

THE PREPARATION OF CHLOROPHYLL*

F. M. SCHERTZ

Introduction

This paper is published in response to repeated requests for information regarding the preparation of pure chlorophyll. It also shows the relative purity of the samples of chlorophyll which have been used as the basis of investigations conducted in this laboratory. It is hoped that the method here described will be helpful to those contemplating a study of this important pigment. Chlorophyll is so important that it is felt that every effort should be put forth by workers to obtain a product of undoubted purity. An effort has been made to present the details of the preparation so that the difficult parts may be mastered by most investigators after a little preliminary work on their part.

Material used

The choice of material to be used in the preparation of chlorophyll is very important for in the leaves of many species of plants chlorophyll decomposes during the process of drying. This is true of leaves of the following plants: alfalfa, spinach, cowpea, and many others. In fact very few leaves will dry entirely satisfactorily. Consequently, in order to save much valuable time and material it is best to use the leaves of only such species as have proven entirely satisfactory. Leaves of *Urtica dioica* have been shown by experience to be best, while those of *Urticastrum divaricata* are very good.

The leaves for our preparation were collected during the active growing season (April or May) and were then spread out on screens to dry, away from direct sunlight. A temperature of 30–40° C. was used for drying. Twenty-four hours are usually sufficient. Electric fans and hot plates were used to hasten the drying process. As soon as the leaves were dry enough to crumble easily in the hands, they were ground finely in a pebble mill. The material should not be ground too finely otherwise the extraction will be more difficult. The method of extraction and purification as given here is the method which has been found to remove the impurities most satisfactorily. All of the operations in the preparation of chlorophyll should be carried out as quickly as possible.

* Soil-Fertility Investigations, U. S. Department of Agriculture, Washington, D. C.

Method of extraction and purification

Two moistened filter-papers are placed in a 25-cm. suction funnel (this size of funnel is used because the layer of meal should not be more than 4–5 cm. thick), and sucked down firmly. One kg. of the leaf meal is loosely placed into the funnel and 2 liters of 80 per cent. acetone (by volume) are poured on and allowed to sink into the meal till some of the extract runs from the funnel below. Suction is applied and the extract is sucked off into a filter-flask. Suction at no time should make the acetone extract boil or the filtration will be hindered. More acetone (1 liter of 80 per cent.) is added and sucked off. This is repeated till all of the green pigment is extracted from the leaf meal. About 3–6 liters of the 80 per cent. acetone are required for the extraction. Finally, the decolorized leaf meal is sucked dry to remove all of the acetone extract from the leaf powder.

The vivid green extract is transferred into 1.5 l. of petroleum ether (B. P. 30–70° C.) in a 6-liter separatory funnel (a). The petroleum ether will take up the four chloroplast pigments and a weakly yellowish green aqueous acetone layer will separate below. This aqueous acetone layer is run in a thin stream through a 4-l. separatory funnel (b) which contains 1 l. of petroleum ether. The petroleum ether in this funnel will remove practically all of the chloroplast pigments. The aqueous acetone from which the chloroplast pigments have been separated is poured into a 6-liter separatory funnel (c) which contains 0.5 l. of petroleum ether. This last separatory funnel of petroleum ether is to break emulsions which will form in the process described in this paper and to remove any traces of chlorophyll which may have passed through the other funnels.

The petroleum ether in the three funnels is now washed with a liter of 80 per cent. acetone which removes impurities and takes away none of the chlorophyll. The washing is carried out by allowing the acetone to run in a fine stream through the petroleum ether solutions. The petroleum ether by taking up acetone has increased considerably in volume. The acetone is removed by allowing a liter of distilled water to flow into *a* and then on down through *b* from a liter separatory funnel (*d*) above *a*. By the above method, much of the accompanying substances are removed, the greater per cent. of the acetone is separated and emulsions are prevented.

The solutions are never shaken at any time throughout this procedure, for emulsions once formed are difficult to break and this method prevents their formation to any great extent. All of the wash liquors from *b* are passed through funnel *c* from which they are run off. A large per cent. of the acetone may be recovered from the aqueous acetone by distillation, and used again.

The acetone is not quantitatively washed from the petroleum ether solutions, for the chlorophylls and xanthophyll would precipitate, and the purification would be made much more difficult; for then the xanthophyll could not be separated from the petroleum ether by the methyl alcohol washings.

The xanthophyll is now separated from the chlorophylls and carotin by allowing 4 liters or more of 80 per cent. (by volume) of methyl alcohol to run down in a fine stream through the petroleum ether in the separatory funnels. The last liter of methyl alcohol may be cautiously shaken with the petroleum ether solution of chlorophyll. The methyl alcoholic extract of xanthophyll is run out of each separatory funnel into the next as rapidly as it separates from the petroleum ether layer, for in this way more of the xanthophyll will be removed from the petroleum ether layers and a purer chlorophyll product will result. The methyl alcoholic extracts may be worked up for xanthophyll by the methods described by WILLSTÄTTER and STOLL.

The last traces of methyl alcohol and acetone are now removed by allowing a fine stream of water to run from separatory funnel *d* down through the petroleum ether solutions. In all, 8 to 12 liters of distilled water are required for this washing. The petroleum ether gradually loses its fluorescence, becomes turbid and the chlorophylls precipitate. Toward the end of the washing, emulsions will form but these may be broken by dissolving salt (NaCl) in the wash water from funnel *b* and then the saline solution is poured into separatory funnel *c*, where most of the emulsion will disappear. If the emulsion still persists it may be broken by running off the aqueous layer from funnel *c* and adding more salt. The saturated solution is returned to funnel *c* and this process is repeated till most of the water is removed from the petroleum ether layer.

The petroleum ether suspension of chlorophyll is now shaken with about 250 gm. of anhydrous Na_2SO_4 which removes the last traces of water and makes the solution filterable. The petroleum ether and accompanying impurities are now separated from the chlorophyll by filtering through a layer of talc on a 25 cm. suction funnel. The layer of chlorophyll which forms upon the talc is constantly broken by stirring with a nickel spatula, otherwise filtration would proceed very slowly. The petroleum ether filtrate obtained contains very little chlorophyll, practically all of the carotin and only a very little of the xanthophyll. From this filtrate carotin may be obtained. The suction applied in this filtration should be very moderate else the filtration will be retarded. Finally, wash the mass of chlorophyll and talc with 500 cc. or more of petroleum ether. Then, apply strong suction to remove as much of the petroleum ether as possible.

A. The acetone method of purification

The chlorophyll-containing talc is removed from the suction funnel and placed in a beaker where it is stirred for a short time with pure acetone (500 cc.). The acetone solution of chlorophyll is now filtered from the mass by placing the whole upon a suction funnel (15 cm. in diameter) and washing the talc with acetone till all of the chlorophyll is removed.

The acetone solution of chlorophyll is poured into 1 l. of petroleum ether in separatory funnel *a* and 500 cc. of petroleum ether is added to each of the funnels *b* and *c*. Water is now added to the chlorophyll solution until the acetone layer separates. This layer is run through the petroleum ether in *b* and *c*, after which the acetone may be recovered. The acetone is now all washed from the petroleum ether solutions by washing as above with 8–12 liters of distilled water. The chlorophyll precipitates and is obtained by filtering the dried (with anhydrous Na_2SO_4) petroleum ether solution through talc upon a suction funnel (15 cm. in diameter). The precipitated chlorophyll is washed with 0.5 to 1 l. of petroleum ether. The petroleum ether mother liquor here should run off slightly yellowish at first and finally only faintly green.

The chlorophyll-containing talc is now freed of the petroleum ether by allowing the suction to continue for a few minutes. The material may be allowed to stand over night in this condition. This purification has removed traces of carotin and xanthophyll. The chlorophyll is now purified by the petroleum ether method which removes practically all of the impurities.

B. The petroleum ether method of purification

The chlorophyll-containing talc is now removed from the funnel and placed in a beaker where it is stirred for a short time with alcohol-free ether (500 cc.).¹

As soon as the chlorophyll has gone into solution it is filtered through a thin layer of talc on a separatory funnel (15 cm.) which is just large enough to hold the mass of material. The talc is washed with ether till all of the chlorophyll is removed.

The ether (1 l.) is now evaporated from the chlorophyll solution until a sirupy mass remains. The evaporation is carried out by placing the container in a hot water bath. The level of the hot water is always kept below the level of the ether in the container. When the volume of the chlorophyll solution is reduced to about 100 cc. the ether is then removed by reduced pressure till the mass is sirupy. About a liter of petroleum ether is added a little at a time and the flask is shaken violently till the chlorophyll precipitates.

¹ The ether used here is prepared by washing U.S.P. ether with water several times and then distilling over CaCl_2 .

The precipitated chlorophyll is now removed from the mother liquor by filtering upon talc and is then washed with about 500 cc. of petroleum ether.

It is extracted again with 500 cc. of purified ether and the solution is concentrated at 30–40° C. to a sirupy mass which is then dried in a beaker in a vacuum desiccator. When dry the steel blue shiny mass of chlorophyll may be easily pulverized and weighed.

TABLE I

YIELD OF CHLOROPHYLL ($\alpha + \beta$) OBTAINED FROM LEAVES FROM DIFFERENT SOURCES

SOURCE	METHOD OF PURIFICATION	YIELD IN GM. PER KGM. OF DRY LEAVES
Minnesota, 1924	Ether	2.0
“	Ether	1.4
Missouri, 1924.....	Ether	6.9
“	Ether	7.1
“	Ether	8.3
“	Ether	8.0
“	Ether	7.9
“	Ether	8.4
“	Ether	8.2
“	Acetone	7.1
Washington, D. C., 1924	Acetone	4.5
“	Acetone	4.3
“	Acetone	3.8
“	Acetone	5.6

Purification of chlorophyll by the methods described here has given the writer his purest product. Consequently, it is submitted as a method of preparing very pure chlorophyll.

Results of extraction by the above methods

Samples of stinging nettle leaves were obtained from Minnesota for extraction and the amount of chlorophyll obtained per kg. from them is compared with that from stinging nettle leaves collected at Washington, D. C. Chlorophyll was also extracted from the leaves of wood nettles from Missouri.²

The amount of chlorophyll obtained per kg. of the dried leaves is recorded in table I.

The yields of chlorophyll as given in this table were not all obtained by the method which has just been described above. The first nine were pre-

² The author is greatly indebted to Dr. CARL G. DEUBER, formerly of the University of Missouri, now of Yale University, for collecting and drying these leaves.

pared by the method given by WILLSTÄTTER and STOLL and the remainder were prepared by the same method except that they were purified by the acetone method instead of being precipitated by petroleum ether as given by WILLSTÄTTER and STOLL in their monograph on chlorophyll.

The quantity of chlorophyll per kilogram from the leaves from Minnesota was the lowest of all and also the pigment which was obtained was off color when dissolved in ether. The wood nettle leaves obtained from Missouri gave the best results and the chlorophyll obtained was the purest.

Using 0.0513 grams of each sample of chlorophyll³ (or 0.10 gm. per liter) tests were made for total yellow pigments, and with the colorimeter also to determine which samples contained the least impurities. Those containing the least impurities when saponified gave the lowest readings on the colorimeter. The carotinoids were separated from the chlorophyllins and the results are recorded in table II.

The results shown in table II are for only the samples which were used in the final work on chlorophyll. Many other samples not described or used in this work showed that changes had taken place in the chlorophyll during its extraction and purification, or that the original leaf material was not of the best grade. Samples which did not show a pure green color when dissolved in ether or which failed to give a good phase test were not used.

In the measurements of chlorophyll in columns 3 and 7 of table II, the readings are in millimeters, and the comparisons are made with the following combination of Lovibond slides, nos. 3, 4, and 5 blue, plus 10 and 20 yellow, using a Duboseq colorimeter. The concentration of chlorophyll in the solutions measured is 0.1000 gm. per liter.

The carotinoids, shown in columns 4 and 8 of table II, were estimated colorimetrically as carotin. The amounts given were the amounts found in each 0.05 gm. sample of chlorophyll.⁴

The readings for samples no. 4 and no. 6 show that most of the carotinoids were removed by the acetone method of preparation, as can be seen by the figures in column 3, table II. All of the samples given in column 3 were then purified by the acetone method as given in this paper. Examination of the figures in the last four columns of table II shows that the method was not only valuable in removing the carotinoids but the purity of the chlorophyll samples was greatly improved. (*Cf.* columns 3 and 7 for chlorophyllin content.)

³ The samples of chlorophyll used here were dried in a vacuum desiccator for two weeks.

⁴ SCHERTZ, F. M. The quantitative determination of carotin by means of the spectrophotometer and the colorimeter. *Jour. Agr. Res.* 26: 383-400. 1923.

TABLE II
 PURITY OF CHLOROPHYLL SAMPLES AS SHOWN BY TESTS OF THEIR CAROTINOID AND CHLOROPHYLL CONTENT, AND THE RESULTS OF
 PURIFICATION BY THE ACETONE METHOD

SAMPLE NUMBER	CHLOROPHYLL YIELD PER KILOGRAM OF DRY LEAVES	CHLOROPHYLL-LIN CONTENT (COLOR-IMETER)	CAROTINOID CONTENT	WEIGHT OF CHLOROPHYLL AFTER ACETONE PURIFICATION	LOSS IN PURIFYING	CHLOROPHYLL-LIN AFTER ACETONE TREATMENT (COLORIMETER)	CAROTINOIDS AFTER ACETONE TREATMENT
1	gm. 8.0	mm. 24.9	mg. 0.73	gm. 5.6	Per cent. 30.0	mm. 17.7	mg. 0.50
2	8.4	16.5	0.73	6.6	21.0	16.1	0.43
3	8.2	23.2	0.77	6.2	24.0	17.4	0.46
4*	4.3	27.5	0.10	2.9	32.0	16.8	less than 0.10
5	6.9	23.1	0.30	5.7	17.0	15.0	less than 0.10
6*	7.1	24.8	0.10	5.0	29.0	17.8	less than 0.10

* Samples 4 and 6 were prepared by the acetone method while the others were obtained by the ether method.

Further evidence may be submitted in favor of incorporating the acetone method of purification with the method for preparing fairly pure chlorophyll. In all, five samples of chlorophyll were obtained by substituting the acetone method for the ether purification as used by WILLSTÄTTER and STOLL. One of the samples of chlorophyll, when extracted and prepared by the acetone method contained 0.20 mg. of carotinoids while the other four samples contained less than 0.10 mg. per 0.0513 gm. sample of chlorophyll. The average result for fifteen samples obtained by the ether method of WILLSTÄTTER and STOLL was 0.81 mg. of carotinoids per 0.0513 gm. of chlorophyll. The method as modified, then, should be far superior to the method given by WILLSTÄTTER, because not only will the carotinoid content of the samples obtained be less, but the chlorophyll content will be much greater, as shown by the figures in table II.

The samples used here would have been further purified if any method had been available whereby a purer sample could be detected. The error in colorimetric estimation was too great to make any further purification worth while and spectrophotometric methods used with the yellow pigments are not applicable to a solution of mixed pigments. Consequently, these six samples after further testing were regarded as pure chlorophyll, though it is highly probable that they contain a small percentage of impurities.

Before any quantitative experiments could be carried on with the chlorophyll prepared it was necessary to know the loss in weight on heating to 100° C. so that the correct amount of chlorophyll could be weighed out for any given experiment.

Table III shows the weight of the samples of chlorophyll before and after drying in an electric oven over night at 100° C. after the samples had been previously dried in a vacuum desiccator for two weeks. The loss in the samples was generally 0.0007 gm. consequently, 0.0507 grams, which represents 0.0500 gm. of dried chlorophyll, was used in each experiment.⁵ In all of the acetone purification experiments in table II, 0.0507 gm. of chlorophyll was used, instead of 0.0513 gm. which was used by WILLSTÄTTER.

The first six samples in table III are those already described in this paper. Samples 7 and 8 in table III were prepared after the ash content of the first six were known. These two samples were prepared by the method described in this paper. The ash content was then determined, the data being given in the last column of table III. These two samples were then again purified by dissolving in ether and precipitating by the addition of petroleum ether. The ash content was then found to be exactly the same

⁵ Chlorophyll cannot be dried at 100° C. and then used, for the heat alters the chlorophyll molecule. Chlorophyll after drying is brownish green instead of a pure green when dissolved in ether. If a dried sample is saponified the result is a dirty brownish green chlorophyllin instead of a clear bright green.

TABLE III

LOSS IN WEIGHT BY DRYING CHLOROPHYLL AT 100° C. AFTER DRYING IN A VACUUM DESIC-
CATOR FOR TWO WEEKS OR MORE, AND THE ASH CONTENT OF
CHLOROPHYLL PREPARATIONS

SAMPLE NUMBER	WEIGHT BEFORE DRYING	WEIGHT AFTER DRYING	LOSS IN DRYING	ASH CONTENT OF CHLOROPHYLL
	gm.	gm.	gm.	Per cent.
1	0.0513	0.0506	0.0007	3.9
2	0.0519	0.0513	0.0007	4.2
3	0.0520	0.0513	0.0007	3.6
4	0.0514	0.0507	0.0007	3.4
5	0.0520	0.0513	0.0007	4.5
6	0.0507	0.0495	0.0012	4.1
7	4.1
8	4.0

as that already determined. Hence, no further attempts to improve the purity of the chlorophyll were made.

Only one of the samples reported in table III gave the theoretical amount (4.5 per cent.) of ash and this sample was not purified as much as some of the others. No explanation is offered as to why the ash content varies in the samples. It might be suggested that the magnesium of the chlorophyll had been replaced by hydrogen but this was not true, for each of the samples of chlorophyll was carefully tested in the spectroscope for bands due to pheophytin. No trace of pheophytin was found in any of the samples. This test is easy to make for the spectroscopical bands of chlorophyll and of pheophytin are quite different.

The ash of two of the samples were tested for their MgO content. The ash from sample no. 1 was found to yield 100 per cent. MgO while that from no. 5 was found to be 92 per cent. MgO.⁶ No other tests to determine the MgO content of the samples were made.

The ash content of samples of chlorophyll prepared by WILLSTÄTTER and his students will now be considered. WILLSTÄTTER and ISLER⁷ prepared pure chlorophyll α and β . The amount of ash (MgO) found for the samples of chlorophyll α was 4.3, 4.6, and 4.2 per cent. and for chlorophyll β it was 4.1, 4.4, 4.4, and 4.2 per cent. WILLSTÄTTER and HUG⁸ give only 3 results for preparations of pure chlorophyll. They are 4.5, 4.6 and 4.9 per cent.

⁶ The author is indebted to R. B. DEEMER of this laboratory for these determinations.

⁷ WILLSTÄTTER, R., and ISLER, MAX. Untersuchungen über Chlorophyll. XX. Über die zwei Komponenten des Chlorophylls. Liebig's Annalen. 390: 269-339. 1912.

⁸ WILLSTÄTTER, R., and HUG, ERNST. Untersuchungen über Chlorophyll. XV. Isolierung des Chlorophylls. Liebig's Annalen. 380: 177-211. 1911.

Their preliminary results were 3.1, 3.2, 3.9, 3.7, and 3.6 per cent. The pure chlorophyll which WILLSTÄTTER and HUG prepared was obtained by a method which was essentially different than that described in this paper. The leaf meal was given a preliminary washing with benzol and with petroleum ether. The chlorophyll was then extracted with ethyl alcohol, transferred to petroleum ether and then purified. Results of analysis for ash are not given in WILLSTÄTTER and STOLL'S monograph on chlorophyll, in which they describe the method which has been modified and described in this paper. Why the results given by WILLSTÄTTER and HUG are higher than the theoretical and why the results given in this paper are lower than the theoretical result, 4.5 per cent., is as yet unexplained.

Tests used in determining purity

PHYTOL CONTENT

Instead of directly determining the phytol content of the chlorophyll, its purity was determined by extracting an ethereal solution of the pigment with 22 per cent. HCl. If the chlorophyll has been altered by the splitting off of the phytol this fact will be indicated because any chlorophyllide present will dissolve in the 22 per cent. HCl. This test is very sensitive.

None of the ethereal solutions of chlorophyll gave any color when extracted with 22 per cent. HCl, hence none of the phytol was split off. If phytol were split off the MgO content would have been too high.

YELLOW PIGMENTS

In the results given in this paper it is seen that none of the chlorophyll preparations was wholly free from yellow pigments, nor has the author succeeded yet in preparing one which is absolutely free from carotinoids.

Most of the preparations here described contained much less than 1 per cent. of the carotinoids; the carotinoid present is xanthophyll.

THE PHASE TEST

The brown phase appeared on saponification with methyl alcoholic potash. Allomerized chlorophyll would not give this test and the solution remains brown with a mixture of pure and allomerized chlorophyll.

THE SPECTRUM

An analysis of the spectrum of the preparations was made to see whether any of the groups in the chlorophyll molecule had been changed in the process of preparation. This observation is far more sensitive in detecting impurities in the sample than would be a chemical analysis of the chlorophyll. If chlorophyll is altered by the action of acid, the presence of pheo-

phytin is observed in the spectrum, for then two absorption bands appear; one before the Fraunhofer line E and the other between the lines E and F. No such pheophytin absorption bands were found in the spectra of these preparations. The absence of these bands is evidence of purity of the samples.

The magnesium complex of all the preparations was found to be unaltered.

COLOR TESTS

In order to tell which preparations of chlorophyll were good, their ether extracts were compared as to color. Several samples were compared at one time and only those showing a pure green color were considered good. Traces of allomerization could be easily detected in this manner.

Allomerization was further tested for by saponifying these ethereal solutions of chlorophyll. Good chlorophyll showed a pure, clear green while allomerized chlorophyll was of a dirty green color, after the saponification.

Summary

1. A method is described for the extraction and purification of chlorophyll ($\alpha + \beta$).

2. From 7–8 grams of chlorophyll may be obtained from 1 kg. of nettle leaves.

3. Purification of chlorophyll by the acetone method described is recommended because by its use most of the carotinoids are removed and the chlorophyll was found to be much purer. The method as outlined is recommended for obtaining very pure chlorophyll. If large yields of chlorophyll, not so pure, are desired, then the method may be easily modified to meet the needs of the worker.

4. 0.0500 gram samples lost about 0.0007 gm. on drying at 100° C.

5. The chlorophyll preparations described here yielded on an average 3.98 per cent. of ash. Two samples of the ash were analyzed and found to contain 92 and 100 per cent. MgO.

6. The tests used in determining the purity of chlorophyll are described.

U. S. DEPARTMENT OF AGRICULTURE,
WASHINGTON, D. C.