Estimation of Zinc Pheophytins, Chlorophylls, and Pheophytins in Mixtures in Diethyl Ether or 80% Acetone by Spectrophotometry and Fluorometry

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Procedures are described for the estimation of any one or more of the components present in mixtures in diethyl ether or 80% acetone comprised of the pigments chlorophylls a and b, pheophytins a and b, and the metallo-pigment complexes, zinc pheophytins a and b. Methods are described for component estimation from equations derived for spectrophotometric and fluorometric analyses. Fluorometric estimation may be made at concentrations of 1×10^{-2} of those required for spectrophotometric estimation.

The presence of zinc complexes in the pigment of certain processed foods has been reported by a number of investigators (Jones et al., 1972). The spectral curves of zinc pheophytins a and b have been published and the similarity between these curves and those for the chlorophylls a and b, respectively, have been indicated (Fishbach and Newburger, 1943; Jones et al., 1968). The observed spectral similarity of the zinc pheophytins and the chlorophylls has prevented the direct estimation of these pigments in mixtures from absorbances at their absorption maxima. Studies of the estimation of the chlorophylls and the pheophytins in mixtures by spectrophotometry have been reported by Vernon (1960) and White et al. (1963). Similar studies of the estimation of these pigments by fluorometry have been reported by White et al. (1972).

The thin-layer chromatographic identification of zinc complexes in pigment mixtures (Jones et al., 1972) demonstrated a greater acid stability of zinc pheophytins than of the chlorophylls. From this observation it was recognized that the differential stability of the zinc complexes and the chlorophylls in acidified solutions might provide a means for either the spectrophotometric or the fluorometric estimation of zinc pheophytins, chlorophylls, and pheophytins in mixtures in diethyl ether or 80% acetone. This is a report of studies conducted for estimation of the different pigments mentioned above in mixtures.

EXPERIMENTAL SECTION

Solutions of the purified chlorophylls, pheophytins, and the zinc pheophytins were prepared as previously described (Jones et al., 1968). Absorptivities of zinc pheophytins a and b in 80% acetone were established from their absorptivities in diethyl ether and their absorbances in both solvents at the same concentration. For the fluorometric methods the relative fluorescence intensity values for the pigments in diethyl ether and 80% acetone were determined according to the procedure of White et al. (1972).

Standard solutions of the pure pigments in diethyl ether and 80% acetone were prepared for reference. The concentration of each pigment standard was calculated from its absorbance and absorptivity. Model solutions or working standards of pigment mixtures were made by adding aliquots of the pigments to volumetric flasks and making to volume with diethyl ether or 80% acetone. The concentration of these model solutions, designated samples, was at a level 2.5 times that suitable for spectrophotometric analysis.

To convert the chlorophylls but not the zinc pheophytins to pheophytins, phosphoric acid was added to the diethyl ether solutions and oxalic acid was added to 80% acetone solutions of the pigments. To convert chlorophylls and zinc pheophytins to pheophytins, hydrochloric acid was added to both the diethyl ether and the acetone solutions. Outlined in Charts I and II are the schemes followed for dilution and acid treatment of pigment solutions given coincident with spectrophotometric and fluorometric analysis according to directions specified.

Spectral curves of the mixtures were read on a Beckman DK-2A spectrophotometer. Precautions were taken in making these readings as outlined for the spectrophotometric estimation of the copper complexes (White et al., 1977). The fluorometric estimation of the chlorophylls, pheophytins, and zinc pheophytins was based upon the

Chart I. Scheme for Spectrophotometric Estimation of Pigments in Mixtures in Diethyl Ether and Acetone (80%)

Spectrophotometric Method

Solution 1
- Treatment:
  - With either diethyl ether or 80% acetone as solvent dilute 10 ml of sample[a] to 25 ml. Designate diethyl ether solution as S1E. Run spectral curve for S1E and read at 660.4 and 642.0 nm. Designate 80% acetone solution as S1A. Run spectral curve for S1A and read at 664.0 and 646.5 nm.
- Mixture may contain chlorophylls a and b, C_a and C_b; pheophytins a and b, Py_a and Py_b; zinc pheophytins a and b, $ZnPy_a$ and $ZnPy_b$.

Solution 2
- Treatment:
  - With diethyl ether as solvent, to 10 ml of sample add 0.05 ml of 1.48 M H_3PO_4, let stand 90 min, dilute to 25 ml. Designate as S2E. Run spectral curve for S2E and read at 660.4, 642.0, 653.9, and 635.3 nm.
  - With 80% acetone as solvent, to 10 ml of sample add 1 ml of 0.5 M oxalic acid in acetone, let stand 30 min, dilute to 25 ml. Designate as S2A. Run spectral curve for S2A and read at 664.0, 646.5, 659.0, and 642.0 nm.
- Mixture will contain pheophytins originally present and pheophytins formed from chlorophylls originally present and will contain zinc pheophytins if originally present.

Solution 3
- Treatment:
  - With diethyl ether as solvent, to 10 ml of sample add 0.05 ml of 12 N HCl, let stand 30 min, dilute to 25 ml, add Na_2SO_4, and mix. Designate as S3E. Run spectral curve for S3E and read at 653.9, 635.3, 667.2, and 654.4 nm.
  - With 80% acetone as solvent, to 10 ml of sample add 0.32 ml of 12 N HCl, let stand 30 min, add 0.23 ml of ethanolamine, dilute to 25 ml. Designate as S3A. Run spectral curve for S3A and read at 659.0, 642.0, 665.8, and 653.4 nm.
- Mixture will contain the total pheophytins, Py_at and Py_bt, representing chlorophyll conversion and zinc conversion products plus original pheophytins.

[a] The term sample designates the model solution prepared for analysis.

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Chart II. Scheme for Fluorometric Estimation of Pigments in Diethyl Ether and Acetone (80%)

Fluorometric Method[a]

Solution 1
Dilute aliquots of S1E and S1A 100 times. Designate as S1EF and S1AF, respectively. Read with filter systems 440, 460, and 405.

Solution 2
Dilute aliquots of S2E and S2A 100 times. Designate as S2EF and S2AF, respectively. Read with filter systems 440, 460, and 405.

Solution 3
Dilute aliquots of S3E and S3A 100 times. Designate as S3EF and S3AF, respectively. Read with filter systems 440, 460, and 405.

[a] Filter systems: 440—at primary, interference, nominally 440; at secondary, interference, nominally 650 + #2-60[b]; aperature 3×; 460—at primary, interference, nominally 460; at secondary, interference, nominally 650 + #2-60[b]; aperature 10×; 405—at primary, interference, nominally 405; at secondary, interference, nominally 680 + #2-60[b]; aperature 10×. [b] #2-60 designates Corning Series, glass, sharp cut, transmission less than 0.5% at 599 nm, greater than 37% at 619 nm.

Table I. Absorptivities (Micromoles^{-1} Centimeter^{2}) of Chlorophylls, Pheophytins, and Zinc Pheophytins in Diethyl Ether

Pigment[a]	Max C_a, 660.4 nm	Max C_b, 642.0 nm	Max Py_a, 667.2 nm	Max Py_b, 654.4 nm	Max $ZnPy_a$, 653.9 nm	Max $ZnPy_b$, 635.3 nm
C_a	90.3	13.2				
C_b	3.86	59.0				
Py_a	37.0	5.21	55.9	17.0	15.9	4.61
Py_b	23.5	10.4	7.24	36.7	36.6	4.53
$ZnPy_a$					90.3	13.2
$ZnPy_b$					3.73	60.2

[a] Symbols used are: C_a, chlorophyll a; C_b, chlorophyll b; Py_a, pheophytin a; Py_b, pheophytin b; $ZnPy_a$, zinc pheophytin a; $ZnPy_b$, zinc pheophytin b.

procedure by White et al. (1972).

RESULTS AND DISCUSSION

To permit the fluorometric estimation of the zinc pheophytins the relative fluorescence intensity values for the pigments in diethyl ether and 80% acetone were determined as described for the chlorophylls and pheophytins by White et al. (1972). Equations for estimation of the zinc pheophytins were developed from the above-mentioned relative fluorescence intensity values in a manner similar to that of White et al. (1972). Findings based on these fluorometric observations will be discussed later.

Shown in Table I are the absorptivities in diethyl ether for the chlorophylls and pheophytins as reported by White et al. (1977) and those for the zinc pheophytins as reported by Jones et al. (1968). In Table II are presented the corresponding absorptivities for the chlorophylls, pheophytins, and zinc pheophytins in 80% acetone, as determined in this laboratory.

Chlorophyll a and b concentrations in samples in diethyl ether were spectrophotometrically estimated from the changes in readings of spectral curves of solutions 1 (S1E) and 2 (S2E) described in Chart I at the chlorophyll a and chlorophyll b peak points, respectively, a procedure suggested by a study by Vernon (1960). Equations 1 and 2 for the estimation of chlorophylls a and b (White et al., 1977) are as follows.

Concentrations of zinc pheophytin a and b in the sample were calculated from the changes in readings at the zinc

Table II. Absorptivities (Micromoles^{-1} Centimeter^{2}) of Chlorophylls, Pheophytins, and Zinc Pheophytins in 80% Acetone

Pigment[a]	Max C_a, 664.0 nm	Max C_b, 646.5 nm	Max Py_a, 665.8 nm	Max Py_b, 653.4 nm	Max $ZnPy_a$, 659.0 nm	Max $ZnPy_b$, 642.0 nm
C_a	77.0	17.6				
C_b	9.05	46.9				
Py_a	45.3	8.81	46.4	18.5	33.7	6.42
Py_b	12.4	20.1	9.36	31.0	23.3	11.9
$ZnPy_a$					77.3	18.4
$ZnPy_b$					8.63	51.3

[a] Symbols used are: C_a, chlorophyll a; C_b, chlorophyll b; Py_a, pheophytin a; Py_b, pheophytin b; $ZnPy_a$, zinc pheophytin a; and $ZnPy_b$, zinc pheophytin b.

$$[C_a] = 17.7\Delta A^{660.4} + 7.15\Delta A^{642.0} \quad (1)$$

$$[C_b] = 19.4\Delta A^{642.0} - 2.92\Delta A^{660.4} \quad (2)$$

pheophytin a and b spectral peaks as obtained from spectral curves of solutions S2E and S3E (Chart I) according to the procedure discussed for the chlorophylls. The following equations were derived from absorptivities in Table I:

$$[ZnPy_a] = 12.6\Delta A^{653.9} + 7.43\Delta A^{635.3} \quad (3)$$

$$[ZnPy_b] = 16.8\Delta A^{635.3} - 1.94\Delta A^{653.9} \quad (4)$$

The concentrations of total pheophytins, designated Py_at and Py_bt in solution S3E (Chart I), representing the original quantities of pheophytins present and in addition those from chlorophylls and zinc pheophytins formed by solution acidification, were calculated from the readings of the spectral curve of solution S3E (Chart I) at the pheophytin a and pheophytin b peak points. The following equations were derived from the absorptivities in Table I:

$$[Py_at] = 19.0A^{667.2} - 3.75A^{654.4} \quad (5)$$

$$[Py_bt] = 29.0A^{654.4} - 8.82A^{667.2} \quad (6)$$

The free pheophytin concentrations in the original solution, S1E (Chart I), were calculated from the following equations:

$$[Py_a] = [Py_at] - [C_a] - [ZnPy_a] \quad (7)$$

$$[Py_b] = [Py_bt] - [C_b] - [ZnPy_b] \quad (8)$$

Directions for the analysis of 80% acetone solutions of the pigments under investigation are included in the scheme in Chart II. Acidification of 80% acetone solution aliquots with HCl in the amounts specified (solution 3, Chart I) was found to cause erroneous spectrophotometric readings of pheophytin a, due to a shift of about 2 nm in λ_{max}. Further study indicated that neutralization of the acid in the acetone solution after the conversion effectively avoided this error. Ethanolamine was added to neutralize the HCl.

The following equations (eq 9–16) were derived for the spectrophotometric estimation of pigments in 80% acetone in a manner similar to that described for derivation of equations for estimation of pigments in diethyl ether. Equations 9 through 14 were derived from the absorptivities in Table II.

The recovery data from the spectrophotometric and fluorometric analysis of pigment mixtures in diethyl ether are presented in Table III. The overall conclusion drawn from a review of the statistics presented was that the proposed procedures proved to be very satisfactory for the

Table III. Estimation of Zinc Pheophytins by Spectrophotometry and Fluorometry[a]

Pigment and method[b]		a (intercept)[c]	b (slope)[c]	R^2	Pigment concn[d] Range	Recovery, %
$ZnPy_a$	S	−0.231**	1.021**	0.999	(3.27-8.43)	97 ± 2
	F	0.926*	0.767**	0.953	(3.27-8.43) × 10^{-2}	96 ± 9
$ZnPy_b$	S	−0.156*	1.014**	0.997	(1.44-3.38)	94 ± 3
	F	0.487**	0.595**	0.932	(1.44-3.38) × 10^{-2}	83 ± 10
C_a	S	0.021NS	0.988**	0.999	(1.0-4.30)	100 ± 1
	F	0.022NS	1.002**	0.999	(1.0-4.30) × 10^{-2}	101 ± 1
C_b	S	0.001NS	0.968**	0.999	(0.5-2.59)	97 ± 0
	F	0.036*	0.885**	0.999	(0.5-2.59) × 10^{-2}	94 ± 2
Py_at	S	0.788**	0.923**	0.995	(8.08-10.77)	101 ± 1
	F	0.110NS	0.980**	0.954	(8.08-10.77) × 10^{-2}	99 ± 0
Py_bt	S	0.831*	0.848**	0.950	(3.38-4.93)	106 ± 4
	F	−0.059NS	1.0150**	0.917	(3.38-4.93) × 10^{-2}	100 ± 0
Py_a	S	0.284**	0.961**	0.999	(1.0-5.97)	113 ± 12
	F	0.431NS	0.886	0.949	(1.0-5.97) × 10^{-2}	114 ± 18
Py_b	S	0.398**	0.995**	0.997	(0.5-3.26)	145 ± 33
	F	0.835**	0.820**	0.949	(0.5-3.26) × 10^{-2}	219 ± 111

[a] Recovery data from mixtures of pure pigments in diethyl ether. Statistics for regression of amount recovered (Y) upon amount added (X). Regression equation = $\hat{Y} = a + bX$. Regressions are based on 12 X-Y pairs. [b] Method: S = spectrophotometric; F = fluorometric. [c] NS indicates nonsignificance; * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level. [d] Pigment concentration expressed in micromoles per liter.

Table IV. Estimation of Zinc Pheophytins by Spectrophotometry and Fluorometry[a]

Pigment and method[b]		a (intercept)[c]	b (slope)[c]	R^2	Pigment concn[d] Range	Recovery, %
$ZnPy_a$	S	0.106NS	0.936**	0.998	3.96-8.08	95 ± 1
	F	0.538NS	0.762**	0.938	(3.96-8.08) × 10^{-2}	86 ± 4
$ZnPy_b$	S	0.679*	0.612**	0.728	1.99-3.19	89 ± 7
	F	1.256**	0.393*	0.448	(1.99-3.19) × 10^{-2}	90 ± 11
C_a	S	0.000NS	0.956**	0.999	1.00-4.17	96 ± 0
	F	0.000NS	0.907**	0.980	(0.00-4.17) × 10^{-2}	91 ± 0
C_b	S	0.000NS	0.963**	0.998	0.50-2.12	96 ± 0
	F	0.000NS	0.982**	0.971	(0.00-2.12) × 10^{-2}	98 ± 0
Py_at	S	8.280*	−0.022NS	0.000	7.77-8.17	101 ± 3
	F	8.482**	−0.032NS	0.001	(7.77-8.17) × 10^{-2}	103 ± 3
Py_bt	S	0.626NS	0.879**	0.899	3.18-4.12	105 ± 3
	F	0.596NS	1.218**	0.866	(3.18-4.12) × 10^{-2}	137 ± 2

[a] Recovery data from mixtures of pure pigments in acetone (80%). Statistics for regression of amount recovered (Y) upon amount added (X). Regression equation = $\hat{Y} = a + bX$. Regressions are based on 12 X-Y pairs. [b] Method: S = spectrophotometric; F = fluorometric. [c] NS indicates nonsignificance; * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level. [d] Pigment concentration expressed in micromoles per liter.

$$[C_a] = 30.5\Delta A^{664.0} + 3.81\Delta A^{646.5} \quad (9)$$

$$[C_b] = 36.1\Delta A^{646.5} - 10.0\Delta A^{664.0} \quad (10)$$

$$[ZnPy_a] = 20.8\Delta A^{659.0} + 7.75\Delta A^{642.0} \quad (11)$$

$$[ZnPy_b] = 23.0\Delta A^{642.0} - 6.33\Delta A^{659.0} \quad (12)$$

$$[Py_at] = 24.5A^{665.8} - 7.40A^{653.4} \quad (13)$$

$$[Py_bt] = 36.7A^{653.4} - 14.6A^{665.8} \quad (14)$$

$$[Py_a] = [Py_at] - [C_a] - [ZnPy_a] \quad (15)$$

$$[Py_b] = [Py_bt] - [C_b] - [ZnPy_b] \quad (16)$$

estimation of the various components of the model mixtures with the exception of those for Py_a and Py_b. The high R^2 values indicated very good agreement of the results in the estimation of a given pigment by a specified method.

As indicated by eq 7 and 8, the values for Py_a and Py_b, respectively, may be in considerable error due to errors in the estimation of the corresponding zinc pheophytins, chlorophylls, or total pheophytin components. For example, the observed large over-recovery value for Py_b may be explained by the somewhat low recoveries of $ZnPy_b$, C_b, and, for the spectrophotometric procedure in particular, a slightly high recovery of total pheophytin b (Py_bt).

The recovery data of pigment components in 80% acetone solution are presented in Table IV. These data clearly indicated that the procedures described provided a reasonably good method for distinguishing between the chlorophylls a and b and the zinc pheophytins a and b in mixtures in 80% acetone. In this study free pheophytins a and b were not added to the pigment mixtures to be analyzed. Therefore, there were no values for Py_a and Py_b in Table IV.

Attention is directed to the regression line equations in Table IV for the pigment component designated Py_at. These lines had high intercepts, very small and negative slopes, and very small R^2 values. The explanation for these atypical equations was considered to be related to the experimental procedures followed. The purpose of this study at the time it was initiated was to investigate the possibility of estimating zinc pheophytins and the chlorophylls in mixtures. The pigment solutions ranges were selected specifically to fall in those ranges which were roughly optimal for the spectrophotometric estimation of chlorophyll a and zinc pheophytin a or of chlorophyll b and zinc pheophytin b. For this reason the Py_at components, in particular, of solution 3 (Chart I) were present at a relatively high level which spanned only a very narrow range. The range for Py_at was, in fact, so limited that regression coefficients obtained represent the slope of a line through a cluster of observations at the upper limit of the range at which this pigment might be estimated.

Table V. Results of Tests for Significance of Differences between Slopes for Different Analytical Procedures[a]

Pigment	Mean square diff between slopes (1)	Pooled within error mean squares (2)	$F = (1)/(2)$[b]
	Diethyl Ether		
$ZnPy_a$	1.6288	0.0920	17.70NS
$ZnPy_b$	0.9337	0.0194	48.13*
C_a	0.0039	0.0035	1.11NS
C_b	0.0432	0.0006	72.00**
Py_at	0.0187	0.0353	0.53NS
Py_bt	0.0718	0.0302	2.38NS
Py_a	0.1724	0.1689	1.02NS
Py_b	0.1545	0.0414	3.73NS
	80% Acetone		
$ZnPy_a$	0.6312	0.0841	7.51*
$ZnPy_b$	0.1180	0.0816	1.45NS
C_a	0.0546	0.0408	1.34NS
C_b	0.0021	0.0813	0.11NS
Py_at	0	0.0234	0 NS
Py_bt	0.0949	0.0262	3.62NS

[a] Pigments in diethyl ether and 80% acetone. [b] NS indicates nonsignificance; * indicates significance at the 0.05 level; ** indicates significance at the 0.01 level.

To evaluate further the instrumental methods of analysis, comparisons were made of the regression coefficients for each pigment based on estimation by spectrophotometry and fluorometry. The results of these comparisons are shown in Table V. With the exception of the difference in slopes for $ZnPy_b$ and C_b in diethyl ether and $ZnPy_a$ in 80% acetone, all differences were nonsignificant. Therefore, the conclusion was drawn that the proposed fluorometric procedure (F) was essentially as reliable as the spectrophotometric method (S) and was suitable for estimation of zinc complexes in diethyl ether and in 80% acetone solutions in a range of concentrations 1/100 that required for spectrophotometric procedures. The large F value for C_b in diethyl ether as shown in Table V is highly significant because of the very small value for "pooled within error mean square" rather than because of a large value for "mean square difference between slopes".

No attempt has been made to estimate pheophorbides, chlorophyllides, and the zinc pheophorbides in mixtures. If proper precautions were taken, the authors would expect that these components of mixtures containing nonphytylated pigment could be estimated with reasonable accuracy by the procedure previously published (White et al., 1963). Pheophorbides are highly subject to loss by adsorption on the laboratory glassware and precautions against such loss should be followed, as previously set forth by White et al. (1972). Also, as indicated earlier, pheophorbides in mixtures subjected to analysis for pheophytins will appear as pheophytins unless the pigment mixture is extracted for pheophorbide removal before the pheophytin estimation step.

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Experimental Formation of Zinc and Copper Complexes of Chlorophyll Derivatives in Vegetable Tissue by Thermal Processing

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Zinc and copper complexes of the pigments pheophytins a and b and/or pheophorbides a and b were experimentally formed in green vegetable tissue by prescribed thermal treatments after the addition of zinc or copper salts. Estimates of the pigment components present in 80% acetone extracts of the vegetable tissue before and after treatment are presented. Procedures for such estimates were based on spectrophotometry if copper complexes were absent or on a combined spectrophotometric–fluorometric method if copper complexes were present. The concentration levels for zinc and copper salts at which appreciable complexing occurred were about 10 and 1 μmol per μmol of total pigment, respectively.

The intentional formation of zinc or copper complexes in vegetable tissue to which zinc or copper salts were added has been observed. Fishbach and Newburger (1943) and Fishbach (1943) reported the presence of a zinc-containing pigment in canned okra to which a weak solution of zinc chloride had been added during the can-filling operation prior to thermal treatment. The purpose of the addition of zinc chloride was to reduce or prevent the loss of the typical green color of fresh okra during canning. Schanderl et al. (1965) observed regreening of some stored, thermally processed lots of commercially preserved vegetables. They investigated the experimental formation of zinc and copper

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