COUNTERCURRENT SEPARATION OF CHLOROPHYLL, CAROTENE AND OTHER COMPONENTS FROM AN EXTRACT OF DEHYDRATED ALFALFA

by

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INTRODUCTION

The separation of chlorophyll and carotene of plant materials has considerable fundamental and practical importance. Interest in this laboratory has centered around stabilization of alfalfa carotenoids and the preparation and storage of carotene concentrates. Some work has been done also, on the counter-current and chromatographic separations of these components from extracts of dehydrated alfalfa. Because of its pro-vitamin role, carotene is an important constituent. Chlorophyll has been, and is now widely used as a component of drugs, deodorizers, and as a coloring agent. Other substances are extracted from alfalfa meal in addition to chlorophyll and carotene. Since the presence of these other components could possibly affect the solubility, distribution and use of the pigments, studies of their occurrence in the chlorophyll and carotene fractions would be desirable.

A number of constituents of alfalfa, other than carotene and chlorophyll, have been identified by other workers. Examples of such constituents are minerals, saponins, hydrolyzable sugars, and paraffinic and alcoholic waxes.

The purpose of the present investigation was not to identify further the constituents of alfalfa, but to note the distribution of chlorophyll, carotene, waxes, and other components in the various fractions as they are obtained from the counter-current apparatus as used in this laboratory. Attempts were made to improve the efficiency of separation of chlorophyll and carotene using a system of 90 percent ethanol in water and Skellysolve-B.

REVIEW OF LITERATURE

There are several methods used to obtain chlorophyll free of other associated plant materials. In the Berkman process (2), the fresh plant material is extracted with water, and the colloidal suspension is separated with a centrifuge. Drying gives a rather pure product. If a petroleum ether extract of plant material is washed repeatedly with water to remove water soluble components, the chlorophyll will precipitate and can be filtered on a talc mat as reported by Scherts (24). Valley Vitamins Inc., of McAllen, Texas, employed a chromatographic method to separate chlorophyll, carotene and xanthophyll from a hexane extract of dehydrated alfalfa. Activated charcoal was used as an absorbent in this process reported by Shearon and Gee (26).

Another method of separating carotene and chlorophyll is based on distribution between immiscible solvents. Willstätter et al., (28) used this method to separate chlorophyll a and b, using alcohol and hexane as the solvent system. Equipment similar to the Craig apparatus (8) can be used for counter-current extractions of small amounts of material. The Craig apparatus uses as many as 59 separate stages and makes possible a rather high degree of purification. Lancaster, et al., (15) were able to get some separation of chlorophylls a and b and good separation of carotene using a Craig apparatus with 90 percent ethanol and hexane as solvents.

The method of Johnson and Talbot (12) specifies that a certain quantity of material be introduced into a series of mixers and separators, with two solvents moving in opposite directions. Kies and Davis (13) used what they termed a "cascade distribution process" in which

the solute phase was held stationary and the mobile phase passed through it.

Of the solvent systems studied by Green (9) of this laboratory, the systems of 98 percent methanol and Skellysolve-B and 90 percent ethanol and Skellysolve-B were the most efficient. Green determined the effect of temperature and the effect of varying concentrations of chlorophyll on these two systems. He also studied the rate of equilibration of chlorophyll and the effect of diluting the alcoholic phase with water.

In separations of chlorophyll and carotene from crude plant extracts, other plant constituents distribute themselves in the two solvent phases according to their solubilities. For example, alcoholic and paraffinic waxes are present in varying amounts depending on the plant material used and the extracting solvent. Much work has been done on separation and characterization of wax components.

Chibnall, et al. (6), devised a method of separating primary and secondary alcohols from the unsaponifiable portion of plant waxes. This method involved esterification with phthalic anhydride. When the reaction mixture was treated with sodium carbonate, the primary alcohol phthalates precipitated. Upon evaporation of the ether solution, dissolving the residue in hot ethyl alcohol and cooling, ketones and paraffins precipitated while the secondary alcohol phthalates remained in solution. Ketones were separated from paraffins due to the fact that their ketoxines are more soluble than paraffins when precipitated from petroleum ether with acetone.

Other attempts have been made to separate long chain alcohols by

distillation of their acetates under reduced pressure. Some workers were able to distill the free alcohols. Schuette and Baldinius (25) separated the paraffins C_{31}, C_{29}, and C_{33}, from candelilla wax by adsorption on an alumina column.

Piper, et al. (22), reported that cocksfoot grass contained n-hexacosanol ($C_{26}H_{53}OH$) and some n-tetracosanol ($C_{24}H_{49}OH$). A paraffin (m.p. 66°C.) of rye grass was thought to contain n-triacontane. Pollard, et al. (23) reported obtaining n-octacosanol from wheat. A paraffin melting at 66°C. was also obtained. Koonce and Brown (14) obtained octacosanol (C_{28}), triacontanol (C_{30}), and dotriacontanol (C_{32}) from carnauba wax. Using cabbage leaves as source material, Channon and Chibnall (4) isolated n-nonacosane ($C_{29}H_{60}$) and di-n-tetradecyl ketone $(C_{14}H_{29})_2CO$.

Chibnall and co-workers (5) found that alfalfa wax was composed of n-triacontanol ($C_{30}H_{61}OH$) and a mixture of long chain paraffins, although Jacobsen (10,11) had stated previously the discovery of two ketones which he named myristone $(C_{13}H_{27})_2CO$ and alfalfone $(C_{21}H_{42}O)$.

EXPERIMENTAL

Chlorophyll and Carotene Separation

Method of Extraction. A Soxhlet type extraction apparatus, similar to the one used previously by Green (9), was employed for the extraction of the alfalfa meal. One kilogram of meal was extracted with five to six liters of 90 percent ethanol. The meal was placed in five small sacks containing 200 grams each. The sacks were tied and placed in a wide-mouthed extraction jar which was mounted beneath a reflux condenser

and connected by a sidearm siphon to a five liter, round-bottomed, solvent supply flask. The alcohol was allowed to heat on the steam cone for five to six hours or until it siphoned over 12-15 times. This effected almost complete extraction of chlorophyll and carotene from the alfalfa meal, as indicated by the absence of color in the siphoning solution.

Distribution Apparatus. The distribution apparatus, except for a few modifications, was the same as that used by Green. Thirty-three mm. pyrex tubing was used for columns. Both columns were 185 cm. long. Each column had to be jointed in order to get the desired length. This was accomplished by wrapping the joint tightly with friction tape and then covering thickly with plaster of paris. This method successfully prevented leakage at the joint. Overhead five liter supply flasks fed the solvents into the column by siphon flow.

Differences between this apparatus and the one used by Green were:

(1) vent for column I

(2) sidearm delivery tube for column I

(3) smaller diameter distribution columns

(4) higher heads for stock liquids

(5) capillary inlets

A sidearm was placed on column I about 10 cm. from the top for the purpose of conducting Skellysolve-B I solution from column I to column II. Two-hole stoppers were used at both ends of both columns. One of the openings in the stopper at the top of column I was used as a vent, which facilitated draining the column and controlled the increase in volume of the liquid due to inflowing solvents. The Skellysolve-B,

the lighter of the two solvents, was maintained at a head of 110 cm. above the surface of the liquid in column I, and was led into the first column at its base through a capillary opening. Nine mm. pyrex tubing was drawn out to make the capillaries. The 90 percent ethanolic extract was maintained at a head of 70 cm. above the liquid in column I, and also entered the column through a capillary opening. Inlets extended 10-15 cm. into the columns to make possible a satisfactory separation before leaving the column. The effective mixing distances in the two columns were 150 cm. for column I and 165 cm. for column II. Column I held 1510 mls. when full and column II contained 1600 mls. when filled. The alcohol I solution was drawn off at the base of column and the Skellysolve-B I solution flowed from the sidearm near the top of column I into column II at its base. In column II, the Skellysolve-B I extract flowed against a fresh supply of 90 percent ethanol which entered at the top of the column, and was maintained at a head of 58 cm. above the liquid in that column.

Operation of Apparatus. In this work, higher heads of Skellysolve-B and alcohol solutions were used than used by Green. This was due partly to the fact that this apparatus used capillaries which required higher pressures for desirable flow. The Skellysolve-B I fraction, upon leaving the top of column I was conducted to the base of column II, where it flowed against a fresh supply of 90 percent ethanol entering at the top. The rate at which alcohol I left column I was regulated by means of a screw clamp. Rates of flow of Skellysolve-B into column I and of alcohol II out of column II were regulated also by means of screw clamps. The original alcoholic extract was allowed to flow into column I as

EXPLANATION OF PLATE I

Distribution Apparatus

A. Skellysolve-B supply flask for column I.

B. 90 percent ethanol extract supply flask for column I.

C. 90 percent ethanol supply flask for column II.

D. Receiving carboy for alcohol I fraction.

E. Receiving flask for alcohol II fraction.

F. Receiving bottle for Skellysolve-B II fraction.

G. Skellysolve-B fraction outlet from column I.

H. Column I.

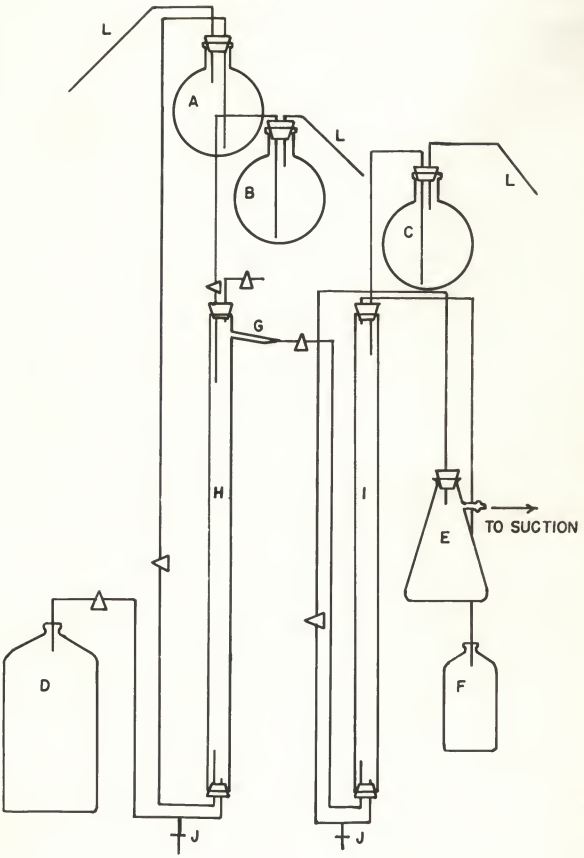
I. Column II.

J. Drains for columns I and II.

K. Air tubes to start siphon flow from supply flasks.

△ Screw type clamps.

PART I



rapidly as gravity and the size of the capillary opening would allow. The alcohol I fraction was caught in a 19 liter glass carboy. Skelly-solve-B I concentrations could be sampled when desired by means of a small drain close to the exit of the sidearm from column I. The Skelly-solve-B II solution was caught in four-liter bottles, and the alcohol solution from the second column was captured in a four-liter filter flask. The sidearm of the flask was connected to an aspirator, the suction from which facilitated the movement of solvents through column II. The meniscus marking the boundary line between the two solvents was detected by moving a 150 watt bulb up and down behind the column while observing from the front. It was necessary to check the meniscus location frequently and to make frequent adjustments of the screw clamps, particularly the one allowing alcohol to escape from column I and the one allowing Skellysolve-B to enter column I.

The use of capillary inlets facilitated greater dispersion of droplets throughout the column, and the rapidly flowing solutions gave considerable turbulence. It was found easier to regulate the solvent flow through smaller openings than through nine mm. tubing.

The purpose was to obtain as complete separation as possible with the distribution apparatus. Carotene is considerably more soluble in Skellysolve-B than in 90 percent ethanol, whereas chlorophyll is more soluble in the alcohol phase. Very good separation of carotene and fairly good separation of chlorophyll was effected in one pass of the solvents through column I. To improve the separation of chlorophyll, a fresh supply of 90 percent ethanol was used in the second column to recover as much of the chlorophyll as possible without sacrificing any

more carotene than necessary.

Optimum solvent ratios and maximum possible separations have been developed for true counter-current systems in which the solute is introduced at the junction of the two phases. Since the solute is present as a soluble component of one phase, these calculations are not applicable.

Solvents are mutually saturated with one another prior to distribution, and this minimized changes in volume before and after contact.

Method of Analysis. Aliquots of the various fractions were analysed for chlorophyll and carotene. Samples of chlorophyll were prepared as follows: Two mls. of the alcohol I or alcohol II fraction was withdrawn and pipetted into a 500 ml. separatory funnel. Fifty mls. of diethyl ether was added along with an ample supply of wash water. The funnel was shaken lightly until the ether layer lost its cloudy appearance. After the layers were separated sharply by a meniscus, the lower water solution was drained off and discarded. The remaining ether solution was passed through a gravity filter funnel containing some anhydrous sodium sulfate to remove traces of moisture. From the funnel, the ether solution was drained into a 100 ml. volumetric flask. The last traces of chlorophyll were washed from the filter paper into the flask and then diluted to the mark with diethyl ether.

Preparation of Skellysolve-B I and Skellysolve-B II sample solutions was carried out by introducing two ml. aliquots of the Skellysolve-B fraction directly into a 100 ml. volumetric flask and diluting to the mark with diethyl ether.

Spectrophotometric analysis of all chlorophyll samples was carried out using a Beckman Model D.U. Spectrophotometer with 1 cm. Corex cells. Absorbancy was measured at two wavelengths and substituted into the equation:

Total chl. (mg/ml) = $7.12 \log \frac{I^o}{I}(6600\text{Å}) + 16.8 \log \frac{I^o}{I}(6425\text{Å})$, as recommended by the A. O. A. C. (1). In this way the chlorophyll concentration can be determined readily. The concentration of the original chlorophyll extract was calculated in the same way using a 1 ml. aliquot. The presence of carotene does not interfere with the chlorophyll analysis, whereas chlorophyll would interfere with an accurate carotene determination. Therefore, in order to analyze for carotene, it is necessary to have a means of separating chlorophyll and carotene.

A carotene sample was prepared as follows: A 10 ml. aliquot of original extract of any of the Skellysolve-B or alcohol fractions was introduced into a separatory funnel, and shaken with 50 mls. of wash water. The water or water-alcohol layer was discarded and the remaining Skellysolve-B solution drawn off into a 125 ml. erlenmeyer flask. This Skellysolve-B solution of chlorophyll and carotene was then poured onto a chromatographic column consisting of one part by volume of Westvaco powdered magnesia no. 2641, and two parts by volume of Hyflo Super-Cel. The columns were packed to a depth of about six inches, and covered by a one-fourth inch layer of anhydrous sodium sulfate. Under suction, the pigments were drawn through and adsorbed on the magnesia. A solution of five percent acetone in Skellysolve-B was used as an eluting agent to remove the carotene. The carotene eluate was drawn off into

a 250 ml. volumetric flask and diluted to the mark with Skellysolve-B. This solution was then analyzed spectrophotometrically with the Beckman Spectrophotometer at 4360Å. The absorbancy obtained was substituted into the equation:

Concentration carotene (mg/ml) = $\log \frac{I^{o}}{I}$ (4360Å) /196

Effect of Varying the Relative Proportion of Solvents. Since there was some question about a 1:1 ratio of solvents for the most satisfactory extraction, an experiment was devised to determine the ratio of solvents which would give the best results. Since, in a 1:1 ratio of solvents, the Skellysolve-B removes practically all of the carotene in a single contact, the ratios involving less Skellysolve-B than 90 percent alcohol were given particular attention. The ratios of alcohol to Skellysolve-B tried were: 1:1, 2:1, 4:1 and 8:1. Ratios of more than 8:1 were impractical since it was difficult to regulate a constant flow of Skellysolve-B which was approximately 1/8 of the alcohol flow.

A quantity of original 90 percent ethanolic extract of alfalfa meal was saturated with Skellysolve-B and then analyzed spectrophotometrically for chlorophyll and carotene. In this way the concentrations in mg/ml of both pigments were determined. Two hundred ml. samples of this saturated extract were then shaken with 200, 100, 50, and 25 mls. of Skellysolve-B in separatory funnels. The solutions were separated, their volumes determined and their chlorophyll and carotene content determined spectrophotometrically. From this information it was possible to calculate the percentage of chlorophyll and carotene in each phase.

The distribution ratios were calculated for each solvent ratio:

Table 1. Relative distribution of pigment solutes in the Skellysolve-B and alcohol fractions using different proportions of solvent.

Table 1.

Ratio of alcohol/ Skellysolve-B	% in alcohol	% in Skellysolve-B	distribution ratio
Chlorophyll			
1:1	48.7%	52.1%	.903
2:1	58.3%	46.0%	1.290
4:1	69.8%	33.2%	2.026
8:1	78.1%	22.4%	3.515
Carotene			
1:1	0-1%	98.8%	.0113
2:1	2.7%	99.6%	.0113
4:1	4.5%	100.0%	.0173
8:1	8.5%	96.5%	.0409

From the above data, it was evident that the most desirable proportions of solvents were 4:1 and 8:1. If the flow of solvents could be as easily manipulated using an 8:1 ratio as it could in using a 4:1 ratio, 8:1 would be the more desirable ratio. From a practical standpoint however, 4:1 was chosen for subsequent separations.

Although techniques of preparing samples were the same throughout, occasionally the total pigment accounted for would amount to 103-104 percent of the original. On other occasions, the total would amount to 94-96 percent. Since this was noted both with chlorophyll and carotene

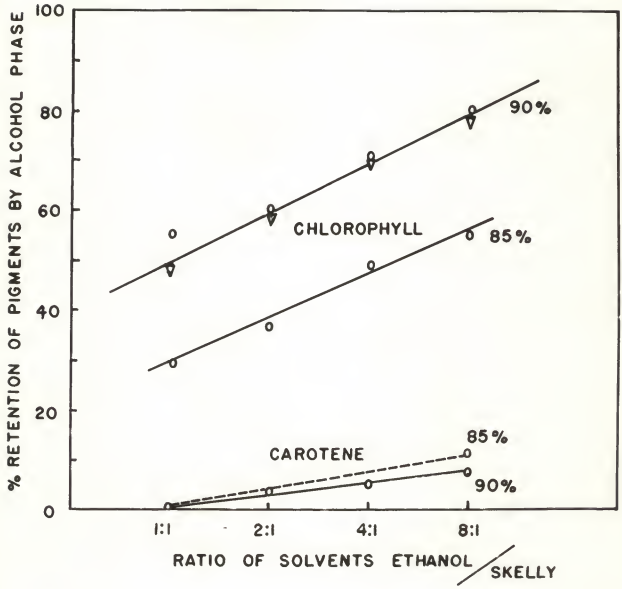


Figure 1. - Retention of pigments by the alcohol phase with different proportions of solvent.

in a large number of individual cases, it was concluded that the accuracy of the method was approximately $\pm$ four percent of the total value.

Method of Obtaining Representative Samples. Since the concentration of pigments would change with time, considerable difficulty was experienced in obtaining a representative sample during the operation of the distribution apparatus. Originally, Skellysolve-B I samples were taken when it seemed apparent that the rates of flow were well adjusted so that the meniscus was not falling or rising. This usually did not occur until the run was at least one-half completed. Since the concentration increased with time up to a maximum, and then dropped off, the sample taken as representative was more concentrated than the average concentration of the total fraction. Calculations based on these data obviously gave yields in excess of the original amounts available.

An experiment was conducted to see how the concentration of chlorophyll and carotene varied with time during the course of a separation. In this experiment, samples of the Skellysolve-B I fraction were taken every 10 minutes during the course of the run. Thus a total of about 10 samples were taken in a period of about one hour and forty minutes. These samples were analyzed for chlorophyll and carotene in the usual manner and the concentrations plotted against time. In all cases, the concentrations rose to a maximum, and then diminished.

Occasionally, difficulty would be experienced in maintaining the meniscus at a constant level, and in such cases, the meniscus invariably moved upward, decreasing the relative amount of alcohol. When this occurred, it was sometimes necessary to turn off the alcohol and Skellysolve-B inlets and drain the excess alcohol off at the base of the column

into the alcohol I carboy. The meniscus was then lowered to its normal position by allowing more Skellysolve-B to enter. In these instances, the concentrations of carotene and chlorophyll in the Skellysolve-B I samples were necessarily lowered due to the dilution involved in adding Skellysolve-B. This accounted for the double peak shown on the accompanying graph. An uninterrupted run showed only one concentration peak. The concentrations of the samples taken at different time intervals were averaged and this average concentration was taken as representative of the entire Skellysolve-B I fraction. This method was the only one which could give reasonably accurate results as to the concentration of the Skellysolve-B I solution in a continuous two-column separation. If a one-column run was being carried out, the Skellysolve-B I could be collected in bottles, and the contents of each bottle analyzed separately. It was necessary to know the concentration of the Skellysolve-B I fraction in order to know the efficiency of extraction in the second column, unless the Skellysolve-B I was assumed to contain all the chlorophyll not contained in the alcohol I fraction.

Emulsions. The sampling technique mentioned above would be meaningless if emulsified solutions were allowed to leave the column at the outlets. Rates of flow and concentrations were adjusted to keep emulsions at a minimum. It was found that 85 percent ethanol produced considerably more emulsion than 90 percent ethanol. When using 85 percent ethanol, the entire column was filled with emulsion. The concentration of solutes and the rates of flow also made a difference. The concentration of 0.23 mg/ml of chlorophyll, or less, was found to be suitable with the alcohol inlet flowing at a maximum rate. Higher concentrations than this could

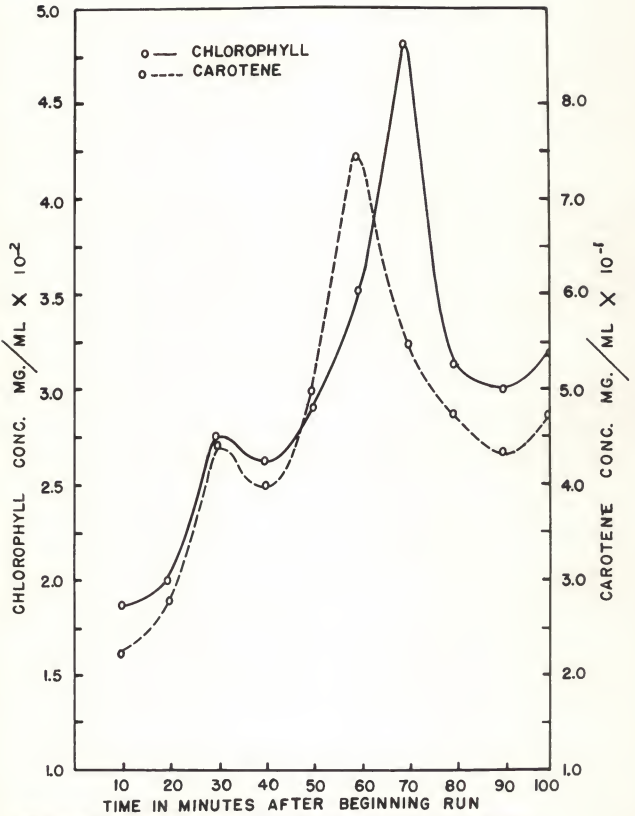


Figure 3. Variation of the pigment concentration of the Skelly-solve-B fraction with time during an interrupted run.

be used if the rate of flow of alcohol was decreased from the maximum. In all cases there was emulsion, but under these specified conditions it was not prohibitive.

Commercial de-emulsifiers were tried in this system to see if they had a significant effect in breaking emulsion. The de-emulsifiers used were weighed out (100 ppm) and dissolved in one phase, then manually shaken with the other phase in eight inch test tubes. The time required for the emulsion to break was noted in each case. Anti-foamers used were Dow-Corning's Antifoam AF Emulsion, 200 Fluid, and Antifoam A. Monsanto de-foamers used were M-5262 and Sterox 110. None of these products helped significantly in the system employed.

Destruction of Pigments. There is a certain degree of photochemical destruction of plant pigments. Pepkowitz (19) studied the light destruction of carotene in relation to time and found that it was considerable under his experimental conditions. In this study, the percentage of chlorophyll and carotene which could not be accounted for, was assumed to have been destroyed. With this stipulation, the destruction of chlorophyll apparently did not exceed one percent. The destruction of carotene in column I did not exceed six percent and destruction of carotene in columns I and II did not exceed eight percent over a time period of one to two hours. However, these figures lack significance when one considers the error limits of $\pm$ four percent.

Efficiency of Extraction. In a single column "pass", the alcoholic solution retained an average of 64.2 percent of the total chlorophyll. An average of 35.6 percent was removed by the counter-flowing Skelly-solve-B. This accounted for approximately all of the chlorophyll.

The maximum retention of chlorophyll by the alcohol I fraction in a single column separation was 68 percent. The highest single value obtained for the chlorophyll content of the Skellysolve-B I fraction was 39 percent. Values of total chlorophyll accounted for, varied from 94-104 percent, with an average of all runs of 99.3 percent. Due to the existence of several variables in the system, this accuracy was considered good.

The carotene extraction in column I by the Skellysolve-B solution was generally good, the average of all runs being 90.1 percent. A high of 98 percent and a low of 80 percent were noted as extreme maximum and minimum values. A maximum of 9.8 percent of carotene was retained by the original alcohol I extract.

The purpose of the fresh 90 percent alcohol supply in the second column was to recover as much of the chlorophyll in the Skellysolve-B as possible. The average of the alcohol extractions of chlorophyll in the second column was 75 percent. The highest alcohol extraction noted for a single run was 81.5 percent. The average percentage of the chlorophyll retained by the Skellysolve-B II solution was 27 percent.

The carotene content of the Skellysolve-B solution was reduced in passing through column II. An average of 89 percent of the available carotene of the Skellysolve-B I fraction was retained in the Skellysolve-B II fraction. This means that 80 percent of the total carotene found its way eventually to the Skellysolve-B II fraction. About six percent was left in the alcohol I fraction and four to fifteen percent was left in the alcohol II fraction.

The ratio of solvents employed in column II determined how much chlorophyll could be retained by the alcohol phase and also how much carotene could be lost by the Skellysolve-B fraction. Actually, 72-73 percent of the chlorophyll in the Skellysolve-B I phase was taken when the ratio of solvents was approximately 2:1. When a ratio of solvents of 4:1 was maintained, the chlorophyll retained amounted to about 81-82 percent. On the other hand, with a 2:1 ratio in the second column, the alcohol took an average of four to five percent carotene, while the alcohol in a 4:1 ratio took about 15 percent of the carotene.

The following table summarizes total results of single and double column extractions:

Table 2. Effect of varying the solvent ratio in the second column upon the distribution of pigments.

Column and ratio used	% chlorophyll in alcohol	% chlorophyll in Skelly-solve-B	% carotene in alcohol	% carotene in Skelly-solve-B
Column I (4:1 ratio)	64.2%	35.6%	6.0%	90.1%
Column II (2:1 ratio)	(.725 35.6)= 25.8%	(.305 35.6)= 10.8%	3.5%	89.0%
TOTAL	90.0%	10.8%	9.11%	80.1%
Column I (4:1 ratio)	64.2%	35.6%	6.0%	90.1%
Column II (4:1 ratio)	(.815 35.6)= 29.0%	(.193 35.6)= 6.9%	15.0%	86.0%
TOTAL	93.2%	6.9%	21.0%	77.5%

The best retention of chlorophyll with both columns was 93.9 percent and the best carotene extraction by Skellysolve-B with both columns was 83.4 percent.

Identification of Waxes, Alcohols and Other Components in the Fractions of the Phasic System

Method Used in Isolating Waxes and Alcohols. Five one-kilogram batches of alfalfa were extracted on a Soxhlet extractor for 12-15 cycles or until the siphoning alcohol was almost colorless. This total volume of solution was analyzed for chlorophyll and carotene. The solution was then saturated with Skellysolve-B and diluted to a desirable concentration of 0.22-0.23 mg/ml of chlorophyll with Skellysolve-B-saturated 90 percent ethanol. The concentration of the final solution could be calculated since the total milligrams of chlorophyll present and the volume of the diluting solvents were known. The total volume was determined by the concentration of pigment extracted with the original ethanolic solution and was usually between 25-35 liters. This procedure was repeated four times on a total of 20 kilograms of alfalfa meal. Two batches of extract were introduced into column I of the counter-current apparatus against a volume of Skellysolve-B one-fourth that of the ethanolic extract. This gave the same solvent ratio as used in previous work with chlorophyll and carotene. The alcohol solution was caught in a 19 liter graduated glass carboy and the Skellysolve-B I fraction was caught in one gallon bottles. These solutions were analyzed for chlorophyll and carotene.

The Skellysolve-B I fraction was then given the following treatment in an effort to isolate waxy constituents.

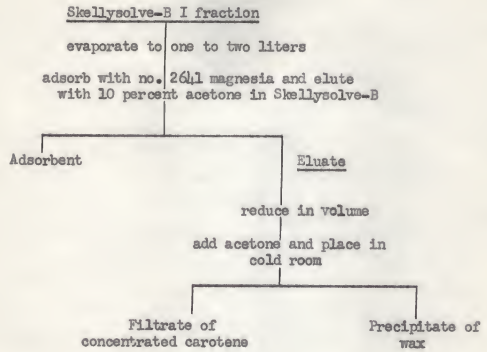
(1) The initial volume of five to six liters of Skellysolve-B was evaporated to one to two liters, and enough Westvaco activated magnesia no. 2641 was stirred into the beaker to cause an orange carotene coloration to rise above the adsorbent as it settled.

(2) The adsorbent and solution were then placed in a large Buchner funnel attached to a four-liter suction flask, and the adsorbent eluted with ten percent acetone in Skellysolve-B until the eluate became light yellow in color. This required approximately 15 liters of eluting solution.

(3) The eluate was reduced in volume on the steam plate to a heavy dark-colored oil to which was added between two and three volumes of acetone. The acetone solution was then placed in a sharp freeze room (-23°C) to precipitate the waxes.

This was a modification of the method used by Blair et al.(3). Blair did additional work on the characterization of these waxes and isolated also an ester from the wax mixture. However, the purpose of this part of the present investigation was merely to determine as completely as possible the amounts of paraffins, and alcohols in the fractions as they were obtained from the counter-current apparatus.

The method used in flow diagram form was:



The alcohol I fractions were given the following treatment:

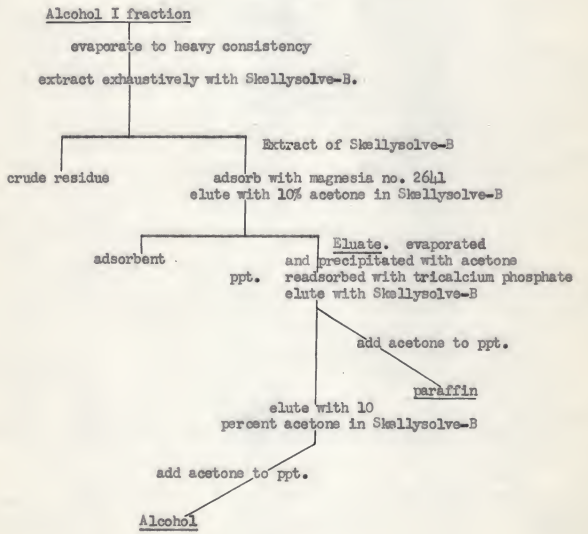
(1) The alcohol I fraction was evaporated to a thick tarry residue. This residue was then extracted repeatedly with hot Skellysolve-B. The Skellysolve-B extract was adsorbed with Westvaco magnesia no. 26&1, after which the mixture was eluted with 10 percent acetone in Skellysolve-B until the eluate came over a faint yellow. This elute was reduced in volume on the steam plate to an oil and precipitated by addition of acetone and cooling at -23°C.

(2) The precipitate was filtered, air-dried and weighed.

(3) The dried material was again placed in solution by heating in Skellysolve-B. Tricalcium phosphate was added until no coloration remained in the supernatant liquid. This adsor-

bent solvent mixture was then placed on a large Buchner funnel, and eluted with about eight liters of Skellysolve-B, followed by another eight liters of 10 percent acetone in Skellysolve-B. Both eluates were reduced in volume on the steam plate and precipitated in the usual manner from hot acetone. The Skellysolve-B eluate yielded paraffin material and the 10 percent acetone in Skellysolve-B eluate yielded the alcohol portion.

The method used in flow diagram form was:



Comparison of Results with Those of Previous Workers. Blair, et al. (3) extracted about 30 kilograms of alfalfa meal with Skellysolve-B and adsorbed the extract with a mixture consisting of a 1:1 volume ratio of tricalcium phosphate and supercel, which was packed into a radial chromatographic device. A carotene fraction was eluted with Skellysolve-B, and a chlorophyll fraction eluted using five percent isopropyl alcohol.

Blair then subjected his carotene waxes to fractional crystallization and found a paraffin mixture melting from 60-65°C. A portion of the carotene wax was insoluble in hot Skellysolve-B and hot acetone and melted at 73-75°C. This was found to be an ester.

The waxes from the chlorophyll fractions were separated into two components by introducing them onto a tricalcium phosphate column and eluting a paraffin fraction with Skellysolve-B and an alcohol fraction with 10 percent acetone in Skellysolve-B. The paraffin component was found to melt over the range of 65-69°C, while the alcohol component melted at 82-83°C. The alcohol fraction was then resolved further on a magnesia column and the alcohol, n-triacontanol, identified along with a mixture of alcohols consisting of 80 percent C_{30} and 20 percent C_{28}. The alcohol, n-triacontanol, was positively identified by X-ray diffraction studies.

The amounts of waxes obtained in the present investigation were less than those obtained by Blair. The wax from the Skellysolve-B extract had a melting point range of 63-69°C. Recrystallized, carotene-free samples of wax from the Skellysolve-B I and Skellysolve-B II fractions showed softening at about 67-68°C., and melted at 78-80°C. The

crude wax dissolved completely in hot Skellysolve-B indicating that no ester was present, or present in imperceptible amounts. Infrared analysis of this wax revealed no absorption peak corresponding to the ester grouping. The fact that the recrystallized wax melted over a 10-12° range, indicated that alcohols were undoubtedly present with the paraffins. Two grams of the wax was introduced onto a tricalcium phosphate column and eluted first with Skellysolve-B, then with 10 percent acetone in Skellysolve-B. A total of one and one-tenth grams of paraffin (m. p. 68-74°C.) was obtained from the Skellysolve-B eluate and 0.44 grams of alcohol (m. p. 82-86°C.) was obtained from the 10 percent acetone in Skellysolve-B eluate. Thus it was concluded that approximately 29 percent of the Skellysolve-B wax was composed of a mixture of alcohols.

Of the Skellysolve-B samples analyzed for crude wax, the greatest yield obtained from five kilograms of alfalfa meal was 4.15 grams. Approximately 30 percent of this weight was impurity. The weight of wax in all fractions was only about 44 percent of that obtained by Blair who used a different extracting solvent.

After evaporation of the alcohol fraction and extraction with Skellysolve-B, the extract was adsorbed by stirring the extract with magnesia, placed on a column, and eluted with 10 percent acetone. The acetone-skelly eluate was then evaporated to dryness and precipitated from hot acetone. The precipitate of alcohol was air-dried and weighed before being readsorbed and eluted for paraffin and alcoholic components. The greatest quantity of crude precipitate obtained by this method amounted to 1.30 grams with approximately 45 percent of that amount represent-

ing impurity. In all cases, the weights of waxes reported are not quantitatively exact, since they indicate only wax which could be removed from the filter paper following filtration. The melting point range of the crude alcohol I wax was 75-141°C. Upon adsorption of the Skellysolve-B solution of the crude precipitate on tricalcium phosphate, and elution with Skellysolve-B, a paraffin mixture was obtained which melted at 65-71°C. The 10 percent acetone in Skellysolve-B eluate yielded an alcohol mixture which melted over the range of 85-149°C. Fractional crystallization of the crude alcohol I wax yielded an impure solid melting at 140-151°C. This higher melting material represented only a very small part of the total wax in the fraction. The highest melting component of the chlorophyll fraction reported by Blair, et al. (3) was n-triacontanol, melting at 86.3-86.5°C. The paraffin fraction resulting from tricalcium phosphate adsorption represented 10-15 percent of the weight of the crude alcohol I wax, while the alcohol fraction represented 40-50 percent. Alcohol II fractions yielded only very small amounts of wax varying from 0.12-0.16 grams when a 2:1 ratio of solvents was used in column II, to 0.50 grams when a 4:1 ratio was used.

Composition of a Water Soluble Crude Residue of Alcoholic Alfalfa Extract. When the alcohol I fractions were reduced to tarry residue, and extracted on the steam plate with Skellysolve-B, an insoluble material which was semi-solid at 100°C. and hard at room temperatures, remained. This crude residue was cooled, and ground with a mortar and pestle to form a powder. The powder was placed in a Soxhlet extractor and the final traces of Skellysolve-B soluble materials removed by refluxing

four to five hours. The material was ground again, sieved to remove any bits of paper from the Soxhlet sleeve, and dried overnight in a vacuum oven at 60°C. The alcohol fractions from two successive single column distributions yielded 228 and 220 grams of this material.

Alcohol II fractions yielded very little tarry residue as compared to the alcohol I fractions, and the residues were almost completely dissolved in hot Skellysolve-B.

Two one-gram samples of crude residue from the alcohol I fraction of the first column distribution (228 grams) were weighed out into crucibles and subjected to 600°C. temperatures in a muffle furnace for 24-36 hours, or until their weights remained constant. Crude residue I contained 6.15 and 6.41 percent mineral as shown by two samples. These two values represent a 6.28 percent average. Likewise, two one-gram samples were taken from crude residue II (220 grams) and treated in a similar manner. The yields were 6.19 and 6.29 percent and represent an average of 6.24 percent. Other crude residues from other runs gave results closely similar to those listed above, and indicate that the mineral content of the residues was quite constant when subjected to identical treatment.

The crude residues studied were found to be hygroscopic in nature. This property was due possibly to saponin content. Peterson (20,21) reported the presence of a factor in alfalfa meal which inhibited growth in chicks. He suggested that this factor could be saponin. This growth inhibition, however, was nullified by administering cholesterol and fat. Lindahl, et al. (16) reported the actual isolation of saponins by the following procedure: The alfalfa meal was extracted with boiling water,

then concentrated under vacuum to 50 percent solids. Sufficient 95 percent alcohol was then added to give a final concentration of 80 percent alcohol. The purpose of the alcohol was to precipitate proteins, salts, etc. The alcoholic solution was then decanted and evaporated again to 50 percent solids in vacuo. The residue was then boiled with powdered cholesterol, the complexed saponin filtered off, washed with water, dried and dissolved in pyridine. A precipitate of saponin was obtained upon addition of anhydrous diethyl ether. These workers reported 0.5 percent yield from the original weight of alfalfa.

Since in this investigation, the extracting solvent was 90 percent ethanol, one could expect the crude residue to contain saponin. Five gram samples of the crude residue were weighed out and about 50 mls. of water added to each sample. This mixture was evaporated on the steam bath until the liquid was largely driven off. Sufficient 95 percent ethanol was added to give an approximate concentration of 80 percent alcohol. The solution was filtered, and the filtrate reduced in volume to about 50 percent solids, after which cholesterol was added and boiled for several minutes. A heavy brown paste resulted which was washed with water and subjected to centrifugation. The solid material was transferred to an evaporating dish and dried under vacuum at 60°C. The dried material was placed in pyridine solution and again filtered, since there was considerable material which did not go into solution. The clear pyridine solution was then precipitated with anhydrous ether, the precipitate was filtered off and transferred rapidly to the vacuum oven, dried and weighed. Of the samples run on crude residue I, an average of 24.45 percent saponin was found. Of those runs on crude residue II, an average yield of 24.85 percent saponin was obtained. These fig-

ures show that 1.07 percent of the original weight of alfalfa was saponin. Due to the fact that the saponin was strongly hygroscopic, it was not possible to ascertain accurately the purity of the product. Routine analysis of the crude residue showed 1.51 percent nitrogen.

The crude residue gave no test with Benedict's or Fehling's solution. This would indicate the absence of reducing sugars. Attempts were made to hydrolyze the crude residue and then to test for reducing power. One gram samples of the dried residue were placed in 50 mls. of water and enough saturated lead acetate was added to produce a flocculent precipitate. The mixture was agitated and allowed to stand for 15 minutes. The supernatant was tested with an additional drop of lead acetate to determine whether or not precipitation of proteins, tannins and etc. was complete. When no further precipitation occurred, the precipitate was filtered off, and the lead in the filtrate precipitated by addition of potassium oxalate. The precipitate of lead oxalate was filtered off and again the filtrate tested for lead ions by adding a small crystal of potassium oxalate. The solution was then neutralized with hydrochloric acid, and an additional five mls. of concentrated hydrochloric acid added. The residue was then refluxed for one hour over low heat to effect hydrolysis. The hydrolyzed solution was neutralized with sodium carbonate, filtered into a 100 ml. volumetric flask and diluted to the mark. A 50 ml. aliquot was placed in a 400 ml. beaker with 25 mls. of cupric sulfate and 25 mls. of alkaline tartrate solution. The beaker was covered with a watchglass and placed in a constant temperature bath maintained at 80°C. for 30 minutes. The cuprous oxide was removed by passing the solution through a Gooch crucible lined with

an asbestos mat. The cuprous oxide was washed with water and the asbestos mat and cuprous oxide were returned to the reduction beaker. The inside of the crucible was rinsed with 10 mls. of ferric ammonium sulfate in sulfuric acid. The rinsings were caught in the reduction beaker. The asbestos mat was carefully broken up so that all of the cuprous oxide went into solution. The crucible was given a final washing with 25 mls. of hot water, and the rinsings retained. 125 mls. of hot water and one drop of a mixture containing 0.15 grams of orthophenanthroline monohydrate and 0.07 grams of ferrous sulfate in 10 mls. of water, were added and then titrated with 0.05 N potassium permanganate. Increasing amounts of permanganate were used with increasing time of hydrolysis up to 24 hours. The results were as follows:

Table 3. Change in reducing power of crude residue with time of hydrolysis.

Time (hrs.)	Mls. of permanganate	% reducing sugar expressed as glucose
1	40.6	12.49
1 1/2	44.6	13.77
24	53.6	16.50

The hydrolyzed solution was concentrated and a sample taken and treated with phenylhydrazine hydrochloride and sodium acetate. An osazone formed which melted at 202-203°C. Although glucose, fructose, and mannose all form the same osazone, glucose seemed to be the most likely possibility.

Since more reducing sugar was freed with longer periods of hydrolysis, it was probable that a glycoside or a mixture of glycosides was being hydrolyzed. If this were true, an aglycon would also be freed and should be present in the solution. Ten gram samples of crude residue were cleared of protein, tannins etc. in the usual way, after which the residue was hydrolyzed with hydrochloric acid. The resulting hydrolyzate was extracted with ether a few times in a separatory funnel and the ether extract reduced in volume and refrigerated. The only material precipitating which could be detected was oxalic acid, which was due to added reagents used in clearing. Apparently the aglycon was present in quantities too small to be isolated successfully.

The hydrolyzed water solution of the crude residue was steam distilled to check for volatile components. The procedure recommended by Shriner and Fuson (27) for water soluble mixtures was used. The acidic hydrolyzate was steam distilled to see if any volatile acids were present. The distillate was then made alkaline and again steam distilled. No amines, acids or volatile neutral compounds were discovered.

Identification of Known Components in a Total Solids Study of the Alcoholic and Skellysolve-B Fractions. Two 100 ml. samples of each of the Skellysolve-B and chlorophyll fractions were pipetted from the receiving flasks into small, tared beakers. The easily volatilized solvent was stripped off by heating on the steam plate. When the samples reached dryness, they were placed in the vacuum oven and heated overnight at 60°C. The beakers were then reweighed, and the weight of residues determined by difference. The weight of chlorophyll, carotene, waxes, and crude residue components were calculated for each fraction. In this way it was possible to calculate what percent of the residue was due to

the known constituents. These results were compiled in Table 4.

DISCUSSION

In this study, both liquid phases obtained by counter-current separation, were analyzed for chlorophyll and carotene. To assume that all carotene or chlorophyll not present in the Skellysolve-B phase, was in the alcohol phase would be somewhat erroneous. It was found that there was minor destruction of pigments, depending on the time required for a run. Destruction of chlorophyll was somewhat less than that of carotene.

It was discovered that a better separation of chlorophyll and carotene could be obtained with a solvent ratio other than 1:1. This was possible because the Skellysolve-B shows a very strong selectivity for carotene even when used in one-fourth to one-eighth the volumes of the aqueous phase. Although an 8:1 proportion of alcohol to Skellysolve-B gave a higher percentage of chlorophyll in the alcoholic phase, less carotene was extracted by the Skellysolve-B. When higher ratios of alcohol to Skellysolve-B were employed, a point was reached where the desirability of a high chlorophyll concentration in the alcoholic phase was offset by a lowered concentration of carotene in Skellysolve-B. This optimum ratio was about 4:1.

Considerable trouble was experienced in getting representative samples of the various fractions, especially the Skellysolve-B fraction. In a continuous two-column run, the only way to check the Skellysolve-B I fraction for concentration of pigments was to draw off samples at regular time intervals and determine the concentration of

Table 4. Known and Unknown Solid Content of Alcohol and Skelly Fractions.

Individual Component	Original Extract	Alcohol I fraction	Skelly I fraction	Alcohol II fraction	Skelly II fraction
chlorophyll (total milligrams)	5,000.0	3,400.0	1,850.0	1,510.0	362.0
carotene "	324.0	16.0	291.0	44.0	249.0
paraffin wax "	960.0	70.0	890.0	100.0	790.0
alcoholic wax "	710.0	220.0	490.0	170.0	320.0
crude residue "	144,000.0	144,000.0	———	———	———
mineral "	8,980.0	8,980.0	———	———	———
saponin "	35,400.0	35,400.0	———	———	———
carbohydrate as glucose "	23,700.0	23,700.0	———	———	———
total nitrogen "	2,020.0	2,020.0	———	———	———
unknown residue	73,400.1	73,400.1	———	———	———
Total solids accounted for (total milligrams)	151,000.0	147,000.0	3,520.0	1,820.0	1,720.0
Total solids	322,000.0	299,000.0	23,700.0	10,900.0	11,800.0
Percent of known solids	48.2	49.3	14.8	16.7	14.7

chlorophyll and carotene. Since the concentration of pigments increased with time to a maximum and then dropped off, it would be purely chance if any one sample was indicative of the average concentration.

Ninety percent ethyl alcohol in water was found inferior to Skellysolve-B for the purpose of wax extraction, since only about 40 percent as much wax was removed from a comparable quantity of meal.

Proper control of the flow of solvents through the columns required rather frequent adjustment of screw clamps, particularly the alcohol I outlet. If too much suction was used on the alcohol II receiving vessel, water was pulled back into the system. The use of capillary inlets gave fine particle dispersion. The use of spiral baffles or a stirring device might conceivably allow for better contact, but a stirrer would probably create too much emulsion. However, the apparatus as described herein gave good separation. When the solvents (in a 4:1 ratio) were shaken in a separatory funnel, and then allowed to reach equilibrium, the alcohol phase contained 69.8 percent of the available chlorophyll. The average retention by the alcoholic phase in the column (with the same 4:1 ratio) was 64.2 percent, with an observed high of 68 percent. The differences were small, varying from two to six percent.

The most successful solvent system ratio consisted of a 4:1 ratio of alcohol to Skellysolve-B in column I and a 2:1 alcohol to Skellysolve-B ratio in column II. With this arrangement, 90.0 percent of the total chlorophyll found its way into the alcoholic phase, and 80.1 percent of the carotene was taken by the Skellysolve-B.

The waxes obtained gave melting points closely comparable to those reported by Blair, et al. (3). The Skellysolve-B fraction of wax was

not entirely paraffin. Some 30 percent of its bulk was high molecular weight alcohols. The alcohol I fraction contained 40-50 percent alcoholic waxes. The crude alcohol I wax melted over a higher range due to the existence of small amounts of a wax component which was more soluble in cold acetone than the rest of the wax. This portion, in impure form, gave a melting point of 140-151°C, and precipitated from the filtrate obtained from previous precipitations. It is not likely that this wax component was extracted by the Skellysolve-B solvent employed by Blair, since he reported no such high melting fraction.

The crude residue obtained by evaporating the alcohol I fraction to dryness was very hygroscopic and could not be left exposed to the air for very long periods of time. Samples were weighed as rapidly as possible and the residue retained in a dessicator or kept tightly stoppered. The water solution of the crude residue was cleared of proteins, and tannins by means of lead acetate and potassium oxalate. When this "cleared" solution was hydrolyzed with acid and extracted with ether, a white precipitate was obtained from the ether extract. At first, it was thought that this solid might possibly be an aglycon freed by acid hydrolysis of plant glycosides. However, further study revealed the precipitate to be oxalic acid, which was due to the reagents used in clearing. If alcohol was used to precipitate proteins instead of lead acetate, the difficulty of precipitating oxalic acid could be avoided. It is very probable that extraction and hydrolysis of larger amounts of residue would yield an aglycon in quantities which could be isolated and characterized.

Since the presence of waxes and other components could affect the

distribution of chlorophyll and carotene, a study of total solids was made. This study revealed the fact that less than 50 percent of the fraction residues could be accounted for as known material. Table 4 shows that smaller percentages of the total solids of the Skellysolve-B fractions and the alcohol II fractions could be identified, than in the original and alcohol I fractions, since no crude residue could be isolated from these fractions. The crude residue constituted a major share of the total solids of original and alcohol I solutions. This table also shows greater amounts of paraffin than alcoholic waxes in the Skellysolve-B fractions, and more alcoholic waxes than paraffins in the alcohol fractions. In all cases, of course, the actual amounts of wax present were very small.

It is believed that this study would be useful to anyone endeavoring to separate these plant pigments by a counter-current method using the same or similar solvents.

SUMMARY

A study was made to determine the most effective solvent ratio for counter-current distributions with the phasic apparatus used previously in this laboratory. The most satisfactory proportion tried consisted of four parts by volume of 90 percent ethanol to one part by volume of Skellysolve-B. Excellent separations were obtained with this system.

Further work revealed also, that the concentration of chlorophyll and carotene in the Skellysolve-B fraction varied with time during the course of a run. The concentration of both pigments rose to a maximum

and then dropped off. Hence, it was necessary to remove samples at regular intervals and analyze them in order to determine an average concentration which would be representative of the total fraction.

The efficiency of separation of the two-column apparatus was such that 90 percent of the chlorophyll remained in the alcohol phase, and 80.1 percent of the carotene was removed by the Skellysolve-B. Up to 1.69 times as much chlorophyll was obtained by using the two-column apparatus than by using a single column contact.

The apparatus itself was modified to the extent that higher heads and capillary inlets were used. The inlets gave finer dispersion and hence better contact and separation.

An effort was made to determine partially the composition of a crude residue obtained from the alcohol I fraction. Forty-eight and nine-tenths percent of the total weight of this residue was identified. It seemed feasible that an aglycon would be produced by acid hydrolysis of the crude residue, since successively larger amounts of sugar were produced with longer periods of hydrolysis. However, no aglycons were isolated nor identified.

Total solid determinations were made on representative samples of each fraction. Forty-eight and two-tenths percent of the residue from the original alcoholic extract was identified, whereas lesser amounts of the total solids of the Skellysolve-B fractions were accounted for.

The plant waxes present were identified and classified as paraffins or alcohols. Their distribution in the various fractions was studied. The presence of a high melting wax was indicated by a melting point 60° higher than any previously reported.

It is possible and likely that further work on this subject would reveal the identity of the wax and also the existence of an aglycon.

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