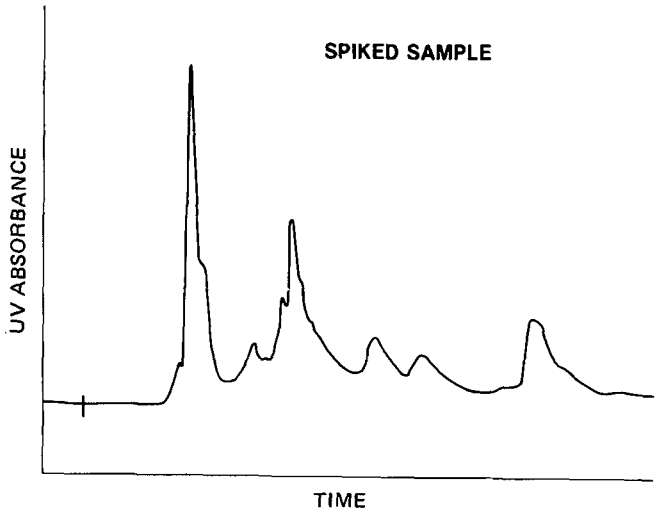


FIG. 5. Chromatogram showing the clove fraction that had been spiked with gallic acid and eugenol.

ACKNOWLEDGMENTS

The author thanks Ahmed Mabrouk for his help; Edmon Wong, Department of Scientific and Industrial Research, Palmerston, New Zealand; Aaron Bluhm for the IR spectrometry; Maurice Bazinet and Donald Robertson for the mass spectrometry; Solomon Bishov for testing the first fractions for antioxidant activity, and Chris Filippi and his colleagues at Waters Associates for help in HPLC.

REFERENCES

1. Chipault, J.R.; G.R. Mizuno, J.M. Hawkins and W.O. Lundberg, Food Res. 17:46 (1952).
2. Chipault, J.R.; G.R. Mizuno and W.O. Lundberg, Ibid. 20:443 (1955).
3. Chipault, J.R.; G.R. Mizuno and W.O. Lundberg, Food Tech. 10:209 (1956).
4. Chipault, J.R., Food Eng. April 1957, 134.
5. Herrmann, K., Z. Lebensm.-Unters.-Forsch. 116:224 (1962).
6. Herrmann, K., Fette Seifen Anstrichm. 75:499 (1973).
7. Brieskorn, C.H.; A. Fuchs, J.B. Bredenberg, J.D. McChesney and E. Wenkert, J. Org. Chem. 29:2293 (1964).
8. Brieskorn, C.H., and H.J. Doemling, Zeit. Lebensm.-Unter. Forsch. 141:10 (1969).
9. Brieskorn, C.H., and H.J. Doemling, Arch. der Pharmazie, 302:641 (1969).
10. Hiraharam, F.; Y. Takai and H. Iwao, Jap. J. Nutrition 32:1 (1974).
11. Watanabe, Y., and Y. Ayano, Ibid. 27:181 (1974).
12. Saito, Y., Abura Kagaku, 26:754 (1977).
13. Chang, S.S.; B. Ostric-Matijasevic, O.A.L. Hsieh and C.-L. Huang, J. Food Sci. 42:1102 (1977).
14. Chang, S.S.; B. Ostric-Matijasevic, C.-L. Huang and O.A.L. Hsieh, U.S. Patent No. 3950266 (1976).
15. Wu, J.W.; M.-H. Lee, C.-T. Ho and S.S. Chang, JAOCS 59:339 (1982).
16. Bishov, S.J.; A.S. Henick and R.B. Koch, Food Res. 25:174 (1960).
17. Bishov, S.J.; A.S. Henick and R.B. Koch, J. Food Sci. 26:178 (1961).
18. Bishov, S.J., and A.S. Henick, JAOCS, 43:477 (1966).
19. Bishov, S.J.; Y. Masuoka and A.S. Henick, Food Tech. 21: 148A (1967).
20. Bishov, S.J., and A.S. Henick, J. Food Sci. 37:873 (1972).
21. Bishov, S.J., and A.S. Henick, Ibid. 40:345 (1975).
22. Bishov, S.J.; Y. Masuoka and J.G. Kapsalis, J. Food Proc. and Pres. 1:153 (1977).
23. Vogel, A.I., Practical Organic Chemistry, Third Enlarged Edition, p. 163, John Wiley and Sons, Inc. (1962).
24. Stahl, E., A Laboratory Handbook, pgs. 41-44 and 686-706, Springer-Verlag, New York (1969).
25. Walters, D., J. Anal. Tox. 1:218 (1977).
26. Barton, G.M., J. Chromatog. 20:189 (1965).

[Received June 12, 1984]

♣Detection of Chlorophyll Derivatives in Soybean Oil by HPLC

M.S. FRASER and **G. FRANKL**, Technical Center, Hunt-Wesson Foods, Inc., 1645 W. Valencia Dr., Fullerton, CA 92634

ABSTRACT

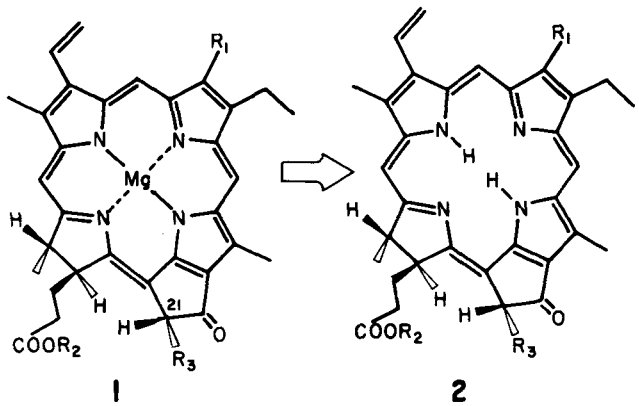
Chlorophyll derivatives have been isolated from a degummed soybean oil by cellulose column chromatography and resolved by reversed phase HPLC. The HPLC separation was performed on a Zorbax ODS column using acetone-methanol (75:25) as the mobile phase. Seven major components were detected by visible (650 nm) light absorption. Pheophytin A is the predominant component of the mixture (40-45% of the total). Pheophytin A', pyropheophytin A, and three unidentified pigments having spectral features resembling pheophytin A comprise the other major pigments. No evidence was found for the presence of chlorophylls A and B in this oil.

INTRODUCTION

The presence in soybean oil of green pigments of the chlorophyll type is of interest not only because of their impact on finished product color but also because of their potential role in oxidative stability (1-9). The quantities of these pigments in soybean oil and other plant extracts usually are determined by spectrophotometric (10-12) or fluorometric (10,13) measurements. Oil processors routinely obtain "apparent chlorophyll" values by the AOCS spectrophotometric method (14). Pritchett et al., in a study of the influence of processing on the chlorophyll pigments, concluded that a normal crude soybean oil contains about 1500 μg/l chlorophyll and a well-processed oil as little as 15 μg/l chlorophyll (15).

The positions of the characteristic visible absorption bands have been used to identify the responsible pigments. Initially, the visible absorption band at 660-670 nm in the spectrum of crude soybean oil was attributed to chlorophyll A or its derivatives (15). After careful consideration of the influence of solvent on the absorption maxima, O'Conner et al. concluded that the absorption bands at 610 and 670 nm observed in the spectrum of a degummed soybean oil were due to pheophytin A (16).

Identifications based solely on spectral data must be regarded with caution. The absorption spectra of certain chlorophyll derivatives are indistinguishable from one another. The structures of chlorophyll A (chl A), chlorophyll B (chl B) and certain derivatives of each which might plausibly arise via hydrolysis reactions occurring during handling and processing of soybean oil are shown in Figure 1. Figure 1 also presents the positions and intensities of the characteristic visible absorption bands (17-20). Of the derivatives of chl A, pheophytin A (pheo A), pheophorbide A and pyropheophytin A (pyropheo A) each have maxima



No.	Name	R_1	R_2	R_3	$\lambda_{max}^{nm}(\epsilon)^a$	Ref.
1a	chlorophyll A[b]	$-CH_3$	-phytyl[c]	$-CO_2CH_3$	430(94700) 663 (75000)	17
2a	pheophytin A	$-CH_3$	-phytyl	$-CO_2CH_3$	409(101800) 666(44500)	18
2b	pheophorbide A	$-CH_3$	-H	$-CO_2CH_3$	409(119200) 667(55200)	19
2c	pyropheophytin A	$-CH_3$	-phytyl	-H	409(102400) 667(49000)	20
1b	chlorophyll B	-CHO	-phytyl	$-CO_2CH_3$	455(131000) 645(47100)	17
2d	pheophytin B	-CHO	-phytyl	$-CO_2CH_3$	434.5(145000) 654(27800)	18
2e	pheophorbide B	-CHO	-H	$-CO_2CH_3$	439(154000) 653(39800)	19
2f	pyropheophorbide B	-CHO	-phytyl	-H		

FIG. 1. Structures and spectral features of chlorophylls A and B and certain derivatives. [a]Spectra were taken of acetone solutions. ϵ is molar extinction coefficient. [b]Chl A′, the C-21 epimer of chl A, has λ_{max}(Et_2O) at 428.5 and 661 nm (21) whereas chl A has λ_{max} (Et_2O) at 428.5 and 660.5 nm (22). [c]Phytyl = $-CH_2-CH=C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$.

at about 408 and 667 nm (in acetone). In addition, several of the derivatives described in Figure 1 may undergo an epimerization reaction (at C-21). This reaction does not affect the absorption spectrum appreciably; thus, chl A′, the epimer of chl A, and chl A each have absorption maxima (in diethyl ether) at 428.5 and 661 nm (21,22).

Chlorophyll derivatives can be separated from one another by thin layer (23,24) or column (10) chromatography. Various straight- (25,26) and reversed-phase (27-30) HPLC methods also achieve effective separations.

The present paper discusses the initial steps in the development of an HPLC method for determining the various green pigments in soybean oil. Key features of the method involve the isolation of the pigments by cellulose column chromatography followed by HPLC analysis. Because chl A (and similar Mg-containing compounds) might generate artifacts during the column chromatography step, a procedure for its conversion to pheo A prior to chromatography is considered. Various green pigments required for the development of these methods were prepared from spinach.

Application of the HPLC method developed herein results in the detection of a number of chlorophyll derivatives in a degummed soybean oil.

EXPERIMENTAL

All commercially available chemicals were used without further purification. A typical phosphoric acid degumming process (31) was used to produce the degummed oils A and B. The fully processed soybean oil (oil C) was commercially refined, bleached and deodorized (31). Visible spectra were obtained with a Cary 219 spectrophotometer. The HPLC system employed an Altex Model 100 pump, a Zorbax ODS (4.6 x 250 mm, DuPont) column, a Schoeffel SF 770 Spectroflow spectrophotometer equipped with a GM 770 monochromator, and HPLC grade solvents.

Isolation of Green Pigments from Spinach

Chlorophyll A and Chlorophyll B. Fresh spinach leaves (12 g) were blended (Waring blender) with acetone (300 ml), the resultant filtered, and the nearly colorless pulp washed with additional acetone (200 ml). A portion (100 ml) of the combined acetone filtrates was diluted with water (300 ml), extracted with hexane (2 x 100 ml), and the combined hexane extracts dried (Na_2SO_4, anhydrous), filtered and concentrated to a small volume by rotary evaporation at temperatures below 50 C.

A portion of the concentrated pigment solution was chromatographed on a small (3 g) cellulose (Machery-Nagle 300 HR) column using an initial mobile phase of hexane-acetone (95:5). After about 50 ml of this mobile phase had eluted, two well-resolved green bands were observable on the stationary phase. Hexane-acetone (90:10) was used to elute each band sequentially. Fractions containing the major portion of each pigment were concentrated and stored in the refrigerator. The first- and second-eluting green pigments were identified as chl A and chl B, respectively, on the basis of TLC and spectral properties (Table I).

Stock solutions prepared from each of these pigments, as well as from the pigments noted below, were analyzed by spectroscopy and their respective concentrations were calculated using molar absorptivity values derived from Vernon's spectral data (11). In a typical case, a concentrated stock solution of chl A was prepared by diluting a portion of the chl A containing fraction to 5.0 ml with acetone. An aliquot (0.1 ml) of the stock solution was added to 3.0 ml of acetone-water (80:20) and its visible spectrum obtained.

Pheophytin A. A second portion of spinach extract was chromatographed on a similar cellulose column using hexane as the initial mobile phase followed by hexane-acetone solvents of increasing acetone content. Under these conditions, the pigments spent a substantially longer time on the column and the first-eluting green band was identi-

TABLE I

Spectral and Chromatographic Properties of Green Pigments Isolated from Spinach

Pigment[a]	λ_{max}(nm)[b]	TLC(R_f)[c]		HPLC (R_t min)[d]	
		Observed	Ref. 23	System 1	System 2
chl A	443,665[e]	0.77	0.84	4.0	8.9
chl B[f]	460,649[e]	0.72	0.68	3.4	7.7
pheo A	409,666[e]	0.90	0.88		13.6
pyropheo A	409,666[g]			8.0	19.4
pyropheo B	436,655[g]			6.8	16.4

[a]Each pigment was obtained by column chromatographic resolution of spinach extract (see Experimental).

[b]The solvent for the spectral determinations was acetone-water (80:20).

[c]Cellulose plates developed with 90:10 pet ether-pyridine (23).

[d]Samples were chromatographed on a Zorbax ODS column using either System 1 (mobile phase = acetone-methanol 80:20 at 1.0 ml/min) or System 2 (acetone-methanol 75:25 at 0.5 ml/min) conditions. In either case, the components were detected at 650 nm using the maximum sensitivity (0.01 absorbance units full scale) of the spectrophotometric detector.

[e]The positions and relative intensities of these bands agree well with Vernon's data (11).

[f]This preparation contains a significant amount of a second component (R_t = 3.6 min with System 1).

[g]The spectra agree well with those of the analogous pheophytin compounds (11).

fied as pheo A. The chromatographic fraction was concentrated and stored as described earlier.

A stock solution of pheo A was prepared by diluting a small portion of the chromatographic fraction to 10 ml with acetone-methanol (75:25). The pheo A concentration of this stock solution was 1.86×10^{-5}M. The influence of solvent on the visible spectrum of pheo A was determined by diluting aliquots (0.5 ml) of the stock solution to 10 ml with the solvent of interest. Spectra were obtained in the following solvent media: 1) acetone; 2) acetone-water (80:20); 3) acetone-methanol (75:25); 4) hexane; and 5) soybean oil C (fully processed). The pheo A concentration of these spectral samples was 9.33×10^{-7}M.

Pyropheophytin A and pyropheophytin B. A 1-lb can of cooked spinach was drained and blended with 200 ml of an 85:15 mixture of acetone and phosphate buffer (pH = 7.5; 0.1 M). After filtration, a portion (100 ml) of the filtrate was diluted with water (200 ml) and the resultant extracted with hexane (2 × 100 ml). Following an unsuccessful attempt to precipitate the pigments with 1,4-dioxane and water, the hexane extract was washed with water, dried, filtered and concentrated. About 25% of this concentrate was chromatographed on a powdered sucrose (20 g) column developed with hexane. Two major green bands were sequentially eluted and collected. The first was identified tentatively as pyropheophytin A (pyropheo A) and the second as pyropheophytin B (pyropheo B) on the basis of their visible spectra and HPLC retention properties (Table I).

Separation of Chlorophyll Type Pigments by HPLC

Stock solutions of each of the pigments isolated from spinach were analyzed by HPLC. The pigment concentrations of these solutions generally were above 1×10^{-6} M. At such concentrations, chromatographic peaks of reasonable size were detected at 650 nm when 5-20 µl injections were made. Preliminary measurements of the retention time of individual pigments were made with a mobile phase of acetone-methanol (80:20) with a flow rate of 1.0 ml/min (System 1). The following stock solutions were analyzed: 1) chl A (2.63×10^{-6}M); 2) chl B (1.04×10^{-6}M); 3) pyropheo A (1.84×10^{-4}M), and 4) pyropheo B (4.93×10^{-5}M).

A test mixture containing each of the above pigments was used to investigate the resolution obtained with eluents of different polarity composed of acetone-methanol and acetone-methanol-water in various proportions and with variable flow rates. The amount of each component in the test mixture was: chl A (1.05×10^{-5}M); chl B (6.2×10^{-6}M); pyropheo A (9.2×10^{-5}M); and pyropheo B (2.46×10^{-5}M). An adequate separation among these components was obtained with a mobile phase of acetone-methanol (75:25) with a flow rate of 0.5 ml/min (System 2). These conditions were used throughout the remainder of this study.

The particular details relating to the use of this HPLC analysis on specific pigment-containing fractions isolated from either soybean oil or spinach are noted in the legends to the respective figures.

Conversion of Chlorophylls to Pheophytins

Acid treatment of chlorophyll test mixtures. A portion (10 ml) of an acetone extract of fresh spinach was evaporated to a small volume, taken up to 50 ml with hexane and shaken for 1 min with an equal volume of 9.2% HCl solution. The hexane layer was washed with water (3 × 50 ml) and an aliquot (5 ml) withdrawn, evaporated and taken up to 5.0 ml with acetone-water (80:20). The spectral and chromatographic properties of this sample were compared with those of a control sample prepared by direct dilution of 1 ml of the original fresh spinach extract to 5.0 ml with acetone-water (80:20). In a variation of this treatment designed to serve as a model for the conversion of chlorophylls to pheophytins in the presence of soybean oil, the hexane layer also contained 10 ml of fully processed soybean oil C. The treated material was manipulated into acetone solution and compared with a control composed of the same amounts of oil and spinach extract. For HPLC measurements, 20 µl of the final solutions were analyzed.

Since the fresh spinach extract contained, according to HPLC analysis, primarily chl A and chl B, the concentrations of each could be determined from spectral data by use of Equations 1a and 1b, respectively, in which A_{649} and A_{665} are the observed absorbance readings at 649 and 665 nm and the molar absorptivities employed for each pigment at each wavelength, calculated from spectral data (11) obtained for acetone-water (80:20) solutions, are as follows: $\epsilon^{649}_{\text{chl A}} = 1.82 \times 10^4 M^{-1}$; $\epsilon^{665}_{\text{chl A}} = 7.08 \times 10^4 M^{-1}$; $\epsilon^{649}_{\text{chl B}} = 4.17 \times 10^4 M^{-1}$, and $\epsilon^{665}_{\text{chl B}} = 8.57 \times 10^3 M^{-1}$. With the

initial concentrations of chl A and chl B known, Equation 2 was used to estimate the magnitude of the absorbance change at 665 nm expected for complete conversion to pheo A and pheo B. For Equation 2, the molar absorptivities used were $\epsilon^{665}_{\text{pheo A}} = 4.30 \times 10^4 M^{-1}$ and $\epsilon^{665}_{\text{pheo B}} = 8.01 \times 10^3 M^{-1}$. The actual degree of conversion obtained experimentally was calculated using Equation 3 and the absorbance values at 665 nm for the initial and final spectral samples.

The yield of pheo A from chl A also was estimated from HPLC measurements according to Equation 4 in which the area of the pheo A peak in the acid treated sample is compared with that of the chl A peak in the untreated control. The amount of unreacted chl A was determined by direct comparison of the areas of the chl A peak in the treated and untreated samples.

$$[\text{chl A}] = (A_{649} \times \epsilon^{665}_{\text{chl B}} - A_{665} \times \epsilon^{649}_{\text{chl B}})/D \quad [1a]$$

$$[\text{chl B}] = (A_{665} \times \epsilon^{649}_{\text{chl A}} - A_{649} \times \epsilon^{665}_{\text{chl A}})/D \quad [1b]$$

where $D = (\epsilon^{649}_{\text{chl A}} \times \epsilon^{665}_{\text{chl B}} - \epsilon^{665}_{\text{chl A}} \times \epsilon^{649}_{\text{chl B}})$

$$\Delta A^{665}_{\text{calc}} = [\text{chl A}](\epsilon^{665}_{\text{chl a}} - \epsilon^{665}_{\text{pheo A}}) + [\text{chl B}](\epsilon^{665}_{\text{chl B}} - \epsilon^{665}_{\text{pheo B}}) \quad [2]$$

$$\text{Pheophytin Yield (\%)} = (A^{665}_{\text{init}} - A^{665}_{\text{final}})(100)/\Delta A^{665}_{\text{calc}} \quad [3]$$

$$\text{Pheo A Yield (\%)} = (\text{Area}^{\text{pheo A}}_{\text{final}} - \text{Area}^{\text{pheo A}}_{\text{init}})(100)/(\text{Area}^{\text{chl A}}_{\text{init}})(\epsilon^{665}_{\text{pheo A}}/\epsilon^{665}_{\text{chl A}}) \quad [4]$$

Acid treatment of degummed soybean oil. Degummed soybean oil A (4.0 g) was dissolved in acetone (final solution volume = 10 ml). Half of this solution was set aside as the control and the remainder was taken up to 50 ml with hexane, shaken with HCl and manipulated (as above) into a final volume of 5.0 ml (acetone). Degummed soybean oil B (5.9 g) was treated similarly. Visible spectra were obtained for each sample.

Isolation of Green Pigments from Soybean Oil by Column Chromatography

Cellulose. In a typical case, degummed soybean oil A (3.0 g) was dissolved in hexane (total solution volume = 10 ml) and the resultant chromatographed on a cellulose (40 g, Machery-Nagle 300HR) column with hexane as initial mobile phase. Elution of the triglycerides, as determined by monitoring the column effluent with a PYE Unicam flame ionization detector, generally required about 150 ml of hexane. After most of the triglycerides had eluted, the green pigments were eluted with 200 ml of hexane-acetone (90:10). The pigment fraction was evaporated to a small volume, then taken up to 10 ml with acetone. Visible spectra of the initial and recovered samples were taken. Samples for HPLC analysis were concentrated to final volumes of 1.0 ml or below (acetone solvent). Several experiments were done using various amounts of sample and adsorbant.

Silica gel and other adsorbants. Several experiments were accomplished in which soybean oil A (2-6 g) in hexane solution (5-10 ml) was chromatographed on silica gel (Grace, Grade 923, mesh 100-200, 30 g), Florisil (Wilshire Chem. Co., 60/100 PR mesh, 30 g), Hi-Flosil (Applied Science, 30 g) or other adsorbants such as aluminum oxide (BioRad AG-7 100-200 mesh, neutral, 50 g) or magnesia (50 g). In each case, the initial mobile phase was either hexane or hexane-acetone mixtures containing 2-4% acetone and, after the triglycerides had eluted, the pigments were recovered with more polar eluents.

Recovery of pheo A from a model oil by silica gel chromatography. A model oil was prepared by diluting 0.1 ml of a pheo A preparation and 4.0 g of soybean oil C (a fully processed oil having a negligible amount of "apparent chlorophyll") to 10.0 ml with hexane. The pheo A concentration of the mixture was 3.15×10^{-6}M. A portion (5 ml) was chromatographed on silica gel (100 g) beginning with hexane-acetone (95:5) as eluent. After triglyceride elution (100 ml), the pigments were eluted with 200 ml of hexane-acetone (50:50). The pigment fraction was manipulated first into 5 ml of hexane solution for spectral analysis and finally into 1.0 ml of acetone solution for HPLC analysis.

Ion exchange chromatography. A small (5 g) Dowex 50W-X4 column was activated (32) by sequential treatment with NaOH (10%), water and HCl (9.2%), then equilibrated first with acetone-water (85:15) and finally with anhydrous acetone. Degummed soybean oil A (5.0 g) was dissolved in 25 ml of acetone solution and the resultant chromatographed. After the triglycerides had eluted (about 35 ml), the eluent was changed to acetone-water (85:15). Five ml fractions were taken and each spectrally analyzed to determine the presence of the green pigments. The triglyceride-free pigment containing fractions were combined, diluted with water (200 ml), extracted with hexane (3 x 50 ml) and the combined extracts dried (Na_2SO_4) and filtered. The filtrate, together with acetone from an additional rinsing of the Na_2SO_4, was then concentrated to a small volume and finally taken up to 1.0 ml with acetone.

Recovery of added pheo A from a model oil solution by ion exchange chromatography. An acetone solution (total volume = 10 ml) containing pheo A and soybean oil C (5.0 g) was prepared. A portion (5 ml) was set aside as a control and the remainder was diluted to 25 ml with acetone and chromatographed on the Dowex column. The pigment fraction was mixed with hexane, gently rotary evaporated to remove most of the acetone, then extracted with water. The hexane layer was then dried, filtered, evaporated and the residue taken up to 5.0 ml with acetone. After spectral analysis, the acetone solution was evaporated to a final volume of 1.0 ml.

RESULTS AND DISCUSSION

At the beginning of this investigation, it seemed likely that the primary green pigments in crude and degummed soybean oils were chl A and pheo A (15,16). In addition, if the situation in soybean oil is similar to that in rapeseed oil (33), one might also expect to detect small amounts of chl B and pheo B. Thus, the minimal capabilities of an effective chromatographic method are to be able to resolve these four compounds. Several reversed phase HPLC methods are effective in achieving such separations (27-30), and each appears potentially useful for quantitative analysis. Therefore, this type of HPLC was selected for use in this study as a way of detecting various green pigments in unbleached soybean oil.

Reasonably pure preparations of each of several green pigments were necessary for the development of an HPLC method. This was achieved by column chromatographic resolution of the pigment mixtures obtained by acetone extraction of either fresh or canned spinach. The major green pigment of each preparation was identified tentatively on the basis of chromatographic (TLC) and spectral data summarized in Table I. HPLC analysis of each preparation using a Zorbax ODS column (System 1 conditions) provided the retention time of the primary component (Table I) and revealed the purity of each preparation. Except for the chl B sample, which was contaminated with a substantial amount of a component of slightly longer

retention time (R_t = 3.6 min), possibly chl B′, each preparation was composed primarily of a single green pigment. The retention time assignment for chl B has been supported by additional HPLC evidence (see below).

Development of an adequate HPLC separation among the compounds of interest involved the preparation of a suitable test mixture and comparison of the separations obtained with several mobile phases. Preliminary results indicated that ternary mobile phases containing water such as that used by Rebeiz et al. (27) led to very long retention times on this Zorbax ODS column for the components of interest. Analysis times were shorter when an acetone-methanol (80:20) mobile phase was used. However, the separation of the chl A and chl B components was unsatisfactory. An acceptable separation was obtained with acetone-methanol (75:25) with a flow rate of 0.5 ml/min. Figure 2 illustrates a typical chromatogram obtained under the latter conditions. The major peaks in Figure 2 are assigned, by assuming that their order of elution is the same as that seen for acetone-methanol (80:20, System 1, Table I), as follows: 1) chl B; 3) chl A; 7) pyropheo B; and 8) pyropheo A. Minor peaks 2 (chl B′) and 4 (chl A′) are assigned on the basis of their slightly longer retention times than the chl B and chl A parents (29). Support for the chl B and chl A assignments comes from the observation that these are the only two green pigments present in fresh spinach leaves (29) and that immediate HPLC analysis of an initial acetone extract of fresh spinach leaves shows peaks only at these assigned retention times. Acid treatment of chl B and chl A produces pheo B and pheo A, respectively; compounds identified as peaks 5 and 6 in Figure 2. In general, the order of elution of the pigments is in agreement with that reported for similar reversed phase separations (27,29). The only discrepancy is our observation that pyropheo B elutes after pheo A, which is contrary to that noted by Schwartz et al. (29).

Preliminary HPLC experiments indicated that direct analysis of degummed soybean oils A and B is not feasible with our system. The small quantities of chlorophyll-type pigments in soybean oils (15), together with the detection limits of the spectrophotometric detector, require that the pigment concentration in the HPLC samples be at least 5- to 10-fold larger than in the degummed oils. This requirement might be avoided by use of a more sensitive fluorescence detector (27). In the present study, however, concentration of the pigments has been achieved by column chromatography whereby the pigments are first separated from the triglycerides and the resulting fractions are evaporated to appropriate volumes.

Early experiments conducted with silica gel or cellulose adsorbants indicated that chl A and chl B are not entirely stable on these solids. To avoid the production of artifacts from the reaction of chl A and B, a procedure was sought which could be used to convert the chlorophylls to derivatives having adequate stability in the presence of these adsorbants. It was also desirable that the procedure allow estimation of the original quantities of chl A and chl B in the soybean oil. An attractive method for achieving these objectives is the acid catalyzed conversion of chlorophylls to pheophytins. This derivatization procedure (see Experimental) was tested by adding an extract containing chl A and chl B to hexane, treating the resultant with acid, then analyzing the treated material spectrally and chromatographically (HPLC). The conversion of chl A and chl B to pheo A and pheo B is illustrated (Fig. 3) by analysis of the original fresh spinach extract (top curve) and the acid treated material (bottom curve). Several experiments of this kind were conducted. In one instance, a model solution of chl A and B in soybean oil C was treated to ensure that the presence of triglycerides and other oil components

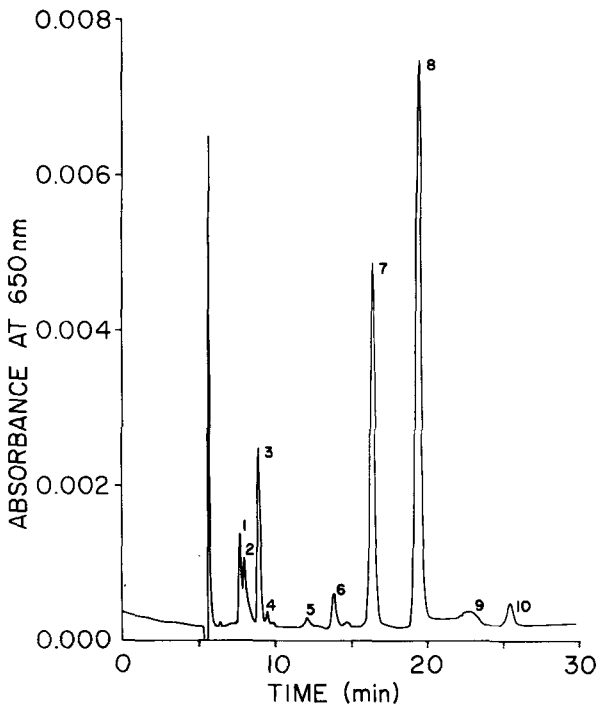


FIG. 2. Reversed phase HPLC separation of the green pigments of a test mixture prepared from components isolated from spinach (see Experimental). Chromatographic conditions include use of a Zorbax ODS column, an acetone-methanol (75:25) mobile phase pumped at 0.5 ml/min and detection at a spectrophotometer setting of 0.01 aufs. Peak designations (see text) are: 1) chl B; 2) chl B′; 3) chl A; 4) chl A′; 5) pheo B; 6) pheo A; 7) pyropheo B; 8) pyropheo A, and 9) and 10) unknowns.

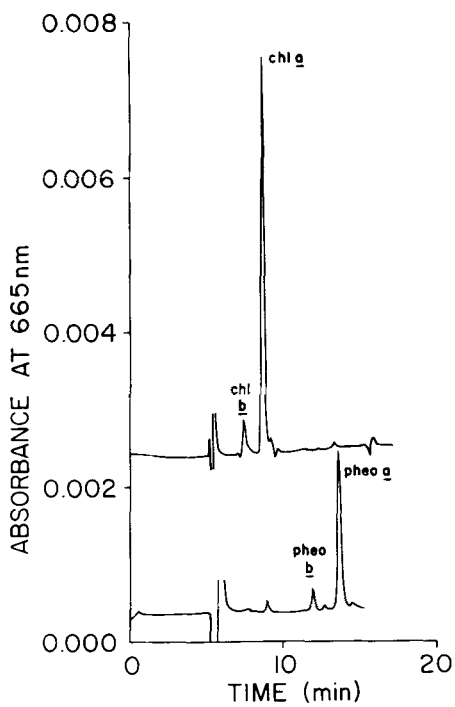


FIG. 3. Reversed phase HPLC chromatogram of a sample (top curve) prepared by diluting a fresh spinach extract to 5.0 ml with acetone-water (80:20) and of a sample (bottom curve) obtained by acid treatment (see Experimental) of a similar extract. Chromatographic conditions are as described in Figure 2, except the analytical wavelength is 665 nm and the injection size 20 μl.

TABLE II

Conversion of Chlorophylls to Pheophytins With Acid

Run	Sample	A_{665}	A_{649}	Spectral analysis[a] [chl A][c] (M X 10^6)	[chl B][c]	Pheo[c] Yield, %	HPLC analysis[b] Peak area (mm^2) chl B	chl A	pheo B	pheo A	Pheo A[c] Yield, %
1	Control	0.310	0.156	4.16	1.94		9.7	122.8	0	3.2	
1	Treated	0.199				100	0	3.2	7.2	71.4	89
2	Control	0.302	0.158	4.03	2.04		11.6	128.3		2.3	
2	Treated	0.209				84			9.0	66.2	78
3	Control	0.300	0.155	4.01	1.98			130.9			
3	Treated	0.204				86			8.4	84.0	100

[a]Solvents for the final samples were: acetone-water (80:20), Run 1; acetone-soybean oil C (80:20), Run 2; acetone-methanol (75:25), Run 3. Control samples contained 1 ml of an acetone extract containing chl A and chl B prepared from fresh spinach in a final volume of 5 ml. The procedure used for acid treatment (see Experimental) involved dissolving the acetone extract in hexane, HCl (9.2%) treatment, and manipulation to the same final 5 ml volume.

[b]Chromatographic conditions are the same as those given in Figure 2 except that the analytical wavelength was 665 nm and the injection size 20 µl. Retention times (min) of the identified peaks are: chl B (7.5); chl A (8.7); pheo B (11.5), and pheo A (13.3).

[c]These quantities were calculated using Equations 1a ([chl A]), 1b ([chl B]), 3 (pheophytin yield) and 4 (pheo A yield).

do not affect the derivatization reaction. The spectral and chromatographic results (Table II) indicate that the conversion of chl A to pheo A proceeds in high yield (about 90%), that little (0-4%) chl A remains unreacted, and that pheo A is not suffering further hydrolysis under these conditions (no HPLC evidence for the generation of more polar pigments).

When degummed soybean oil A was treated with acid according to this procedure, no spectral changes were observed; both the initial and acid treated samples had $A^{668} = 0.039$ (in acetone). Degummed soybean oil B behaved similarly: $A^{668}_{init} = 0.041$; $A^{668}_{final} = 0.039$ (in acetone). The maximum amount of chl A which might plausibly be undetected by this technique has been estimated as 1.4×10^{-7} M (about 15% of the estimated total pigments in oil A). This estimate was made by: 1) assuming a given precision for the spectral measurements; 2) approximating the total green pigments in the treated sample (calculated from the spectrum as pheo A) and assuming the same total for the initial sample, and 3) calculating the magnitudes of the spectral changes accompanying conversion of various quantities of chl A. In the chromatographic isolation of green pigments from oil A, therefore, it has been presumed that the oil contains negligible quantities of chl A, and other related magnesium-containing derivatives, thus no pretreatment step is used. Lack of detection of chl A in a degummed soybean oil has been observed previously (16).

Column chromatography, using either adsorbants or an ion exchange resin, has been evaluated as a potential method for isolating the pigments from the triglycerides of soybean oil. Table III summarizes a series of separations obtained on cellulose and silica gel adsorbants and on a cation exchange resin. A near quantitative (95%) recovery of green pigments is obtained when a small amount of oil (1.0 g) is chromatographed on a cellulose column using a cellulose/oil ratio of 30. Separation of pigments from the triglycerides is impaired (65% pigment recovery) with a larger sample size (5.0 g oil) and a cellulose/oil ratio of 6. Better resolution was obtained using a silica gel column. An 80% recovery of pigments was realized when 5.0 g of a degummed soybean oil was chromatographed on a 60 g silica gel column. Furthermore, a quantitative recovery of added pheo A from 5.0 g of a model oil was observed with a larger (100 g) column. The pheo A preparation used in this experiment contained, by HPLC analysis, a small amount of pheo A′. The pigments recovered, when reanalyzed by HPLC, were comprised of the same two components in similar proportions. There was no evidence that the silica gel chromatography had caused the formation of any new pigments. When the pigments isolated from a degummed soybean oil (Run 5, Table III) were concentrated and analyzed by HPLC, the chromatogram was complicated by a number of negative peaks. For this reason, the use of silica gel columns was abandoned. Several attempts were made to use an ion exchange resin (32) to achieve the desired results. Preliminary results (Run 8, Table III) suggested a high recovery of pigments, but further examination of a model oil containing added pheo A revealed the instability of pheo A to this procedure. HPLC of the recovered pigment fraction showed that only 19% of the original pheo A was present and a significant quantity of a more polar compound (possible pheophorbide A) was formed. The apparent discrepancy between the recovery noted in Runs 8 and 7 (Table III) probably is due to the fact that Run 7 included a step in which the column eluent (in hexane) was extracted with water. Compounds such as pheophorbide A have appreciable solubility in water (34). Ion exchange chromatography was not further pursued because of this problem. For these reasons, cellulose column chromatography was selected as the method for isolating green pigments from soybean oil for the purpose of preparing samples having pigment concentrations suitable for HPLC detection. The cellulose isolation step may either be used directly for soybean oils shown to be free of chl A (and similar Mg-containing pigments) or after acid treatment of oils containing chl A. This method has the advantages that most of the pigments (65%) can be separated from the triglycerides, that the concentrated samples do not show negative peaks on HPLC analysis, and that there is little likelihood that the pheophytin-type pigments are affected by this procedure. The latter point has not been studied directly herein. However, the results of analogous experiments with silica gel column chromatography are presumed to hold true for the milder adsorbant cellulose. The one drawback of the cellulose procedure, as practiced here, is the lack of quantitative pigment recovery. This shortcoming will have to be overcome in the development of a quantitative procedure.

HPLC analysis of the pigment fraction (vol. = 0.5 ml) isolated from degummed soybean oil A by cellulose column chromatography (Table III, Run 2) reveals the presence of about seven components (Fig. 4). Peaks 5 (pheo A), 6

TABLE III

Isolation of Green Pigments from Soybean Oils by Column Chromatography

Run	Oil	Oil amount (g)	Column	Absorbance[a]		Pigment recovery (%)[b]
				A_{init}	A_{Recov}	
1	A	1.0[d]	cellulose (30 g)	0.019[c]	0.018[c]	95
2	A	5.0[e]	cellulose (30 g)	0.050[e]	0.026[g]	62[f]
3	A	5.0[e]	cellulose (30 g)			70
4	A	3.0[e]	cellulose (40 g)	0.027[g]	0.023[g]	85
5	A	5.0[e]	silica gel (60 g)	0.045[g]	0.036[g,h]	80
6	C + pheo A[i]	2.0[d]	silica gel (100 g)	0.228[d,n]	0.230[d,n]	101 (97)[j]
7	C + pheo A[i]	5.0[k]	Dowex 50W-X4 (5 g)	0.688[l]	0.390[l]	57 (19)[j]
8	A	5.0[m]	Dowex 50W-X4 (5 g)	0.045[g]	0.036[g]	80

[a]The reported absorbance value has been corrected by subtracting from the observed value a "baseline" reading at the same wavelength. This "baseline" reading was determined graphically by drawing a line connecting the observed readings at 630 and 700 nm and noting the absorbance value of this line at the analytical wavelength. Except for Run 7, all of the solutions for which A_{init} values are determined contain the amount of oil shown in column 3. Since pheo A has similar A^{665} values in oil and in acetone solution, no spectral correction for the presence of oil is applied.

[b]Calculated as follows: Recovery (%) = $(100)(A_{recov})/A_{init}$.

[c]Solution volume = 5 ml ($CHCl_3$), λ = 668 nm.

[d]Solution volume = 5 ml (hexane).

[e]Solution vol. = 10 ml (hexane).

[f]Since control experiments showed that $A^{acet}_{665}/A^{oil}_{668}$ = 0.85 for pheo A in acetone and oil solution, the solvent dependent differences were corrected for as follows: Recovery (%) = $(100)(A_{recov})/(0.85)(A_{init})$.

[g]Solution vol. = 10 ml (acetone), λ = 665 nm.

[h]Sum of the absorbances of several sub-fractions.

[i]A portion of a pheo A stock solution was added (see Experimental).

[j]Values in parentheses are yields (%) determined by HPLC.

[k]Solution vol. = 25 ml (acetone).

[l]Solution vol. = 5 ml (acetone), λ = 665 nm.

[m]Solution vol. = 10 ml (acetone).

[n]λ = 670 nm.

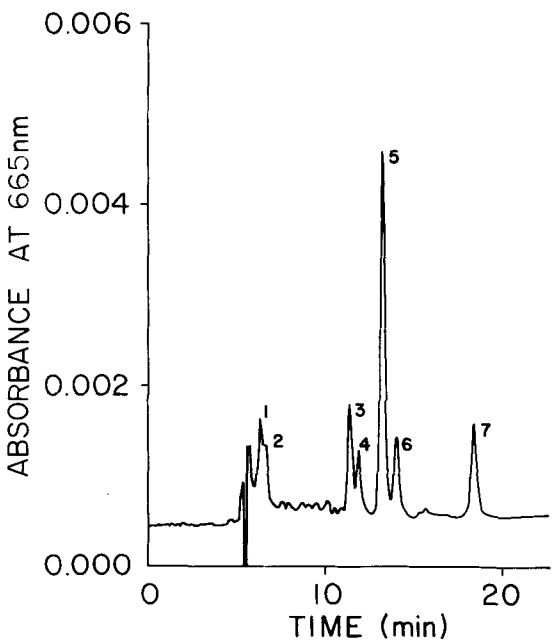


FIG. 4. Reversed phase HPLC chromatogram of the green pigment fraction (vol. = 0.5 ml, acetone solvent) isolated from 5.0 g of degummed soybean oil A by cellulose column chromatography (Table III, Run 2). The HPLC conditions are similar to those given for Figure 2, except that the analytical wavelength is 665 nm and the injection size 20 µl. Peak designations are: 1-4) unknown; 5) pheo A; 6) pheo A′, and 7) pyropheo A.

(pheo A′), and 7 (pyropheo A) each may be tentatively identified on the basis of retention time similarities with the corresponding pigment isolated from spinach. Peaks 1 and 2 are nearly unretained under these HPLC conditions and probably are relatively polar compounds. It seems possible that these might be pheophorbides. The pheo A degradation product observed during HPLC analysis of the pigment fraction recovered after ion exchange chromatography had a retention time similar to component 1. Since the ion exchange resin probably degraded pheo A by an acid catalyzed hydrolysis reaction, the presence of derivatives having a free carboxyl group (such as pheophorbide) would not be surprising. Components 3 and 4 have retention times similar to pheo B and pheo B′, respectively. However, further HPLC studies (see below) conducted at other analytical wavelengths are not consistent with these designations. Compounds 3 and 4 remain unidentified. The chromatogram shows no evidence of detectable quantities of either chl A or B. This observation is consistent with earlier results showing the lack of spectral change upon the treatment of this soybean oil with acid.

Since spectral evidence (15,16) suggests that pigments with a pheo A-type spectrum predominate in soybean oils, it was of interest to determine the spectral characteristics of each of the seven compounds detected by HPLC (Fig. 4). This was done by monitoring the HPLC separation of a similar sample at several key wavelengths. The results are shown in Table IV. All of the detected components (compound 4 is absent in this sample) have A_{665}/A_{650} values in the 2.8-3.9 range: values similar to that of pheo A (3.9) and dissimilar to pyropheo B (0.46), a compound having a similar spectrum to pheo B. Peaks 3, 5 and 6 have A_{410}/A_{650} = 7-11; again more in agreement with a pheo A-type (A_{410}/A_{650} = 9.3) than a pheo B-type (A_{410}/A_{650} = 1.9) spectrum. Values for compounds 1 and 2 were not obtainable because of interferences caused by co-elution of components absorbing strongly in the 410 nm region. These

TABLE IV

Effect of Analytical Wavelength on the Response of Isolated Pigments

Peak no.[a]	R_t (min)[b]	Component	Peak height (H,mm)			H_{410}/H_{650}[c]	H_{665}/H_{650}[c]
		λ(nm) =	410	650	665		
1	6.6		e	10	30		3
2	6.9		e	5	15		3
3	12.1		54	5	18	10.8	3.5
5	14.0	pheo A	d	26	101		3.9
6	14.9	pheo A′	63	6	20	10.5	3.3
7	19.6	pyropheo A	72	10	28	7.2	2.8

[a]The HPLC sample, in a final volume = 0.3 ml (acetone), was isolated by cellulose column chromatography from degummed soybean oil A (3.0 g). In this experiment, the fraction contained only 20% of the oil pigments and no peak 4 (peak designations are as in Fig. 3) was observed.

[b]Chromatographic conditions for the HPLC analysis are similar to those given in Figure 2 except that 20 μl injections and various analytical wavelengths were used.

[c]Pheo A (in acetone-methanol 75:25) has A_{410}/A_{650} = 9.3 and A_{665}/A_{650} = 3.9 whereas pyropheo B (in acetone-water 80:20) has A_{410}/A_{650} = 1.9 and A_{665}/A_{650} = 0.46.

[d]The HPLC peak was too large (off-scale) to measure in this run.

[e]Co-elution of strongly absorbing components interferes with these values.

TABLE V

Relative Amounts of Green Pigments Detected by HPLC

Chrom. no.	Area (sq. in.)[a] Peak[c] 1&2	3	4	5	6	7	$A_{pheo\ A}/A_{tot}$	[pheo A][b] (M × 10^5)
1[d]	0.08	0.07	0.05	0.24	0.06	0.09	0.41	1.07
2[d]	0.10	0.04		0.25	0.07	0.09	0.46	1.10
3[d]	0.05	0.03	0.01	0.12	0.04	0.04	0.41	0.53

[a]Calculated using a planimeter. The chromatographic run was monitored at 665 nm, and 20 μl injections were used; otherwise, conditions are as in Figure 2.

[b]Calculated from a calibration curve (see Fig. 5).

[c]Designations correspond to those of Figure 4.

[d]Each of these samples was isolated by cellulose column chromatography from degummed soybean oil A. The amount of oil chromatographed, the percentage of pigments recovered in the HPLC sample, and the volume of the HPLC sample are: 1) 5.0 g, 62%, 0.5 ml (Table III, Run 2); 2) 3.0 g, 20%, 0.3 ml, and 3) 5.0 g, 70%, 1.0 ml (Table III, Run 3).

results suggest that all of the detected compounds are spectrally similar to pheo A. Compound 3, which has a retention time similar to pheo B, does not have spectral characteristics consistent with this designation. All of the detected compounds are probably derived from either chl A or pheo A, which is itself a derivative of chl A. The observation of a group of pigments all spectrally similar to pheo A is consistent with earlier spectra of soybean oils (15,16), but requires a modification of the initial idea that only pheo A is present.

Pheo A is the predominant component present in isolates prepared from degummed soybean oil A and is responsible for 40-45% of the response measured at 665 nm. The relative amounts of the seven detected components are shown in Table V. Since the column chromatography step recovers only about 60-70% of the pigments present in this soybean oil, it is possible that the distribution in the oil may be quantitatively different. It is even possible that certain components in the oil might not have been present in the isolate. Although these possibilities cannot be entirely resolved at this point, it seems likely that their existence will not substantially alter the conclusions. This is supported by HPLC analysis of isolates prepared by silica gel column chromatography. Pigment recoveries in these cases were usually 80-90% and, although the HPLC chromatograms were complicated by negative peaks, the basic patterns noted in Figure 4 (and elsewhere) were perceptible with no additional components being observed.

A first step towards obtaining quantitative estimates of the amounts of green pigments detected has been taken in the case of pheo A. The relationship between the concentration of pheo A in a pheo A preparation isolated from spinach and the magnitude of its HPLC peak under specified conditions has been obtained. Figure 5 shows a calibra-

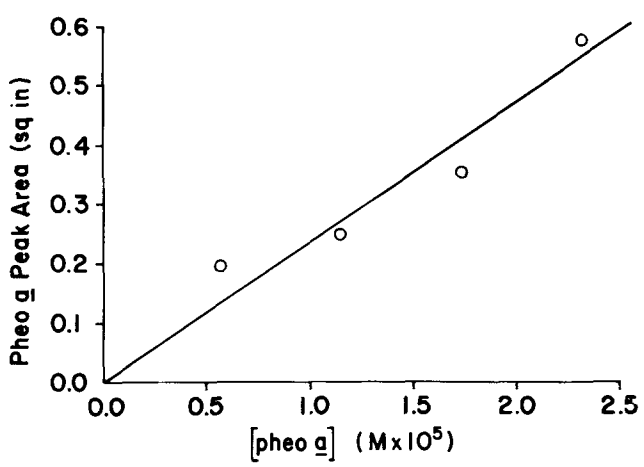


FIG. 5. Calibration curve relating the spectrophotometrically determined concentration of pheo A to the area of its peak on HPLC analysis. The pheo A preparation was isolated from spinach and contained, by HPLC analysis, about 90% pheo A and 10% pheo A′. The spectrally determined concentration of pheo A is corrected for the pheo A′ impurity. HPLC conditions are the same as given for Figure 2, except the analytical wavelength is 665 nm and the injection size 20 μl.

tion curve assembled from the data. There is a straight line relationship between these quantities over the conentration range studied. The pheo A concentrations of several samples studied by HPLC are given in Table V. Development of a quantitative procedure for all the components requires the identification of each detected component, the refinement of the chromatographic isolation step so that quantitative pigment recovery is achieved, and the accomplishment of a series of recovery studies designed to ensure that there is no interconversion of pigments during isolation or handling.

The practical significance of the presence of a number of different chlorophyll derivatives in degummed soybean oil cannot be fully estimated at present. From a bleaching efficiency point of view, it would be of interest to determine the relative sensitivities of each of the pigments to conventional bleaching treatments. From an oil stability view, it is interesting to note the recent findings of Endo et al. (35) that derivatives such as pheo A and pheophorbide A may be more prooxidant than chl A in the photooxidation of methyl linoleate.

REFERENCES

1. Rawls, M.R., and P.J. van Santen, JAOCS 47:121 (1970).
2. Clements, A.H., R.H. van den Engh, D.J. Frost and K. Hoogenhout, JAOCS 50:325 (1973).
3. Carlsson, D.J., T. Suprunchuk and D.M. Wiles, JAOCS 53:656 (1976).
4. Sattar, A., and J.M. Deman, JAOCS 53:473 (1976).
5. Chan, H.W.-S., JAOCS 54:100 (1977).
6. Terao, J., and S. Matsushita, *JAOCS 54:234 (1977).*
7. Frankel, E.N., W.E. Neff and T.R. Bessler, Lipids 14:961 (1979).
8. Terao, J., and S. Matsushita, J. Food Processing and Preservation 3:329 (1980).
9. Terao, J.; R. Yamauchi, H. Murakami and S. Matsushita, J. Food Processing and Preservation 4:79 (1980).
10. Holden, M., in "Chemistry and Biochemistry of Plant Pigments," 2nd edition, edited by T.W. Goodwin, Vol. 2, Academic Press, New York, 1976, pp. 1-37.
11. Vernon, L.P., Anal. Chem. 32:1144 (1960).
12. White, R.C.; I.D. Jones and E. Gibbs, J. Food Sci. 28:431 (1963).
13. White, R.C.; I.D. Jones, E. Gibbs and L.S. Butler, J. Agr. Food Chem. 20:773 (1972).
14. AOCS Official and Tentative Methods, American Oil Chemists' Society, Champaign, IL, 1973, Method Cc 13d-55.
15. Pritchett, W.C.; W.G. Taylor and D.M. Carroll, JAOCS 24:225 (1947).
16. O'Connor, R.T.; E.T. Field, M.E. Jefferson and F.G. Dollear, JAOCS 26:710 (1949).
17. Mackinney, G., J. Biol. Chem. 132:91 (1940).
18. Wilson, J.R.; M-D. Nutting and G.F. Bailey, Anal. Chem. 34:1331 (1962).
19. Wasielewski, M.R., and W.A. Svec, J. Org. Chem. 45:1969 (1980).
20. Pennington, F.C.; H.H. Strain, W.A. Svec and J.J. Katz, J. Amer. Chem. Soc. 86:1418 (1964).
21. Strain, H.H., and W.A. Svec, in "The Chlorophylls," L.P. Vernon and G.R. Seely, eds., Academic Press, New York, NY, 1966, p. 21.
22. Strain, H.H.; M.R. Thomas and J.J. Katz, Biochim. Biophys. Acta 75:306 (1963).
23. Sievers, G., and P.H. *Hynninen, J. Chrom. 134:359 (1977).*
24. Shiraki, M.; M. Yoshiura and K. Iriyama, Chem. Lett. 103 (1978).
25. Yoshiura, M.; K. Iriyama and M. Shiraki, Chem. Lett. 281 (1978).
26. De Jong, D.W., and W.G. Woodlief, J. Agric. Food Chem. 26: 1281 (1978).
27. Rebeiz, C.A.; M.B. Bazzaz and F. Belanger, Chrom. Review 4:8 (1978).
28. Liebezeit, G., J. High Resolution Chrom. and Chrom. Comm. 3:531 (1980).
29. Schwartz, S.J.; S.L. Woo and J.H. von Elbe, J. Agric. Food Chem. 29:533 (1981).
30. Braumann, T., Biochim. Biophys. Acta 637:8 (1981).
31. Norris, F.A., and K.F. Mattil, in "Bailey's Industrial Oil and Fat Products," 3rd Ed., edited by D. Swern, John Wiley and Sons, New York, NY, 1964.
32. Wilson, J.R., and M.-D. Nutting, Anal. Chem. 35:144 (1963).
33. Niewiadomski, H.; I. Bratkowska and E. Mossakowska, JAOCS 42:731 (1965).
34. Aronoff, S., in "Adv. in Food Research," Vol. IV, edited by E.M. Mrak and G.F. Stewart, Acad. Press, New York, NY, 1953, pp. 133-184.
35. Endo, Y.; R. Usuki and T. Kaneda, JAOCS 61:781 (1984).

[Received August 14, 1984]