. and Engin. Chem. 16: 35–40, illus.

(16) ———
1924. THE INFLUENCE OF CONSTITUTIONAL VARIATION IN DYES UPON THEIR RELATIVE ABSORPTION IN AQUEOUS AND ALCOHOLIC SOLUTIONS. Jour. Amer. Chem. Soc. 46: 2118–2124.

(17) ———
1924. THE INFLUENCE OF CONSTITUTIONAL VARIATION UPON THE ABSORPTION AND STABILITY TO HYDROGEN IONS OF CERTAIN HALOGENATED DERIVATIVES OF FLUORESCEIN. Jour. Amer. Chem. Soc. 46: 2270–2275, illus.

(18) ———
1924. THE SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATIONS AND OF THE APPARENT DISSOCIATION CONSTANTS OF INDICATORS. I. THE METHODS. Jour. Amer. Chem. Soc. 46: 627–631, illus.

(19) HOLMES, W. C.—Continued.
1925. THE SPECTROPHOTOMETRIC IDENTIFICATION OF DYES. II—BASIC FUCHSINS. Indus. and Engin. Chem. 17: 59–60, illus.
(20) ———
1925. THE REDUCTION PRODUCTS OF AZO DYES. Amer. Dyestuff Rptr. 14: 647–650, 686, 705, 732, 740, 753–754, 776.
(21) ———
1925. SPECTROPHOTOMETRIC IDENTIFICATION OF DYES. III—BASIC VIOLETS OF THE TRIPHENYLMETHANE GROUP. Indus. and Engin. Chem. 17: 918–919, illus.
(22) ———
1925. ANALYSIS OF DYE MIXTURES BY MEANS OF TITANOUS CHLORIDE. Amer. Dyestuffs Rptr. 14: [415]–418.
(23) ———
1926. THE CHEMICAL NATURE OF METACHROMASY. Stain Technol. 1: 116–122.
(24) ———
1926. THE SPECTROPHOTOMETRIC EVALUATION OF DYE MIXTURES. Amer. Dyestuff Rptr. 15: 189–191, illus.
(25) ———
1927. THE CHOICE OF SOLVENT IN SPECTROPHOTOMETRIC DYE ANALYSIS. Amer. Dyestuff Rptr. 15: 247–248, illus.
(26) ———
1927–29. STAIN SOLUBILITIES. II, III, IV. Stain Technol. 2: 68–70, 1927; 3: 12–13, 1928; 4: 73–74, 1929.
(27) ———
1927. TAUTOMERISM OF AMINATED DYES WITH VARIATION IN CONCENTRATION. Amer. Dyestuff Rptr. 16: 429–432, illus.
(28) ———
1927. COLLOIDAL PHENOMENA IN DYE SOLUTIONS. (Note) Jour. Amer. Chem. Soc. 49: 790–791.
(29) ———
1927. THE SPECTROPHOTOMETRIC DETECTION OF BORON. Jour. Assoc. Off. Agr. Chem. 10: 522–523.
(30) ———
1928. THE SPECTROPHOTOMETRIC EVALUATION OF MIXTURES OF METHYLENE BLUE AND TRIMETHYL THIONIN. Stain Technol. 3: 45–48, illus.
(31) ———
1930. THE ABSORPTION SPECTRA OF DYES. *In* International Critical Tables of Numerical Data, Physics, Chemistry and Technology. v. 7, p. 173–211. New York and London.
(32) ——— and BERLINER, J. F. T.
1927. DYE SOLUTION PHENOMENA, WITH VARIATION IN HYDROGEN ION CONCENTRATION WITH PARTICULAR REFERENCE TO DYE IDENTIFICATION. Amer. Dyestuff Rptr. 16: [81]–84, illus.
(33) ——— and DARLING, J. F.
1924. THE HYDROLYSIS OF AURAMINE. Jour. Amer. Chem. Soc. 46: 2343–2348, illus.
(34) ——— and PETERSON, A. R.
1930. ABSORPTION RATIOS OF BIOLOGICAL STAINS. Stain Technol. 5: 65–72.
(35) ——— and PETERSON, A. R.
1930. THE ANALYSIS OF NEUTRAL RED AND OF THE PYRONINS. Stain Technol. 5: 91.
(36) ——— and PETERSON, A. R.
1931. THE ATMOSPHERIC DEALKYLATION OF CRESYL BLUE. Stain Technol. 6: 79.
(37) ——— and SNYDER, E. F.
1925. THE SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATIONS AND OF THE APPARENT DISSOCIATION CONSTANTS OF INDICATORS. II. THYMOL BLUE. Jour. Amer. Chem. Soc. 47: 221–226, illus.
(38) ——— and SNYDER, E. F.
1925. THE SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATIONS AND OF THE APPARENT DISSOCIATION CONSTANTS OF INDICATORS. III. BROMOCRESOL GREEN. Jour. Amer. Chem. Soc. 47: 226–229, illus.

(39) ——— and SNYDER, E. F.
1925. SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATIONS AND OF THE APPARENT DISSOCIATION CONSTANTS OF INDICATORS. IV. 1-NAPHTHOL-2-SODIUM SULFONATE INDOPHENOL. Jour. Amer. Chem. Soc. 47: 2232–2236, illus.

(40) ——— and SNYDER, E. F.
1925. SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATIONS AND OF THE APPARENT DISSOCIATION CONSTANTS OF INDICATORS. V. FAST GREEN FCF. Jour. Amer. Chem. Soc. 50: 1907–1910, illus.

(41) ——— and SNYDER, E. F.
1928. THE ATMOSPHERIC OXIDATION, OR DEALKYLATION, OF AQUEOUS SOLUTIONS OF METHYLENE BLUE. Stain Technol. 4: 7–10.

(42) KAYSER, H.
1905. HANDBUCH DER SPECTROSCOPIE. v. 3, 604 p., illus. Leipzig.

(43) KNECHT, E., and HIBBERT, E.
1925. NEW REDUCTION METHODS IN VOLUMETRIC ANALYSIS. A MONOGRAPH. Ed. 2, 134 p., illus. London and New York.

(44) KRÜSS, G., and KRÜSS, H.
1891. KOLORIMETRIE UND QUANTITATIVE SPEKTRALANALYSE IN IHRER ANWENDUNG IN DER CHEMIE. 291 p., illus. Hamburg and Leipzig.

(45) KUNDT, A.
1878. UEBER DEN EINFLUSS DES LÖSUNGSMITTELS AUF DIE ABSORPTIONSSPECTRA GELÖSTER ABSORBIRENDER MEDIEN. Ann. Phys. [Leipzig] 4: 34–54.

(46) LAMBERT, J. H.
1760. PHOTOMETRIA, SIVE DE MENSURA ET GRADIBUS LUMINIS COLORUM ET UMBRAE. illus. Augsburg.

(47) MEEK, D. B.
1917. THE ABSORPTION SPECTRA OF SOME POLYHYDROXYANTHRAQUINONE DYES IN CONCENTRATED SULPHURIC ACID SOLUTION AND IN THE STATE OF VAPOUR. Jour. Chem. Soc. [London] Trans. 111 (2): 969–988.

(48) MOIR, J.
1922. THE CALCULATION OF THE COLOUR OF THE AZO-DYES AND RELATED COLOURED SUBSTANCES. Jour. Chem. Soc. [London] Trans. 121 (2): 1555–1562.

(49) ———
1923. THE COLOUR OF MONOCYCLIC SUBSTANCES CALCULATED BY ASSIGNING AN ABSORPTION BAND TO EACH POSSIBLE TAUTOMERIC FORM. Jour. Chem. Soc. [London] Trans. 123 (2): 2792–2803.

(50) PALKIN, S., and WALES, H.
1924. THE IDENTIFICATION OF PHENOLS BY MEANS OF THE SPECTROSCOPE. Jour. Amer. Chem. Soc. 46: 1488—1493, illus.

(51) PIHLBLAD, N.
1912. ÜBER EINEN NEUEN BEWEIS FÜR DIE KÖRPERLICHE EXISTENZ DER MOLEKÜLE. Ztschr. Phys. Chem. 81: 417—430, illus.

(52) ROWE, F. M., ed.
1924. COLOUR INDEX. 370 p. Bradford, Yorkshire.

(53) SCHULTZ, G.
1928–31. FARBSTOFFTABELLEN. Aufl. 7, neu bearb. und erweitert von L. L. Lehmann, 764 p., Leipzig.

(54) SHEPPARD, S. E.
1909. INFLUENCE OF THEIR STATE IN SOLUTION UPON THE ABSORPTION SPECTRA OF DISSOLVED DYES. Roy. Soc. [London] Proc. A 82: 256.

(55) STENGER, F.
1888. UEBER DIE GESETZMÄSSIGKEITEN IM ABSORPTIONSPECTRUM EINES KÖRPERS. Ann. Phys. u. Chem. 33: [577]–586.

(56) THIEL, A., DASSLER, A., and WULFKEN, F.
1924. ÜBER AZO-INDIKATOREN VOM TYPUS DES METHYLGELB, METHYLORANGE UND METHYLROT. Fortschr.Chem. Phys. u. Phys. Chem. v. 18, pt. 3.

(57) WALES, H., and PALKIN, S.
1926. IDENTIFICATION OF PHENOLS BY MEANS OF THE SPECTROSCOPE. II. Jour. Amer. Chem. Soc. 48: 810–813.

(58) WATSON, E. R.
1918. COLOUR IN RELATION TO CHEMICAL CONSTITUTION. 197 p., illus. London and New York.

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