A STUDY OF THE PHASIC SEPARATION OF CHLOROPHYLL AND CAROTENE

by

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INTRODUCTION

Much work has been done in this laboratory on the stability of carotene in dehydrated alfalfa (13), the preparation and storage of its concentrates (14) and upon other related subjects. Of the three principal plant pigments, carotene, xanthophyll and chlorophyll, interest has been centered on carotene because of its pro-vitamin A properties. The recent prominence given to chlorophyll products in drugs and deodorizers has created considerable interest concerning procedures for the isolation of chlorophyll and its derivatives. Therefore, the separation of chlorophyll and carotene from plant tissue and the separation of one from the other has become increasingly important.

Chlorophyll, in most of the higher plants, exists in a 3-1 or a 2-1 ratio of chlorophyll a to chlorophyll b. The b component differs from the a in having a formyl group replacing a methyl group, giving it two less hydrogens and one more oxygen. Hence the empirical formula for chlorophyll a is $C_{55}H_{72}O_5N_4Mg$ and that of chlorophyll b is $C_{55}H_{70}O_6N_4Mg$. Both forms undergo similar reactions and are quite easily decomposed in the presence of heat, light, acid and air. Drying also causes degradation to various decomposition products.

In commercial compounds the water soluble chlorophyllins are usually used, due to their relative ease of preparation. At present the chlorophyll derivative most frequently used is the sodium copper chlorophyllin (17). Perhaps if chlorophyll

itself was more easily isolated, it would be a more favorable constituent for some commercial products than its water soluble salts.

In the past few years countercurrent distribution has become increasingly important. Therefore phasic separation by countercurrent distribution might be one method of separating carotene and chlorophyll. The purpose of this investigation was to determine distribution coefficients for chlorophyll and carotene in different systems and to study their separation in a distribution apparatus constructed in this laboratory.

REVIEW OF LITERATURE

There are four possible ways of obtaining chlorophyll in more or less pure form:

The Berkman Process (2) in which the fresh plant material is ground with a small amount of water. The colloidal suspension obtained is then subjected to various filtrations, centrifugations and drying steps which leaves the chlorophyll in a fairly pure state.

By chromatography of plant extracts, such as was employed by Valley Vitamins, Inc., of McAllen, Texas (16).

By exhaustive washing with water of a petroleum ether plant extract. This removes all the water soluble materials and causes the precipitation of chlorophyll, which may then be

filtered on talc or powdered sugar as in the method of Zscheile and Comar (20) or Schertz (15).

Separation of the plant pigments by distribution between immiscible solvents.

Willstatter and Stoll (19) used this method successfully to separate chlorophyll a and b by partitioning them between petroleum ether and aqueous methyl alcohol. However chromatography, using various adsorbents, has largely replaced Willstatter's procedure for separating the a and b fractions.

Little has been done with this fourth method. However the recent development of systematic extraction procedures, such as those made possible by the countercurrent distribution apparatus of Craig (5), makes it apparent that the method could be adopted to the industrial separation of plant pigments.

The application of the Craig apparatus, which incorporates a large number of individual stages, is limited to the treatment of small amounts of solutes. Ability to deal with larger quantities of material is possible with the apparatus designed by Johnson and Talbot (8). Their apparatus is still on the laboratory scale however and apparently has not been used on plant extracts.

Only two experiments have been reported in the literature, in which plant extracts were treated in a countercurrent system. Kies and Davis (9) mention that their apparatus has been used successfully in the purification of plant extracts. The distribution described in their paper is termed a "cascade

distribution process". It can not be considered a countercurrent distribution in the same sense as the Craig procedure since one phase containing the solute is held stationary and the mobile phase is passed through it.

Lancaster, Lancaster and Dutton (10) using a 25 tube Craig apparatus on a system of chlorophyll a and b, and carotene, with hexane and 90 percent ethanol as solvents, were able to get a good separation of the carotene and a partial separation of the chlorophyll a from the b. However their primary interest was to study the operation of the countercurrent distribution apparatus and to compare the degree of separation obtained with that predicted by their modification of the formula of Martin and Synge (11) and that calculated by use of the binomial expansion.

Methods for carrying out extraction or distribution processes fall into two distinct classes, multiple or successive contact methods and countercurrent contact methods (6,7). Watanabe and Morikawa (18) developed a process called "pseudo countercurrent extraction" which is a hybrid of the two distinct classes.

EXPERIMENTAL

Effect of Temperature on Equilibrium

Previous experiments in this laboratory indicated that the distribution constant; $K = \frac{Cx}{Cy}$, where Cx equals the concentration in the aqueous solvent and Cy equals the concentration in the petroleum ether, for chlorophyll and carotene; might vary considerably with temperature. Six combinations of solvents; 98 percent methyl alcohol, 90 percent methyl alcohol, 90 percent ethyl alcohol, 75 percent isopropyl alcohol, 50 percent n-propyl alcohol, and 60 percent acetone with Skellysolve B were used in rather extensive tests to verify these data. The rather high percentage of water in the three last named solvents was necessary in order to get good separation into two phases.

The chlorophyll was prepared by the method of Schertz (15) and the carotene used was a mixture of crystalline alpha and beta carotene (General Biochemicals, Inc.).

Three solutions of chlorophyll in Skellysolve B were made up to a concentration of approximately 90 mg/l for experiments at 0°, 10°, and 25° C. Also three solutions of carotene in Skellysolve B were made up to an approximate concentration of 30 mg/l. One of the chlorophyll solutions and one of the carotene solutions were placed at each of the three temperatures to allow them to reach that temperature before use. Each of the six aqueous solvents mentioned above were also placed at each of

the three temperatures until the solvent reached that temperature. Each one was then saturated with pure Skellysolve B. Twenty ml of the Skellysolve B solution of chlorophyll was placed in an eight inch test tube with 20 ml of one of the aqueous solvents. This was repeated with each of the aqueous solvents. The test tubes were stoppered with clean rubber stoppers, shaken and allowed to stand overnight to allow sufficient time for equilibrium to be reached. Samples were taken from the Skellysolve B layer and analyzed. Duplicates were run on each of the six samples at each of the three temperatures. The same procedure was followed using the Skellysolve B solutions of carotene.

Analysis for chlorophyll was made as follows: five ml of the Skellysolve B solution was withdrawn into a 100 ml volumetric flask and diluted to volume with diethyl ether. This dilution resulted in a single phase solution for subsequent spectrophotometric determination for total chlorophyll. All spectral work was done with a Beckman Model DU Spectrophotometer using one cm Corex cells. By substitution in the equation: Total chlorophyll (mg/l) = 7.12 $\log \frac{I_o}{I}$ (at 6600Å) + 16.8 $\log \frac{I_o}{I}$ (at 6425Å), as recommended by the A.O.A.C. (1), the chlorophyll concentration can be readily calculated. Concentration of the original solution was determined in a similar manner. The difference in these concentrations was the concentration in the aqueous solvent layer.

Carotene analysis was made as follows: five ml of the Skellysolve B layer was diluted to volume with Skellysolve B in a 100 ml volumetric flask. This solution was then read on a Beckman Spectrophotometer at 4360Å. From the formula (1), $C = \log \frac{I_0}{I} (4360Å)/196$, the concentration is given in grams per liter of solution.

Samples which contained a mixture of chlorophyll and carotene were also set up to determine if any interaction of pigments altered the distribution coefficients. Chlorophyll from the mixture was determined as previously stated since carotene does not interfere with its spectrophotometric determination. However, it was necessary to separate the carotene for its subsequent analysis. This was done by the chromatographic adsorption of a ten ml sample of the Skellysolve B layer on a column of Westvaco Powdered Magnesia, #2641, and Hyflo Super Cell (1-2 ratio by volume) and elution of the carotene with a four percent acetone in Skellysolve B solution into a 250 ml volumetric flask.

The volume of the Skellysolve B layer was observed to increase on mixing of the two immiscible solvents. This volume change was noted and used in future calculations. The total volume of the system was assumed to remain constant at 40 ml.

Table 1 shows the results of this experiment. The dilution caused by the increase in the Skellysolve B layer volume has been considered in calculating the percent extraction and K values.

Table 1. Effect of temperature on distribution of chlorophyll and carotene between Skellysolve B and various immiscible solvents.

Skellysolve B plus:	Percent of solvent in water	Percent extracted from Skellysolve B layer: Chlorophyll	Carotene	Mixture: Chlorophyll	Carotene
25° C.					
methyl alc.	98	58.7	5.3	55.8	2.5
methyl	90	19.4	1.9	18.7	4.0
ethyl	90	36.9	5.6	38.8	1.5
iso-pr.	75	4.1	4.5	0.1	2.2
n-prop.	50	6.2	8.6	2.5	0.0
acetone	60	0.0	2.3	2.1	1.9
10° C.					
methyl alc.	98	59.7	3.5	55.2	0.5
methyl	90	15.4	1.9	13.7	3.5
ethyl	90	39.1	5.1	38.1	0.0
iso-pr.	75	4.0	5.0	4.0	0.0
n-prop.	50	3.4	6.8	0.9	2.8
acetone	60	2.8	3.5	0.0	2.6
0° C.					
methyl alc.	98	57.3	0.7	56.2	0.0
methyl	90	11.9	0.0	10.0	1.5
ethyl	90	37.4	4.5	37.1	0.0
iso-pr.	75	3.8	5.6	4.4	0.0
n-prop.	50	1.0	3.5	1.0	5.8
acetone	60	0.0	6.8	2.5	3.5

The isopropyl alcohol, n-propyl alcohol and acetone systems gave very little extraction of either pigment due to the large percentage of water which was required to make them immiscible with the Skellysolve B. This in turn caused a large increase in the Skellysolve B volume, which indicated that the alcohol and/or acetone layers contained more water by percent than originally, due to the alcohol and/or acetone passing into the Skellysolve B layer. Apparently there is little change in the

percentage extraction due to the temperature difference, using these three solvents. It is obvious that they are unsatisfactory for separating plant pigments in countercurrent distribution systems.

Ethyl and methyl alcohol, however, show favorable separation and a few trends of the effect of temperature on distribution and the effect of one solute on the other may be observed. For clarity Table 2 shows the distribution constant K for the different systems of ethyl and methyl alcohol. Both phases were of the same volume.

Table 2. K values for chlorophyll and carotene in systems of ethyl alcohol- and methyl alcohol-Skellysolve B.

Temp. °C.	Percent of solvent in water	Alcohol	K values			
			Chlorophyll		Carotene	
			Pure system	Mixture	Pure system	Mixture
25	98	methyl	1.421	1.262	0.0560	0.0256
10	98	↓	1.481	1.230	0.0363	0.0050
0	98	↓	1.341	1.283	0.0071	0.0000
25	90	methyl	0.241	0.230	0.0194	0.0417
10	90	↓	0.182	0.159	0.0194	0.0363
0	90	↓	0.135	0.111	0.0000	0.0152
25	90	ethyl	0.585	0.634	0.0593	0.0152
10	90	↓	0.642	0.616	0.0538	0.0000
0	90	↓	0.597	0.590	0.0472	0.0000

Few trends are apparent in the pure chlorophyll systems. In the case of 90 percent methyl alcohol a decreasing concentration occurs in the alcohol layer with decreasing temperature. In the

mixture of carotene and chlorophyll this was observed in both the 90 percent methyl and 90 percent ethyl alcohol systems. The inconsistency of this change and its small value make it quite insignificant. In all cases the concentration of carotene in the alcohol layer decreased with decreasing temperature. However, the concentration was small in all cases and may not be significant.

Changes in the K value for the pure chlorophyll and carotene systems, when compared with K values for the mixture are small but still quite important. In all but a few exceptions, carotene in 90 percent methyl alcohol at all temperatures and chlorophyll in ethyl alcohol at 25°, the pigments were more concentrated in the alcohol phase in the pure system than in the mixture. This indicates that one pigment affects the distribution of the other.

The exceptions noted above may be due to unavoidable evaporation. This evaporation would have considerable effect upon the concentration of the small volumes which were used. Duplicates were used in all cases and were in close agreement.

The noninflammability of trichloroethylene is a recommendation for using it in a distribution system. Therefore, attempts were made to use it in a solvent system for separation of plant pigments. Trichloroethylene is miscible in all proportions with petroleum ether. Various alcohols and acetone needed a rather high percentage of water to produce two phases. Seventy-five percent methyl, 75 percent ethyl, 50 percent isopropyl and 50

percent n-propyl alcohol and 60 percent acetone produce good separation of layers with the trichloroethylene. However the aqueous solvents are now the lighter of the two.

All solvents were allowed to reach their respective temperatures of 25°, 10°, and 0° C. before mixing. The pigments were dissolved in trichloroethylene and the aqueous solvents were previously saturated with trichloroethylene. The change in volume of the two layers when mixed was noted and the experiment was conducted as previously described. As expected, considering the high concentration of water in the alcohol and acetone layers, very little of either pigment was extracted from the trichloroethylene layer. Indeed in all cases the extraction ranged between zero and three percent.

Preparation of Chlorophyll by Chromatographic Adsorption

It was necessary to prepare more pure chlorophyll for subsequent experiments. Chlorophyll is produced by the method of Schertz (15) in very low yield and upon chromatographing a solution of this chlorophyll, some yellow pigment is found to be still present. It was desirable therefore to obtain chlorophyll by another procedure.

Chromatography using a suitable adsorbent should offer a suitable method of producing pure chlorophyll. Powdered sugar (20) and an adsorbent mixture composed of a four to one (by volume) ratio of talc to urea (12) have been used, but these have

only moderate adsorbing qualities. Other adsorbents such as powdered magnesia adsorb so strongly that it is very difficult to remove the chlorophylls from the chromatogram. However it was found that magnesia on long standing becomes partially deactivated and loses its ability to hold the chlorophylls so tightly. Some difference in the amount of pigment an adsorbent would hold and the ease of eluting the pigments were observed in different batches of the adsorbent. In all cases however the aged adsorbent could be made practically colorless by the use of proper eluting agents.

The adsorbent mixture was composed of a two to one (by volume) ratio of Hyflo Super Cell and aged Westvaco Powdered Magnesia #2641. The plant extract was obtained by extracting dehydrated alfalfa meal with Skellysolve B in a laboratory Soxhlet extractor capable of holding 1.0-1.1 kilogram of the dry meal. Plant extracts were obtained in this way for all subsequent experiments where the crude extract was used.

Two types of columns were used. One was of 51 mm Pyrex tubing, four feet in length. The column was packed with adsorbent to a height of 12-15 inches. It was very difficult to get this column properly packed to avoid channeling. The second type of column was a large gradually tapering percolator funnel, 20 inches high and of approximately four liters capacity. This column was very satisfactory; flow of solvent through the adsorbent was rapid, channeling seldom bothered and a larger quantity of adsorbent could be used.

A cotton plug was placed in the bottom of the column, adsorbent added until it was at a height of nine to ten inches when packed, and vacuum applied. A thin layer of anhydrous Na_2SO_4 was then placed on top and the column wetted with Skellysolve B. The Skellysolve B extract was then carefully added until one third of the adsorbent was colored. The first washing of the adsorbent was carried out with a quantity of Skellysolve B in order to begin the separation of pigment bands. This was followed by one liter of five percent acetone in Skellysolve B, then one liter of ten percent acetone in Skellysolve B.

Carotene was eluted on the first application of acetone. As the concentration of acetone was increased the more tightly adsorbed yellow pigments were eluted.

The washing of the column was continued with 15 percent acetone in Skellysolve B. The last carotenoid band to become noticeable was a dull orange. Directly above this was a narrow brown band, then the green chlorophyll and above this the brown pheophytin band at the top of the column. After the dull orange band had been completely developed, time could be saved by drawing the solvent from the adsorbent and removing the adsorbent from the column. This removal of the adsorbent was facilitated by the flared top of the column. The yellow and brown layers at the bottom of the adsorbent were separated from the chlorophyll and upper pheophytin band with a spatula. The upper pheophytin band was not removed since there was no clear cut boundary between it and the chlorophyll.

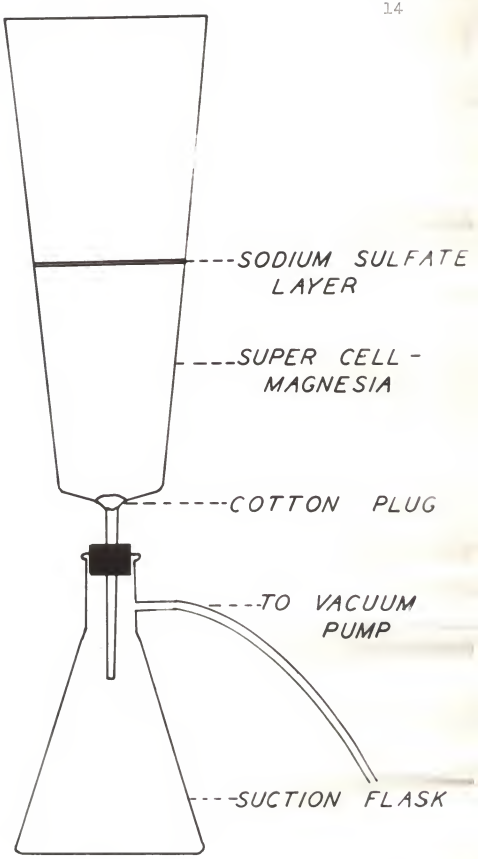


FIGURE I. DIAGRAM OF CHROMATOGRAPHIC APPARATUS USED IN SEPARATION OF CHLOROPHYLL

The column was cleaned, a small amount (approx. 1") of fresh adsorbent was placed in the bottom and the chlorophyll containing magnesia was replaced in the column. Elution followed with a solution containing 20 percent ethyl alcohol (95 percent), 20 percent acetone and 60 percent Skellysolve B. The first solvent coming through sometimes contained a little yellow pigment which was removed before the chlorophyll eluate entered the receiving flask. Both the chlorophyll and pheophytin were eluted by this solvent mixture. It was necessary therefore to cut off the eluate after the major portion of chlorophyll had been removed and the pheophytin had started to enter the flask.

The chlorophyll eluate was washed carefully with water to remove the ethyl alcohol and acetone and then dried over anhydrous Na_2SO_4. It was combined with similar eluates from two other columns and readsorbed according to the procedure used with the original extract. Repeated washing with 15 percent acetone in Skellysolve B will leave the chlorophyll at the top of the adsorbent and the impurities below. Again the adsorbent was removed from the column and the chlorophyll containing band was replaced on a thin layer of fresh adsorbent. Elution was made with the alcohol, acetone, Skellysolve B mixture as previously described. The solution was washed with water, dried with anhydrous Na_2SO_4, evaporated to a syrup in a vacuum desiccator and stored in a stoppered bottle at -10° C. or lower.

If care is used very little contamination is obtained in the Skellysolve B solution of chlorophyll. Exposure of the

solution, especially the purified solution, to heat or light must be avoided, since such extracts of chlorophyll have been found to be especially susceptible to decomposition (21).

To determine the purity of the solution and to prove that the chlorophyll had not undergone change the following tests were made:

A sample of the concentrate was dissolved in ether and log $\frac{Io}{I}$ obtained at 6600 Å and 6425 Å. The concentration of chlorophyll a and chlorophyll b was calculated by the formulas (4): chlorophyll a (mg/l) = 9.93 log $\frac{Io}{I}$ (6600) - .777 log $\frac{Io}{I}$ (6425) and chlorophyll b (mg/l) = 17.6 log $\frac{Io}{I}$ (6425) - 2.81 log $\frac{Io}{I}$ (6600). Total chlorophyll was determined by the formula previously given.

Total chlorophyll = 19.75 mg/l
Chlorophyll a = 13.21 mg/l
Chlorophyll b = 6.57 mg/l
19.78

It may be seen that the total chlorophyll and the summation of a and b values are nearly identical. Also, the ratio of a to b is nearly two to one. This is the ratio found in most plants.

A small amount of a Skellysolve B solution of the purified chlorophyll was adsorbed on a talc-urea column, where it was seen that only a minimum of yellow pigments and pheophytin was present. The a and b components were separated and purified twice more by chromatography on similar columns (12). An adsorption curve was then run on each component. The two adsorption peaks for each component agree very closely in height and

wave length to those given by Zscheile and Comar (20) as shown in Fig. 2.

Rate of Equilibration of Chlorophyll in an Immiscible System

An experiment was devised to determine the rate at which chlorophyll attained equilibrium between two immiscible solvents. The experiment was conducted in a 25° C. constant temperature room. The system, 90 percent ethyl alcohol and pure chlorophyll in a Skellysolve B solution was used. The solvents were mutually saturated with each other prior to mixing. Ten ml of the 90 percent ethyl alcohol was placed in an eight inch test tube and ten ml of the chlorophyll in Skellysolve B solution carefully placed on top to avoid mixing. The test tube was stoppered and placed in a Burrell, Model BB, wrist action shaker. Samples were shaken at a rate of six vibrations per second for various lengths of time. At the end of a specified period shaking was stopped, the layers were allowed to separate and a sample was taken from the Skellysolve B layer. Analysis was made as previously described for chlorophyll. Table 3a shows the results of the first experiment.

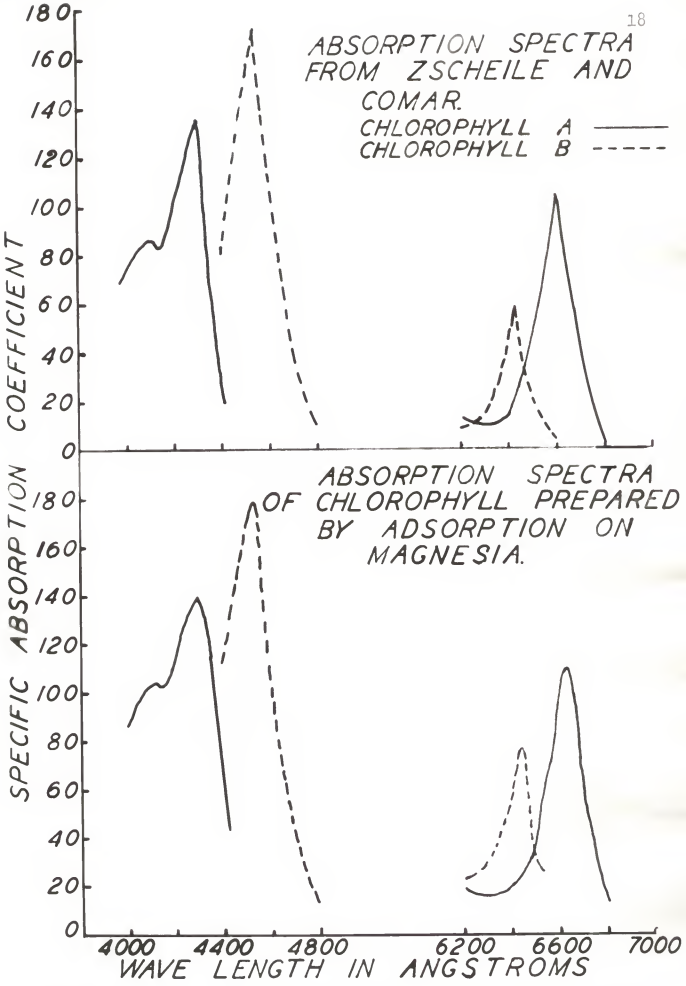


FIGURE 2. ABSORPTION SPECTRA FOR CHLOROPHYLL A AND B.

Table 3a. Effect of time of shaking samples on percent extraction of chlorophyll from Skellysolve B layer (original concentration of chlorophyll solution = 92.3 mg/l)

Sample	Time of mixing	Percent extracted
1	30 seconds	43.8
2	2 minutes	40.9
3	10	41.2
4	20	40.0
5	60	41.8
6	120	41.7

From the results of this experiment it is seen that equilibrium is reached in less than 30 seconds. The experiment was repeated using shorter periods of shaking. Table 3b shows that equilibrium of the chlorophyll between these two solvents must be extremely rapid. The data are presented graphically in Fig. 3.

Table 3b. Effect of time of shaking samples on percent extraction of chlorophyll from the Skellysolve B layer (original concentration of chlorophyll solution = 259.6 mg/l).

Sample	Time of mixing	Percent extracted
1	5 seconds	41.2
2	10	42.1
3	15	42.6
4	30	42.4
5	45	41.9
6	60	43.0
7	120	43.1

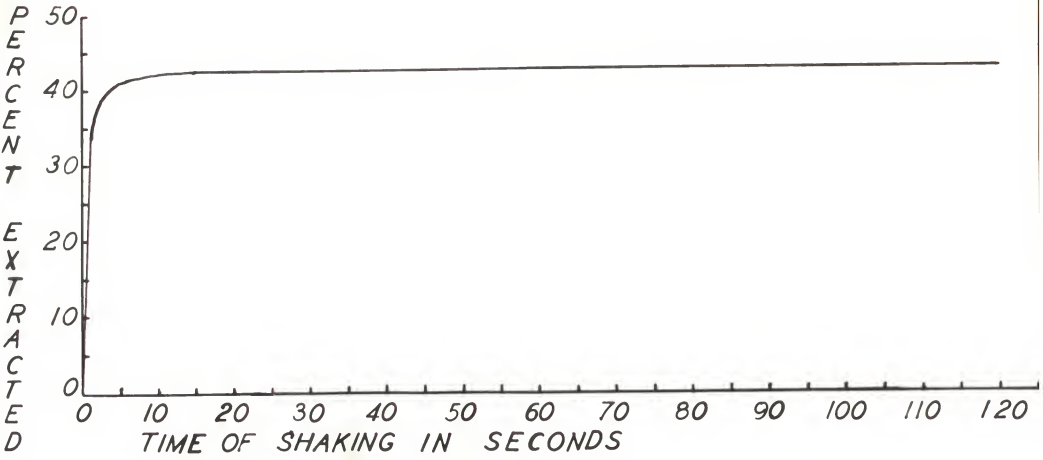


FIGURE 3. TIME OF SHAKING SAMPLES AGAINST PERCENT OF CHLOROPHYLL EXTRACTED.

Effect of Addition of Water to Skelly-Alcohol Systems

In order to determine the effect of an increasing percentage of water in the aqueous solvent on the amount of chlorophyll extraction from the Skellysolve B layer, the following experiment was devised.

Solutions of methyl alcohol containing varying amounts of water were saturated with Skellysolve B at 25° C. Six samples of Skellysolve B containing chlorophyll at a concentration of approximately 190 mg/l were saturated with one of the aqueous solvents. Ten ml of the aqueous solvent were placed with ten ml of the corresponding Skellysolve B solution in an eight inch test tube. After the Skellysolve B layer had reached equilibrium, it was analyzed as previously described. Similar samples were prepared with ethyl alcohol. Results are shown in Table 4.

The results shown in Table 4 are also plotted in Fig. 4. Essentially a straight line is obtained indicating that the distribution of chlorophyll in the aqueous solvent is directly proportional to the amount of water present in the solvent.

Table 4. Effect of water in aqueous phase on percent extraction of chlorophyll from the Skellysolve B layer (concentration = 190 mg/l).

Sample	Percent alcohol	Percent of chlorophyll extracted from the Skellysolve B layer
1	98 methyl	59.4
2	95	46.9
3	90	22.1
4	85	7.0
5	80	0.0
6	70	0.0
7	92 ethyl	48.4
8	90	44.6
9	85	30.0
10	80	18.5
11	70	0.5
12	50	0.05

Effect of Concentration on Distribution of Chlorophyll in an Immiscible System

Johnson and Talbot (8) found that in the system, oxalic acid in water and butanol, the distribution coefficients varied from 1.770, using a concentrated solution, to 2.706 with one of lesser concentration. Experiments were devised in order to determine if such a concentration effect was noticeable in a system containing plant pigments. Chlorophyll solutions of Skellysolve B, varying in concentration from 762.5 mg/l to 45.18 mg/l were mixed with equal volumes (ten ml) of 90 percent ethyl alcohol in eight inch test tubes. Solvents were mutually saturated at 25° C., prior to mixing. Skellysolve B solutions

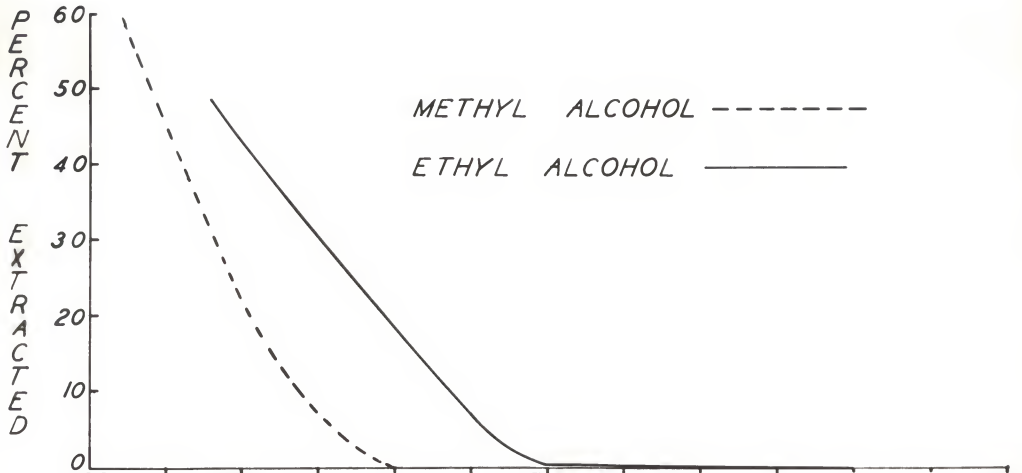


FIGURE 4. PERCENT OF ALCOHOL AGAINST PERCENT OF CHLOROPHYLL EXTRACTED

of crude alfalfa meal extract were also used. The concentration of these solutions varied from 688.0 mg/l to 37.5 mg/l. After reaching equilibrium samples of the Skellysolve B layer were taken and analyzed as before. In the more concentrated mixtures one ml of the Skellysolve B solution was diluted to 100 ml with diethyl ether for subsequent spectrophotometric determination. Table 5 gives the results of this experiment.

Table 5. Relation of concentration of chlorophyll to its distribution coefficient in a mixture of 90 percent ethyl alcohol and Skellysolve B.

Sample No. and solution	Concentration in mg/l of original solution	Percent extracted	K(conc. aq.)/(conc. Skelly)
	Pure chlorophyll		
1	762.5	43.5	0.770
2	450.5	43.9	0.781
3	280.8	46.5	0.880
4	109.9	43.5	0.770
5	45.2	45.5	0.836
	Chlorophyll of crude extract		
1	688.0	33.5	0.503
2	464.5	33.3	0.498
3	299.1	34.0	0.514
4	112.2	34.7	0.529
5	37.5	36.3	0.569

In the above calculations no correction was made for the change in volume of the Skellysolve layer. Therefore, the percent extraction appears higher than that reported earlier in

the paper. Apparently there is no significant or consistent change in the value of K as the concentration changes. Additional experiments confirmed these results.

It may be noted that there is a considerable difference in the percent of chlorophyll extracted from the pure chlorophyll solution and that extracted from the crude extract. This effect is probably due to the presence of other fat soluble substances in the crude extract of alfalfa.

Countercurrent Distribution

Description of Apparatus. The apparatus constructed and used in this laboratory for the separation of carotene from chlorophyll employed a combination of successive contact and countercurrent extraction. A diagram of the essential parts of the apparatus is shown in Plate I.

The two tubes in which contact of the immiscible liquids was made, consisted of 51 mm Pyrex tubing, approximately 200 cm in length. They were stoppered at each end with No. 10, two-hole stoppers. The Skellysolve B was held in a five liter round bottom flask and maintained at a head of 80 cm above the top of the columns. From this source, the Skellysolve B solution was siphoned through eight mm Pyrex tubing to the bottom of column 1. The alcohol for column 1 did not require as high a head as the Skellysolve B, because it has a greater density. It was maintained approximately 50 cm above the top of column 1. This

EXPLANATION OF PLATE I

Distribution Apparatus

A. Column 1.

B. Column 2.

C. Column 1 alcohol outlet tube.

D. Skellysolve B transfer tube.

E. Column 2 alcohol outlet tube.

F. Skellysolve B outlet tube.

G. Container for alcohol from column 1.

H. Suction flask container for alcohol from column 2.

I. Container for extracted Skellysolve B.

J. Skellysolve B inlet tube.

K. Column 1 alcohol inlet tube.

L. Column 2 alcohol inlet tube.

M. Skellysolve B extract source.

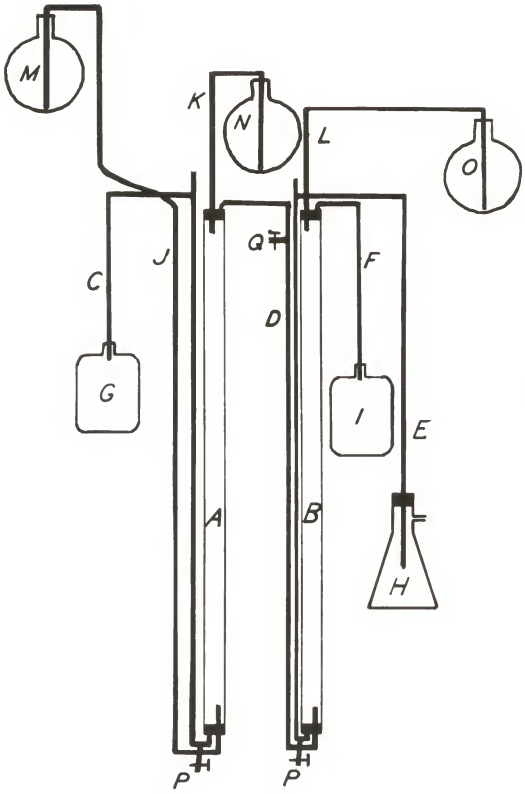
N. Column 1 alcohol source.

O. Column 2 alcohol source

P. Column drains.

Q. Sampling drain for column 1.

PLATE I



solvent was siphoned, using eight mm tubing, to the top of column 1.

The alcohol supply for column 2 was at a height of approximately 40 cm above the top of this column. The alcohol outlets for both columns, the Skellysolve B outlet for column 2 and the tubing transferring Skellysolve B from column 1 to 2 were all constructed of 8 mm Pyrex tubing. All inlet tubes extended five to seven cm beyond the stoppers into the columns. This gave an effective mixing distance of 188 cm in each column. All outlet tubes were even with the rubber stoppers. These five to seven cm spaces at each end of the column allowed the solvents to separate.

The alcohol outlet for column 1 and the Skellysolve B outlet for column 2 led to one gallon colored glass bottles. The alcohol outlet from column 2 led to a four liter suction flask. A T tube with rubber tubing and pinch clamp attached was located at the bottom of each column to permit the columns to be drained. Volumes of the columns were 3415 $\pm$ 10 ml, and required approximately 1700 ml of each solvent to fill one column. A T tube with pinch clamp on the Skellysolve B transfer tube between columns allowed samples to be taken from Column 1 at any time. Rubber tubing was used for all connections. The solvents slowly attack the rubber tubing which made it necessary to replace them occasionally. The entire apparatus was attached by means of clamps to an iron framework built for that purpose.

Operation of Apparatus. In the operation of the apparatus the flow of the Skellysolve B solution and of the alcohol was started in column 1. Pinch clamps regulated the rate of flow of both solvents. Skellysolve B bubbles were dispersed up through the lower alcohol layer and the alcohol settled down through the upper Skellysolve B layer. When the column was filled, alcohol was forced by way of the outlet tubing into a one gallon bottle. The Skellysolve B was forced from the top of column 1 to the bottom of column 2 through the connecting tubing.

As Skellysolve B entered the second column, fresh alcohol was allowed to flow into the top of the column. When the column became full, the separated Skellysolve B and alcohol layers passed by means of outlet tubings into collection bottles. It was found necessary to substitute a suction flask with a slight decrease in pressure for the alcohol container in order to cause the solvents to flow properly in column 2. It was preferable to use this arrangement rather than to extend the head of the solvents or to use a pump. With this arrangement essentially all solvents were caused to flow by gravity and the rates of flow were controlled by pinch clamps and limited by the size of the tubing.

It was impossible to consider this a true countercurrent distribution since the solute was not added at the middle of the column but at one end with one of the solvents. Also, no portion of the column could be designated as a distinct plate

or tube. It was impossible therefore to make the usual calculations such as maximum separation and optimum solvent ratio which are possible for true countercurrent systems (3,6). Since it was not merely multiple contact, different results from those predicted upon that basis should be obtained.

Efficiency of Extraction, System of Pure Chlorophyll and Carotene. Experiments were conducted to determine the efficiency of extraction with this apparatus. Several trials were necessary for the operator to become accustomed to using the apparatus. Controlling the rate of solvent flow was the most difficult task since the several screw type pinch clamps required almost continual adjustment. Equal volumes of solvents were used and therefore the ideal situation was to keep the interface at the midpoint of the columns. Emulsions which formed at the interface varied in their intensity with the concentration of solute used and the rate of solvent flow.

Systems used in the "countercurrent apparatus" were: a Skellysolve B solution of pure chlorophyll and 90 percent ethyl alcohol, a Skellysolve B solution of pure chlorophyll and carotene with 90 percent ethyl alcohol, and a Skellysolve B extract of alfalfa meal with 90 percent ethyl alcohol and with 98 percent methyl alcohol.

When using pure chlorophyll only column 1 was used in order to facilitate a saving of material. Four and one half to five liters of each solvent were usually used. Experiments were conducted at room temperature which varied from 25-27° C.

Analysis was made by withdrawing samples from the Skellysolve B outlet tube at different times. The first two to three liters were not included since it would not be a representative sample. Solvents were mutually saturated before distribution began. Even then there was an increase in volume of the Skellysolve B layer which was measured and found to be 111 percent of the original volume at 25° C. Maximum extraction obtained was 63.0 percent. This figure was obtained by subtracting the amount due to the dilution caused by the increased volume of the Skellysolve B layer.

When using the Skellysolve B solution of chlorophyll and carotene with 90 percent ethyl alcohol, maximum extraction was 60.6 percent of the chlorophyll, while that of the carotene was 7.3 percent.

Efficiency of Extraction, System of Crude Skellysolve B Extract of Alfalfa Meal. Extensive trials were made using the Skellysolve B extract of alfalfa meal in the apparatus with 90 percent ethyl alcohol. In this case both columns were used. Seven liters of Skellysolve B extract were used and of this volume approximately 5.2 liters were transferred to the second column. Seven liters of alcohol were used for the first column and 5.2 liters for the second. Room temperature varied from 25-30° C. Samples were taken from both columns at various times after the first three liters of Skellysolve B had passed through the system.

It was found that in the second column, the change in volume of the Skellysolve B layer was negligible.

The averages of the samples taken from the best three trials using both columns are given in Table 6. The average percent extraction of chlorophyll from the crude extract shown in Table 5 is 34.34. Considering volume change, this would be 27.2 percent. Efficiency of extraction of the distribution apparatus is obtained by dividing the percent of extraction with the apparatus by the percent of extraction by the single contact method.

Table 6. Average extraction from the three best trials through the distribution column.

Trial	Percent of chlorophyll extraction by column 1	Efficiency	Percent of chlorophyll extraction by column 2	Efficiency	Percent of overall chlorophyll extraction	Percent of overall carotene extraction
1	48.9	1.80	47.4	1.74	73.1	8.58
2	45.2	1.66	45.6	1.68	70.2	6.26
3	46.8	1.72	45.5	1.67	71.1	5.55
Av.	47.0	1.72	46.2	1.70	71.5	6.80

By using 98 percent methyl alcohol and crude Skellysolve B extract in single contact experiments, the average percentage extraction was found to be 44.6. Using this system the Skellysolve B layer increased to 103 percent of its original volume. Therefore corrected percent extraction was 43.0 percent.

The results of the best trial using 98 percent methyl alcohol and crude Skelly extract were as follows: For chlorophyll:

60.0 percent extraction by column 1, 59.9 percent extraction by column 2, 83.9 percent overall extraction, and overall efficiency was 1.24. For carotene, the overall extraction was 7.86 percent.

When the apparatus was in operation it was necessary to use illumination behind the columns to follow the movement of the droplets and to locate the interface, especially when concentrated solutions were used.

Emulsions. It was thought that the formation of emulsions may have a detrimental effect on the desired separation. Experiments were made to determine if certain antifoam agents exhibited anti-emulsifying properties. Dow-Corning Antifoam A was found to be slightly soluble in Skellysolve B. The anti-foam agent was added, within the recommended concentration ranges, to alternate test tubes containing equal volumes of Skellysolve B extract and 90 percent ethyl alcohol. The test tubes were stoppered and a test tube that contained the Antifoam A and one that did not contain it were shaken simultaneously. The rate of dispersion of the emulsion was observed and in all cases, samples containing the antifoam agent retained their emulsions slightly longer than those without it. This indicated the ineffectiveness of this antifoam agent as an anti-emulsifier. Nearly identical results were obtained when Monsanto's AE1 antifoam agent was used in a similar experiment.

Effect of Rate of Flow. The efficiency of the extraction was checked for varied rates of flow through the columns. The results of these experiments did not vary greatly. On the

average those trials which took approximately one hour for a seven liter supply of Skellysolve B to pass through the columns gave the best extraction. This would be at a rate of approximately two ml per second. Rates above and below this value tended to give slightly poorer results.

Since smaller droplets should attain equilibrium more rapidly with each succeeding elevation, attempts were made to cause a finer dispersion of bubbles of solvent from both the Skellysolve B and alcohol inlet tubes. Attempts to use glass wool packing at each end of the column and to use narrower inlet tubes for this purpose were both unsuccessful since they offered too much of a hindrance to the siphon fed solvent flow. If the solvents were pumped into the system perhaps these additions would be successful.

DISCUSSION

In determining the effect of temperature variation on the distribution coefficients of carotene and chlorophyll, there were several factors which may have considerable effect upon the results. It was found necessary to use considerable care when saturating the aqueous solvent with the Skellysolve B. Both liquids should have reached the temperature used before mixing and saturation should be made just to the point where the second phase appears. To add an excess would invalidate the results since a three component system of alcohol, water and

Skellysolve B would be established between the water and Skellysolve B. The aqueous layer would thus contain a decreased fraction of alcohol. An increased fraction of water in the alcohol phase was shown to have a large effect on the amount of pigment extracted by that phase. Care should be used to avoid evaporation of solvents as much as possible. It was found advantageous to analyze samples used in these experiments after allowing only the minimum amount of time for equilibrium to be reached in order to decrease the error of evaporation.

Perhaps a difference in the character of chlorophyll prepared by various methods and exposed to varying conditions exerts some influence upon its distribution between immiscible solvents. These sources of possible error make results difficult to reproduce. It was necessary to run the experiment several times in order to produce the reasonably constant values given for the distribution of carotene and chlorophyll in the various systems at 0°, 10°, and 25° C. The differences in value for changing temperature is too small to make the operation of a countercurrent system at temperatures different from room temperature practical.

Chlorophyll, produced by the method of Schertz, was obtained in low yield and was found to contain impurities. These impurities were thought to be due largely to drying the purified product. The most successful preparation of chlorophyll was found to be the procedure described in this paper. When the aged powdered magnesia was used a good quantity of pigment

could be adsorbed and then removed from the column. The magnesia evidently becomes deactivated to some extent upon standing and occasional exposure to air. This makes it a suitable adsorbent for this work.

It was found that there was no appreciable difference in the concentration range of 45-762 mg per liter upon the distribution coefficients. It is generally known that most saturated solutions are so concentrated that the mass law no longer holds and the distribution coefficients change with concentration. Perhaps the concentration ranges tested were not near enough to saturation to observe this effect.

In the operation of the distribution apparatus, there are several difficulties which must be overcome. There is some evaporation of solvents but this can be minimized by keeping the openings to all supply flasks and receiving bottles as small as possible. The amount of vacuum applied to the second column alcohol receiving flask must be accurately controlled to keep the solvents flowing properly in this column. All screw type pinch clamps had to be adjusted often to obtain an equal flow of both solvents and to keep their rate of flow as constant as possible. The average efficiency of extraction of the apparatus, the ratio of its extraction to that obtained by the single contact method, for the different systems with chlorophyll were as follows: pure chlorophyll with Skellysolve B and 90 percent ethyl alcohol, 1.64; chlorophyll and carotene in the same system, 1.56; for crude extract in the same system, 1.71; and for crude

extract in Skellysolve B and 98 percent methyl alcohol, 1.40. The crude extract in Skellysolve B and 90 percent ethyl alcohol gave the most efficient extraction. This is explained because trials with this system were made after a better technique of operating the apparatus was developed.

The data presented in Table 5 show that the percent of extraction of pure chlorophyll is ten percent greater than that of chlorophyll from crude extract. By calculating the volume change of the Skellysolve B for both cases it is seen that the extraction from the crude extract was only 70.6 percent of that obtained from the pure solution. Table 2 shows that the presence of carotene has a small effect upon chlorophyll extraction. This indicates that the other fat soluble substances exert considerable influence on chlorophyll extraction in a liquid-liquid system.

The rate of equilibration of chlorophyll between Skellysolve B and alcohol was shown to be very rapid when the two phases were intimately mixed. Efficiency of the apparatus could undoubtedly be increased if the droplets of solvent which pass through the opposite phase are made smaller. However, in this apparatus it was impossible to obtain smaller droplets than were naturally formed.

The ideal situation would be to have very minute bubbles which equilibrated themselves with each succeeding increase in elevation and at the same time move so fast that there would be as many "plates" or "stages" as possible. Perhaps the efficiency

of the apparatus would be enhanced by adding spiral baffles which would increase the effective length of the column.

This type of extraction gives a rapid separation of the bulk of each pigment and should prove especially successful for the industrial separation of chlorophyll and carotene.

SUMMARY

The distribution coefficients of chlorophyll and carotene in several immiscible solvent systems were determined at three different temperatures. Skellysolve B with 90 percent ethyl alcohol or with 98 percent methyl alcohol show definite possibilities as satisfactory systems for the phasic separation of chlorophyll and carotene. The higher alcohols were found to require such a large amount of water, in order to make them immiscible with Skellysolve B, that they were unsatisfactory as solvents for the plant pigments.

A procedure for preparing pure chlorophyll by chromatography, using aged powdered magnesia as the adsorbent, was developed. Proof of the purity of the product obtained by this procedure was gained by its readsorption on a talc-urea column and subsequent separation of the a and b fractions. The absorption curves for these two fractions agreed closely with those given by Zscheile.

Experiments to obtain the rate of attaining equilibration of chlorophyll in these immiscible systems indicated that this

rate was very rapid. The effect of different amounts of water in the aqueous solvent layer on the extraction of chlorophyll was studied. It was shown that increasing amounts of water in the alcohol drastically reduced the amount of chlorophyll contained in that layer. It was found that change of concentration of solute within the concentration ranges studied had no significant effect upon the distribution coefficients of chlorophyll in Skellysolve B and ethyl or methyl alcohol.

A distribution apparatus, employing a combination of countercurrent and multiple contact extraction principles, was used in order to determine its effectiveness in separating chlorophyll and carotene. It was found that up to 1.71 times as much chlorophyll could be extracted from the Skellysolve B layer by using the apparatus as by the single contact method.

It should be possible to add improvements to the apparatus which would further increase its extraction efficiency. The results obtained were encouraging and indicate the advisability of further work on the project.

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A STUDY OF THE PHASIC SEPARATION OF CHLOROPHYLL AND CAROTENE

by

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Work in this laboratory has centered primarily on carotene research because of its pro-vitamin A properties. However the recent prominence given to chlorophyll products in drugs and deodorizers has caused a great deal of interest concerning procedures for the isolation of chlorophyll and its derivatives. Therefore the separation of chlorophyll and carotene from plant tissue and the separation of one from the other has become increasingly important. The increasing importance of countercurrent distribution has given encouragement to the possibility of commercially separating chlorophyll and carotene by phasic distribution.

The purpose of this investigation was to determine distribution coefficients for chlorophyll and carotene in different systems and to study their separation in a distribution apparatus constructed in this laboratory.

In the subsequent experiments, pigment concentrations were determined by methods recommended by the A.O.A.C. All spectral work was done with a Beckman Model DU Spectrophotometer.

The distribution coefficients of chlorophyll and carotene in several immiscible solvent systems were determined at three different temperatures. No appreciable change of the coefficients with change of temperature was noted. Skellysolve B with 90 percent ethyl alcohol or with 98 percent methyl alcohol show definite possibilities as satisfactory systems for the phasic separation of the plant pigments. The higher alcohols required

such a large amount of water to make them immiscible with Skellysolve B that they were unsatisfactory.

A procedure for preparing pure chlorophyll by chromatography, using aged powdered magnesia as the adsorbent, was developed. Proof of the purity of the product was obtained from the absorption spectra of the separated a and b components.

Experiments run to obtain the rate of equilibration of chlorophyll in the immiscible systems used showed this equilibration to be very fast. It was found that increasing amounts of water in the aqueous solvent drastically reduced the amount of chlorophyll extracted by that solvent from the Skellysolve B layer. Changing the concentration of plant extract had no significant effect on the distribution coefficients of chlorophyll. However, it was shown that the distribution in the alcohol layer of chlorophyll from a crude alfalfa meal extract was only 70.6 percent of that obtained from a solution of pure chlorophyll. This indicates the effect of the other fat soluble substances on the distribution of chlorophyll in an immiscible system.

A distribution apparatus, employing a combination of countercurrent and multiple contact extraction principles, was used to determine its effectiveness in separating chlorophyll and carotene. It was found that up to 1.71 times as much chlorophyll could be extracted from the Skellysolve B layer by using one column of the apparatus as could be obtained by the single contact method.

Several different solvent systems were used in the apparatus. Results indicate that this method could be very successful for the industrial separation of chlorophyll and carotene.