SPECTROPHOTOMETER
EDUCATIONAL MANUAL
TABLE OF CONTENTS

Elements of Spectrophotometry .............................................................. 1
Computation on Dilution Factors ......................................................... 3
Fig. 1 – Spectral Transmission Curve .................................................. 5
Fig. 2 – Spectral Transmittance Curves of Evans Blue in various concentrations .................. 6
Fig. 3 – Percent of light transmitted vs. Concentration at a given wavelength of Evans Blue .... 7
Fig. 4 – Negative logarithm of the transmission (Absorbance) vs. Concentration at a given wavelength of Evans Blue .................. 8
Notes on Spectrophotometric Techniques ............................................ 9
Blank Solutions ................................................................................. 9
Optimum Wavelength ...................................................................... 9

Section on Laboratory Experiments taken from "Laboratory Manual of Instrumental Analysis" by C. M. Riley 1959. Reprinted by permission of C. M. Riley and McGraw Hill.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The Spectronic 20 Colorimeter and Absorption Spectra</td>
</tr>
<tr>
<td>2.</td>
<td>The Beer-Lambert Law</td>
</tr>
<tr>
<td>3.</td>
<td>Simultaneous Spectrophotometric Analysis of a Two-Component Mixture</td>
</tr>
<tr>
<td>4.</td>
<td>Desirable and Undesirable Colorimetric Properties</td>
</tr>
<tr>
<td>5.</td>
<td>The pK_a of an Indicator</td>
</tr>
<tr>
<td>6.</td>
<td>Photometric Method of Titration</td>
</tr>
<tr>
<td>7.</td>
<td>&quot;Precision&quot; or &quot;Differential&quot; Spectrophotometry</td>
</tr>
<tr>
<td>8.</td>
<td>Tristimulus Colorimetry</td>
</tr>
<tr>
<td>9.</td>
<td>Spectrophotometric Determination of Stability Constants and the Formula of Complex Ions</td>
</tr>
</tbody>
</table>
Elements of Spectrophotometry

Everyone can tell whether tea is "strong" or "weak" by its color, or in another manner of speaking, by the amount of light it transmits. This is the beginning of spectrophotometry. We soon find, however, that room light is not sufficient for most quantitative chemical determinations. One discovers that he must examine his material at one or more wavelengths. In fact, this is often carried out beyond the range where the eye has any sensitivity at all in the ultraviolet and infrared regions of the spectrum.

Spectrophotometric measurements are, in principle, easily performed. The solution under examination is placed in a test tube or cuvette, the proper wavelength is selected, and the test tube then placed in the light path so that this wavelength passes through the solution and its container. Any transmitted light is directed onto a photosensitive device which converts the radiant energy into electrical energy. The electrical current generated can be measured in any number of ways, the most common method being an ordinary meter. By measuring the electrical current produced when the solution is in the light path and comparing it to the current produced by say, distilled water, one obtains the percent transmission of the solution. One can then choose a second wavelength and repeat the measurement a second time, most likely obtaining a slightly different value. If this procedure is repeated at intervals over the wavelength range of interest, one obtains a spectral transmission curve (see Fig. 1).

It may not be clear how knowledge of the spectral transmission characteristics of a solution helps us quantitatively. In Fig. 2, we see spectral transmission curves of a substance in various concentrations. It is seen that there is a difference in the amount of light absorbed for each concentration of our solution. If we construct a graph of the percent of light transmitted for each concentration at a given wavelength, we obtain the curve seen in Fig. 3.

This does not seem very helpful. If, however, we plot the negative logarithm of the transmission at any one wavelength we now obtain a straight line plot (see Fig. 4).

This negative log has been given the name absorbance; it is important because of its linear relationship to concentration.

Conformance to a straight line is implied by "Beer's Law" which mathematically ties together absorbance, concentration, and optical path length. Specifically, the absorbance (A) is equal to the product of a proportionality constant called absorbtivity (a), the path length (b), and the concentration of the sample (c). Hence, the formulation $A = abc$. 
Although the readings on the Spectronic 20 are obtained as "% transmission," the definition of absorbance is conveniently based on "fractional transmission" ($T_f$) where, for instance, 40.8% transmission equals 0.408 fractional transmission, or 40.8% $T$ equals 0.408 $T_f$. One can calculate absorbance from transmission readings using the following relationship:

$$A = \log_{10} \frac{1}{T_f}$$

By combining the expressions as given in the preceding paragraphs, one may relate concentration to transmission:

$$abc = A = \log_{10} \frac{1}{T_f}$$

For instance, if \% $T = 40.8$ then one finds the $A = +0.389$. If the absorbtivity ($a$) is known to be 0.0022 (mg/100 ml)$^{-1}$ (mm)$^{-1}$ and the path length ($b$) is 11.7 (the nominal inside diameter of 1/2" test tubes), one calculates:

$$A = abc$$

$$0.389 = 0.0022(\text{mg/100 ml})^{-1}(\text{mm})^{-1} \times 11.7 \text{ mm} \times c$$

hence, $c = 15.11$ mg/100 ml.

There is an easier way, however, to carry this calculation out without ever calculating the absorbtivity or the path length directly. In the case of the sample and the known standard, one has:

1) $A_{\text{sample}} = a.b.c.$ sample
2) $A_{\text{standard}} = a.b.c.$ standard

and if one divides one equation by the other one obtains the ratio:

$$3) \frac{A_{\text{sample}}}{A_{\text{standard}}} = \frac{c_{\text{sample}}}{c_{\text{standard}}}$$

so that

$$4) c_{\text{sample}} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \cdot c_{\text{standard}}$$

The only requirement is that the path length must be equal in both cases (equivalent to using the same test tube or matched test tubes) and "$a$", the absorbtivity, must be the same in both measurements; in other words Beer's Law must apply to our system. It should be emphasized that this is not always the case. One should check several points with standard solutions to make sure that this condition is met.
Note also that $\frac{c_{\text{stand}}}{A_{\text{stand}}}$ is a constant. Hence, this can be calculated first and used as a multiplier, if a series of unknowns must be computed.

*(Note that the Spectronic 20 has a wavelength range of 340 to 950 m\(\mu\), whereas the visible portion of the spectrum is normally limited to 400 to 700 m\(\mu\).)*

** An equivalent expression is $A = -\log \frac{T_f}{T}$. If one prefers to work in $\% T$ instead of $T_f$, the expression becomes $A = 2 - \log \frac{\% T}{100}$. A table has been included so that the user may convert from percent transmission to absorbance without reference to log table.

† In order to obtain a value for absorbtivity measurements must be made using standard samples. By making up a known concentration of standards, by measuring the cell thickness and by calculating the absorbance from the measured transmission, one can calculate the absorbtivity.

**COMPUTATION ON DILUTION FACTORS**

Almost all blood samples or urine samples require dilution prior to analysis. It is usually necessary to express the concentrations in terms of the undiluted blood or urine. To do this, any actual dilutions must be taken into consideration, which can be done with the simple formula:

$$5) \frac{V' \cdot c'}{V_{\text{sample}}} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \cdot \frac{V' \cdot c'}{V_{\text{standard}}}$$

where the "primed" symbols are before dilution and the "unprimed" symbols after dilution.

Note that this includes the dilution of both standards and sample. If the effective dilution of the sample and standard are the same,

$$6) \frac{V'}{V_{\text{sample}}} = \frac{V'}{V_{\text{standard}}}$$

the equation reduces to:

$$7) c'_{\text{sample}} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \cdot c'_{\text{standard}}$$

This is merely a special case of equation (5) and is valid only for equal dilutions of sample and standard, even though it resembles equation (4).

Urines require additional information. Quantitative analysis of urines is usually performed on a 24 hour collection. In reporting constituents of urine, the concentration per 100 ml should be given as well as the 24 hour volume so that total grams (or moles or millimoles or milliequivalents) passed in 24 hours may be determined.
FIG. 1—Spectral Transmission Curve
FIG. 2—Spectral Transmittance Curves of Evans Blue in various concentrations
FIG. 3—Percent of light transmitted vs. Concentration at a given wavelength of Evans Blue
FIG. 4—Negative logarithm of the transmission (Absorbance) vs. Concentration at a given wavelength of Evans Blue
Errors in the use of photometers may be classified into two groups: experimental errors and instrumental errors. In spite of the numerous possible sources of instrumental errors, well designed photometers are capable of a precision of 1/2\% or better. Only with very careful work does this small value become the limiting error. Such errors as Beer's Law deviations, faulty reagents, weighing and dilution errors, instability of color, temperature, light, air or temporal effects, and personal errors contribute more to the inaccuracy of colorimetric measurements than do instrumental errors. It is important that once optimum conditions for a colorimetric method have been determined, they be strictly adhered to.

Mention must be made of a commonly overlooked source of error. It can be shown that there is an optimum transmission range for any spectrophotometer over which small errors in reading the scale will have minimal effect on the apparent concentration. It is recommended that readings be restricted to the range between 20\% transmission and 70\% transmission (equivalent to an absorbance range between 0.7 and 0.16) wherever practicable. For readings outside of this range, this source of error becomes larger and the determination should be repeated with sample concentrations which will bring the transmission within the recommended range.

BLANK SOLUTIONS

So far we have tacitly assumed that there is nothing else in solution which affects the transmission other than the sample itself. Quite clearly, however, the transmission as measured on the spectrophotometer is the result of all substances in solution which absorb at any particular wavelength.

Seldom does one find a solution whose "background" transmission contributes absolutely nothing to the transmission of the solution. In addition, some of the reagents may contain trace impurities of the substances being sought. To obviate this difficulty, one should always run a "blank" determination where the entire analysis is carried out in duplicate except that the addition of sample is omitted. The "true" concentration of the unknown is the concentration as measured on the sample less the concentration of the blank.

OPTIMUM WAVELENGTH

Ordinarily it is said that the optimum wavelength for an analytical determination is at an absorption maximum. The final selection of the proper wavelength for a given method depends on several considerations in addition to the wavelength of maximum sensitivity. The most desirable wavelength is the one which, at a given solution-depth, shows agreement with Beer's Law over as wide a range as possible of the concentrations apt to be encountered in the clinical lab and which also permits this range to be read within the most accurate region of the photometer scale. It is sufficient for our purpose to remind the reader that if Beer's Law is "obeyed" a graph of absorbance plotted against concentration will result in a straight line.
Many laboratory workers are under the impression that only those methods which conform to Beer's Law will yield satisfactory results. Nevertheless, numerous useful methods such as the alkaline picrate method for creatinine, do not follow Beer's Law exactly at any concentration ordinarily encountered. It is, of course, convenient to employ methods which conform to Beer's Law, in order that calculations can be made on the basis that the absorbancies of unknowns and standards are directly proportional to their concentrations. However, satisfactory analysis can be obtained using techniques which do not follow Beer's Law, provided there are also analyses on standard solutions slightly greater than and slightly less than the concentration of the unknown.

It should be emphasized that the table of concentration values listed under each method are typical values and for best results a working curve should be established in each laboratory for each method. To this end, methods of standardization are indicated with each procedure. Standards are essential, in any case, as quality control samples.

References should be consulted for further information on Spectrophotometry and Colorimetry. Do not overlook the Reference Manual as supplied with the Spectronic 20 for detailed operating instructions. In addition, a Spectrophotometer Educational Manual (B&L #33-29-09) is available at modest cost which contains many experiments elucidating the theory and uses of colorimetry.

A booklet on Fundamentals of Spectrophotometry is also available from Bausch & Lomb. A book by B. W. Rice entitled "Principles and Methods of Clinical Chemistry for Medical Technologists," Charles C. Thomas, Publisher, Springfield, Illinois (1960) will be a worthwhile addition to the clinical laboratory.

The procedures described in the present Manual have been chosen as among those most frequently performed in hospital clinical laboratories at the present time. As far as possible, methods are presented in a uniform format, in order to make for ease in carrying out analytical directions and in preparing reagents. It should be stressed at this point that it is important to consult the references cited for a more adequate insight into the methods.

Additional methods and replacement methods are contemplated as clinical techniques improve. Comments and criticisms on any of the present methods are solicited. By returning the attached card, your name will be kept on our mailing list to receive information about any future revisions which may occur.
EXPERIMENT 1

THE SPECTRONIC 20 COLORIMETER AND ABSORPTION SPECTRA

Apparatus.

B&L Spectronic 20 Colorimeter. The instrument has a diffraction grating monochromator and an electronic detection, amplification and measuring system. It operates in a wavelength range of 340 to 650 m\(\mu\). (Range may be extended to 950 m\(\mu\) or above by adding a red filter and changing phototubes).

Cuvettes. Two matched cuvettes; one special cuvette\(^1\) for observing the light path color.

Volumetric flask. 25 ml

Pipet. 10 ml

Stock Solution.

Chromium (III) nitrate solution. 0.0500 M.

Discussion of the Spectronic 20.

White light emanating from the tungsten lamp of the Spectronic 20 passes through an entrance slit and is dispersed by a diffraction grating. From the dispersed beam, a narrow band of a selected wavelength is passed through a second slit and into the sample solution being measured. Any of this light which is not absorbed by the solution, but which passes through it, falls upon the phototube of the instrument where the intensity of the transmitted light is measured electronically.

The diffraction grating is a precision replica grating having 600 grooves to the millimeter and accurately spaced. The white light falling upon the grating is dispersed into a horizontal fan of beams (violet and ultra-violet) at one end and the long wavelengths (red and infrared) at the other.

The spectrum of light falls on a dark screen with a slit cut in it. Only that portion of the spectrum which happens to fall on the slit goes through into the sample, and we can project any part of the spectrum onto the slit that we wish simply by turning the grating. The grating is turned by the knob on the top of the instrument (the wavelength control knob). Attached to this knob is a dial calibrated in wavelengths. This dial may be set to the wavelength wanted.

The wavelengths are given in millimicrons. \([1 \text{ m}\mu = 10^{-7} \text{ cm.}]\) (The slit of the instrument passes a band of wavelengths 20 millimicrons wide.) Because of the linear diffraction of the grating, this bandwidth of 20 m\(\mu\) is constant over the entire wavelength region.

\(^1\) The special cuvette described in this experiment is one fabricated by the experimenter. It may be duplicated by using a 1/2" test cuvette (B&L Cat. No. 33-29-27) and a piece of white chalk which has been cut diagonally. After light passes through the exit slit of the monochromator, those bands passed are visible on the diagonal face of the "chalk "screen". 
DIAGRAMS OF THE SPECTRONIC 20 LIGHT-PATH: TOP VIEW; FRONT VIEW.
The colorimeter is turned on by rotating the amplifier control (left-hand knob) clockwise. This should be done at least 20 minutes before measurements are made. After the instrument has warmed up, the amplifier control knob may be adjusted so that the meter needle will read "0" on the percentage transmission scale when no light is striking the phototube.

The right-hand knob regulates the amount of light passing through the second slit to the phototube. The need for this light control knob arises from the fact that the light source does not emit light of different wavelengths at equal intensities and the phototube is not equally responsive to light of varying wavelength. Besides this, the "blank" solution (the medium in which the substance being measured is located) may itself absorb light of certain wavelengths. In order to measure the absorbance due to only a particular species in solution, such "side effects" which affect the %T reading must be compensated for. Therefore, after the colorimeter has been "zeroed" (by means of the amplifier control knob), a "blank" solution is placed in the light path and the light control knob is rotated until the dial reads 100 %T, to achieve this desired compensation. If a sample solution is now placed in the light path, any change in the %T reading is due to the particular light-absorbing species in the sample and the %T reading is a measure of the quantity of that species present.

Of course, whenever a change in wavelength is made, however slight, the 0 %T and 100 %T must then be reset, since the amount of "compensation" needed varies with wavelength. The meter needle should go to 0 %T whenever the cuvette is removed from the sample holder, because removing the cuvette releases an occluder, which drops into the light beam and prevents the beam from reaching the phototube. Adjusting the zero point on the scale is therefore always done with the sample holder empty.

The phototube is a cesium-antimony surface type photoemissive cell (type S-4). The relative response of the phototube to a beam of monochromatic light of constant intensity is:

<table>
<thead>
<tr>
<th>Wavelength ( \lambda )</th>
<th>Response (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>350 ( \mu )</td>
<td>90 %</td>
</tr>
<tr>
<td>375</td>
<td>98</td>
</tr>
<tr>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>425</td>
<td>98</td>
</tr>
<tr>
<td>450</td>
<td>91</td>
</tr>
<tr>
<td>475</td>
<td>81</td>
</tr>
<tr>
<td>500</td>
<td>68</td>
</tr>
<tr>
<td>512</td>
<td>61</td>
</tr>
<tr>
<td>525</td>
<td>53</td>
</tr>
<tr>
<td>550</td>
<td>37</td>
</tr>
<tr>
<td>575</td>
<td>21</td>
</tr>
<tr>
<td>600</td>
<td>10</td>
</tr>
<tr>
<td>612</td>
<td>7</td>
</tr>
<tr>
<td>625</td>
<td>5</td>
</tr>
</tbody>
</table>
Thus it is seen that the phototube is much more sensitive to light of wavelength 400 μm than to light of wavelength 600 μm. This means that the phototube will require a greater flux of 600 μm than of 400 μm monochromatic light, in order for the same %T reading to be registered upon the colorimeter dial.

The handling of the cuvettes is extremely important. Often two cuvettes are used interchangeably: one for the "blank" solution and one for the samples to be measured. Yet any variation in the cuvette (such as a change in cuvette width or the curvature of the glass, stains, smudges or scratches) will cause varying results. Thus it is essential, in dealing with cuvettes, to follow several invariant rules:

1. Do not handle the lower portion of a cuvette (through which the light beam will pass).
2. Always rinse the cuvette with several portions of the solution before taking a measurement.
3. Wipe off any liquid drops, or smudges on the lower half of the cuvette, with a clean Scott Wiper before placing the cuvette in the instrument. NEVER wipe cuvettes with towels or handkerchiefs.
4. When inserting a cuvette into the sample holder:
   a. To avoid any possible scratching of the cuvette in the optical path, insert the cuvette with the index line facing toward the front of the instrument.
   b. After the cuvette is seated, line up the index lines exactly.

The cuvette should be removed in the reverse manner.

5. When using two cuvettes interchangeably, use one of the cuvettes always for the "blank" solution and the other cuvette always for the various samples being measured. Under apparatus these are called "matched" tubes.

These rules must be observed in all experimental work with the colorimeter. The equipment used in these experiments is expensive and requires a reasonable amount of care. Sloppy technique will not be tolerated.

Theory.

The colorimeter, or spectrophotometer, is an important analytical instrument that makes possible a quantitative measurement of the light passing through a clear solution. The first step in such an analysis is to determine the optimum wavelength (i.e., color of light) to use in the analysis.
Theory - Continued.

The wavelength that is selected must be appreciably absorbed by the substance under analysis or else a measurement of transmitted light will not be a significant measure of the concentration of the desired substance. On the other hand, the substance must not absorb too much of the wavelength chosen or else the transmitted light might be of too weak an intensity to measure accurately. It can be shown mathematically that the best compromise between too much and too little absorption comes in the region around 36.8% T. (This is the region where the error in reading the absorbance is least in comparison with the actual absorbance of the substance.)

This, however, is not the only consideration. The wavelength of light that is chosen must fall in a region where %T is not changing rapidly with change in wavelength. This is because the colorimeter cannot isolate a single wavelength but rather isolates a band of wavelengths. However, if all the wavelengths in this narrow band are absorbed to nearly the same extent by the substance, then the situation we have is nearly the same as the one we would have were we able to isolate a single wavelength to use. Thus we choose a flat portion of the %T vs. wavelength plot (absorption spectrum, described below) in selecting a wavelength for analysis. (There are some fine points and extensions to this matter which will be considered later in the course.)

To obtain the information required by the above considerations, we measure (and plot) the %T of a solution as a function of wavelength of the incident light across all the region of the spectrum available to us. Such a plot is called an absorption spectrum. In the experiment at hand, the absorption spectrum of chromium (III) nitrate solution is obtained, involving the use of the visible range: 375 to 625 μm.

Procedure.

The lab procedure for this experiment is brief - on purpose: to allow you ample time to become thoroughly familiar with the Spectronic 20 Colorimeter, how it functions and how it is operated.

A. Operation and Response of the Colorimeter.

Plug in and turn on the colorimeter. Adjust the amplifier control knob (left front) until the meter needle reads 0 %T, and allow the colorimeter to warm up for twenty minutes.

In the meanwhile prepare 0.0200 M Cr(III) solution by pipetting 10 ml. of the 0.0500 M stock solution into a 25 ml. volumetric flask and diluting to the mark. Mix well by inverting the flask about fifteen times.

After the instrument has warmed up, re-zero the instrument if necessary. Be sure the sample holder is in place, by pressing straight down on the holder. The top of the holder is shut whenever you adjust the instrument and take readings, to eliminate as much stray light as possible.
A. Operation and Response of the Colorimeter - Continued.

Put approximately 3 ml. of distilled water into a rinsed cuvette, and wipe dry with a clean Scott Wiper. (NOTE the 5 rules concerning the use of cuvettes.) Turn the light control knob (right front) counterclockwise as far as it will turn, to diminish the amount of light passing to the phototube. Insert the water-filled cuvette into the sample holder, and align the index lines exactly.

Turn the wavelength control knob to 510 μm.

Rotate the light control knob clockwise until the meter needle registers about 90 on the %T scale.

By rotating the wavelength knob, scan the visible spectrum and note how the response (measured by the position of the meter needle) varies with wavelength.

Determine the wavelength of light to which the instrument is most responsive. (It will be near 510 μm.) Adjust the %T to read 100 at this wavelength. Then, without readjusting either the amplifier control knob or the light control knob, run a "spectral curve" for the relative response of the colorimeter, reading from the %T scale. Take readings at the following wavelengths: 350, 375, 400, 425, 450, 475, 500, 512, 525, 550, 575, 600, 612 and 625 μm.

B. The Visible Spectrum.

Now place into the sample holder the special cuvette for observing the light-path color. Rotate the cuvette until the light path strikes the sloping surface of the chalk. Observe and record the color of the beam every 50 μm from 650 μm to 350 μm. It may be necessary to rotate the light control knob to increase or decrease the intensity of the light. Do not allow the meter needle to read off scale.

Adjust the wavelength to 600 μm. Note the variation in color across the band of light. (See question 4 at end of experiment.) How many different wavelengths of light comprise the band you see?

Adjust the wavelength to 550 μm. Rotate the light control knob and observe the change in light intensity. The amount of light passing into the sample is regulated by a mobile metal strip into which a narrow V-shaped aperture has been cut. As the strip is moved back and forth in the light path (by turning the light control knob), more or less light is allowed to pass between the sides of the V. Note the variation in light intensity across the band, with the far edge of the light band having the greatest intensity. (See question 5 at end of experiment.)

C. Absorption Spectrum of Cr(NO₃)₃ Solution.

Determine the absorption spectrum of the 0.0200 M Cr(III) solution, prepared in part A, in the following manner.

Set the wavelength dial at 375.
C. Absorption Spectrum of Cr(NO₃)₃ Solution - Continued.

Adjust the instrument to read 0 % T and 100 % T, when no cuvette, and when the water-filled cuvette, respectively, is in the sample holder. From this point on through experiment 3, be sure to use the same cuvette at all times for the distilled water “blank.”

Place approximately 3 ml. of the prepared 0.0200 M Cr(III) solution in your second cuvette. Wipe and insert into the sample holder. Read the % T of the solution from the dial.

Although the meter is electronically protected from burn-out, it is best to turn the light control counterclockwise before changing to another wavelength.

Turn the wavelength dial to 400. Again set the “0” and the “100” using the “blank” solution. Place the 0.0200 M solution in the holder again and read the % T at this wavelength.

Continue this procedure at wavelengths 405, 415, 425, 440, 455, 470, 490, 500, 520, 530, 540, 550, 570, 575, 580, 600 and 625 mµ.

Empty and rinse the cuvettes thoroughly with water (NEVER with cleaning solution); but be sure to keep the two cuvettes distinguished, so that the same one cuvette will be used for the “blank” and the other one cuvette will be used to measure all “samples” during the following experiments 2 and 3.

Treatment of Results.

PARTS A, B.

On a piece of graph paper plot “relative overall response” of your colorimeter vs. λ, using the data obtained by you in lab. Plot wavelength horizontally.

On the same graph plot a curve representing the “relative response of the phototube” as a function of λ, using the data supplied in the discussion at the beginning of the experiment.

Along the top of the graph indicate the colors observed for the various wavelengths of light.

It will be noted that the two curves plotted above do not coincide. While the relative response of the phototube is great toward light of wavelength 400, the relative response of the entire instrument toward this wavelength is low. And the colorimeter registers much greater relative response at 525 mµ than would be expected from a consideration of phototube response alone. The difference lies with the light source. Although, for example, the photocell is highly responsive to 400 mµ light, the light source is so weak an emitter of 400 mµ light that the net response of the colorimeter to that wavelength light is poor.
Treatment of Results.

PARTS A, B. - Continued.

From the above two curves, calculate the relative intensity of the colorimeter lamp's emission over the visible range of the spectrum, in the following manner. At each wavelength studied, divide the "relative overall response" by the "relative phototube response." This gives a series of numbers which indicate the "relative lamp intensity" at various wavelengths, the largest of the numbers being in the neighborhood of 3.0. To convert these relative numbers to a scale where the maximum is approximately 100, multiply each of the numbers by the factor \( \frac{100}{3} \). Thus:

\[
\frac{\text{instrument response}}{\text{phototube response}} \times \frac{100}{3} \quad \text{relative lamp intensity.}
\]

(The exact value obtained for a particular wavelength is unimportant; the importance lies in how the value changes as you go from wavelength to wavelength.)

Finally, plot a curve, on the same graph as above, representing the "relative intensity of the lamp" as a function of wavelength.

PART C.

On a second piece of graph paper, plot the absorption spectrum (\% T vs. \( \lambda \)) of the 0.0200 M Cr(III) solution.

Using the log scale on a slide rule, convert the % T values obtained for the solution into absorbance readings (A 2 = log \% T). List the A values in your notebook for future reference.

Questions.

1. To what color of light is the instrument most responsive? What color of light is emitted most strongly by the tungsten lamp?

2. In what way do the various knobs on the colorimeter affect the light beam which is passed through the sample holder?

3. "The effective bandwidth is only 20 millimicrons and is constant over the entire wavelength region." What is meant by the term "bandwidth?"

4. When light is diffracted from a grating, the sine of the angle of diffraction is directly proportional to the wavelength. That is,

\[
\sin \Theta \propto \lambda.
\]

Which color light is diffracted at a greater angle, red light or yellow light?
Questions - Continued.

5. If both red and yellow light pass through the second slit of the colorimeter at the same time, the intensity of which color light will be affected most by the V-shape of the light-control aperture, and why? (It might well be pointed out that the diffraction grating is so arranged in the Spectronic 20 to give horizontal dispersion of light; i.e., the color of the dispersed light varies horizontally but not vertically.)

6. When a solution is red in color, does the solution absorb red light strongly or transmit red light strongly?

7. By referring to your Cr(III) absorption spectrum, suggest a desirable wavelength for analyzing Cr(III) nitrate solutions having a concentration range of 0.02-0.04 M. Explain briefly why this same wavelength might be undesirable for analyzing a Cr(III) nitrate solution, if the concentration were appreciably greater or less than 0.02-0.04 M.

8. In calculating the relative lamp intensity of the colorimeter at various wavelengths, it was tacitly assumed that the water and the glass of the "blank" cuvette absorbed none of the light (in the region of wavelengths used). Do you think this assumption is justified, and why?

9. (This one is just to think about.)

"The effective bandwidth... is constant over the entire wavelength region." For this statement to be true, the spectrum produced by the diffraction grating must be linear (equal angles between consecutive wavelengths), and the light intensity must be regulated by varying the vertical height of the light beam rather than by changing the width of the beam. How are these two conditions consistent with the original statement?
EXPERIMENT 2

BEER'S LAW

Apparatus.

Bausch and Lomb Spectronic 20 Colorimeter.
2 Cuvettes.
Pipets.  5, 10 and 20 ml.
Volumetric flasks.  Five 25 ml. (one for unknown)
Beaker.  100 ml.

Stock Solution.

Chromium (III) nitrate solution.  0.0500 M.

Theory.

In order to determine the concentration of a solution quantitatively by measuring the amount of light it transmits, one obviously needs some workable relationship between the solution's concentration and its transmission of light. The Beer-Lambert Law, sometimes known more simply as "Beer's Law," provides such a relationship. This law is a modification of an earlier law derived by Lambert, which applied to the transmission of monochromatic light by homogeneous solids.

Lambert concluded that each unit length of material through which light passes absorbs the same fraction of entering light. If \( P \) (or \( I \)) represents intensity of transmitted light and \( P_0 \) (or \( I_0 \)) represents intensity of incident light, then the change in \( P \) is proportional to the intensity of incident light multiplied by the change in thickness \( \ell \) of the material through which the light passes. Mathematically,

\[
\frac{dP}{P} = -k \, d\ell
\]

The proportionality constant is \( k \) and the negative sign arises from the fact that \( P \) becomes smaller when \( \ell \) becomes larger.

Rearranging and integrating:

\[
\int_P^{P_0} \frac{dP}{P} = -k \int_0^\ell d\ell
\]

\[
\therefore \ln \frac{P}{P_0} = -k \ell \quad \text{or} \quad \log \frac{P}{P_0} = - \frac{k}{2.303} \ell .
\]
Theory - Continued.

Beer modified the law to apply to solutions. He found that doubling the concentration of light-absorbing molecules in a solution produced the same effect as doubling the thickness. The modified law (the Beer-Lambert Law, aforementioned) may be expressed mathematically as

\[ \log \frac{P}{P_0} = -\epsilon \ell \, C. \]

In this expression, the concentration \( C \) of the solution is generally expressed in moles per liter, by convention; \( \epsilon \) is then called the molar extinction coefficient and is a constant (corresponding to \( \frac{k}{2.303} \) in the former expression), characteristic of the absorbing substance and of the particular wavelength of light used. The cell width \( \ell \) is expressed in centimeters, and is essentially constant in most experimental work. Thus \( \log \frac{P}{P_0} \) is seen to be directly proportional to concentration.

If \( \log \frac{P}{P_0} \) is plotted against concentration for a solution which obeyed the Beer-Lambert Law, a straight line results, whose slope is \(-\epsilon \ell\). \( \frac{P}{P_0} \) is called the transmittancy of the solution.

More often, in experimental work dealing with optical methods of analysis, the terms percent transmittance \( \% T = \frac{P}{P_0} \times 100 \) and absorbance \( A = \log \frac{P_0}{P} = 2 \log \% T \) are used. Absorbance is also called optical density. As with \( \log \frac{P}{P_0} \), both absorbance and \( \log \% T \) are directly proportional to concentration when a solution obeys the Beer-Lambert Law. This may be shown by slightly modifying the Beer-Lambert equation given above and then observing the direct proportionality with concentration. For absorbance, the desired equation results simply by multiplying through by the factor \((-1)\); i.e.,

\[ A = \epsilon \ell \, C. \]
Graphs of absorbance vs. conc., or log %T vs. conc., are known as Beer's Law plots. They are made by measuring the light absorbed by solutions of varying concentration. The cell width and the wavelength of the light are maintained constant. If a linear plot can be obtained (showing that the Beer-Lambert relationship holds for the solution at that wavelength), the plot may then be used in determining the concentration of unknown solutions.

Unfortunately, however, the condition of monochromatic light upon which the Beer-Lambert Law is based is not obtainable in the laboratory. Since more than one wavelength of light passes through a solution at the same moment, deviations from the law are observed over much of the available spectrum, and non-linearity is observed in the Beer's Law plots. An absorbance reading in such a case will indicate a concentration which may be quite different from the actual concentration of the solution. The object, therefore, of much preliminary laboratory work in optical analysis is to find a suitable wavelength band where the deviation from the law will be only slight or negligible.

The spectral curve, obtained in expt. 1, serves as a guide in our search for a desirable wavelength for Cr(III) analysis. The Beer's Law plots, to be obtained in the present experiment, will show the relative desirability of different wavelengths chosen from various portions of the spectrum. From these plots it can be seen that the choice of a wavelength for analysis is to some extent dependent upon the approximate concentration of the solution.

Procedure.

The colorimeter should be turned on at least twenty minutes before any measurements are taken.

Obtain approximately 75 ml. of the Cr(III) stock solution (0.0500 M) in a small beaker. Make up solutions that are 0.0100, 0.0200, 0.0300 and 0.0400 M in chromium, by pipetting 5, 10, 15 and 20 ml. respectively into four 25 ml. volumetric flasks and diluting each to the mark with distilled water.

By referring to your plot of %T vs. \( \lambda \) for the 0.0200 M chromium solution, select six wavelengths at which to study absorbance as a function of concentration. Include in your selection:

1. Wavelengths at the two minima of the curve.
2. A wavelength where %T is at a maximum.
3. A wavelength corresponding to a steeply rising portion of the curve.
4. A wavelength corresponding to a steeply descending portion of the curve.
5. The 625 m\( \lambda \) wavelength.

Obtain Beer's Law plots for Cr(NO\(_3\))\(_3\) solution for each of the selected wavelengths, in the following manner:

Turn the wavelength dial to one of the chosen wavelengths.
Procedure - Continued.

Adjust the colorimeter for 0 and 100 % T, using the same cuvette for the distilled water "blank" as in expt. 1. Remove the cuvette from the sample holder and see if the meter returns to "0" (after 10 seconds). If not, reset the "0" and then repeat the adjustment for 100 % T. Continue this until "0" and "100" are obtained with the cell out, and in, respectively.

Measure and record the % T and absorbance (optical density) of the 0.0200 M Cr(III) solution at the selected wavelengths, using the same cuvette with this solution as you used in expt. 1. (The spectral curve obtained in expt. 1 should indicate similar % T values for the wavelengths of light selected above.) BE SURE to set the instrument at 0 and 100 % T with the "blank" after each change of the wavelength setting, as described previously.

Discard the 0.0200 M sample and rinse the cuvette several times with small portions of your 0.0100 M solution. Place about 3 ml. of the 0.0100 M solution in the cuvette and record % T and absorbance at each of the 6 wavelengths. Do this also for the other 2 prepared solutions [0.0300 and 0.0400 M Cr(III)] and for the stock solution [0.0500 M Cr(III)], rinsing the cuvette thoroughly with the next solution before making a measurement.

Obtain an unknown Cr(III) solution and dilute to the 25 ml. mark. Determine the % T and A of this solution at each of the 6 wavelengths.

Finally rinse the cuvettes thoroughly with water, turn OFF the colorimeter and replace the cover. Before leaving lab, consider your answer to question 3 below.

Treatment of Results.

Plot \( \log \% T \) versus concentration and absorbance versus concentration for each set of data obtained at each of the six wavelengths. Although absorbance readings are made directly from the colorimeter dial, it is better to convert the % T readings to absorbance by slide rule calculation and then to use these calculated values for plotting. [The \( \log \% T \) vs. conc. curves may conveniently be plotted upon a piece of Coleman semi-log paper supplied in the lab, plotting % T directly along the vertical axis.]

Draw a horizontal line across your Beer's Law plots at 80 % T and 20 % T. Measurements of % T should be restricted within these two limits, because % T readings greater than "80" and less than "20" allow too large an error in concentration, in comparison with the actual concentration of the substance being measured.

From an appropriate Beer's Law curve, determine the concentration (in moles per liter) of your unknown Cr(III) solution.
Treatement of Results - Continued.

Questions.

1. Briefly account for the appearance of the Beer's Law plots obtained in this experiment; i.e., where do the plots adhere to or deviate from Beer's Law, and why? (Summarize for each of the six wavelengths.)

2. At which of the six wavelengths is the extinction coefficient the smallest?

3. Estimate how accurately you can read % T off the colorimeter dial; i.e., ± % T.

Estimate how accurately you can determine the wavelength of light passing through the sample; i.e., ± μm.

4. Assuming that you could reproducibly read the % T scale to ± 1 % T at each of the six selected wavelengths, how accurately could you report the concentration of a 0.0150 M Cr(III) solution at each wavelength (e.g., 0.0150 M ± 0.0050 at 968 μm) of a 0.0550 M solution?

[Helpful hint - Use your Beer's Law plots of log % T vs. conc., plotted on the semi-log paper.]

5. What wavelengths would be most desirable to use in analyzing a solution approximately 0.015 M and a solution approximately 0.055 M in Cr(III), and why? Consider both adherence to Beer's Law and inherent error in the final concentration reported.

6. (A question just to think about.)

Starting with the Beer-Lambert relationship

\[ A = \log \frac{P}{P_0} = \varepsilon \ell C \]

a. Show that \( A = 2 - \log \% T \).

b. Show that, in order for the relationship to be dimensionally correct, the molar extinction coefficient \( \varepsilon \) must have the dimensions liters per mole per centimeter.

c. Show how to determine the slope that a Beer's Law plot would have at some given wavelength, if you have at your disposal only an absorption spectrum of a solution of known concentration and the knowledge that at the given wavelength Beer's Law is obeyed. (Assume constant cuvette length.)
EXPERIMENT 3

SIMULTANEOUS SPECTROPHOTOMETRIC ANALYSIS
OF A TWO-COMPONENT MIXTURE

Apparatus.

Bausch and Lomb Spectronic 20 Colorimeter.
2 Cuvettes.
Pipets. 5, 10 and 20 ml.
Volumetric flasks. Five 25 ml. (one for unknown)
Beakers. Two 100 ml.

Stock Solutions.

Chromium (III) nitrate. 0.0500 M.
Cobalt (II) nitrate. 0.1880 M.

Theory.

When a solution of two colored (light-absorbing) substances is prepared, often the presence of the second component will cause a change to occur in the light-absorbing properties of the first component. A situation of this sort will be observed in experiment 4. With such solutions the absorbance by the components is not additive (due to their interaction), and an experiment, as the one to follow, cannot be carried out upon the system.

However there are many instances in which the two components do not react or interact in any way with one another, and thus neither affects the light-absorbing properties of the other. The absorbance of light by such components is additive. That is to say, the total absorbancy of the two-component solution is just the sum of the absorbancies which the two substances would have individually, if the substances were in separate solutions under similar conditions and had the same concentrations as in the mixture. When this is true for the components of a solution, then there is a possibility of analyzing solutions of these substances spectrophotometrically for each component.

Because upsetting interactions often do arise, sometimes when they are least expected, the necessary first step to take when a multiple-component analysis is being considered is to investigate the nature of the absorption spectra of the various substances, while the substances are separate and while they are in solution together. Such an investigation is carried out in the procedure below for the case of the Co(II)-Cr(III) system. As it happens, the absorbancies of these two colored components are additive, and the possibility of a two-component analysis may be considered further.

Now if there were some wavelength of light not at all absorbed by cobalt while being strongly absorbed by the chromium, and some other wavelength of light where the converse were true, then these two wavelengths could be used in analyzing for the two components in the same way that one does one-component analysis — just as if the other component were not there at all. This, unfortunately, is not the case.
Theory - Continued.

However, if we can find one wavelength which is weakly absorbed by the cobalt while being strongly absorbed by the chromium, and some other wavelength where the converse is true, there is still a good possibility of accomplishing the analysis since the absorbancies are found to be additive. Such a pair of wavelengths does exist for the Co(II)-Cr(III) system: one near 510 μm and the other near 575 μm. That these wavelengths also occur at flat portions of the spectra is added good fortune. The analysis is made in the manner indicated below.

In experiment 2, the proportional relationship between the absorbance of a substance and its concentration was shown to be

\[ A = \varepsilon \lambda C. \]

By using the same cuvette for each sample being analyzed, the factor \( \lambda \) is kept constant, so that we may combine the two constants \( \varepsilon \) and \( \lambda \) into a single extinction constant \( k \), thus:

\[ A = k C. \]

In this equation \( k \) is a proportionality factor which relates \( A \) and \( C \) for some particular substance at some particular wavelength. On a Beer's Law plot of \( A \) (vertically) vs. \( C \) (horizontally), \( k \) is the slope of the line on the graph.

For a solution containing \( n \) light-absorbing components whose absorbancies are additive, the total absorbance of the solution at some wavelength may be represented as the sum of all the individual absorbancies (of the 1st component through the \( n \)th):

\[ A_i = \sum_{j=1}^{n} k_{ij} C_j, \quad i = 1 \]

The subscript \( j \) refers to components just as \( i \) refers to wavelengths of light used. This general equation may be written more explicitly as

\[ A_1 = k_{11} C_1 + k_{12} C_2 + \ldots + k_{1n} C_n. \]

For a second wavelength \( i=2 \), the explicit equation would be

\[ A_2 = k_{21} C_1 + k_{22} C_2 + \ldots + k_{2n} C_n, \]

expressing the fact that the total absorbance by the second wavelength \( A_2 \) is equal to the absorbance of that wavelength by substance 1 of concentration \( C_1 \) plus the absorbance of that wavelength by substance 2 of concentration \( C_2 \), etc.
Theory - Continued.

When a two-component solution is analyzed, only the first two “kC” terms have any meaning. Using two different wavelengths of light provides us with two equations in two unknowns. By solving the equations simultaneously, one may determine the concentrations of the two components. The various k's are determined from Beer's Law plots for the separate components at the two wavelengths chosen for the analysis.

Procedure.

A. Additivity of Absorbancy of Cr(III) and Co(II) Nitrate Solutions.

Prepare the following solutions while the Spectronic 20 is warming up for use:

I. In a small beaker obtain approximately 70 ml. of 0.1880 M cobalt (II) nitrate stock solution. From this prepare 0.0376 M, 0.0752 M and 0.1504 M Co solutions in your 25 ml. volumetric flask.

II. Obtain in another small beaker about 25 ml. of 0.0500 M chromium (III) nitrate stock solution. Using these stock solutions, prepare 25.00 ml. of a solution that is 0.0200 M in Cr and 0.0752 M in Co.

Determine the absorption spectrum of the 0.0752 M Co solution and of the two-component (Cr-Co) solution, taking readings at wavelengths of 375, 400, 425, 440, 455, 470, 480, 490, 500, 520, 530, 540, 550, 570, 580, 600 and 625 mu. The values of absorbance, A, are required, but it is better to read values of %T and convert these values to A by slide rule. %T readings must be used to obtain the higher values of A.

Plot (absorbance vs. mu) on one graph the absorption curves for (a) Co, (b) Cr from experiment 1, and (c) the Cr-Co mixture.

Now, for each wavelength, add the values of A for the single component curves and plot these points on the same graph as above. These points, representing the sum of the first two curves, should fall closely on the curve for the mixture, showing that the non-interaction condition is fulfilled.

B. Determining the k's from Beer's Law Plots.

By referring to the Cr and the Co absorption spectra, find two desirable wavelengths at which to carry out an analysis of a Cr-Co mixture.

Obtain Beer's Law plots (A vs. conc.) for Co(NO₃)₂ at the chosen wavelengths, using the various Co(II) solutions prepared at the beginning of lab. The Beer's Law plots obtained during experiment 2 may suffice for the Cr(III) solution; if the wavelengths you choose do not correspond to any of the expt. 2 graphs, you may wish to run two additional Beer's Law curves at the chosen wavelengths.
B. Determining the k's from Beer's Law Plots - Continued.

From the slope of the Beer's Law plots, determine the values of k for the Cr and the Co solutions at the two wavelengths chosen. Note question 6 (c) of expt. 2. The calculated k values correspond to $k_{11}, k_{12}, k_{21}$ and $k_{22}$ in the equations given above.

(In determining the k's in this manner, we are essentially determining the extinction coefficients of each of the pure solutions at the two wavelengths, since the cell length is maintained constant.)

C. Analysis of a Cr-Co Mixture.

Obtain an unknown Cr-Co mixture and dilute to the mark. Determine the absorbance of the unknown mixture at each of the chosen wavelengths.

Calculate the concentration (moles per liter) of each of the components in the unknown, by setting up simultaneous equations and solving for the two unknowns. One method of calculation is indicated in question 3, below.

Questions.

1. If a Cr(III) solution contained a very high concentration of a pure-blue material (e.g., saturated Cu(NO$_3$)$_2$), suggest how one might accurately analyze the solution for Cr(III) in the concentration range of 0.0300 M to 0.0600 M.

2. [Image of absorption spectra for solutions of substances I and II at two different concentrations.]

Above are the absorption spectra from 400 to 600 mp of two substances (I and II) which do not react or interact in any way.

a. If you were to analyze an unknown mixture of I and II of approximately equal concentrations to those above, why would it be unwise to choose for your analysis $\lambda_1$ and $\lambda_2$? $\lambda_1$ and $\lambda_4$? $\lambda_4$ and $\lambda_5$? $\lambda_2$ and $\lambda_5$?

b. If you were to analyze a solution of substance I in which a small quantity of substance II was present as an impurity, what wavelength(s) would you consider first, in planning the analysis, and why?

c. Why is a two-component analysis possible using $\lambda_2$ and $\lambda_4$, but not possible using $\lambda_1$ and $\lambda_6$?
3. (optional)
The simultaneous equations for total absorbance $A_i$ given in the discussion may be rewritten in a form to give the concentrations directly. For two components ($j = 1$ and $j = 2$) and two wavelengths ($i = 1$ and $i = 2$) the general form of the rewritten equation is

$$C_j = \sum_{i=1}^{2} p_{ji} A_i \quad j = 1, 2$$

where the $p_{ji}$ factors may be calculated from the $k_{ij}$ factors in the previous expressions.

a. Write out the two equations explicitly.

b. Find algebraic expressions for the $p_{ji}$ in terms of the $k_{ij}$. (One way to do this is to solve the earlier equations simultaneously for the $C_j$ and then to equate equivalent coefficients.)

c. Compute numerical values for the $p_{ji}$ using the results of (b) and the experimental values of the $k_{ij}$. Substitute these numerical values into the equations in (a) above, and solve directly for the concentrations of cobalt and chromium in your unknown.

d. In non-mathematical terms summarize what essentially has been done in steps (a) through (c).
EXPERIMENT 4

DESIRABLE AND UNDESIRABLE COLORIMETRIC PROPERTIES

Apparatus.

Bausch and Lomb Spectronic 20 Colorimeter.
2 Cuvettes.
Pipets. 1 (in 0.1 ml’s), 1, 2, 3, 4, 5, 10 and 20 ml.
Volumetric flasks. Four 100 ml.
Beakers. Three 100 ml.
Graduated cylinder. 100 ml.
Medicine droppers. Two

Solutions.

Ferric chloride. \(10^{-3}\) M, in 0.5 M HCl
Ammonium thiocyanate. Saturated.
Ammonium thiocyanate. 0.5 M
Sodium acetate. 2 M
1,10-phenanthroline. 0.3%
Sodium hydroxide. Conc.
HCl Hydrochloric acid. Conc. (12 N)
Aqueous ammonia. Conc.
Hydroxylamine hydrochloride. 10 % (freshly prepared)

Approx. quantities needed for all pipette rinsings and measurements:

<table>
<thead>
<tr>
<th>PART I</th>
<th>PART II</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ml.</td>
<td>80 ml.</td>
</tr>
<tr>
<td>30 ml.</td>
<td>-</td>
</tr>
<tr>
<td>100 ml.</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>dropwise</td>
</tr>
<tr>
<td>-</td>
<td>35 ml.</td>
</tr>
<tr>
<td>dropwise</td>
<td>dropwise</td>
</tr>
<tr>
<td>12 ml.</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>dropwise</td>
</tr>
<tr>
<td>-</td>
<td>20 ml.</td>
</tr>
</tbody>
</table>

Reagents.

Sodium fluoride.
Sodium oxalate.
Sodium tartrate.
Potassium dihydrogen phosphate.
Congo red paper.

Theory.

In the usual (direct) spectrophotometric methods of analysis, the absorbance of the substance (or a derivative thereof) is measured. In the cases of Cr(III) and Co(II) previously studied, the properties of the substances permitted accurate analysis, without the use of other reagents. For instance, these substances had stable colors, conformed well enough to Beer’s Law and had sufficiently large extinction coefficients at certain wavelengths to allow direct analysis. However, many instances arise in which the substance does not possess properties which are conducive to accurate optical measurement. Such a substance must be converted, by reaction with some suitable chromogenic reagent, into a new species having desirable properties, before a direct analysis can be made. The color-producing reaction may be illustrated by the equation:

\[
\text{Sample} + \text{Chromogenic reagent} \rightarrow \text{Product.}
\]
Theory - Continued.

In this experiment we will consider some of the properties that are desirable for a chromogenic reagent and for the species which is optically measured.

Whether the sample is colored itself or requires addition of a color-forming reagent, the solution intended for colorimetric measurement should ideally possess these five properties:

1. Stability for sufficient time to permit accurate measurement. Instability, which usually results in fading, is sometimes the result of air oxidation, photochemical decomposition, effects of acidity, temperature, solvents, or other conditions. Sometimes, by modifying the conditions, greater stability can be obtained.

2. Intense color (large extinction coefficient). This can be controlled by altering the solvent, in many cases, and by selecting a reagent of suitable sensitivity.

3. Freedom from effects of minor variations in pH, temperature, or other conditions.

4. Solubility of the colored product.

5. Conformity of the colored system to Beer's Law.

When a color-forming reagent is needed, the reagent selected should possess as many of the following properties as possible:

1. Stability in solution. Reagents which deteriorate in a few hours, ferment, or develop mold on storage usually have to be prepared frequently and a new calibration curve must be obtained for each new batch of reagent.

2. Rapid color development.

3. Stoichiometric reaction with the desired constituent. If the reaction goes to completion, the exact amount of reagent added, if colorless, is not critical, and an excess may be used to allow for a wide range of concentrations and possible interference by side reactions which consume the reagent. The color intensity will then be proportional to the concentration, assuming that the colored product itself obeys Beer's Law. Sometimes, when unfavorable equilibrium conditions exist, a large excess of reagent may be added to shift the equilibrium to the desired direction.

4. Transparency (an extinction coefficient of zero) in the spectral region involved in the measurement.

5. Selectivity or specificity, so that the color is a measure of the desired constituent only.
Theory - Continued.

6. Freedom from interference by other constituents which might convert the reagents or desired constituent to an unreactive form or complex leading to incomplete color development.

7. Capacity to function in the all-round best solvent. The choice of solvent in which to make a color measurement is sometimes an important consideration.

The above gives a brief summary of desirable properties for solutions and reagents, if they are to be useful in colorimetric analyses. For a more detailed discussion, including specific examples of reagents which have been found desirable in analyses, see: Mellon, *Analytical Absorption Spectroscopy*, pp. 20-23 and following. Some of the more important conditions mentioned in the above discussion will be studied in this laboratory experiment.

Procedure.

Read the whole of the procedure before starting this experiment, so as to be able to utilize your lab time most effectively. Allow the colorimeter to warm up before using.

I. THE IRON (III) THIOCYANATE SYSTEM.

A. Effect of Standing Time on Absolute Absorbance.

Pipette 5 ml. stock iron solution and 3 ml. satd. NH₄SCN into a clean 100 ml. volumetric flask. Measure the absorbance immediately at 480 mp using distilled water as the "blank" solution. Measure the absorbance again at approximately 30 minute intervals for a period of about 2 hours. Record the time and the absorbance for each measurement. In the intervening time, proceed with experiment part IB, (or IA. - if IA. is started at this time, bear in mind that the measurements on the two systems are to be made at different wavelengths and thus that 0 and 100 % T must be readjusted before each reading).

Plot absorbance vs. time (in minutes) for all measurements made.

B. Effect of Reagent Excess on Absolute Absorbance.

While the above measurements are being made, continue the experiment by preparing and measuring solutions of Fe(III) containing varying amounts of thiocyanate. The solutions are prepared by pipetting the following quantities of stock Fe(III) and 0.5 M NH₄SCN solutions into 100 ml volumetric flasks, and diluting to the mark with distilled water.
B. Effect of Reagent Excess on Absolute Absorbance - Continued.

<table>
<thead>
<tr>
<th>ml of $10^{-3}$ M Fe(III)</th>
<th>ml of 0.5 M $\text{NH}_4\text{SCN}$</th>
<th>Ratio SCN/Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.3</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>300</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>1000</td>
</tr>
<tr>
<td>5</td>
<td>20.0</td>
<td>2000</td>
</tr>
<tr>
<td>5</td>
<td>30.0</td>
<td>3000</td>
</tr>
</tbody>
</table>

The larger quantities (20 and 30 ml) may be carefully measured in a graduated cylinder. Because of the fading which occurs as noticed in part I.A., it is desirable to make up one solution at a time, measuring the absorbance at 480 mp as soon after preparation as possible. Water is used as the "blank" solution for these measurements.

Plot absorbance vs. SCN/Fe ratio for all the measurements made in this part of the experiment.

C. Effect of pH on Absolute Absorbance.

NOTE: Because of the fading of the color of the complex with time, the solutions of different pH should be prepared one at a time and the measurements obtained likewise, before proceeding to the next solution.

pH $\sim$ 0. Recall that the Fe(III) stock solution is 0.5 M in HCl. To 4 ml. of Fe(III) accurately pipetted into a 100 ml. volumetric flask, add 2 ml. saturated $\text{NH}_4\text{SCN}$ and 8 ml. of conc. HCl, so that the final hydrogen ion concentration will be $\sim$ 1 M after dilution to 100 ml. Mix thoroughly and immediately measure the absorbance of the solution at 480 mp.

pH $\sim$ 1. To 4 ml. stock iron in a 100 ml. volumetric flask, add 2 ml. satd. $\text{NH}_4\text{SCN}$ and 13 drops conc. HCl, and dilute to the mark with distilled water. Obtain the absorbance of the solution at 480 mp. GO IMMEDIATELY TO PART ID, BELOW.

pH $>$ 1. Prepare solutions of various pH values, in the following manner. To 4 ml. of the stock iron and 2 ml. satd. $\text{NH}_4\text{SCN}$ in 100 ml. volumetric flasks, add respectively 0 drops, 7 drops, and 9 drops of 4 M NaOH solution. Dilute to the mark, and measure the absorbancy of each solution at 480 mp. Test the pH of each solution with pH paper.

Plot absorbance vs. pH (approx.) for all of the measurements made in this part of the experiment.
D. Effects of Anions on Absolute Absorbance.

After you measure the absorbancy of the pH ~ 1 solution in part I.C., add a very small portion (about the size of a grain of sugar) of NaF to the solution in the cuvette. Place your thumb over the opening at the top and shake vigorously. Do NOT use a stirring rod. Now measure again the absorbance of the solution. If no effect is observed, add another very small portion of NaF and repeat the shaking and measurement.

IMMEDIATELY rinse the cuvette thoroughly with tap water, then with distilled water, and finally with your pH ~ 1 solution again. Measure the absorbancy of the pure pH ~ 1 solution again, and then add a small quantity of sodium oxalate. Shake as above and note any change in the absorbancy.

Repeat the procedure in the paragraph just above, using sodium tartrate and potassium dihydrogen phosphate, respectively, in place of sodium oxalate.

Report your observations, as to the effect of each of the salts upon the absorbancy of the iron-thiocyanate solution, as part of your discussion in question 1 at end of experiment.

II. IRON (II) ORTHOPHENANTHROLINE SYSTEM.

A. Effect of Standing Time on Absolute Absorbance.

To 4 ml. Fe(III) stock solution in a 100 ml. volumetric flask add 1 ml. of 10% NH$_2$OH·HCl solution. Swirl the flask for a few seconds, allow to stand for 1-2 minutes, and then add 2 ml. 0.3% orthophenanthroline solution (henceforth abbreviated o-phen). Drop in a small piece of Congo red paper and add 2 M NaAc solution until the indicator paper changes from blue to red. (The purpose of the hydroxylamine hydrochloride is to reduce the Fe(III) to Fe(II), which forms a better complex with o-phenanthroline; the sodium acetate adjusts the pH so that the color of the complex will develop more rapidly.) Dilute to the mark with distilled water and mix well. Measure the absorbancy of the solution at 512 mu. Distilled water may be used as the "blank" solution. Repeat the measurement approximately every 30 minutes for a period of 2 to 3 hours.

Plot absorbance vs. time (in minutes) for all measurements obtained.

NOTE: The iron solution prepared above is used also in part II.D. of this experiment.

B. Effect of Reagent Excess on Absolute Absorbance.

Prepare seven other solutions in a manner similar to the above, but add respectively 0.0, 0.2, 0.4, 0.6, 1.0, 3.0 and 4.0 ml. of 0.3% o-phen. Measure the absorbancies of these solutions at 512 mu using distilled water as the "blank." It is not necessary to work as rapidly with these solutions as with the thiocyanate system.
B. Effect of Reagent Excess on Absolute Absorbance - Continued.

Plot absorbance vs. ml. of o-phen. Two intersecting lines should result, with the point of intersection corresponding to the amount of o-phen just needed to complex the iron present in the solution.

C. Effect of pH on Absolute Absorbance.

Measure the absorbance of the following 5 solutions of different pH, using the 512 nm wavelength for all measurements.

pH = 1.7. Pipette 4 ml. stock iron solution and 1 ml. NH₂OH·HCl solution into a 100 ml. volumetric flask. Swirl for a few seconds and allow to stand for 1-2 minutes. Add 2 ml. 0.3% o-phen, dilute to the mark with distilled water and mix well. Measure absorbancy immediately.

pH ~ 2. Repeat as above, but before diluting to the mark add 2 M NaAc solution dropwise until the red color of the complex begins to develop.

pH ~ 5. Use the first absorbance measurement obtained with the solution in part II.A. This same solution is used in part II.D.

pH ~ 9. To 4 ml. stock iron solution in a 100 ml. volumetric flask, add 1 ml. NH₂OH·HCl solution and 2 ml. 0.3% o-phen. Then add conc. NH₃ dropwise until the solution is alkaline to litmus. Dilute to the mark and mix well.

pH ~ 12. To 4 ml. stock iron solution, add 1 ml. NH₂OH·HCl solution, 2 ml. 0.3% o-phen, and 13 drops of 4 M NaOH. Dilute to the mark and mix well.

Plot absorbance vs. pH (approx.) for each of the 5 measurements. The absorbancy of this system is nearly independent of pH only in the region of approx. pH = 3 to approx. pH = 10.

D. Effect of Anions on Absolute Absorbance.

Use the solution prepared in part II.A. In the same manner as in part I.D., study the effect of the four anions (fluoride, oxalate, tartrate, and dihydrogen phosphate) upon the absorbance of the solution. Be sure to rinse the cuvette especially well after testing with the fluoride.

Report your observations, as to the effect of each of the salts upon the absorbancy of the iron-orthophenanthroline system, as part of your discussion in question 1 below.
Treatment of Results (a summary).

I. IRON (III) THIOCYANATE SYSTEM.
   Part A. Plot A vs. time (in minutes).
   Part B. Plot A vs. ratio (SCN/Fe).
   Part C. Plot A vs. pH (approx.).
   Part D. Give a summary of your observations, as part of your discussion in question 1.

II. IRON (II) ORTHOPHENANTHROLINE SYSTEM.
   Part A. Plot A vs. time (in minutes).
   Part B. Plot A vs. ml. of o-phen. Include in the graph a point corresponding to the first or second measurement made in part II.A.
   Part C. Plot A vs. pH (approx.).
   Part D. Give a summary of your observations, as part of your discussion in question 1.

Questions.

1. Discuss clearly but briefly the relative merits of thiocyanate ion and o-phenanthroline as colorimetric reagents for iron. Include in your discussion the following points: (a) time stability, (b) quantity of colorimetric reagent needed, (c) effect of pH, (d) effect of anions, (e) other pertinent factors.

2. a. Interpret qualitatively the shape of the absorbance vs. ml. o-phen curve obtained in part II.B.

   b. If the Fe(II)-o-phen complex had dissociated appreciably, what shape would you expect for the absorbance vs. ml. curve? (Draw a sketch.)

3. It is known that Fe(II) and o-phen form a stable 1:3 complex; i.e.,
   \[ \text{[Fe (o-phen)$_3$]$^{++}$} \]

   From this fact and the data obtained in part II.B., calculate the concentration (moles per liter) of o-phen in the original o-phen solution used in lab. The formula for o-phenanthroline (or 1,10-phenanthroline is

   ![Formula](image)

4. Calculate the sensitivity (in mg per liter per 0.01 absorbance units) for the o-phen method and for the thiocyanate (SCN/Fe ratio of 2000) method for iron analysis.
EXPERIMENT 5

THE pKₐ OF AN INDICATOR

Apparatus.

Bausch and Lomb Spectronic 20 Colorimeter.
2 Cuvettes.
Pipets. 1, 5 and 10 ml.
Volumetric flasks. Four 25 ml.
Beakers. Three 100 ml.

Solutions.

Bromthymol blue. 0.1% in ethanol (20%).
Na₂HPO₄. 0.10 M.
KH₂PO₄. 0.10 M.
Hydrochloric acid. Conc.
Sodium hydroxide. 4 N.

Reference.


Procedure.

NOTE: Bromthymol blue is unstable in acid solution over prolonged periods of time. Therefore, obtain all absorbance measurements with a solution on the same afternoon that you prepare the solution.

A. Absorption Spectra of Bromthymol Blue at Various pH Values.

Obtain 3 complete absorption spectra of bromthymol blue at 3 pH values - a low pH (~1), a high pH (~13) and a solution nearly neutral (pH ~ 7).

pH ~ 1. Carefully pipette 1 ml. bromthymol blue stock solution into a clean 25 ml. volumetric flask. Add a few ml. of distilled water, then 4 drops of concentrated HCl, and finally dilute to the mark with distilled water. Invert several times to effect mixing, and add about 5 ml. of this solution to a cuvette rinsed with this solution. Measure the %T (and calculate the absorbancy) of this solution from 365 to about 575 at 20 mµ intervals. At maxima and minima in the curve, the intervals should be decreased to 10 mµ. Plot absorbances vs. λ (mµ), and indicate the color of the solution.
A. Absorption Spectra of Bromthymol Blue at Various pH Values - Continued.

\[ \text{pH} = 6.9 \]. Pipette 1 ml. indicator into a 25 ml. volumetric flask and add 5 ml. each of 0.10 M Na₂HPO₄ and KH₂PO₄ from a pipet. Dilute to the mark and obtain the spectrum as above. Plot on the same graph as above (A vs. mJl), and indicate color of solution.

\[ \text{pH} \sim 13 \]. To 1 ml. indicator in a 25 ml. volumetric flask, add 12 drops of 4 N NaOH. Dilute to the mark and obtain the spectrum as above. Plot your values on the same graph as above, and indicate color of solution. The three curves should intersect each other at the same point, called an isosbestic point.

B. Absorbancy of Solutions (Differing in pH) at Selected Wavelengths.

Refer to the graph you have made and select 2 wavelengths at which further absorbance measurements will be made. You should select a wavelength to the left of the isosbestic point and one to the right of this point. Choose wavelengths where the acid and base forms of the indicator show a maximum difference in their absorbance.

Measurements will be made on solutions of 7 different pH values other than the 3 solutions studied thus far.

<table>
<thead>
<tr>
<th>ml. indicator</th>
<th>ml. H₂PO₄⁻</th>
<th>ml. HPO₄⁻</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>1.0</td>
<td>5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(2)</td>
<td>1.0</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(3)</td>
<td>1.0</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td>(4)</td>
<td>1.0</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>(5)</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>(6)</td>
<td>1.0</td>
<td>1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>(7)</td>
<td>1.0</td>
<td>0.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Pipette the above quantities for a given pH into a 25 ml. volumetric flask and dilute to the mark. Measure the absorbancy of each solution at the 2 selected wavelengths. (Remember to readjust the instrument to 0 and 100 % T whenever you change the wavelength setting.) Calculate the pH of the above solutions given the fact that the second ionization constant for H₃PO₄ is $1.3 \times 10^{-7}$ (pK₂ = 6.9).

Treatment of Data, and Commentary.

Combining the absorbance values at the two selected wavelengths (obtained in part A) with the data obtained in part B, plot absorbance (vertically) vs. pH (horizontally) for each of the two wavelengths studied. Connect the points with a smooth curve. The mid-point of each curve corresponds to equal concentrations of the acid and the base form of the indicator. From each graph determine the pKₐ of the indicator. The two values thus obtained for the pKₐ may differ slightly.

Draw two horizontal lines across each of your A vs. pH plots: one corresponding to the absorbancy of the pH ~ 1 solution of the indicator, and the other corresponding to the absorbancy of the pH ~ 13 solution. The first line (I) gives the
Treatment of Data, and Commentary - Continued.

absorbancy of the indicator when present entirely in the acid form. The second line (II) gives the absorbancy of the indicator when present entirely in the base form. Any deviation of the actual absorbancy from these two lines is a measure of the extent to which one form of the indicator has been converted to the other form.

Consider the A reading obtained for the pH = 6.2 solution (diagram, page 2). By subtracting the absorbancy at pH = 6.2 from the absorbancy at pH 1 (the acid line, I, of the curve), one can obtain a measure of the amount of In⁻ in the solution of pH = 6.2. By subtracting the absorbancy at pH 13 (the base line, II, of the curve) from the absorbancy at pH = 6.2, one can obtain a comparable measure of the amount of HIn in the pH = 6.2 solution. The ratio of In⁻/HIn may be obtained by dividing the results of the two calculations above. (The ratio could also be obtained by measuring with a ruler the relative lengths of the two arrows indicated on the diagram.)

Determine the In⁻/HIn ratio corresponding to each of the points plotted on one of your two absorbance vs. pH graphs, and plot log(In⁻/HIn) (vertically) vs. pH on a graph. That point where the line crosses the zero axis corresponds to equal concentrations of base and acid form of the indicator. From the pH at which the line crosses the zero axis on your plot, determine the pKᵦ of the indicator. Report your three values for the pKᵦ.

Why does a plot of log(In⁻/HIn) vs. pH give a straight line? The answer is quite simple. Bromthymol blue is a monobasic indicator, which dissociates according to the general equation:

$$HIn \rightleftharpoons H^+ + In^-.$$
The equilibrium constant is
\[ K_a = \frac{[H^+] \cdot [In^-]}{[HIn]} \].

Taking logarithms:
\[ \log K_a = \log H^+ + \log \frac{In^-}{HIn} \],
or
\[ \log \frac{In^-}{HIn} = -\log H^+ + \log K_a = pH - pK_a. \]

This resulting equation is in the form \( y = mx + b \), since \( pK_a \) is a constant. Therefore, by plotting the \( "y" \) term versus the \( "x" \) term, a straight line would be expected, having a slope of \( m = 1 \). When \( In^- = HIn \), or when \( \log (In^-/HIn) \) equals zero, then \( pH = pK_a \). This relationship is utilized above in determining the \( pK_a \) of the indicator.

Questions.

1. a. What is meant by an isosbestic point?
   
   b. If the extinction coefficients of \( HIn \) and of \( In^- \) were determined at a \( \lambda \) corresponding to an isosbestic point, how would they compare in value?
   
   c. What factors could contribute to the inability of an operator to obtain an isosbestic point in a study of a system of this nature?
   
   d. Could this experiment be applied to an indicator for which no isosbestic point was obtained in the available spectral range? Explain briefly.

2. If you were given a series of bromthymol blue solutions of varying pH and were asked to determine the total amount of the indicator present in each solution, what wavelength(s) would you use for the analysis, and why? (No pH meter available.)

3. Suppose the indicator under study were a dibasic acid which dissociated according to the general equation:
\[ H_2In \rightleftharpoons 2H^+ + In^- \]

with both hydrogens coming off simultaneously in a single step (a rare case).
Questions - Continued.

3. Continued.

a. If \( \log \left( \frac{[\text{In}^-]}{[\text{H}_2\text{In}]} \right) \) were plotted against pH, what would the slope of the resulting line be?

b. When the line crosses the zero axis, in this case does \( \text{pH} = \text{pK}_a \)? (Show your reasoning.)

4. If the indicator dissociated according to the general equation (not a rare case):

\[
(\text{HIn})_2 \rightleftharpoons 2 \text{H}^+ + 2 \text{In}^-,
\]

what would you plot in order to get a straight line, and what would the slope of that line be? (Show your reasoning.)

5. (This one is to think about.)

Reference books list the range of bromthymol blue as an indicator from pH 6.0 to 7.8. Can you account for the difference between the true \( \text{pK}_a \) and an "apparent" \( \text{pK}_a \) deduced from this range?
EXPERIMENT 6
PHOTOMETRIC METHOD OF TITRATION

**Apparatus.**

Bausch and Lomb Spectronic 20 Colorimeter, with blue-sensitive phototube (type S-4).
3 Cuvettes, including special cuvette for observing light path color.
Red-sensitive phototube (IP 40).
Red filter.
Stirring bar.
Magnetic stirrer.
Ring stand.
Burette clamp.
Burette.
Beaker 250 ml.
Graduated cylinder. 100 ml.
Pipet. 5 ml.

**Solutions.**

EDTA (Ethylenediamine tetraacetic acid – disodium salt). 0.250 M. [100 g./liter solution]
Cu-Bi (0.05 to 0.1 M in each) solutions, acidified with 0.5 M HNO₃.
Labelled A, B, C, D, . . . (to be assigned)

**Theory.**

In the photometric method of equivalence point detection in titrations, use is made of the difference in the extinction coefficients (at the analytical wavelength selected) of the various species present. The appearance or disappearance of an absorbing species will give a linear, or concentration-dependent, change in absorbance which will yield two straight lines that intersect at the equivalence point. In this respect photometric titration curves resemble in shape the curves obtained in conductimetry and amperometry (in which the conductance and the current, respectively, also vary linearly with concentration).

The selection of the analytical wavelength requires much care, for there are at least three components present which may absorb light: the original substance, the titrant, and the resulting product or products. The usual procedure is to select some wavelength at which only one component absorbs. However, the mere fact that only one component absorbs at a particular wavelength does not necessarily mean that that particular wavelength should be selected for the analysis; for, if the absorbance is very intense, the %T readings may be limited to the undesirable 0-20 %T region, where comparatively large errors in measuring absorbance would overshadow the inherent accuracy of the photometric titration.
Theory - Continued.

For a successful photometric titration it is necessary that the measured species adhere to Beer's Law, and the necessary chemical and instrumental precautions must be observed to maintain the relation $A = \varepsilon_1 C$.

Some typical photometric titration curves are shown below for the reaction:

$$X + T \rightarrow P,$$

where $T$ is the titrant, $X$ the component to be determined and $P$ the product(s) of the reaction.

Case I. $X$ and $P$ both absorb.
Case II. $X$ and $T$ both absorb.
Case III. Only $T$ absorbs.
Case IV. One (or more) of the products, $P$, absorbs.
Case V. $P$ and $T$ absorb and $\varepsilon_T > \varepsilon_P$.
Case VI. $P$ and $T$ absorb and $\varepsilon_T < \varepsilon_P$.

Case II above offers the greater accuracy due to the acute angle of interaction, which facilitates locating the equivalence point. (This is similar to the plot obtained for an $\text{H}_2\text{SO}_4$-$\text{Ba(OH)}_2$ conductimetric titration.)

Procedure.

I. THE RED AND VERY NEAR INFRARED REGION OF THE SPECTRUM

A. The 2nd Order Visible Spectrum; Effect of Red Filter.

In the sample holder of the colorimeter place the special cuvette for observing the light-path color. Making observations at 50 m$m$ intervals, record the color of light observed from 750 to 1000 m$m$. The intensity of the beam should be regulated as high as possible in observing these colors.
A. The 2nd Order Visible Spectrum; Effect of Red Filter - Continued.

Compare the colors at 400 and 800 m\(\mu\), 475 and 950 m\(\mu\), and 500 and 1000 m\(\mu\).

[CAUTION: The instrument is much more sensitive to the shorter wavelength light; adjust the right-front knob accordingly.] The colors seen at the above pairs of wavelengths may appear somewhat different, due to the much lower intensity of the "longer wavelength" light; therefore try to compare only the average color observed.

Now hold the red filter (NOTE: do not touch the filter except by its edge; otherwise difficulty-removed fingerprints will remain) over the "special cuvette" and, looking at the chalk surface through the red filter, observe the visible spectrum from 350 to 650 m\(\mu\). Record the range of wavelengths obscured by the filter and the range not obscured. Carefully replace the filter in the lens paper and temporarily set it aside.


Remove the special cuvette and unplug the instrument. Carefully tilt the instrument back and unscrew the back plate. Remove the original (blue-sensitive) phototube—not the light bulb—by carefully pulling on the tube while wiggling it back and forth. Note that the inner surface of the curved "target" in the phototube is directed toward the sample holder. Insert the red-sensitive phototube (IP40) in place of the blue-sensitive tube. Any fingerprint smudges should be wiped off the phototubes with a clean Scott Wiper. Store the blue-sensitive tube in the box which originally contained the IP40 phototube. After making sure that the phototube is firmly seated in the colorimeter, shut the back plate, right the instrument and plug it in again.

Record the instrument response from 350 to 1000 m\(\mu\) at 50 m\(\mu\) intervals, except between 600 and 700 m\(\mu\) where a 25 m\(\mu\) interval should be used. This instrument response should be determined as in experiment 1. The intensity of the light beam is reduced (by turning the right-front knob) and the spectrum is scanned to determine the wavelength of maximum sensitivity. At this wavelength the instrument is set to 100 \% T with distilled water in the cuvette and the remaining readings are taken without changing the intensity control.


Again unplug the instrument and tilt it back. Unscrew the back plate and place the red filter carefully in position. Replace the back plate, taking care that the filter does not drop out of its slot.

Again record the instrument response, as in part B, from 350 to 1000 m\(\mu\).

The red-sensitive phototube and red filter combination are also used in the colorimeter during part II of this experiment.
Procedure - Continued.

II. PHOTOMETRIC TITRATION OF Bi-Cu MIXTURE

The copper-EDTA complex has a maximum absorption at 725 μm, and this wavelength should be used as the analytical wavelength. (See absorption spectrum data given in question 3 at end of experiment.) The other solute particles involved during the titration are all either non-absorbing or only very slightly absorbing with respect to 725 μm light.

Rinse and fill your burette with 0.250 M EDTA stock solution.

Pipet 10.0 ml. of Cu-Bi "unknown" solution, which will be available at your desk, into a clean 250 ml. beaker. (Note the letter of your unknown solution.) Dilute with approximately 140 ml. of water; keep a record of the exact amount which you add. Mix well, using the mechanical stirrer, before taking a measurement on the solution. CAUTION: The solution must be mixed thoroughly before every new measurement; at least a half-minute of stirring time should be allowed.

Using a 5 ml. pipet, or more simply by pouring directly out of the beaker, rinse your sample cuvette thoroughly, returning the rinsings to the beaker. Then refill your sample cuvette, and read the percent transmittance of this solution at 725 μm against a distilled water "blank."

Repeat the 100 % T adjustment and the % T reading on the sample, until suitable agreement is found. This is important.

Return the sample to the beaker. Add a 0.5 ml. increment of 0.250 M EDTA, and allow thorough mixing. Measure % T as above, repeating the measurement until suitable agreement is obtained.

Repeat the additions of EDTA increments and % T measurements until a total of 9.5 ml. of EDTA has been added. Return all rinsings and samples to the original beaker of solution being titrated.

If time permits, the above titration may be repeated for a duplicate check.

FINALLY, unplug the colorimeter, carefully remove the red filter and the red-sensitive phototube, and replace with the original blue-sensitive phototube.

Treatment of Results.

PART I.

Plot a graph (% T vs. λ) of the response of the colorimeter:
(a) with only the red-sensitive phototube, and
(b) with both the red-sensitive phototube and the red filter.
Include the spectral region obscured by the red filter.

PART II.

Plot the data obtained in the photometric titration (absorbance vs. ml. of 0.250 M EDTA). Three straight lines should result, which give two intersections corresponding to a copper equivalence point and a bismuth equivalence point.
Report.

A. Indicate which Cu-Bi solution you analyzed.

B. Calculate the number of moles of Bi(III) and Cu(II) in 10.0 ml of the Cu-Bi solution.

C. Assume that the unknown solution was made from a copper alloy. What must the empirical formula of the copper alloy have been? (i.e., Cu_{1.00}Bi_{1.71} or Cu_{1.00}Bi_{0.84}, etc.)

NOTE: While no direct statement has been made regarding which inflection point corresponds to Cu(II) and which corresponds to Bi(III), this should be evident from the discussion above and the shape of the titration curve. Be sure you associate the correct volume with the proper metal ion.

Questions.

1. If a solution contained only Bi(III), how could you determine the bismuth concentration photometrically with EDTA, as above? Outline briefly how you would perform this titration.

2. In the discussion above, it was pointed out that adherence to Beer's Law is essential for a successful determination. As in conductimetry, dilution of the sample by adding titrant may produce sufficient deviation so as to require correction.

   a. If \( V = \text{initial volume of solution} \) and \( v = \text{volume of titrant added} \), by which factor: \( \frac{V}{V+v} \), or \( \frac{V+v}{V} \), should you multiply the absorbance reading, in order to correct the absorbance for dilution?

   b. How may a photometric titration be carried out so that we can neglect the dilution caused by adding the titrant?

   c. What \( \% \) error in absorbance (due to dilution) results at your Cu(II) end point? at your Bi(III) end point?

   d. Would a plot of the corrected absorbance vs. volume of titrant be advisable in the titration just performed in lab?

3. The absorption spectrum of Cu(II)-EDTA was obtained using a cuvette of thickness identical to the one you used:

   | Wavelength (\( \mu \)) | A   | Wavelength (\( \mu \)) | A   |
---|-------------------|-----|-------------------|-----|
550 | 0.013            | 725 | 0.210            |
575 | 0.026            | 750 | 0.191            |
600 | 0.045            | 775 | 0.175            |
625 | 0.070            | 800 | 0.145            |
650 | 0.127            | 850 | 0.103            |
675 | 0.164            | 900 | 0.070            |
700 | 0.195            | 950 | 0.040            |
Questions - Continued.

3. Continued.

a. What concentration was employed for this absorption spectrum?

b. The sensitivity of a photometric titration system can be altered simply by the selection of wavelength. Thus for the titration of more concentrated solutions of copper, a wavelength where the molar extinction coefficient is smaller should be employed. Assuming that an absorbance reading of no more than 0.7 (i.e., % T no less than 20%) should be found during a titration, but also assuming that we wish as large a change as possible (e.g., 0.05 to 0.7 absorbance units), what wavelength smaller than 725 μm should be employed in the titration of a 10 ml sample of 1 M copper solution (diluted to 150 ml, before photometric measurement)? [See Fig. 1 in the article by Sweetser and Bricker, Anal. Chem., 24, 1107 (1952).]

4. a. What is the wavelength relationship between 1st and 2nd order spectra?

b. Orange light occurs at a wavelength of 600 μm. At what wavelength will orange light occur because of 2nd order diffraction? At what wavelength will orange light occur because of 3rd order diffraction?

c. Does a prism instrument suffer from this cause of "extraneous light"?

d. What is the purpose of the red filter? Would this red filter be suitable for use at 1300 μm? (Why, or why not?)

e. A substance absorbs strongly at 900 μm but not at all at 450 μm. If no red filter is employed, would you expect Beer's Law to be followed at 900 μm? Explain briefly.
EXPERIMENT 7

"PRECISION" OR "DIFFERENTIAL" SPECTROPHOTOMETRY

Apparatus.

Bausch and Lomb Spectronic 20 Colorimeter.
2 Cuvettes.
Pipets. 1, 2, 3, 4, 5 and 10 ml.
Beaker. 150 ml.
Graduated cylinder. 100 ml.
Volumetric flasks. Nine 25 ml. (including 2 for unknowns: A and B)

Stock Solution.

Chromium nitrate. 0.2500 M.

Unknowns.

Usually from 0.10 to 0.15 M in chromium, when diluted to the mark.

References.

An interesting approach to practical differential colorimetry is the paper by Bastian [Anal. Chem. 21, 972 (1949)]. Other good references include: pp. 50-55, 62-63 of Analytical Methods of Analysis, 3rd Ed., by Willard, Merritt and Dean; and pp. 151-154 of Instrumental Methods of Chemical Analysis by Ewing. The Willard, Merritt and Dean reference gives an especially clear and concise description of each of the three types of precision colorimetry: the high absorbancy method, the low absorbancy method, and the method of ultimate precision; and Ewing includes a quoted explanation of the $\beta$ factor, used to correct the absorbance readings for differences between the cuvettes.

Some of the nomenclature in the above literature references is different from that used in these experiments. $I$ is often used instead of $P$ to indicate light intensity. $\alpha$ ( = extinction coefficient when $C$ is in grams per liter, sometimes called the absorptivity) corresponds to $E$, multiplied by the molecular weight of the substance being measured. $b$ is often used to refer to cell length, herein indicated by $l$. Transmittancy is $T$.

Theory.

The ordinary colorimetric method, although probably more widely used than any other method of analysis, suffers two drawbacks – (1) operation limited to samples transmitting between 20 and 65%, and (2) lower precision than volumetric and gravimetric techniques. For this latter reason, the determination of major components by colorimetry is seldom attempted. These two limitations may often be circumvented,
Theory - Continued.

however, by a differential approach to the spectrophotometric measurement, and results whose accuracy approaches that of volumetric procedures may be readily achieved. This experiment illustrates such an approach.

The four types of spectrophotometric measurements may be classified according to the "standards" used to establish the "0" and "100" points on the percent transmittance scale. These standards actually correspond to known concentrations \( C_1 \) or \( C_2 \) of the sample species: \( C_1 \) being used to set the "0" and \( C_2 \) to set the "100"; \( C \) corresponds to the unknown concentration of the sample species whose value is to be determined by the procedure.

<table>
<thead>
<tr>
<th>Method</th>
<th>&quot;0&quot; % T Standard</th>
<th>&quot;100&quot; % T Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Ordinary</td>
<td>Shutter ( C_1 = \infty )</td>
<td>Solvent ( C_2 = 0 )</td>
</tr>
<tr>
<td>II. High Absorbance</td>
<td>Shutter ( C_1 = \infty )</td>
<td>Solution of definite concentration ( C_2 &lt; C )</td>
</tr>
<tr>
<td>III. Low Absorbance</td>
<td>Solution of definite conc. ( C_1 &gt; C )</td>
<td>Solvent ( C_2 = 0 )</td>
</tr>
<tr>
<td>IV. Ultimate Precision</td>
<td>Solution of definite conc. ( C_1 &gt; C )</td>
<td>Solution of definite conc. ( C_2 &lt; C )</td>
</tr>
</tbody>
</table>

A detailed analysis of each method may be found by consulting the references given above. The following discussion is to provide a general view of the three precision methods (II, III, and IV).

The high absorbancy method is a modification of the ordinary colorimetric method, whereby solutions of high concentration (or more properly, high absorbancy) may be measured with considerably greater accuracy. In the ordinary colorimetric method, two cuvettes (cells) are used, one containing the sample solution under analysis and the other containing pure solvent (the 100% T "blank"). Arrangements are made instrumentally so that the absorbance of the solvent and cuvette is subtracted from the absorbance of the sample, the difference being the absorbance of the desired compound. While this method is applicable to solutions of moderate absorbancy, solutions of high absorbancy cannot be measured accurately. To allow accurate measurements of high-absorbancy solutions, a known solution of the substance to be measured is employed as the 100% T "blank," rather than the pure solvent. The known solution to be used as the "blank" is so chosen as to be slightly more dilute than the unknown. By setting the 100% T with such a known solution, one effectively increases the size of the % T scale (what had once been 2 or 10 % T, for example, may now read 100 % T, while no change occurred with the 0 % T). Accordingly, greater accuracy is possible in the % T readings and the concentration determinations.
Theory - Continued.

In this high absorbancy method, the absorbance read becomes the difference between the absorbance of the sample and the absorbance of the standard, or (where \( k \) represents \( E \)):

\[
A = kC - kC_2 = k(C - C_2).
\]

The low absorbancy method applies to the other extreme in concentration range. Its application is in trace analyses. In this precision method the 100 % T is set, as usual, with distilled water. The 0 % T, however, is set by use of a known solution of the substance being measured, with a concentration that is somewhat greater than that of the unknown solutions. The effective size of the % T scale is thereby increased, similarly to the case above, except that this time weakly-absorbing solutions are favored. With the low absorbancy method, however, unlike the high absorbancy method and ordinary colorimetry, a calibration curve (log % T vs. conc., or A vs. conc.) must be plotted in order to readily determine the concentration of an unknown. because C and log % T are not directly proportional.

The method of ultimate precision involves a combination of the two methods above. The 0 % T is set using a standard solution somewhat more concentrated than the unknown, and the 100 % T is set using a standard solution somewhat less concentrated than the unknown. Because of non-linearity, a calibration curve is required with this method also.

Cell Corrections.

Because of the non-identity of the sample and reference cells, a correction (\( \beta \) factor) is evaluated and applied. A brief description of the beta factor is given here; this is intended to be a guide for use in evaluating the experimental data and should not be considered a full explanation.

Consider two cuvettes which are identical only in the fact that they contain the same solutions \( [ \text{e.g., 0.0300 M Cr(III)}] \). Further denote one of the cuvettes as the sample cuvette by the subscript \( s \), and the other as the reference or "blank" cuvette by the subscript \( r \). If Beer's Law holds, we will have \( A_s = \varepsilon_s l_s C_s \) and

\[
A_r = \varepsilon_r l_r C_r \text{ at some one wavelength, where } A_s \text{ is the absorbance reading obtained with the sample cuvette and } A_r \text{ with the reference cuvette.}
\]

But suppose that \( A_s \neq A_r \), as will be the case in most optical work due to the difference in the optical properties of the two cuvettes. Since the two solutions in the cuvettes are identical, \( C_s = C_r \) and \( \varepsilon_s = \varepsilon_r \). Divide:

\[
\frac{A_r}{A_s} = \frac{\varepsilon_r l_r C_r}{\varepsilon_s l_s C_s} = \frac{l_r}{l_s} = \beta.
\]
Cell Corrections - Continued.

To correct a value of $A'$ (transformed from the %T read) for some other concentration — assuming $\varepsilon$ is constant and thus that Beer's Law is valid —, multiply:

$$A' \times \frac{A_r}{A_s} = A' \times \varepsilon = A^*_s,$$

where $A^*_s$ is the calculated absorbance and $A^*_s$ is the corrected absorbance. The determination of $\varepsilon$ is done in part I of the Procedure.

Procedure. (High Absorbancy Method)

Observe the operating procedure for the Spectronic 20.

**NOTE:** In order to achieve the expected high accuracy inherent in the high absorbancy method, careful technique in every operation is mandatory.

In the volumetric flasks carefully prepare solutions of the following concentrations: 0.0100, 0.0200, 0.0300, ..., 0.1400, and 0.1500 M, by diluting appropriate volumes of the 0.2500 M Cr(III) stock solution with distilled water. Take about 150 ml. of the stock solution to use for preparing all the solutions in this experiment and for rinsing the pipettes. These solutions may be prepared in sets of five, as they are needed. Do not discard the 0.0500 M nor the 0.1000 M solution until you have completed the experiment.

It is very important in this experiment that the "blank" cuvette be kept as the "blank." There are times when both cuvettes will contain Cr(III) solutions (the "blank" consisting of a Cr(III) solution, rather than of distilled water), and some intermixing of cuvettes may result unless care is used. It is also imperative that the cuvettes be placed in the sample holder always in exactly the same position. The analytical wavelength used is about 550 nm. (Do not use the wavelength of ca. 575 nm determined in expt. 1 as the optimum wavelength.) This new wavelength is selected by considering all of the data of expt. 1 — a combination of the $\lambda$ of strong absorption and $\lambda$ of high instrumental response, this latter reason being a major consideration.

I. PRELIMINARY EVALUATION OF CUVETTES

Set 100 %T on the instrument, using distilled water in the "blank" cuvette. ($\lambda = 550$) Empty the cuvette and fill it with the prepared 0.0300 M solution and record the %T. Now fill the "sample" cuvette with the 0.0300 M solution and immediately read the %T obtained with this cuvette. The %T readings should be near 36.8 %T.

During the remainder of the experiment, use the cuvette giving the greater %T reading as the "blank" cuvette. (Mark the cuvette accordingly.)

From your %T readings, calculate the beta factor to be used with the cuvettes.
Procedure. (High Absorbancy Method) - Continued.

II. CALIBRATION CURVES.

A. Using distilled water as the "blank" (with due attention to the proper cell and its position), measure and record %T of each solution 0.0100 through 0.0500 M.

B. Now rinse and fill the "blank" cuvette with the 0.0500 M solution. (NOTE: do not discard the 0.0500 M solution.) Using this solution as the "blank" or reference measure the %T of Cr(III) solutions in the concentration range 0.0500 through 0.1000 M.

C. Make similar measurements on 0.1000 - 0.1500 M solutions, using the 0.1000 M solution as the "blank" or reference. If the instrument cannot be set to 100 %T with the 0.1000 M solution as reference, use the solutions of next lower concentration until one is found which can be set at 100 %T. With high concentration solutions, errors due to fluctuations in the %T readings may be diminished by making rapid consecutive readings on the reference and the sample solutions. (NOTE: do not discard the solution you used above as the reference.) This process theoretically could be continued indefinitely, but in practice solutions of greater than about 0.13 M cannot be balanced to 100 %T using the Spectronic 20.

D. Convert all the %T readings to absorbancy, and correct these A values using your \( \beta \) factor. Plot the corrected A values vs. concentration on a graph. The resulting plot should consist of several sloping lines which are approximately straight and are parallel to one another.

III. UNKNOWNS.

Dilute the unknowns (A and B) to the mark with distilled water and, by using proper "blank" solution(s), determine the %T of each.

From the %T values and using the \( \beta \) factor, determine a corrected absorbance value for each unknown. (NOTE: The \( \beta \) factor must be determined anew each day that %T readings are made; thus, any %T readings obtained on the second day should be corrected using the \( \beta \) factor determined on that day.

Determine the molarity of the two unknowns. REPORT your results to four figures to the right of the decimal point (i.e., 0.1254 M and 0.0975 M for A and B respectively; ect.).

Analysis of Error. (Ordinary and High Absorbancy Methods).

Under the assumption that the system obeys Beer's Law and that the error may be solely attributed to inaccuracy in reading the %T scale, we can make a rather complete yet simple treatment of the expected error in the analysis. This treatment consists of calculating the expected error at each experimental point on your calibration curve, and then noting how the expected error varies from point to point on the curve.
Analysis of Error.  (Ordinary and High Absorbanicy Methods). - Continued.

The first step in the treatment is to evaluate the error in concentration (dC) resulting from an error in reading the percent transmittance scale by a given amount (dT). This error, dC, is the absolute error. It depends strongly upon the portion of the transmittance scale where the reading is made: in the high transmittance region the absolute error is smaller than in the lower transmittance region. The quantitative relationship is easily derived from Beer’s Law:

\[ A = - \log T = \varepsilon l (C - C_0) \]

or:

\[ -A = \log T = -k (C - C_0) \]

\[ 0.43 \ln T = -k (C - C_0) \]

Therefore:

\[ dC = -0.43 \frac{k}{T} dT. \]

Whenever T reads 1 (absorbance = 0), then \( \frac{dC}{dT} = -0.43 \). Since T cannot exceed unity, \( \frac{dC}{dT} \) can never be smaller than -0.43. Note also that the \( k \) above, is the slope of the line which results when you plot absorbance vs. concentration; thus you can easily calculate \( k \) from your calibration curves.

Determine the three \( k \) values corresponding to the three straight lines on your A vs. C graph. (The three \( k \) values should be similar.) Using these \( k \) values and the relationship above, calculate the absolute error for each of your 17 experimental points, assuming an error of 1 % in reading the percentage transmission scale (dT = 0.01).

Of more significance to the analyst, however, is the relative error, which is the absolute error in concentration divided by the actual concentration (and often then multiplied by 100 for relative percent error; i.e., \( \frac{dC}{C} \times 100 \)). Calculate the relative percent error (for 1 % error in reading the percentage transmission scale) for each experimental point, and record each of these values at the proper points on your A vs. conc. graph.

Questions.

1. a. Calculate the relative percent error caused by misreading the %T scale by 1% at the point of minimum error for the ordinary colorimetric method (i.e., at 36.8 %T).

b. Find on your calibration curve (high-absorbanicy technique) the point where the relative percent error is lowest. How much greater precision (i.e., 2-fold, 8.2-fold, ...?) results from operating near this point using the high-absorbanicy technique, rather than diluting the sample so as to operate in the 36.8 %T (optimum) region using the ordinary colorimetric technique?
Questions - Continued.

1. Continued.

c. Which of your 15 standard solutions should be used as the 100 % T reference solution, in order to obtain an optimal analysis: of an unknown Cr(III) solution, which happens to be 0.096 M. of your two unknown Cr(III) solutions?

2. If you were measuring the concentrations of aqueous Cr(III) solutions, using a wavelength of 550 m, which of the four methods of colorimetry, described above, would you use for solutions in the following concentration ranges? (Why?)

   a. Range of $10^{-3}$ to $10^{-2}$ M.
   
   b. Range of $10^{-2}$ to $10^{-1}$ M.
   
   c. Range of $10^{-1}$ to 1 M.
   
   d. Range of 0.04 to 0.05 M.
EXPERIMENT 8
TRISTIMULUS COLORIMETRY

Theory.

In previous experiments color as a property of the substance has been measured, specifically the ratio of incident intensity to the departing intensity. In this experiment color as a property of light and light alone will be investigated. The Committee on Colorimetry of the Optical Society of America gives this definition of color: "Color consists of the characteristics of light other than spatial and temporal inhomogeneities; light being that aspect of radiant energy of which a human observer is aware through the visual sensations ... ."

It has long been known by those who work with colors that three independent colors or primaries are necessary to match a given color. Only rarely will a match be obtained by fewer than three primaries. Since three primaries are necessary to match any given color then it follows that there should be three numbers to describe any color.

After many years of study of matching colors and intensities using hundreds of observers to establish an average observer there has emerged a system by which a color may be described using three numbers. Since it has been shown that any three real monochromatic colors cannot be mixed to give all other colors, three parameters have been established, by which all real colors can be specified.

One of these parameters, Y, indicates the brightness of the color. This is one quality of color to which the eye is sensitive. The other parameters, X and Z, are not associated with brightness. However, from these three parameters or tristimulus values two ratios are obtained \( x = X/(X + Y + Z) \) and \( y = Y/(X + Y + Z) \). Here \( x \) and \( y \) are chromaticity coordinates which indicate the proportional part of \( X \) and \( Y \) which are needed to match the specific color. \( z \) is also a chromaticity coordinate but by definition \( x + y + z = 1 \) so \( z \) is fixed when \( x \) and \( y \) are calculated. For this reason the chromaticity diagram has only \( x \) and \( y \) axes. By agreement these quantities \( x, y, z \) may not have negative values, \( Y, x \) and \( y \) are the three numbers, then, which describe a color specifically.

From these studies there evolved certain multiplying factors, \( M_i \) where \( i \) may be \( X \) or \( Y \) or \( Z \), by which any spectral curve between 380 m\( \mu \) and 780 m\( \mu \) can be converted to the three numbers which identify the color of light; whether it be reflected, transmitted or absorbed light. By multiplying the intensity of light at a specific wavelength by the appropriate \( M \) and adding these products together for all the wavelengths \( X, Y, \) and \( Z \) may be found; or mathematically for example

\[
X = \int_{380}^{780} E M_x \lambda d\lambda
\]

Where: \( E \) represents the percent transmittance, or reflectance.
From this it can be seen that

\[
x = \frac{\int \sum_{i=x}^{z} \frac{780}{380} E M_i \, d\lambda}{\int_{z}^{380} E M_i \, d\lambda}
\]

\[
y = \frac{\int \sum_{i=x}^{z} \frac{780}{380} E M_i \, d\lambda}{\int_{z}^{380} E M_i \, d\lambda}
\]

(2,3)

The International Commission on Illumination (I.C.I.) recommended three standard illuminants. I.C.I. illuminant C is used in this experiment. This illuminant represents average daylight or a color temperature of about 6000°K.

I. REFLECTANCE MEASUREMENTS

The determination of spectral reflectance is an important aspect of appearance specification. However, the apparent color of any material is a strong function of the viewing environment. The viewing environment of the Reflectance Attachment for the Spectronic 20 is a specialized one designed to measure total reflectance less the part of the specular component being absorbed by the sample. This will give readings closely related to appearance but not necessarily the same as that of a different reflectance measuring instrument.

Calculation.

It is readily seen that use of equations (2,3) is tedious and lengthy. Therefore several methods of approximation have been developed, of which two will be discussed here. The "B & L Trichromatic Coefficient Computing Form for Illuminant C", shortened to "B & L Computing Form" is one method for determining color points. On this form there are 2 sets of 10 scales and 1 set of 7 scales, each scale preceded by a wavelength. Each scale has the % intensity reading from 0 to 100% across the top. Directly below the % intensity reading is the product of intensity and the M_i factor for that wavelength and color coordinate. In this part of the experiment for intensity use % R. For each wavelength take the number appearing below the % R, read on instrument, and record it at the right. Each of these number corresponds in the first set of scales to an X value. When the values at the right are added together for each set of scales the result is the color coordinates \( \Sigma X, \Sigma Y, \Sigma Z \) from which x, y and Y, the color coordinates, are calculated as below.

The second method is to take % R readings at the same ten wavelengths and actually do the multiplying (the B&L form does the multiplying for you on the slide rule scales). Using Table 1, place % R readings in column A, and multiply each reading by the corresponding multiplier in the X, Y, and Z columns. The resulting values of X, Y, and Z are totaled to give \( \Sigma X, \Sigma Y, \Sigma Z \). The color coordinates (x,y) are obtained by then dividing \( \Sigma X \) and \( \Sigma Y \) by \( \Sigma X \), \( \Sigma Y \), \( \Sigma Z \).
TABLE I

<table>
<thead>
<tr>
<th>A</th>
<th>(X) Multiplier</th>
<th>(Y) Multiplier</th>
<th>(Z) Multiplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>415</td>
<td>1.97</td>
<td>.05</td>
<td>9.40</td>
</tr>
<tr>
<td>445</td>
<td>12.15</td>
<td>1.04</td>
<td>61.90</td>
</tr>
<tr>
<td>475</td>
<td>4.98</td>
<td>3.93</td>
<td>36.30</td>
</tr>
<tr>
<td>505</td>
<td>.07</td>
<td>12.30</td>
<td>6.40</td>
</tr>
<tr>
<td>535</td>
<td>6.38</td>
<td>25.80</td>
<td>.84</td>
</tr>
<tr>
<td>565</td>
<td>19.90</td>
<td>28.70</td>
<td>.08</td>
</tr>
<tr>
<td>595</td>
<td>27.20</td>
<td>18.85</td>
<td>.03</td>
</tr>
<tr>
<td>625</td>
<td>18.60</td>
<td>8.10</td>
<td>---</td>
</tr>
<tr>
<td>655</td>
<td>5.43</td>
<td>2.22</td>
<td>---</td>
</tr>
<tr>
<td>685</td>
<td>0.76</td>
<td>0.28</td>
<td>---</td>
</tr>
</tbody>
</table>

\[ x = \frac{\Sigma X}{\Sigma X + \Sigma Y + \Sigma Z} \quad y = \frac{\Sigma Y}{\Sigma X + \Sigma Y + \Sigma Z} \]

Procedure.

Dye a good grade hard surface filter paper with three shades of each dye supplied. Use dye in stock strength for one sample, then dilute successive portions by 2/3 and 1/3 stock strength to get lighter shades. The filter paper is best cut to 1-1/2 inch diameter discs before dyeing and the entire disc thoroughly saturated with the dye. While the samples are drying, usually about three to four hours is sufficient, calibrate the secondary standard according to the operating procedure below. This secondary standard is located in the cover of the reflectance attachment on the Spectronic 20. Care should be taken not to get fingerprints on this white porcelain plate. A cake of MgCO₃ is supplied as the primary standard. It is soft and powdery so do not handle unnecessarily.
Operation of Spectronic 20 with Reflectance Attachment.

1. Plug in auxiliary power source and Spectronic 20; turn on and adjust Spectronic 20 to read 0% R. Read as % T on the dial of the Spectronic 20. Allow 15 minutes for warm up.

2. Set instrument to the first of the ten selected wavelengths and readjust 0% R.

3. Raise sample cover slightly and rotate out of the way being careful not to touch the white under surface.

4. Place the MgCO₃ block over the opening so that a flat surface makes contact with the sponge gasket all the way around. Do not allow the block's surface to touch any foreign object during the standardization procedure. Now set 100% R by depressing the shutter lever on the left side of the housing and adjusting the light control knob. If this step is not reproducible, the MgCO₃ block is probably not perfectly smooth and may be improved by gently sliding it back and forth on a flat piece of clean paper. The MgCO₃ block is very fragile and should be handled as little as possible. Never depress the shutter lever when the opening is uncovered.

5. Replace the cover; if necessary, clean the white surface with a Scott wiper moistened with distilled water; dry with clean wiper; depress shutter lever and read and record the % R at the first wavelength. This is the secondary standard.

6. Repeat steps 2-5 for the remaining wavelengths.

7. At the first wavelength, set 0% R and then set the % R found for the secondary standard in step 5.

8. Place sample over opening, close cover, depress shutter lever, read and record % R of the sample. Repeat for each sample and at each wavelength.

9. Determine the color coordinates of reflected light for each sample in the following ways:

   1. One sample using B&L forms.
   2. One sample using Table I.
   3. The remaining sample using either method.

10. Plot the points on the attached chromaticity diagram.
II. TRISTIMULUS COLORIMETRY FOR MASKING AN INDICATOR

A. Determining Color Coordinates and Color Concentrations.

In a 50 ml. volumetric flask pipette 5 ml. of methyl orange stock solution and 5 ml. of 0.2 N HCl and dilute to the mark with distilled water, and in another flask pipette 5 ml. of methyl orange stock solution, and 5 ml. of 0.2 N NaOH and dilute to 50 ml. with distilled water. From these two solutions determine the absorbance of the acid and base forms of the methyl orange indicator at the ten wavelengths in Table I.

Dilute 5 ml. of blue dye and yellow dye stock solutions to 50 ml. with distilled water and obtain the absorbance of these two dye solutions at the ten wavelengths. Look at end of last page.

Before the next laboratory period calculate the complementary color coordinates of the two forms of the indicator and the two dye solutions by either the methods previously described using absorbance instead of % R.

Calculate the complementary color point coordinates of a 50/50 mixture of acid and base forms of the methyl orange in a similar manner but using the absorbance values obtained by adding the absorbance of the two forms at each wavelength and dividing by 2.

Also calculate the coordinates of the I.C.I. illuminate point by taking the absorbance to be equal to 1 at all wavelengths. Next plot all complementary color points on the chromaticity diagram furnished. If the calculations were carried out properly, the complementary color point for the 50/50 mixture of indicator will lie on a line connecting the complementary color points of the acid and base forms but not necessarily half way between.

The color concentrations (J) of the dye solutions and the 50/50 mixture of indicator are calculated by dividing \( \Sigma X + \Sigma Y + \Sigma Z \) for the solution by \( \Sigma X + \Sigma Y + \Sigma Z \) obtained when the absorbance was 1 at all wavelengths. Multiply the resulting J values by the dilution factor and obtain the J value of the stock solutions. The color concentration (in units of absorbance per unit path length) is therefore a measurement of the amount of light absorbed by a solution ("weighted" according to the tristimulus parameters) as compared to a solution that has an absorbance of 1 (per unit path length) at all wavelengths.

The volume ratio of dye solutions to indicator solution necessary for proper masking of the methyl orange is calculated by solving simultaneously the following equations.

\[
(x_{\text{In}} - x_{G,P.})(J_{\text{In}}) + (x_{B} - x_{G,P.})(J_{B}) + (x_{Y} - x_{G,P.})(J_{Y}) = 0
\]

\[
(y_{\text{In}} - y_{G,P.})(J_{\text{In}}) + (y_{B} - y_{G,P.})(J_{B}) + (y_{Y} - y_{G,P.})(J_{Y}) = 0
\]
A. Determining Color Coordinates and Color Concentrations - Continued.

where x and y are the complementary color point coordinates of the indicator (In), blue dye solution (B), yellow dye solution (Y) and the I.C.I. illuminant point or grey point (G.P.) and J is the color concentration of the indicator (In) and dye solutions (B) and (Y). All values of x and y are known, as well as the value of J\textsubscript{In} (it is the value of the stock indicator solution would have if it were a 50/50 mixture of acid and base forms) and then the values of JB and JY are necessary for masking are calculated. To find the ratio of indicator to yellow and blue dye, we divide the values of J\textsubscript{In}, JB and JY that satisfy the two equations by the color concentrations of the corresponding stock solutions.

Next titrate 10 ml. 0.1 M NaAc diluted with 90 ml. of water using 0.2 M HCl by measuring the pH every 1/2 ml. up to 3.5 ml. and every .25 ml. up to 6.5 ml. and then every 1/2 ml. up to 10 ml. Plot pH vs. ml. titrant (HCl).

B. Mixing and Evaluating the Masked Indicator.

Prepare about 25 ml. of mixed indicator by measuring with a burette the proper proportions of indicator and dye solutions.

In a 400 ml. beaker place 10 ml. of 0.1 M NaAc and about 250 ml. water. Adjust the solution to pH 3.0, using a pH meter, by adding 0.2 M NaOH or 0.2 M HCl with an eye dropper. Fill two test tubes 2/3 full with the pH 3.0 solution and mark them with a grease pencil. Now adjust the solution to pH 3.6 and likewise place some of the solution in two other test tubes and mark. Repeat this process at pH 3.0, 3.1, 3.2 . . . until 10 pairs of test tubes are filled. The last pair of test tubes will contain solution of pH 3.9. With one set of 10 test tubes add about 5 drops of methyl orange to each tube (add the same amount to each tube) such that the indicator color will be easily seen at each pH. Repeat this process using about 10 drops of mixed indicator for the other set of test tubes. Record in your notebook the color of each indicator at the various pH’s, and also record these colors (for both indicators) along the pH axis of your titration curve for sodium acetate with HCl.

Titrate a sample of 10 ml. 0.1 M NaAc diluted to 100 ml. with 0.2 M HCl using mixed indicator. When the end point is reached, measure the pH with a meter and by referring to your titration curve determine if the color change occurred at the end point. Repeat the titration using methyl orange. If time permits do 2 or 3 titrations using each indicator. Compare the end point and deviation in parts per thousand using the two indicators.

Questions.

1. If only the blue dye solution had been used to mask the methyl orange, would the colorless point (grey point) occur at a pH higher or lower than the pH you obtained in your experimental data? (Explain briefly).
Questions - Continued.

2. If you had at your disposal, dyes that would produce monochromatic colors, what single wavelength dye would you choose to mask methyl orange at the same ratio $H\text{ln}/I\text{n}$ as you used experimentally?

3. You want to mask methyl red indicator when ratio $I\text{n}/H\text{ln} = 1$ (complementary color point at this ratio is $x = 0.160$, $y = 0.310$) and you have 4 stock dye solutions from which to choose.

<table>
<thead>
<tr>
<th>dye</th>
<th>Complementary Color Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$x = 0.400$  $y = 0.210$</td>
</tr>
<tr>
<td>B</td>
<td>$x = 0.460$  $y = 0.210$</td>
</tr>
<tr>
<td>C</td>
<td>$x = 0.520$  $y = 0.410$</td>
</tr>
<tr>
<td>D</td>
<td>$x = 0.430$  $y = 0.490$</td>
</tr>
</tbody>
</table>

There are six possible combinations of two dyes ($A + B$, $A + C$, $A + D$, $B + C$, $B + D$, $C + D$). Tell which four of the combinations could be used and list them in order with the best choice first.

4. If the $\%$ $R$ of 3 shades of some dye is converted to absorbance by the relation $A = 2 - \log \% R$. Where will the complimentary color points lie with respect to each other? How do you account for this?
EXPERIMENT 9
SPECTROPHOTOMETRIC DETERMINATION OF STABILITY CONSTANTS AND THE FORMULA OF COMPLEX IONS

Apparatus.

Spectronic 20 Colorimeter.
2 Pyrex Cells.
Pipets: 2, 5, 10, 20, and 25 ml.
Volumetric flasks, 2-250 ml. and 10-25 ml.
Beakers. 50 and 125 ml.
Magnetic Stirrer.
Leeds Northrup pH Meter.

Solutions.

0.500 M Cupric nitrate stock solution.
0.500 M Iminodiacetic acid stock solution.

References.


Theory.

I. METHODS FOR DETERMINING THE FORMULA OF A COMPLEX

A. Method of Continuous Variations.

This method is the most widely used and versatile method available for the spectrophotometric determination of complex formula. It can also be used for stability constant determination if there is only one complex formed.

1. The sum of the total analytical concentrations, C, of complex $C_x$, and metal ion, $C_m$, is held constant and only their ratio is varied

$$C_x + C_m = C$$

2. A wavelength of light is selected where the complex absorbs strongly and the ligand and metal ions do not.

3. A plot of the mole fraction of ligand in the mixture, $X_l$, versus absorbance.
Where:
\[
X = \frac{C_x}{C}
\]
\[
\frac{C_x + C_m}{C} = \frac{C}{C}
\]
\[
1 - X = \frac{C_m}{C}
\]
gives the above triangular shaped curve. The legs of the triangle are extrapolated until they cross. The mole fraction at the point of intersection gives the formula of the complex, since here (where the ligand and metal are in proper relative concentrations to give maximum complex formation) for the complex \(MX_n\)
\[
n = \frac{C_x}{C_m} = \frac{X}{1-X}
\]

4. To determine whether there is more than one complex formed in solution (which would lead to erroneous results), the method is usually carried out at several different wavelengths and at several different values of C.

5. In the vicinity of maximum absorbance the actual curve may be observed to deviate somewhat from the extrapolated intersecting lines. From the amount of deviation one can determine the stability constant of the complex. For an explanation of this calculation see the section below on the determination of stability constants.

B. Molar Ratio Method.

This method is very similar to the method of continuous variations. The difference lies in the fact that the total analytical concentration of metal is held constant rather than the sum of the ligand and metal concentration. At a wavelength where only the complex absorbs, the absorbance versus ligand concentration curve is exactly the same as that obtained for a photometric titration where only the product absorbs.

1. The absorbance of solutions of constant total metal ion concentration and varying ligand concentrations is measured.

2. A plot of absorbance versus the ratio of ligand to metal concentration gives a curve like the one below, if only the complex absorbs.
B. Molar Ratio Method - Continued.

3. The straight line portions are extrapolated to where they cross. The ratio at this point is the ratio of ligand to metal ion in the complex.

4. The difference between the extrapolated values and the actual values of the absorbance can be used for stability constant calculation. See the discussion below for this calculation.

5. If the dissociation constant of the complex is too large, then a smooth continuous curve is obtained which cannot be accurately extrapolated to a good value of $R$. In this case the slope ratio method is best.

C. Slope Ratio Method.

This method is most valuable for weak complexes, since the absorbance measurements used with this method involve only solutions containing a large excess of the metal and solutions containing a large excess of the ligand. However, the method is applicable only when one complex species is formed and when Beer's Law is followed, and these are two rather limiting restrictions.

1. The absorbance of solutions containing a large excess of metal ion and various ligand concentrations is measured at a wavelength where only the complex, $M_n X_m$, absorbs.

2. A plot of absorbance versus total ligand concentration gives a straight line (if Beer's Law is obeyed) whose slope, $S_x$, is:

$$S_x = \frac{\varepsilon_c \ell}{n}$$

Where $\varepsilon_c$ is the molar absorptivity of the complex and $\ell$ is the cell length.

3. Similar measurements are made on solutions with a large excess of ligand and various metal ion concentrations. Another plot of absorbance versus metal ion concentration gives a line with the slope, $S_m$, equal to:

$$S_m = \frac{\varepsilon_c \ell}{m}$$
C. Slope Ratio Method - Continued.

4. The formula is then determined since:

\[ \frac{S_m}{S_x} = \frac{n}{m} \]

for the complex \( M_m X_n \)

II. **METHODS FOR DETERMINING THE STABILITY CONSTANT OF A COMPLEX**

A. Continuous Variation and Molar Ratio Methods.

The extrapolated absorbance values, \( A_{\text{expt.}} \), near the "equivalence point" on the molar ratio and continuous variations plots correspond to the total absorbance of the complex if complex formation were complete. Actually the complex is slightly dissociated in this region and the true absorbance read is therefore somewhat lower.

1. With a 1:1 complex (for which this method is generally applicable since only one complex species forms), the ratio of the true absorbance to the extrapolated absorbance is the mole fraction of complex actually formed:

\[ \frac{A}{A_{\text{expt.}}} = \frac{[MX]}{C} \]

Where \( C \) is the total analytical concentration of the metal or ligand (whichever is the limiting concentration at the point in question.) Then,

\[ [MX] = \left( \frac{A}{A_{\text{expt.}}} \right) C \]
\[ [M] = C_m - [MX] = C_m - \left( \frac{A}{A_{\text{expt.}}} \right) C \]
\[ [X] = C_x - [MX] = C_x - \left( \frac{A}{A_{\text{expt.}}} \right) C \]

\[ K_{\text{dissociation}} = \frac{(C_m - \left( \frac{A}{A_{\text{expt.}}} C \right)) (C_x - \frac{A}{A_{\text{expt.}}} C)}{(A / A_{\text{expt.}}) C} \]

\[ = \frac{[M] [X]}{[MX]} \]

where \( C_m \) and \( C_x \) are the total analytical concentrations of metal and ligand, respectively.

B. Bjerrum's Method.

This method is applicable when the ligand is either an acid or a base with known ionization constant, or in general whenever there are two different equilibria which can compete for the metal or ligand and when one of these equilibrium constants is known. With an acid ligand we have protons competing with the metal
B. Bjerrum's Method - Continued.

Ions for the ligand ion; if we add a large excess of metal ion and determine the complex concentration as a function of pH we can, knowing the ionization constants for the ligand, compute the formation constants for the complex. Similarly, we could compute one metal ion against another for the ligand ion and, by using the stability constant of one metal complex, determine the stability constant for the other metal complex.

1. The absorbance of the complex in the presence of a 100-fold excess of metal ion is measured in the pH range where a proton can successfully compete with the metal ion for the complex.

2. A plot of absorbance versus pH, where the complex is the only light-absorbing species gives:

\[
\begin{align*}
A & \quad \text{A}_1 \quad \text{A}_2 \\
\text{pH (increasing \textup{---})} &
\end{align*}
\]

where \( A_1 \) is the absorbance of the metal complex less the limiting absorbance of the solution at low pH, and \( A_2 \) is the absorbance of the metal when completely complexed (at higher pH values.)

3. The ratio of metal complex to acid ligand concentration is:

\[
\frac{[MX]}{[H_aX]} = \frac{A_1}{A_2 - A_1}
\]

and for the competing equilibrium:

\[
M^{+a} + H_aX = MX + aH^+
\]

\[
pK' = - \log \frac{[MX]}{[M^{+a}][H_aX]} - \log [H^+]^a + \log [M^{+a}]
\]

\[
pK' = - \log \frac{A_1}{A_2 - A_1} + a\phi + \log [M^{+a}]
\]
B. Bjerrum's Method - Continued.

4. A plot of \( \log [MX] \) versus pH gives a straight line whose slope is \( a \), since 
   \[ K' \] and \( [M^+a]^a \) are both constant. The value of \( pK' \) is determined from the 
equation above by "plugging in" the value of pH at which \( \log [MX] \) is equal 
to zero, using the proper values of \( a \) and \( \log [M^+a] \).

The advantage of the log plot method for determining the pH value lies in the 
fact that an average value, determined from several pH and absorbance 
values, is obtained. Any association of the acid or complex forms will cause 
a deviation of the log plot from a straight line and consequently this will 
serve as a warning that such behavior exists.

C. "Matching Absorbance" Method (applicable only for determining stability constants).

1. The absorbance of two solutions having different metal to ligand ratios are 
   measured at a wavelength where only the complex absorbs.

2. The solution of higher absorbance is diluted until the absorbance of the two 
   solutions is the same.

3. From the formal concentrations of metal and ligand is solution 1, \( C_{m,1} \) and 
   \( C_{x,1} \), and the formal concentrations of metal and ligand in solution 2, \( C_{m,2} \) 
   and \( C_{x,2} \), after dilution, we can calculate the dissociation constant of the 
   metal-ligand complex.

4. For a 1:1 complex we have:

\[
[M]_1 = C_{m,1} - [MX]_1
\]
\[
[X]_1 = C_{x,1} - [MX]_1
\]
\[
[M]_2 = C_{m,2} - [MX]_2
\]
\[
[X]_2 = C_{x,2} - [MX]_2
\]

When the absorbance of the two solutions are equal then \( [MX]_1 = [MX]_2 = c \) where \( c \) is some unknown concentration.
C. “Matching Absorbance” Method - Continued.

5. The stability constant, $K$, can then be calculated from the two stability constant expressions which contain $c$ and $K$ as unknowns, by eliminating $c$.

$$K_{\text{diss}} = \frac{(C_{m,1} - c) (C_{x,1} - c)}{c} = \frac{(C_{m,2} - c) (C_{x,2} - c)}{c}$$

and $c$ is:

$$c = \frac{(C_{m,1} C_{x,1}) - (C_{m,2} C_{x,2})}{(C_{m,1} + C_{x,1}) - (C_{m,2} + C_{x,2})}$$

Procedure.

A. Continuous Variations: Formula and Stability Constant Determination.

Pipet 10 ml. of stock iminodiacetic acid (IDA) into a 250 ml. volumetric flask and dilute to the mark with distilled water. Also pipet 10 ml. of stock Cu(NO$_3$)$_2$ solution into another 250 ml. volumetric flask and dilute to the mark.

Pipet 2, 5, 7, 10, and 12 ml. of the 0.02 M Cu$^{+2}$ solution made above into 5 - 25 ml. volumetric flasks and dilute to the mark with the 0.02 M IDA solution. Also pipet 2, 5, 7, 10, and 12 ml. of 0.02 M IDA into 5 other 25 ml. volumetric flasks and dilute to the mark with 0.02 M Cu$^{+2}$. Label these 10 flasks with the ml. of IDA they contain.

Adjust each of the 10 solutions to a pH of 2.0, using a pH meter. This should be done in the following manner. After standardizing the pH meter with the standard buffer solution provided, place the contents of one of the 25 ml. flasks into a clean, dry, 50 ml. beaker, drop in a stirring bar and place the beaker on the stirrer. Lower the electrodes carefully into the beaker without allowing them to contact the side of the beaker. Add conc. HNO$_3$ or 50% NaOH as necessary, by filling a dropper with the proper solution and touching the tip of the dropper to the solution without squeezing the bulb; in this manner an extremely small amount of acid or base can be added by diffusion through the tip of the dropper. When the pH has been adjusted to exactly 2.0, return the solution to the volumetric flask. Repeat for each of the remaining solutions.

Measure the absorbance of these 10 solutions at 650 and 700 nm.

If time permits repeat the above using 20 ml. aliquots of the stock solutions to make 0.04 M Cu$^{+2}$ and IDA solutions for making up the 10 solutions to be measured.
Treatment of Results.

A. Continuous Variations.

Plot the absorbance of your Cu-IDA solutions versus the mole fraction of IDA (for example, the solution containing 20 ml of IDA has a mole fraction of 20/25 or 0.80). Draw a straight line from the absorbance of pure IDA to that of pure Cu. For each point on the graph, subtract the absorbance due to free Cu (as represented by the straight line) and replot the absorbance values thus obtained against the mole fraction of IDA. Extrapolate the two straight sides of the curve until they cross. From the value of the fraction, $X_1$ at the intersection, calculate $n$.

From the ratio of the true absorbance to the extrapolated absorbance $A/A_{\text{expt.}}$ calculate the overall dissociation constant, $K'_{d'}$, for four points between $X = 0.4$ and 0.6.

$$K'_{d'} = \frac{[Cu] \cdot \text{NH(C}_2\text{HCOO)}_2]}{\left[\text{Cu}^{++}\right] \cdot \text{NH(C}_2\text{HCOO)}_2]}$$

$$= \frac{[H^+]^2 \frac{A - C}{A_{\text{expt.}}}}{\left(C_m - \frac{A - C}{A_{\text{expt.}}} \right) \left(C_x - \frac{A - C}{A_{\text{expt.}}} \right)}$$

$$\text{Cu}^{++} + \text{CH}_2\text{COOH} \rightleftharpoons \text{CuNH(CH}_2\text{COO)}_2 + 2\text{H}^+$$
A. Continuous Variations - Continued.

where $C_m$ and $C_x$ are the total analytical concentrations of metal and ligand, respectively, and $C$ is the total analytical concentration of the metal or ligand whichever is in limiting concentration. Given that $pK_{1,2} = 11.67$ for the ionization constants of IDA, calculate the average dissociation constant of CuIDA complex.

The literature value is $pK = 10.3$.

Questions.

1. Considering your experimental results in the continuous variations experiment, were both of the stock Cu and IDA solutions of exactly equal concentration or, if not, which was more concentrated?

2. Why is the method of continuous variations more useful for weak complexes than the molar ratio method?

3. By the method of continuous variations it is found that a metal, M, and a ligand, X, form a 1:3 complex MX$_3$. Derive a simple formula for the calculation of the stability constant $K_{\text{diss}}$ at the point of maximum absorbance (assuming that the complex is the only light-absorbing species), in terms of the actual maximum absorbance $A$, the extrapolated maximum absorbance $A_{\text{expt}}$, and the limiting concentration of the complex $C$. (Note that in this case the total analytical concentration of metal in the solution $C_m = C$ and of the ligand $C_x = 3C$; but the actual concentration of metal $[M]$ and of ligand $[X]$ in the solution (due to dissociation of the complex formed) would actually be much less than this.)

4. In Bjerrum’s method, why is it necessary to add a large excess of metal ion?