A PHOTOMETRIC DETERMINATION OF CHLOROPHYLL AND CAROTENE IN EXTRACTS OF GREEN TISSUE

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INTRODUCTION

All life processes are dependent upon the all important green pigment in the higher plants. It has been known for many years that this pigment is in some way related to food manufacture. The ready conversion of the energy of the sunlight to energy stored in the food materials of plants and the part chlorophyll plays in the plant has intrigued investigators for many years.

Quantitative determinations of chlorophyll have been used in numerous studies, such as photochemical reactions, chlorophyll in relation to metabolic processes and other biologically important compounds, genetical studies, effects of pathological conditions, the structure of the chloroplasts, and a host of other studies.

Carotene, too, has been of great interest to workers in the field of vitamins during the past ten years. This yellow pigment accompanying chlorophyll in the green plastid is known to be the precursor of vitamin A. As such, determinations for carotene in plant materials are of considerable value.

New methods, then, for the determination of chlorophyll and carotene in green tissue are still of great interest to plant physiologists and biochemists. Procedures based largely on the methods of Willstaetter and Stoll (68) are long and tedious. Most methods lack either accuracy, reproducibility or are useless when both pigments are to be determined in the same sample. Although chlorophyll determinations are laborious and time consuming, those for carotene are even more so. Consequently, a new method, which would be rapid, reliable and simple to utilize was sought.

The application of the photoelectric colorimeter for chlorophyll and carotene determinations has been suggested although little experimental evidence is available. A number of excellent instruments are now obtainable, and their use has become widespread. However, the lack of suitable filters has limited their acceptance.

REVIEW OF LITERATURE

Until the investigations of Willstaetter, botanical and spectroscopic methods were employed in the study of the occurence, morphology, and detection of the pigments. Then Willstaetter and his co-workers worked out techniques for the separation and purification of these chlorophylls and carotenoids. Meanwhile, studies relative to the physiological significance of all these pigments in plant metabolism have been constantly investigated. During the last decade interest has centered in a study of the physical-chemical constitution of chlorophyll as it exists in the chloroplast. In addition, new analytical methods for the quantitative extraction, separation, and estimation of the pigments have been devised.

The extensive literature on pigments, scattered throughout numerous journal articles, and the contradictory evidence found therein (at least to 1913) make it a difficult task to attempt to review plant pigment studies. The assembled data that preceeded Willstaetter's investigations were so contradictory and confusing that the conclusions drawn from them are now considered only of historical interest. Following this period the best review is given by Palmer (43), although his book was published eighteen years ago. Willstaetter and Stoll's book (68) provides an excellent background for pigment studies, but the review of Priestly's (47) concerning the work of Lubimenko must not be omitted. L Zechmeister

(70) has recently published a very good book on the carotenoids.

Extraction methods

Berzelius (2) was apparently the first to attempt an extraction of chlorophyll with acids or alkalies. There was little or no success with this method, but for many years his renown as a chemist appeared to influence other investigators. Consequently, many workers obtained equally poor results with the same type of extraction. Berzelius did succeed, however, in isolating the yellow pigments from autumn leaves with eighty five percent alcohol, and, thus, extracted water soluble pigments as well as carotenoids.

Alcohol was the most popular solvent for the extraction of the pigments until the time of Willstaetter. Fremy (12), Sorby (56), Timiriazev (60), Filhol (10), Hansen (18), Immendorf (23), Monteverde (37), Kohl (26), Monteverde and Lubimenko (38), and, as late as 1932, Deleano and Dick (9) used this solvent for the extraction of the green and yellow pigments. Hansen (18) used ninetysix percent alcohol in the cold after boiling the leaves in water and drying them.

Arnaud (1), interested largely in extracting the yellow pigments, found that petroleum ether used on air or oven dried material extracted only a small part of the green pigment and xanthophyll. Tswett (62) adopted a modification of this method for extractions preliminary to his chromatographic separation. J. H. C. Smith (54) preferred this treatment and Russell (49) used it for the extraction of carotene from dry alfalfa.

Willstaetter and Stoll (68) suggested aqueous acetone as an extraction medium for all plastid pigments, and it is now being used extensively,

especially where the isolation of both the green and yellow pigments is desired. The extraction methods of Wurmser and Duclaux (69), Schertz (52), Maiwald (33), Sprague and Shive (57), Peterson (45), Bills and McDonald (4), Oltman (42), Miller (36), Russell (49), and Godnew and Kalischewicz (15) are based on Willstaetter's procedure or some modification.

The extraction of carotenoids from seeds was accomplished by Gill (14) with carbon bisulfide. Coward (8) extracted carotenoids satisfactorily with aqueous alcoholic-potassium hydroxide, while Pyke (48) used methyl alcohol-potassium hydroxide with ether for the same purpose. At the suggestion of J. H. C. Smith, Smith and Smith (55) extracted the carotenoids with pyridine. The great objection to the use of this substance is its known toxicity.

Holmes and Leicester (22) extracted carotenoids with chloroform after soaking the tissue with alkali to get rid of chlorophyll. Kuhn and Brockmann (31) extracted green tissue for carotenoids with a petroleum ether-methyl alcohol mixture. In 1934, Guilbert (16) suggested saturated potassium hydroxide - methyl alcohol as an extraction mixture for carotene, thus causing decomposition of the chlorophyll. He then extracted the mixture with ethyl ether. Peterson, Hughes and Freeman (46) in 1937 modified this procedure by substituting petroleum ether for ethyl ether.

Methyl alcohol was employed by Hicks and Panisset (21) and Fleischer (11) for chlorophyll determinations of algal material.

Separation of the green and yellow pigments

The saponification of the chlorophyll and a subsequent separation from the yellow pigments has been the standard procedure for many years. However, attempts to study the yellow pigments were largely confined to etiolated tissue or autumn leaves previous to the adoption of this method. Berzelius (3) extracted autumn leaves with alcohol but considered the yellow pigment as a fat derived from the green pigment.

As early as 1864 Stokes (59) separated the green and yellow pigments by removing the chlorophyll and carotenoids with an aluminum hydroxide preparation. Fremy (13) found that not only aluminum hydroxide but magnesium hydroxide and barium hydroxide solutions were effective in carrying down both pigments. He found that barium hydroxide solution was the most effective and that by the use of alcohol the yellow pigment was removed from the mixture. Timiriazev (60) and Monteverde and Lubimenko (38) employed the same method in their determinations. Contrary to these reports, it was found in the present work that barium hydroxide does not carry down both pigments. It selectively reacts with the chlorophyll leaving the carotenoids in solution. Apparently, these early workers added enough of the barium hydroxide solution to dilute the extract with water to such an extent as to precipitate both pigments.

Sorby (56) separated the green and yellow pigments by the use of the two immiscible solvents, alcohol and carbon bisulfide. Treub (61) repeated this work and obtained similar results. Arnaud (1) took up the carotenoids in carbon bisulfide after the petroleum ether, the extraction medium, was distilled off. Filhol (10) was able to remove the chlorophyll from carotenoids by treatment with animal charcoal. Recently, animal charcoal was tried as a selective adsorbent for chlorophyll, but the carotenoids were carried out of solution along with the green pigment.

The method suggested by Kraus (29) depended on the use of immiscible solvents as eighty five percent alcohol and benzene. Konrad (27) found that the separation was more effective with seventy percent alcohol. Treub (61) suggested that carbon bisulfide be used in place of benzene when chlorophyll was present in a stronger alcoholic solution. Wiesner (66, 67) showed that many vegetable and ethereal oils as well as many aromatic compounds could be used for the separation from alcohol.

Tswett (63) separated the pigments by means of a chromatographic technique. Hansen (18) appears to be the first worker to separate the pigments by alkali saponification, then salting out the soap with NaCl. Kohl (26), Willstaetter and Stoll (68), Wurmser and Duclaux (69), Schertz (52), Maiwald (33), Peterson (45), Coward (8), Holmes and Leicester (22), Kuhn and Brockmann (31), Guilbert (16), Miller (36), Deleano and Dick (9), and Pyke (48), used some modification of the alkali saponification treatment.

Even in a quantitative photometric determination, Oltman (42) used an alkali saponification method, although Godnew and Kalischewicz (15) and Johnston and Weintraub (25) made no separation of the green and yellow pigments. They measured the chlorophyll in the original extract by the selection of the proper filters.

Separation of carotenoids.

Borodin (5) established a basis for the classification of yellow pigments when he found one group more soluble in alcohol than the other. His investigations showed also that the group slightly soluble in alcohol was highly soluble in petroleum ether. Monteverde (37) and Monteverde and Lubimenko (38) used this method of separation in their studies. Since

that time practically all workers with few exceptions have used the petroleum ether - methyl alcohol or ethyl alcohol method as the basis for carotene - xanthophyll separation. Among these were Willstaetter and Stoll (68), Henrici (20), Wurmser and Duclaux (69), Schertz (52), Coward (8), Peterson (45), Deleano and Dick (9), Kuhn and Brockmann (31), Guilbert (16), Miller (36), Pyke (48), and Peterson, Hughes and Freeman (46).

Arnaud (1) found that petroleum ether extracted very little xanthophyll from dry material. Tswett (63) developed a chromatographic technique for the separation of the carotenoids. This method has proven to be of great importance in the study of the chemical nature of these pigments, and is being used extensively today. Recently, Clausen and McCoord (7) have obtained excellent results by substituting diacetone for aqueous alcohol for the carotenoid separations. Hegsted et al (19) have adopted this method.

Quantitative chlorophyll determinations

Actual quantitative determinations of chlorophyll do not seem to have been attempted before 1910. Stokes (59) made optical studies of chlorophyll but no quantitative measurements. Monteverde (37) attempted to measure the relative chlorophyll values by spectographic means.

The spectrophotometer has been used frequently with considerable success. Malarski and Marchlewski (34) treated chlorophyll with hydrochloric or oxalic acid and measured the extinction coefficient of the phaeophytin. Jacobson (24) used the same treatment and compared the sample against an arbitrary standard. Although Wiegert (65) did not actually carry on any work, he pointed out how chlorophyll might be quantitatively estimated by the use of the spectrophotometer. Both Wurmser and Duclaux (69) and Schertz (53) established spectrophotometric methods

for chlorophyll determinations. Zscheile (71) was able to measure both chlorophyll "a" and "b" by this method. Zscheile's work on chlorophyll determinations with the spectrophotometer has been the most precise of any that has been attempted. The photoelectric colorimeter is of greater practical value and it is more rapid. However, it is by no means as accurate an instrument as the spectrophotometer.

Most quantitative methods for the determination of chlorophyll since Willstaetter's work have been colorimetric. Willstaetter and Stoll (68) decomposed the chlorophyll and compared it against methyl or ethyl chlorophyllide both treated in the same manner. He also compared the extracted material against a pure chlorophyll standard. Schertz (52) followed the same general procedure as did Hicks and Panisset (21). Henrici (20) compared her samples with a solution of crude chlorophyll extracted from nettle and, consequently, obtained only qualitative results. A mixture of chlorophyll a and chlorophyll b was used as a colorimetric standard by Maiwald (33).

In 1928 Guthrie (17) suggested an inorganic standard of definite proportions of copper sulfate (5 molecules of water), potassium dichromate, and ammonium hydroxide. This standard has been used rather extensively. In 1929 Sprague and Shive (57) introduced a dye mixture of a solution of malachite green and naphthol yellow which was modified later by Sprague and Troxler (58). However, their standard has been used very little. Lovibond slides have been employed for chlorophyll determinations (48) in numerous cases.

Lubimenko (32) used a spectrocolorimeter with a standard of ethyl chlorophyllide and Fleischer (11) made the same type of determination using a No. 243 Corning red filter of 3.12 mm. thickness.

Oltman (42) appears to have been the first to use a photoelectric colorimeter for chlorophyll determinations. However the chlorophyll determinations were made after saponification rather than on the original sample as did Godnew and Kalischewicz (15) and Johnston and Weintraub (25). Koziminski (28) used an ethyl chlorophyllide standard, and the amount of chlorphyll was calculated by the use of a conversion factor.

Quantitative carotene determinations

The first attempt to determine carotene was made by Wiesner (67), who compared the total alcoholic extract from an etiolated plant with the yellow alcoholic extract from green plant tissue obtained by the Kraus separation.

Arnaud (1) dissolved carotene in carbon bisulfide and using blue glass filters, compared it colorimetrically with 0.001 percent solution of carotene in carbon bisulfide. Kohl (26) compared the unsaponifiable pigment colorimetrically with a standard in the same manner as did Arnaud. Monteverde and Lubimenko (38) followed the same method as did Willstaetter and Stoll (68) although the latter made their comparisons with a 0.2 percent aqueous solution of potassium dichromate, as well as with a petroleum ether solution of pure carotene. Sprague and Shive (57) suggested a dye standard of Naphthol yellow and Orange G. Kuhn and Brockmann (31) suggested azobenzene, while Guilbert (16), Russell (49), and Peterson, Hughes and Freeman (46) used potassium dichromate. Krogis (30) employed the dichromate solution with a phosphate buffer.

Deleano and Dick (9) oxidized the carotene with a known amount of standard dichromate in excess, and then back-titrated with iodine to obtain a measure of the carotene concentration. Recently, Brooke, Tyler,

and Baker (6) used a Cenco photelometer for carotene determinations employing a Corning No. 554 and a Corning No. 038 filter.

Munsey (41) has urged a wide adoption of the photoelectric colorimeter in carotene investigations because of its accuracy and other advantages.

THE PROBLEM

Colorimetric analyses are the most common methods of determining chlorophyll and carotene. The old standard methods are unsatisfactory in many phases of the determinations especially when the determinations of both pigments are desired from the same sample. The need of a rapid, accurate, and simplified method of chlorophyll and carotene determinations becomes evident when many pigment determinations are attempted in the laboratory. Consequently, an effort was made to devise a method satisfying these requirements.

EXPERIMENTAL PROCEDURE

Materials and methods

The principles of photometry

Colorimetry implies different meanings to workers in certain other fields (40). In this paper it refers to the measurement of the concentration of a colored substance by comparing the depth of the color against a standard. The Beer - Lambert law states that the amount of light absorbed by different concentrations of a colored solution is an exponential function of the concentration when the thickness of the solution is constant:

$$I = I_0 \times 10^{-k_1 c}$$

Where I = intensity of light after passing through the solution

Io = incident intensity

k = molecular extinction coefficient (depending on solute and solvent)

l = thickness of solution

c = concentration

$\log_{10} Io/I = E$ (extinction)

Then in matching two solutions

$E_1 = E_2$

$kc_1 l_1 = kc_2 l_2$

or $c_1/c_2 = l_2/l_1$

Thus, the unknown concentration can be calculated by matching the depth of color against a standard. The above law holds very well where an instrument like the spectrophotometer is used. Nearly monochromatic light can be obtained with this instrument. The application of this law to ordinary colorimetric work can not give accurate results since white light is used. The different wave lengths employed introduce complex factors. Photometry is an advance over colorimetry and an approach to the accuracy of spectrophotometry. Filters, as used in photometry, simplify the conditions, since many wave lengths are eliminated, and the light is limited to a narrow region of the spectrum.

A photoelectric instrument does not depend upon the presence of a standard for each determination as does a colorimeter. A calibration curve is determined and can be used thereafter without any standards, although it is wise to recalibrate occasionally.

The most important advantage of the photoelectric colorimeter lies in the objectivity of the instrument. Different observers obtain the same values that are not subject to the vagaries of the human eye in reading

colors. The Sheard-Sanford photelometer is one of the simplest commercially standardized instruments available. The instrument (fig. 1) consists of a light source focused through a variable diaphragm onto a planoconvex lens. The light then passes through one of the cells on the cell carrier containing the pure solvent or solution to be tested. The light emerging from the analyzing cells then passes through the wave length filter and from there to the photronic cell. The current generated in the photronic cell circuit is measured by a sensitive galvanometer. The variable diaphragm is similar to a compur camera shutter. The light rays are rendered essentially parallel by the planoconvex lens. Part of the light reaching the cell is reflected and part is absorbed. Both cells must be of the same thickness to eliminate any errors due to differences in absorption, transmission, or reflection. The cells were one cm. thick and transmitted ninety two percent of the light. The amount of current set up by the photronic cell measures the light intensity. The source of the light is a small auto headlight bulb attached to the 110 volt AC line through a transformer. Although a transformer is used the current is apt to fluctuate due to the use of other electrical equipment. A storage battery may be used to a greater advantage as a source of steady current if facilities are available for recharging the battery.

The greatest limitation to the use of the photoelectric colorimeter is the lack of suitable filters. An ideal filter is one that would have a maximum transmission in the exact region of maximum absorption of the solution that is being examined. Since the ideal one is unavailable, one whose maximum transmission is near the maximum absorption should be selected. In addition, the effective region of transmission should be as narrow as possible. The spectrophotometer is by far the better instrument

to employ, since filters are not necessary, and the region of the spectrum used can be limited by slit adjustments. However, this instrument is not as readily available to the general worker as the photoelectric colorimeter.

Two filter systems were used in these studies. The first, that transmitted in the red, was used for chlorophyll determinations. Since the carotenoids do not absorb at all in this region, the chlorophyll could be determined directly in the original extract without any separation of the pigments. The combination finally chosen was a Corning No. 243 red filter (11, 44) and a Corning No. 396 H. R. blue filter. The latter was needed for the purpose of heat resistance since the infra-red has an effect on the photronic cell. The transmission range of this combination lay between 6100 Å and 7500 Å in the infra-red.

Since carotenoids and chlorophyll have an absorption band in the blue region of the spectrum, it was necessary to remove chlorophyll in order to determine the carotene content. After the chlorophyll had been removed it was necessary only to choose a filter with maximum transmission in the blue region. A Corning No. 554 H. R. was finally selected after trying certain other ones.

Excellent reviews on photometry have appeared by R. H. Müller (40) and M. G. Mellon (35).

Analytical procedure

One very important step which has not been emphasized sufficiently in most quantitative pigment determinations is that of a complete grinding procedure preliminary to extraction. It is imperative that the tissue be thoroughly disintegrated. This applies regardless of the method

of extraction or the solvent used. The temptation is to give a preliminary grinding and then let the solvent do the rest of the work.

In general, the procedure used for extraction is somewhat like that of Ulvin (64). Two grams of fresh tissue are placed in a mortar (glass is preferred since it is easier to clean out) with a small amount of sodium carbonate to neutralize any possible acidity. In this way chlorophyll decomposition is prevented. The tissue is then ground as finely and rapidly as possible. If the tissue is too dry it is moistened with pure acetone. Moreover, the acetone prevents oxidation of the carotene to a certain extent. It is then ground more with quartz sand and moistened frequently with pure acetone. After it is finely macerated, about twenty five cc. of pure acetone are added and the grinding continued for a few more minutes. The extract is decanted through a Buchner funnel, and the residue is again macerated and extracted with eighty five percent acetone (twenty five or thirty cc.). The residue and extract are placed in the Buchner funnel and the residue soaked two or three times before drawing off the liquid. The number of grindings depends upon the tissue used. The experienced worker soon knows when extraction is complete. The presence of water soluble pigments in the acetone extract -- particularly when using eighty five percent acetone often gives the impression of incomplete extraction. If, when using pure acetone, the liquid comes through clear into a test tube, one can be certain of complete extraction. The filtrate collected in a test tube should be shaken with a little petroleum ether when eighty five percent acetone is used, and if no yellow color passes to the petroleum ether layer, carotene is not present. The extract is made up to one hundred cc. or any desired volume with eighty five percent acetone.

The chlorophyll content is then ready to be measured. A part of the extract is placed in the absorption cell of the photoelectric colorimeter, and its transmission is compared with that of distilled water. This value is then interpreted as concentration through the use of the standard curve. Fifty cc. of the acetone extract are taken for the carotene determinations. This is placed in an oil flask or one hundred ml. Erlenmeyer flask with the prepared barium hydroxide. The latter is prepared by hydrating one gram of the anhydrous barium hydroxide (J. T. Baker, Barium Hydrate, anhydrous) or barium oxide in an organic solvent miscible with water. The solid barium compound is put into five cc. of acetone to which five cc. of water is added while stirring. It may also be prepared in one of two other ways. If there were enough water in the extract (and there should be), no previous activation would be necessary. Again, an activated barium hydroxide results when a soluble barium salt reacts with an alkali in the presence of the organic solvent miscible with water.

The acetone extract and barium hydroxide mixture are refluxed for thirty minutes on a hot plate or water bath. The flask and contents are then cooled and filtered. The green sludge remaining on the filter is washed with pure acetone to remove all the carotene. The yellow solution is placed in a separatory funnel to which fifty cc. of petroleum ether (B. P. 30°- 50°C.) is added. The solution must never be shaken but the extraction can be completed by swirling the funnel with a rotary motion. The acetone is washed out of the petroleum ether layer with water and is re-extracted with two fifteen cc. portions of petroleum ether. The extracts are combined with the rest of the petroleum ether. The petroleum ether is then washed with either eighty five and ninety percent methyl or eighty and eighty five percent ethyl alcohol in order to wash out xanthophylls. The alcohol extract is re-extracted with petroleum ether. This extract is

added to the petroleum ether layer which is washed again with a little alcohol, then once with water and then filtered through anhydrous sodium sulfate into a volumetric flask and made to volume. The concentration of carotene in the petroleum ether solution (largely beta-carotene in green tissue) is then determined in the same manner as in the chlorophyll determination described above. The Corning No. 554 H. R. filter was used. Again the value is interpreted from the standard curve. Correction for the presence of chlorophyll may be made, but this is rarely necessary. Occasionally greenish material may be present, but it will be filtered out in the sodium sulfate filtration.

Results

Chlorophyll experiments

The most important single factor in this study was the proper choice of suitable filters. Certain filters and filter combinations were tested, namely, the Cenco No. 3, and combinations of the Corning H.R. 243 plus 0.25 N copper sulfate solution, Corning No. 243 and a Cenco blue glass H. R. filter, and finally the Corning No. 243 and 396 blue filter.

The calibration curve for each filter is represented in figure 2. It is obvious from these data that the Cenco filter is not satisfactory for this particular study. It is desirable that a large change in transmission be induced by a small change in concentration. The Cenco filter shows this characteristic only in an extremely short range. The best curve was obtained with the Corning filter No. 243 plus a solution of 0.25 N copper sulfate. An excellent curve was also obtained with a 0.167 N copper sulfate solution. The advantage of the latter was that a greater light intensity could pass through. However, the inconvenience caused by the prep-

aration of these solutions led to their discontinuance. The other two curves, obtained through combining the Corning No. 243 filter with the Cenco blue glass filter, and combining the Corning No. 243 with the Corning No. 396 filter, were much alike and readily reproducible. Each could be used effectively and for most of the work the No. 396 was selected because it absorbed more of the infra-red. Both the curves exhibited gentle slopes throughout most of the entire range of concentrations employed. The glass filters were much more convenient although slightly less satisfactory than copper sulfate.

The calibration curves for these filters were determined in the following manner. Pure chlorophyll (5x grade obtained from the American Chlorophyll, Inc.) was dissolved in eighty five percent acetone. Sixty two and one-half mg. samples were diluted to a volume yielding a stock solution of one hundred twenty five mg./l. Different concentrations of chlorophyll were prepared from this stock solution by diluting certain aliquots to definite volumes. The photelometer was adjusted to read 100 with the filters in place and distilled water in the standard cell. The cell with the standard (or unknown) was aligned with the light source of the instrument by shifting the carriage. The ammeter reading was then taken. Each photometric value was plotted against its concentration to obtain the resulting curve. This procedure was followed for every determination whether for chlorophyll or carotene, known or unknown. The chlorophyll concentration of any unknown could be determined by interpreting the photometric reading from these standard curves.

Table I shows the data obtained in a study of the accuracy of chlorophyll determinations upon weighed samples of chlorophyll with the use of the four filters. It can be seen readily that the percentage error with

the Cenco filter exhibits a greater range than with the copper sulfate. The poor curve obtained with the Cenco filter accounts for this large error. The results, in general, seem to be more reliable in that range where 45-75 percent of the light is transmitted and the practice has been to dilute within that range. The absolute error tends to be large though the percentage error is small where transmission is below 45 percent.

It became necessary to know exactly what effect the presence of carotene would have upon chlorophyll determinations, although theoretically it should have none. Known quantities of chlorophyll were weighed out and solutions of different concentrations were made. Carotene was added to each of these solutions to make a concentration of twenty seven ppm. The results are shown in table II. The percent variation is very close to the experimental error indicating that there is little, if any, effect. These data agree with the conclusions of Johnston and Weintraub (25) as well as Godnew and Kalischewicz (15).

Photometric and colorimetric chlorophyll determinations were compared on certain green materials. A Duboso colorimeter was used for all colorimetric determinations. Guthrie's standard was first used for determining the chlorophyll concentration, then a pure chlorophyll standard, and finally, a photoelectric colorimeter. The data in table III indicate that the values obtained colorimetrically with a pure chlorophyll standard compare favorably with those when the photelometer was used. However large variations at the higher concentrations were secured with Guthrie's standard.

Carotene experiments

The proper selection of filters or filter combinations is also the important factor in the determination of carotene concentrations. Three filters were tried, namely, a Cenco No. 1, and a Corning No. 554 H. R. filter with and without a Novial A No. 038 filter.

The readings for carotene content could not, however, be made directly upon the crude extract since chlorophyll has an absorption band in the blue region of the spectrum from 4000 Å to 5100 Å, which overlaps that of the absorption by carotene (4180 Å - 4900 Å. Values in ether solution). This necessitated a separation of the green pigment from the yellow.

The data in table IV show that the transmission values at all concentrations (approximate) are similar. The Corning No. 554 plus Corning No. 038, a combination used by Brooke, Tyler, and Baker (6) served only to cut down the transmission without increasing the sensitivity. The Corning number 554 served the purpose as well as any filter.

Eleven and four tenths milligrams of beta-carotene obtained from the S. M. A. Co. were diluted to 500 ml. in petroleum ether at 26°C. Dilutions were made from this stock solution to give the various concentrations required. Photometric readings were made in the same manner as in the chlorophyll determinations. The resulting values were plotted against concentration and the curve (fig. 3) was thereafter used for interpreting unknown values.

A method of correction for the possible presence of chlorophyll in any carotene sample was worked out. Rarely was chlorophyll found present in the carotene solution with the method of separation used. The inset in figure 3 depicts the curve obtained for chlorophyll with the filter No. 554 used for carotene determination. With the aid of both curves in

figures 2 and 3 the correction for the chlorophyll present is made in the following manner: the amount of chlorophyll in the petroleum ether solution of carotene is determined by using filters No. 243 and No. 396. The reading for total percent transmission in the blue region with filter number 554 is then obtained. This is the result for both carotene and chlorophyll. The curve inset in figure 3 indicates the amount of absorption due to chlorophyll if no carotene were present. The actual carotene content is determined by the use of the following formula:

$$Y = \frac{T \times 100}{C}$$

Y = actual percent transmission of carotene.

T = total percent transmission of carotene and chlorophyll in the blue spectrum.

C = percent transmission of chlorophyll concentration in blue spectrum if no carotene were present (from figures 2 and 3).

As reported in the review of the literature, practically all methods used at present depend on the separation of the two pigments by an alkaline saponification of the chlorophyll. This saponification method retrieves carotene in a petroleum ether solution. The means of separation used in this study is based on a new principle which was devised largely by Dr. H. G. Petering of the Michigan State College Chemistry Experiment Station. This involves the separation of the chlorophyll from carotene by precipitating the green pigment with barium hydroxide octahydrate. The carotene could then be filtered off. Magnesium hydroxide and calcium hydroxide were not as efficient as the barium hydroxide. Ordinary barium hydroxide was activated by first placing the material in acetone or alcohol. A subsequent addition of water resulted in a fine, porous mass.[1]

(1)This reaction was later shown to be due to the formation of the barium hydroxide octahydrate.

The material was then added to the chlorophyll solution. Several experiments were conducted to establish the effect of different volumes of water and acetone upon the activity of the barium hydroxide. It was found that there was little difference in these amounts as long as enough water was present to complete the reaction. It also appears that good activity is obtained with either anhydrous barium hydroxide or the octahydrate.[1] The use of eighty five percent acetone seems to be more effective than pure acetone.[2]

Data were gathered, as shown in table V, to determine just how much barium hydroxide is necessary in order to remove the chlorophyll. It is obvious that there is a close relationship between the amount of barium hydroxide, the time of refluxing, and amount of chlorophyll removed. The general practice has been to use one gm. of barium hydroxide (anhydrous) five cc. of acetone, and five cc. of water for each fifty cc. of a one hundred ppm. solution of chlorophyll for a thirty minute reflux period. The amount of $Ba(OH)_2$ used in getting this data is in excess of the amount needed for these pigment concentrations as later shown by Petering and Morgal.

Since a quantitative recovery of carotene is imperative the effect of the barium hydroxide upon the carotene was studied. Experiments were run in which duplicate samples -- one treated, the other not -- were compared. A known amount of carotene was added to an unknown sample, and then none added to a duplicate sample. The difference between the samples with and without the added carotene then represented the amount recovered. The data in table VI show the results of these tests. The results indicate that a complete quantitative recovery of carotene was

(2) Petering and Morgal, (private communication) have shown that eighty five percent acetone is the best concentration since more water will throw out the pigments.

accomplished.

A number of experiments were run in order to determine the accuracy of this method for carotene determinations as compared with the present accepted method (A. O. A. C.). A number of experiments were run in conjunction with Dr. E. J. Benne of the Michigan State College Chemistry Experiment Station. The comparison was made with the Peterson, Hughes and Freeman method (46). The data presented in table VII shows remarkably close agreement between the two methods. Both chlorophyll and carotene were determined on the same extract by this new procedure. The older method does not allow for a chlorophyll determination. Many plant tissues were employed which would present a wide variation in both chlorophyll and carotene content. The greatest percentage variation occured with yellow wax bean pods, which had an extremely small carotene content. A very small loss of pigment in this case would have produced a large error.

Samples of blue grass taken in the fall gave results with the barium treatment twenty to twenty five percent higher than those run by the Peterson, Hughes and Freeman method. This was the only instant of poor agreement between the two methods. Either the acetone extracted more carotene than the alcoholic KOH, or the acetone takes up an impurity other than carotene which is not extracted from the tissue by the Peterson, Hughes and Freeman method. Moon (39) reported that grasses yield red or brown resinous substances which are precipitated during saponification with alcoholic alkali. This makes the extraction of carotene from the residue by petroleum ether very difficult. This may have been the source of the trouble.

Both chromatographic and spectrophotometric studies revealed no im-

purities present in either solution.

DISCUSSION AND CONCLUSIONS

Although many aspects of the procedure as given in this paper resemble other methods, it differs in several important respects. There is a growing belief, well substantiated, that the photoelectric colorimeter is a better instrument than the colorimeter. The important error in colorimetry induced by visual impressions of different individuals is absent in photoelectric colorimetry. The values obtained are the result of objective readings, and tints or shadings need not be interpreted by the eye. The short period employed in testing and the almost immediate results obtained, reduce the chances of pigment decomposition. The time saved is a factor in which all investigators are interested. The use of a standard curve which needs to be checked only occasionally is preferable to the frequent preparation of new standards. The standard equipment employed is an advantage and the spectral filters are more sensitive than those in many other methods.

On the other hand, errors are found in the photometric method. The sensitivity of the photoelectric cell to certain wave lengths varies, and the fluctuation of the current from an AC line will cause a deviation on the ammeter which is a source of error. This can be easily eliminated by the use of a storage battery provided a charger is available. The greatest disadvantage may appear to be the cost of the instrument, but this is slight when compared to the spectrophotometric method.

The use of barium hydroxide octahydrate also presents many advantages over the older methods. There is no doubt that its action is more rapid than the alkaline saponification for chlorophyll breakdown. The use of a

solid material without any special preparation, the small amount of handling, and the ease of separation by filtering, give it an advantage over the alkaline saponification method. Moreover, a complete removal of chlorophyll is obtained. It was found that the filtrate after filtering off the green sludge had no barium present. This simplifies the procedure tremendously since numerous washings are not necessary in ridding the solution of alkali as is the case where alkaline saponification is employed.

A separation of the pigments is not necessary for the determination of chlorophyll, and although a separation is required for the carotene determination, the same sample can be used for both estimations.

An additional advantage lies in the reduction of the number of operations and transfers in this manner reducing the sources of error. Fewer reagents are needed to carry through a determination.

The entire procedure takes about one-half the time ordinarily spent with other procedures, even those in which photoelectric colorimeters have been used.

SUMMARY

1. The literature and methods involved in extraction of pigments, separation of the green and yellow pigments, separation of the carotenoids, and quantitative determinations of chlorophyll and carotene are reviewed.

2. A discussion of the principle of photometry and a description of the photoelectric colorimeter used is given.

3. Chlorophyll was determined photometrically on the crude extract in the red region of the spectrum by means of suitable filters. The preparation of a calibration curve for chlorophyll is described.

4. Data for the accuracy and reliability of the method as compared to colorimetric methods are shown.

5. A study of a few potentially available filter systems was conducted.

6. A report of the preparation of the extract for the carotene determination, the type of filter used, a method of correction for any chlorophyll present in the carotene sample, and the calibration curve is discussed.

7. A study of the method for treating the barium hydroxide for chlorophyll decomposition and its effect on carotene is presented.

8. Comparisons of values obtained by this method with those from the generally accepted method are included.

9. Exact details are presented for an analytical procedure for chlorophyll and carotene in green tissue.

10. A discussion of advantages, disadvantages and errors of the method is presented.

ACKNOWLEDGEMENT

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INDEX OF TABLES

TABLE I

THE ACCURACY OF CHLOROPHYLL DETERMINATIONS

Pure chlorophyll in acetone solution (mg/l.)

Sample No.	Chlorophyll concentration by weight	Chlorophyll concentration photelometer with Corning 243 & Aklo 396	Percent error
1	19.9	20.5	3.0
2	39.7	38.0	-4.2
3	79.4	82.0	3.3
4	99.3	102.5	3.2
5	119.1	124.0	4.1
6	39.7	39.5	-0.5
		A-Corning 243 + N/4Cu. SO_4 filter.	
		B-Corning 243 + Cenco H. R. blue filter	
		C-Cenco No. 3 filter	
7A	24.4	24.8	0.6
7B	24.4	24.0	-0.6
7C	24.4	23.0	-5.7
8A	31.3	33.1	5.7
8B	31.3	31.9	0.9
8C	31.3	30.6	-2.2
9A	42.0	44.0	4.8
9B	42.0	44.0	4.8
9C	42.0	44.0	4.8
10A	52.2	55.6	6.5
10B	52.2	53.7	2.5
10C	52.2	47.2	-9.6
11A	66.5	69.0	3.8
11B	66.5	70.0	5.4
11C	66.5	62.3	-6.3
12A	105.0	100.0	5.0
12B	105.0	103.0	3.0
12C	105.0	93.5	6.5

TABLE II

THE EFFECT OF CAROTENE ON CHLOROPHYLL DETERMINATIONS

Expressed as mg./l.

Sample	Chlorophyll concentration. Checks	Chlorophyll concentrations, containing in solution 27 p.p.m. of carotene.	Percent variation
1	20.5	20.9	1.9
2	124.0	117.0	-5.6
3	6.9	6.5	-5.7
4	6.9	7.3	5.7
5	85.0	85.0	0.0
6	30.7	31.0	1.0

TABLE III

COMPARISONS OF THE COLORIMETRIC AND PHOTOMETRIC METHODS FOR DETERMINATION OF CHLOROPHYLL

	Photelometer	Colorimeter	
Sample	Corning filters No.243 plus 396	Guthrie's standard ($K_2Cr_2O_7$ + $CuSO_4$ + NH_4OH)	Chlorophyll standard
Tomato leaf	141.0 mg/l.	129.6 mg/l.	146.0 mg/l.
Tomato leaf	180.0	179.8	194.0
Nettle leaf	153.0	117.1	156.0
Nettle leaf	128.0	114.4	132.0
Nettle leaf	80.0	75.2	
Alfalfa leaf	57.5	57.2	55.4
Celery leaf	83.0	78.5	
Celery leaf	70.0	72.2	
Celery leaf	103.2	97.5	

TABLE IV

THE EFFECTS OF CERTAIN FILTERS ON

PHOTOMETRIC READINGS OF STANDARD CAROTENE SOLUTIONS

Expressed as p.p.m.

Carotene concentration	Corning No.554	No.554 + Novial A (No.038)	Cenco No.1
4.16	27.1	26.9	25.0
3.33	34.5	34.3	32.2
2.66	41.3	40.7	39.5
2.13	49.0	48.5	47.5
1.70	55.7	55.9	55.0
1.36	62.8	62.3	62.0
1.09	68.8	68.2	68.0
0.54	82.5	83.3	82.5
0.28	92.0	92.3	91.8
0.14	97.0	97.8	98.0

TABLE V

THE AMOUNT OF BARIUM HYDROXIDE REQUIRED UNDER CERTAIN CONDITIONS TO REMOVE CHLOROPHYLL FOR THE CAROTENE DETERMINATIONS

p.p.m. chl.	cc. chl. sol.	Weight $Ba(OH)_2$	cc. Ho for $Ba(OH)_2$	Time in min. of refluxing	Chl. Reading	p.p.m. chl. conc.
100	50	1 gm.	5	30	98.7	0.4
100	50	1 gm.	5	25	98.4	0.5
100	50	1 gm.	5	20	97.2	0.9
100	50	1 gm.	5	15	96.9	1.0
50	50	0.5 gm.	5	30	93.1	2.4
100	50	2.0 gm.	5	20	97.5	0.8
100	50	2 gm.	5	30	99.2	0.2
100	50	3 gm.	5	20	98.2	0.5
100	50	4 gm.	5	10	96.9	1.0
100	50	5 gm.	6	10	100	0.0
100	50	0.5 gm. $Ba(OH)_2$ + 0.5 gm. $Ca(OH)_2$	11	20	94.8	1.7

TABLE VI

CAROTENE RECOVERIES FROM ACETONE SOLUTIONS

FOLLOWING THE BARIUM HYDROXIDE TREATMENTS

Sample	Chlorophyll concentration	Carotene concentration
1	0.0 mg./l.	0.89 mg./l.
2	0.0	0.54
3	0.0	1.40 1.43
4	0.0	0.89
5	0.0	0.63
6	0.0	1.55 1.52

1 & 4 -- 50 cc. acetone and 50 cc. carotene solution.
2 & 5 -- 50 cc. acetone and 50 cc. chlorophyll extract.
3 & 6 -- 50 cc. carotene solution and 50 cc. chlorophyll extract.
Sum of 1 and 2 should equal 3.
Sum of 4 and 5 should equal 6.

Fifty cc. of an acetone solution of carotene oil (S.M.A.) refluxed 15 minutes with 1.5 grams barium hydroxide. Extraction with petroleum ether. Check has no barium hydroxide treatment.

Sample	Concentration of check	Concentration of treated
A	1.05 mg./l.	1.00 mg./l.
B	1.12	1.10
C	1.35	1.44
D	2.19	2.21

Sample — Fifty cc. of an acetone solution of carotene refluxed one-half hour with 1.5 gms. barium hydroxide. No barium hydroxide treatment in check.

Acetone-check	0.67 mg. /l.
Acetone-treated	0.71
Pet.ether check	0.64
Pet.ether treated	0.67

TABLE VII

COMPARISONS OF METHODS FOR CAROTENE DETERMINATIONS

Sample	Date Harvested	Percent water	CAROTENE: Peterson-Hughes-Fr[illegible] Green wt. mg./100gm.	CAROTENE: Peterson-Hughes-Fr[illegible] Dry wt. mg./100gm.	CAROTENE: Barium hydroxide treatment Green wt. mg./100gm.	CAROTENE: Barium hydroxide treatment Dry wt. mg./100gm.	CHLOROPHYLL Green wt. mg./100gm.	CHLOROPHYLL Dry wt. mg./100gm.
Blue grass (Poa pratensis)	8-16-39	64.5	15.8	44.6	15.2	42.9	536.5	1552.0
Alfalfa (Medicago sativa)	8-17-39	72.5	14.7	53.5	14.3	52.0	404.6	1565.8
Yellow wax bean pods(young) (Phaseolus vulgaris)	8-17-39	68.0	0.31	0.97	0.61	1.91	7.7	24.1
Green stringless bean pods(young) (Phaseolus vulgaris)	8-18-39	87.0	0.50	3.85	0.67	5.15	24.3	187.0
Lettuce (Lactuca sp.)	8-25-39	89.0	4.31	39.2	4.55	41.3	121.5	1105.0
Spinach (Spinicia oleracea)	8-25-39	92.0	3.88	48.5	3.70	46.3	132.6	1657.0
Willow leaves (Salix babylonica)	8-28-39	64.0	15.20	42.2	16.70	46.4	52.1	144.8
Artichoke leaves (Helianthus tuberosa)	8-29-39	70.0	13.16	45.4	13.66	47.2	49.3	164.5
Soy bean leaves (Soja max)	9-20-39	72.0	13.0	46.4	14.43	51.5	483.3	1726.1
Celery leaves (Apium graveolens)	9-21-39	84.0	8.05	51.6	8.80	55.0	291.5	1821.9
Celery stalks (Apium graveolens)	9-21-39	90.0	0.35	3.48	0.40	4.01	8.63	86.3
Blue grass (Poa pratensis)	11-13-39	82.0	15.0	83.0	18.4	102.2	---	---
Alfalfa (Medicago sativa)	8-24-39	77.0	11.9	51.7	12.6	54.8	401.9	1747.4

INDEX OF FIGURES

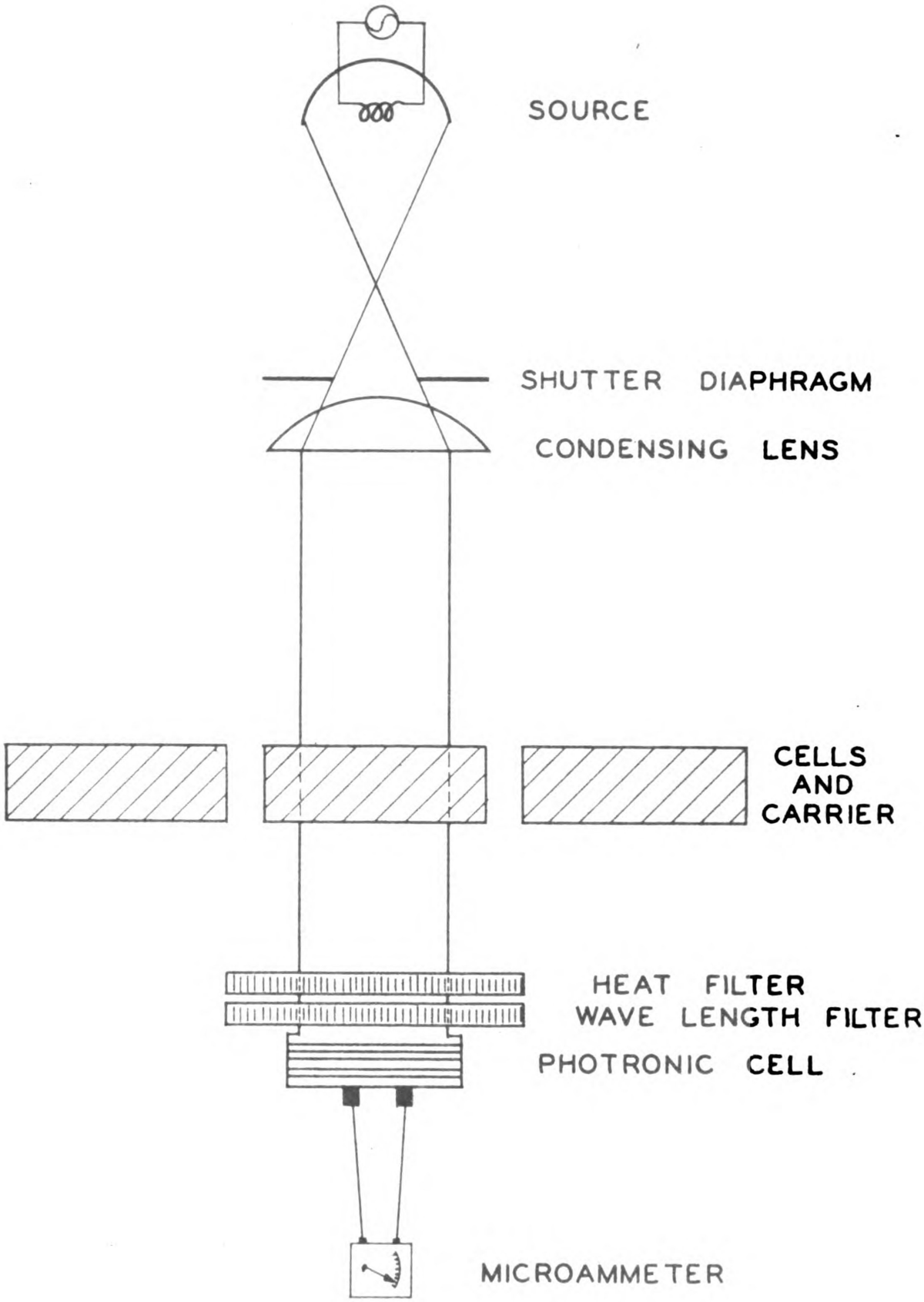

SOURCE
SHUTTER DIAPHRAGM
CONDENSING LENS
CELLS AND CARRIER
HEAT FILTER
WAVE LENGTH FILTER
PHOTRONIC CELL
MICROAMMETER

FIG. 1

FIG. 2

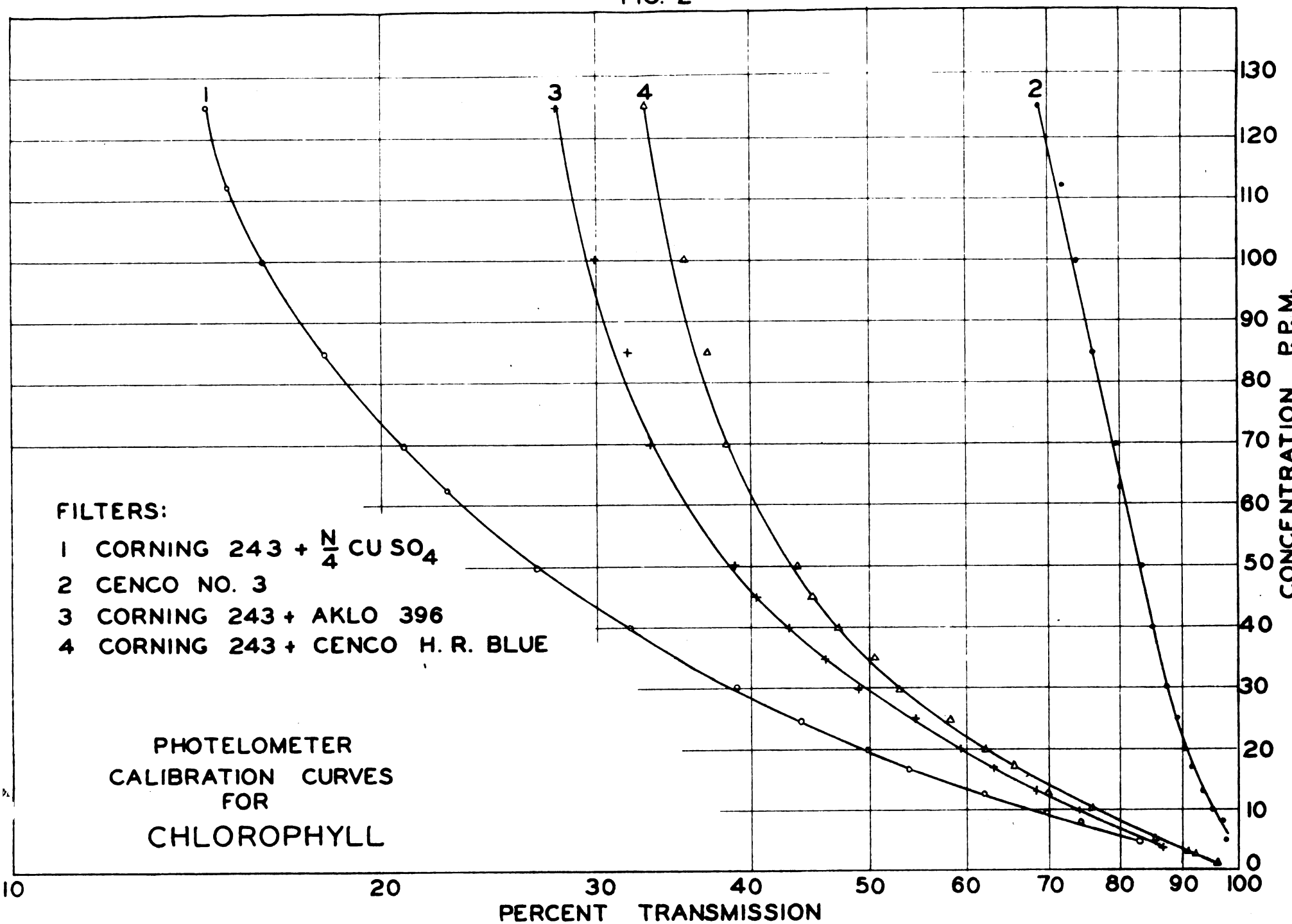
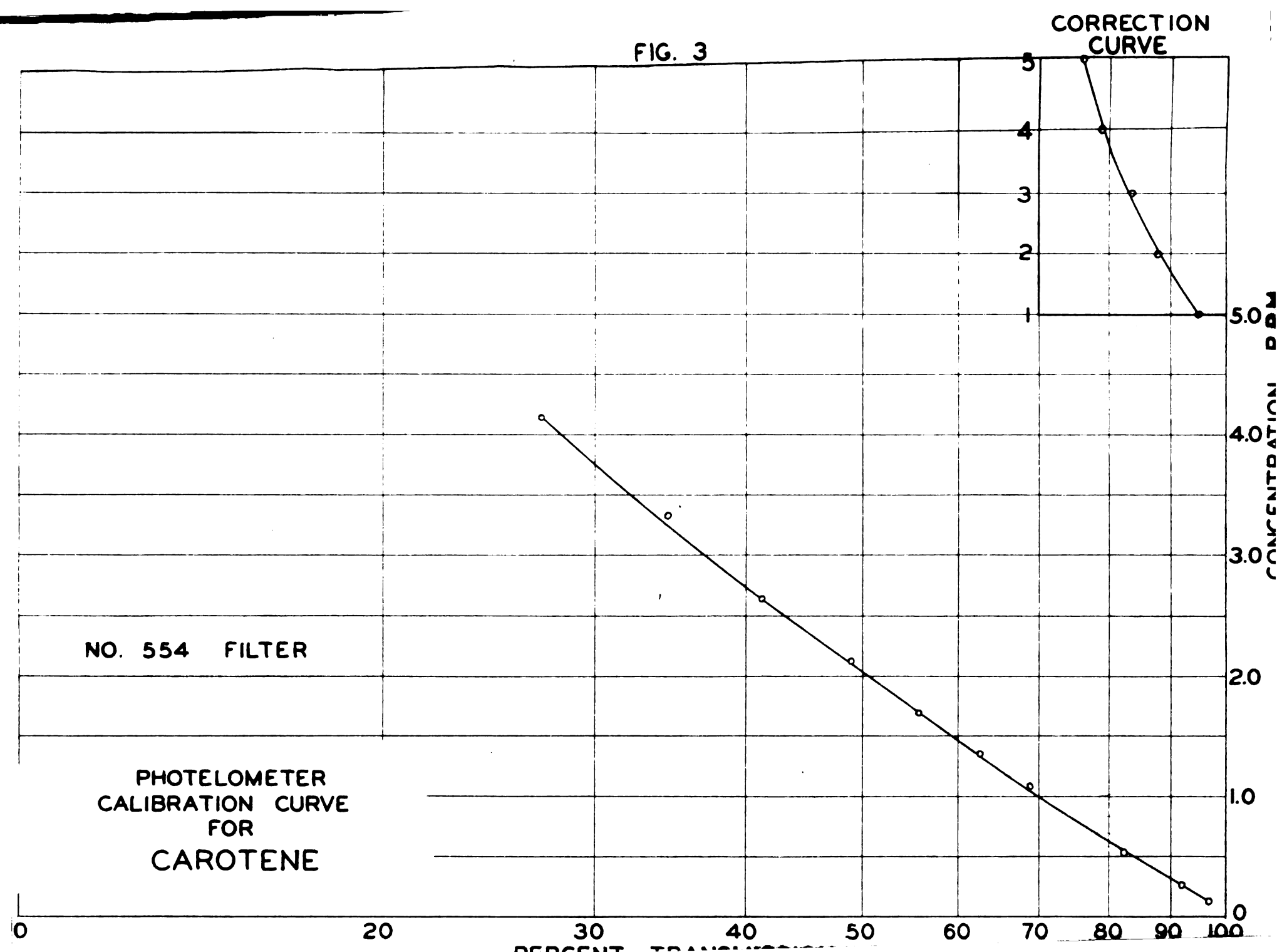

1
3
4
2
FILTERS:
1 CORNING 243 + N/4 CU SO4
2 CENCO NO. 3
3 CORNING 243 + AKLO 396
4 CORNING 243 + CENCO H. R. BLUE
PHOTELOMETER
CALIBRATION CURVES
FOR
CHLOROPHYLL
10
20
30
40
50
60
70
80
90
100
PERCENT TRANSMISSION
0
10
20
30
40
50
60
70
80
90
100
110
120
130
CONCENTRATION P.P.M.

FIG. 3


CORRECTION CURVE
5
4
3
2
1
5.0
4.0
3.0
2.0
1.0
0
CONCENTRATION P.P.M.
NO. 554 FILTER
PHOTELOMETER CALIBRATION CURVE FOR CAROTENE
0
20
30
40
50
60
70
80
90
100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