TABLE I
Comparison of Analytical Results

	New Method		P. and M. Method		T.G.A. Method	
	Mono-glyceride	Glyc-erin	Mono-glyceride	Glyc-erin	Mono-glyceride	Glyc-erin
No. of observations	25	25	14	13	16	16
Minimum	42.5%	4.20%	42.7%	3.60%	41.7%	4.23%
Maximum	44.6%	4.50%	44.6%	4.47%	47.8%	4.37%
Average	43.4%	4.33%	43.8%	4.22%	45.1%	4.31%
Standard deviation	0.70	0.065	0.51	0.27	1.77	0.038

tories,[1] using the new method reported here, the Pohle and Mehlenbacher method (3) and the Toilet Goods Association Method (5). In the Pohle and Mehlenbacher method the sample is dissolved in chloroform, free glycerol is extracted with water, the two solutions are separated, and each oxidized with a solution of periodic acid in 95% acetic acid. In the T.G.A. method the sample is dissolved in ethyl acetate, free glycerol extracted with 10% sodium sulfate solution, the two solutions are separated, and each is oxidized with a solution of periodic acid in 80% acetic acid. In both methods the excess of periodic acid is determined by adding potassium iodide and titrating liberated iodine with sodium thiosulfate. Monoglyceride and glycerin contents are calculated from the amounts of periodic acid consumed. Results of all the analyses are summarized in Table I.

[1] The laboratories of the following companies collaborated with the authors' laboratory: Colgate-Palmolive Company, Emery Industries Inc., Lever Brothers Company, Procter and Gamble Company, and Swift and Company.

Summary

A new method of analyzing mixtures containing monoglyceride and glycerin is presented. It is based upon oxidation of the sample with periodic acid. The new method is more rapid than the older methods because it is not necessary to separate the two layers when the glycerin is extracted from the solution of the sample. Precision of the titrations is improved because in the older methods the sample titration must equal at least 80% of the blank titration, but in the new method the sample titration can be very small and a correspondingly greater difference represents the monoglyceride or glycerin in the sample.

Acknowledgment

This work was sponsored by the Glycerine Division, Association of American Soap and Glycerine Producers, New York, N. Y.

REFERENCES

1. Handschumaker, E., and Linteris, L., J. Am. Oil Chemists' Soc., *24*, 143-145 (1947).
2. Montequi, R., and Doadrio, A., Inform. quim. anal. (Madrid), *6*, 31-40 (1952).
3. Pohle, W. D., and Mehlenbacher, V. C., J. Am. Oil Chemists' Soc., *27*, 54-56 (1950).
4. Pohle, W. D., Mehlenbacher, V. C., and Cook, J. H., Oil and Soap, *22*, 115-119 (1945).
5. Toilet Goods Association Inc., Method No. 26 of the Board of Standards, March 20, 1947, 9 Rockefeller Plaza, New York 20, N. Y.
6. Glycerine Analysis Committee, J. Am. Oil Chemists' Soc., *27*, 412-413 (1950); Am. Oil Chemists' Soc. Standard Method Ea 6-51.

[Received April 13, 1954]

The Determination of Chlorophyll in Oil[1]

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THE green coloring matter in plants, leaves, seeds, etc., is called chlorophyll. The classical work of Willstatter and Stoll (1) has adequately dealt with the structure and properties of the material. The present-day popularity of chlorophyllin preparations has lent glamor to the green stuff and made chlorophyll a household word. To the oil chemist and to the oil processor green in an oil is just another color pigment requiring time and material to remove and adding to the cost of the resulting chlorophyll-free products. Green may be excellent in many preparations, but in a shortening or other edible oil green lends no glamor and must be removed by bleaching. Pritchett, Taylor, and Carroll (2) have discussed "Chlorophyll Removal During Earth Bleaching of Soybean Oil." In this paper the need for chlorophyll removal is adequately covered.

In 1934 Zscheile (3) discussed the preparation and purification of chlorophyll A and B and made quantitative measurements of their absorption spectra. In the same year Long and Stevenson (4) delivered a paper on "A Simple Test to Detect Chlorophyll in Tallow." While no quantitative measurements were made, a simple test for chlorophyll detection was revealed and has been extensively used. In 1941 Zscheile and Comar (5) reported in detail on the absorption spectra of the various chlorophylls and the influence of preparative procedures on their purity. Shortly after Comar (6) published a paper on the analysis of plant extracts for chlorophyll A and B, using a commercial spectrophotometer. Chlorophyll concentrations in extracts were expressed in milligrams of chlorophyll per liter and could be translated into other appropriate terms by simple calculations if desired.

It is interesting to note that extracts of chlorophyll A analyzed by Zscheile and Comar (5) and by Comar (6) show a principal absorption at 660 millimicrons while a tremendous amount of recent work carried out directly on oils shows the absorption peak at 670 millimicrons. It is not within the scope of this paper to discuss why this discrepancy occurs. That the principal absorption of chlorophyll does occur at 670 millimicrons or close to that wavelength when absorption measurements are made directly on green oils is substantiated in a report by Melvin, MacMillan, and Senti (7). If one assumes that chlorophyll B absorbs strongly at about 17.5 millimicrons below chlorophyll A, there is little or no indication of the presence of chlorophyll B in refined or refined and bleached cottonseed or soybean oils. The reason for no apparent chlorophyll B can only be due to its absence in the seeds processed or to its insolubility in expressed or solvent extracted oil. For the purposes of this paper we shall call the green pigment in oil which absorbs at about 670 millimicrons, chlorophyll, and confine ourselves to its

[1] Presented at the annual meeting, American Oil Chemists' Society, San Antonio, Tex., April 12-14, 1954.

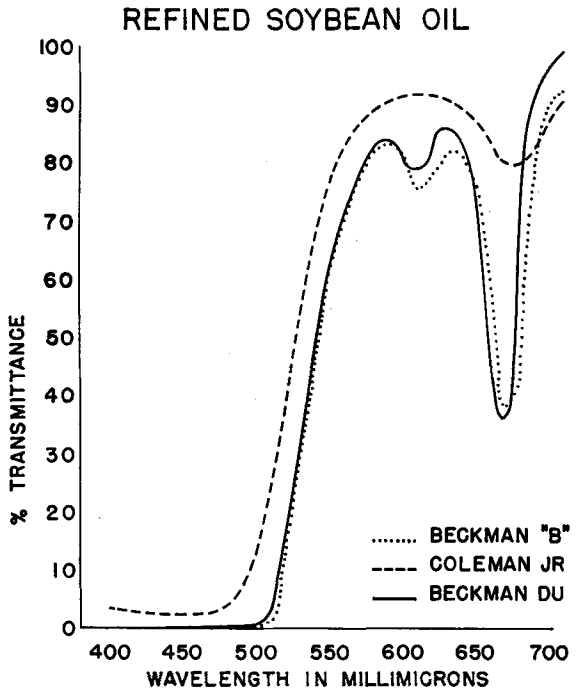

FIG. 1.

estimation in commercial oils such as soybean and cottonseed.

The Problem. Figure 1 shows the typical absorption curve of a refined soybean oil. The chlorophyll absorption occurs at 670 millimicrons, and our problem becomes one of estimating spectrophotometrically the amount of chlorophyll present in the oil, outlining the procedure involved and calculating the reproducibility of the results obtained.

Work Done. The first step in the work was to analyze a sample of commercial chlorophyll to use as a basic material for later studies on oil. A sample of magnesium chlorophyll, obtained from the Valley Vitamin Company Inc. and designated as S-628, was used as the source of chlorophyll for this work. This chlorophyll is sold on a 4½% chlorophyll content basis. The chlorophyll content was determined spectrophotometrically, using the method of Zscheile and Comar.

Some 0.222 g. of the chlorophyll sample were dissolved in 1,000 ml. of a mixture of 70% petroleum ether and 30% ethyl ether. Further dilutions of one part of concentrated solution to 20 volumes of solvent and to 40 volumes of solvent were made. Spectrophotometric curves were run on the two dilute solutions, and the chlorophyll content of the diluted solutions and the original sample were calculated, using Comar's average specific absorption coefficient of 102.

The concentration of chlorophyll in the diluted solutions was found to be .5984 milligrams per liter and .2908 milligrams per liter. This calculates back to a chlorophyll content on the original sample of 5.31%.

The second part of the problem consisted of determining the absorptivity of the chlorophyll in cottonseed and soybean oils. Using the chlorophyll sample which contained by analysis 5.31% chlorophyll, dilutions were made in refined and bleached soybean oil and in two samples of refined and filtered cottonseed oil. The concentration of chlorophyll added in the final samples was the same in every case. It should be noted that, while in spectrophotometric methods of analysis concentration is normally expressed on a weight-volume basis, in all cases in this work concentration is expressed on a weight-weight basis, or in other words, p.p.m. by weight.

Spectrophotometric curves were determined on all of the oils containing chlorophyll and on the original oils to which no chlorophyll was added. The Beckman DU, Beckman B, and Coleman Junior spectrophotometers were used.

Knowing the chlorophyll content in the refined oils and bleached oils and the length of cell, absorptivities were calculated for chlorophyll for each of the three instruments. These absorptivities are:

Beckman DU =.1016
Beckman B =.0717
Coleman Jr. =.0306

Absorptivity, a is equal to absorbance A/CL.

Where concentration is in parts per million, absorbance A is the difference between absorption at 670 millimicrons and one-half the sum of the absorbances at 630 and 710 millimicrons (630 only for Coleman instrument) and L is in centimeters.

Pertinent calculations are shown in Tables I, II, and III. The derived calculations for parts per million chlorophyll are:

$$\text{For the Beckman DU: p.p.m. chlorophyll} = \frac{A670 - \frac{A630 + A710}{2}}{0.1016\ L\ (\text{in cm.})}$$

$$\text{For the Beckman B: p.p.m. chlorophyll} = \frac{A670 - \frac{A630 + A710}{2}}{0.0717\ L\ (\text{in cm.})}$$

$$\text{For the Coleman Jr.: p.p.m. chlorophyll} = \frac{A670 - A630}{0.0306\ L\ (\text{in cm.})}$$

The work carried out above on the Beckman B spectrophotometer was done using an instrument with a stepwise sensitivity control and a blue phototube. At a later date the instrument was equipped with a red phototube and a continuous sensitivity control. The calculations used for the Beckman DU were applicable to this equipment directly.

TABLE I

Chlorophyll in Mg. Chlorophyll M, S-628
Valley Vitamin Inc.

A. 0.222 grams in 1000 ml. solvent (70% petroleum ether, 30% ethyl ether)

a. 1 volume A diluted to 20 volumes with solvent

a_1 1 volume A diluted to 40 volumes with solvent

$$C(a) = \frac{.611 \times 1000}{102.1 \times 10} = \frac{611}{1021} = .5984 \text{ mg./liter}$$

$$C(a_1) = \frac{.297 \times 1000}{102.1 \times 10} = \frac{297}{1021} = .2908 \text{ mg./liter}$$

% chlorophyll in original sample =

$$\text{From (a) \%} = \frac{.5984 \times 20 \times 100}{.222 \times 1000} = 5.39$$

$$\text{From } (a_1)\ \% = \frac{.2908 \times 40 \times 100}{.222 \times 1000} = 5.24$$

Average % = 5.315

The Method in Detail

Parts per Million Chlorophyll

Scope. This method is used to determine p.p.m. of chlorophyll on refined and on refined and bleached

TABLE II

Chlorophyll in Oils Used for Development of Equations

X = 0.222 grams chlorophyll in 1000 grams oil

A = 1 part X to 4 parts oil by weight

B = 1 part X to 9 parts oil by weight

C = 1 part X to 14 parts oil by weight

D = 1 part X to 19 parts oil by weight

E = 1 part X to 39 parts oil by weight

F = Original oil, no added chlorophyll

$$X = \frac{0.222 \times .05315}{1000} = \frac{.01180}{1000} = 11.8 \text{ p.p.m.}$$

$$A = \frac{11.8}{5} = 2.36 \text{ p.p.m.}$$

$$B = \frac{11.8}{10} = 1.18 \text{ p.p.m.}$$

$$C = \frac{11.8}{15} = 0.786 \text{ p.p.m.}$$

$$D = \frac{11.8}{20} = 0.590 \text{ p.p.m.}$$

$$E = \frac{11.8}{40} = 0.295 \text{ p.p.m.}$$

F = Original oil, no added chlorophyll

oils. Parts per million of chlorophyll are calculated from measurements made on a spectrophotometer at 630, 670, and 710 millimicrons. The method is not applicable to hydrogenated oils and finished products since the chlorophyll absorption does not occur at 670 millimicrons in processed oils.

Reagents

CCl_4

Redistilled carbon tetrachloride. The transmittance should not differ from that of distilled water by more than 0.5% at 400 millimicrons.

Apparatus

Beckman B Spectrophotometer

Modified with a continuous sensitivity control.

Red Phototube

This tube contains a frosted glass window and must be used for all measurements above 600 millimicrons.

Sample Cells

Water white, 50 mm. long, 20 mm. wide, 40 mm. deep. Obtained from Pyrocell Manufacturing Company, 207-211 E. 84th street, New York 28, N. Y.

These cells must be matched and show no greater spread than 0.5% transmission when checked with distilled water at 400 millimicrons.

Adjustment of Spectrophotometer. Before using, the Spectrophotometer should be checked carefully, following instructions given in the operating manual obtained with the instrument. The wavelength setting of the instrument should be checked with the blue sensitive phototube in the instrument. With the instrument turned on and with no cells in the cell holder, the maximum sensitivity obtainable with the instrument should occur at approximately 540 millimicrons. It is advisable to check the instrument occasionally against a mercury vapor lamp, using 579.1, 546.1, 435.8, and 404.7 millimicron checking points. Any mercury vapor lamp can be focussed to pass through the instrument in place of the regular incandescent lamp normally used. Transmission peaks should occur at the specified wavelengths.

Operation. Install and adjust the instrument following the instructions given in the manual. Wavelength checks should be made as indicated above and the carbon tetrachloride should be checked against distilled water. Turn on the instrument at least 15 minutes before use.

Place a 50-mm. cell containing the CCl_4 in the cell compartment. Set the instrument to the desired

TABLE III

Calculation of Specific Absorption Coefficient
p.p.m. Chlorophyll Added

mμ	A	B	C	D	E	F
	2.36	1.18	0.786	0.590	0.295	0.00
Beckman DU — 100 mm. cell						
670	2.00	1.350	.945	.730	.387	.058
630	.500	.282	.202	.165	.103	.048
710	.099	.067	.053	.050	.040	.031
$\frac{630+710}{2}$	.300	.175	.128	.108	.072	.040
$670 - \frac{630+710}{2}$	1.700	1.175	.817	.622	.315	.018
Corrected for 0.00 p.p.m.	1.682	1.157	.799	.604	.297	.000
a = A/CL	.07127*	.09805	.10165	.10237	.10068	.....
Average used						.1016
Beckman B — 50 mm. cell						
670	.920	.530	.373	.306	.168	.040
630	.265	.158	.112	.093	.064	.034
710	.110	.080	.060	.053	.042	.028
$\frac{630+710}{2}$	.188	.119	.086	.073	.053	.031
$670 - \frac{630+710}{2}$	.732	.411	.287	.233	.115	.009
Corrected for 0.00 p.p.m.	.723	.402	.278	.224	.106	.000
a = A/CL	.06127*	.06814	.07074	.07593	.07186	.....
Average used						.0717
Coleman Jr., 21.84 mm. cell						
670	.266	.146	.099	.079	.040	.007
630	.118	.065	.045	.037	.021	.008
670-630	.148	.081	.054	.042	.019	.000
Corrected	.148	.081	.054	.042	.019	.....
a = A/CL	.02872	.03075	.03147	.03260	.02950	.....
Average used						.0306

* Not in average.

wavelength using the wavelength knob. Turn the sensitivity knob to position "1" and with the cell aperture closed, adjust the dark current to give a reading of zero. Open the cell aperture and adjust the slit to 0.1 mm. With the continuous sensitivity control adjust the reading to 100%. Again check the dark current and readjust if necessary.

Close the cell aperture and move the cell containing the sample into the light beam. The sample must be clear and brilliant. Open the cell aperture and read the absorption as shown by the meter. Close the cell aperture and change the wavelength setting to the next desired wavelength with the CCl_4 in the light beam. Readjust the dark current and sensitivity control to give 0 and 100% readings. Again put the sample in the light beam. Make all of the desired readings following these procedures.

Parts per million of chlorophyll, using Beckman B with red sensitivity phototube

$$\text{P.p.m. chlorophyll} = \frac{A670 - \frac{A630 + A710}{2}}{0.1016\ L\ (\text{in cm.})}$$

Temperature affects transmission readings made on the Beckman B Spectrophotometer. Every effort should be made to read the transmittances at a uniform temperature, preferably 85°F. ± 5°F. This precaution is particularly important when chlorophyll is being determined.

The Coleman Jr. Spectrophotometer can be used for determining chlorophyll in amounts above 0.1 p.p.m. Readings are made in the 25-mm. cuvette against CCl_4 at 630 and 670 millimicrons.

$$\text{P.p.m. chlorophyll} = \frac{A670 - A630}{0.0668}$$

The Beckman DU Spectrophotometer can be used for determining chlorophyll in exactly the same manner as for the Beckman B. The same calculations apply.

Remarks

We can now apply the method to the analysis of the oil shown in Figure 1. The necessary data are these

Wavelengths	Absorbances		
	DU	B	Coleman
630	.066	.088	.042
670	.440	.420	.098
710	.005	.034	....
Cell length (cm.)	5.0	5.0	2.184
P.p.m. chlorophyll	0.80	0.71	0.84

The analysis of a single sample reveals little or nothing of the precision or the accuracy of the determination. The accuracy of results is a relative matter depending upon the accuracy of the data which goes into the development of the calculations used. In this case accuracy rests solely upon the work of Comar and his associates, who determined the specific absorption of chlorophyll and which we have accepted and used in this work. It is relatively unimportant in work on oil whether an error of 10 or even 25% in accuracy occurs since the problem is chiefly one of removing substantially all of the chlorophyll present. It is important however that two different laboratories arrive at the same result. This is a matter of precision. The precision of the determination has been studied.

Parts per million of chlorophyll is routinely determined on cooperative sample in the Procter and Gamble laboratories. The data obtained have been statistically studied. The data show

For Beckman B and Beckman DU

For refined oils (.34 to 1.43 p.p.m.) $\sigma = .060$ 134 det'ns, 11 samples

For bleached oils (.007 to .063 p.p.m.) $\sigma = .0065$ 110 det'ns, 9 samples

For Coleman Jr.

For refined oils (.36 to 1.30 p.p.m.) $\sigma = .086$ 116 det'ns, 12 samples

In these calculations $\sigma = \sqrt{\frac{\Sigma\,(d)^2}{N - \text{Number of sample series}}}$

No calculations were made for bleached oils, using the Coleman Jr. instrument, since the instrument is entirely unsuitable for measurements below 0.1 p.p.m. chlorophyll.

As might be expected, results obtained by the Beckman DU or Beckman B instruments are more precise than those obtained on the Coleman Jr. The Coleman Jr. can however be used with adequate precision and a considerable saving in time for many measurements on refined oils. The fact that about 10 samples and 10 different instruments were used in the precision measurements should be borne in mind. Obviously much better precision will be obtained in a single laboratory.

Conclusions

A method for determining parts per million of chlorophyll in an oil has been developed. The details of the method are given. The Beckman DU, Beckman B, and Coleman Jr. spectrophotometers have been used. Reliability of results has been determined and standard deviations have been calculated.

REFERENCES

1. Willstatter, R., and Stoll, A., "Investigations on Chlorophyll," Translation by Schertz and Merz, Science Press (1928).
2. Pritchett, W. C., Taylor, W. G., and Carroll, D. M., J. Oil Chemists' Soc., *24*, 225-227, (1947).
3. Zscheile, F. P. Bot. Gaz., *95*, 529-562 (1934).
4. Long, C. P., and Stevenson, H. B., J. Am. oil Chemists' Soc., *12*, 7-8 (1935).
5. Zscheile, F. P., and Comar, C. L., Bot. Gaz., *102*, 463-481 (1941).
6. Comar, C. L., Ind. and Eng. Chem., *14*, 877-879 (1942).
7. Melvin, E. H., MacMillan, Duncan, and Senti, F. R., J. Am. Oil Chemists' Soc., *30*, 255-259 (1953).

[Received April 21, 1954]