mended before values can be considered reliable. Our results suggest that the modified reaction retained the basic characteristics described in the method of Spies and Chambers (1948) except that the fast progress of the reaction makes it more suitable for automated rather than for manual analysis.

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Estimation of Copper Pheophytins, Chlorophylls, and Pheophytins in Mixtures in Diethyl Ether

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A method is described for the estimation of any one or more of the components in mixtures in diethyl ether comprised of the pigments chlorophylls a and b, pheophytins a and b, and the metallo-pigment complexes, copper(II) pheophytins a and b. Estimations are based on calculations from equations derived for a combined spectrophotometric–fluorometric procedure. Calculation adjustments are described which permit estimation of all components by spectrophotometry.

Copper(II) pheophytins and/or copper(II) pheophorbides may be pigment components in processed food and may also be products in living organisms under certain conditions, as indicated by Jones et al. (1972) who have described procedures for the detection of these copper complexes by thin-layer techniques. Procedures for estimation of the chlorophylls and pheophytins in mixtures have been described by Vernon (1960) and White et al. (1963) based on spectrophotometry and by White et al. (1972) based on fluorometry. This is a report of studies of the estimation of the copper(II) pheophytins in mixtures with chlorophylls and pheophytins using spectrophotometry and fluorometry.

EXPERIMENTAL SECTION

Standard solutions in diethyl ether were prepared from the chlorophylls, pheophytins, and copper(II) pheophytins which had been purified as previously described by Jones et al. (1968). The concentration of each pigment standard was calculated from its absorbance and absorptivity. Working standards of pigment mixtures were made by adding aliquots of the six pigments to a volumetric flask and making it to volume. In the working standards, the concentration ratios of the a and b components were maintained at approximately 2:1 and 4:1, respectively. About one-third of the total pigments were the copper complexes.

Spectral curves of the mixtures were read on a Beckman DK-2A spectrophotometer. Measurements were made before and after acidification to convert chlorophylls to pheophytins (0.10 ml of 12 N HCl/50 ml). Acidified samples were permitted to stand 2 h at room temperature in the dark and dried with Na_2SO_4 before reading.

The wavelength maximum, λ_{max}, of each pigment was determined in this laboratory. The wavelength calibration was checked with each run using a hydrogen emission line at 656.3 nm as a reference. Corrections were made when necessary. The effect of acidifying solutions of pheophytins a and b and solutions of copper pheophytins a and b was studied. Absorbances were read at chlorophyll a and b peaks only for samples before acidification and at the peaks of all pigments following acid addition. The absorbance of each mixture was the average of three readings.

The fluorometric characteristics of the copper(II) pheophytins were investigated.

RESULTS AND DISCUSSION

Shown in Table I are the wavelength maxima and the absorptivities of the six pigments. From these values equations were derived for spectrophotometric estimation of each pigment in the mixtures, as described below.

Chlorophyll a and b concentrations in samples in diethyl ether were estimated from the change in the absorbance at the chlorophyll a and chlorophyll b peaks, respectively, a procedure suggested by a study by Vernon (1960). At the chlorophyll a peak wavelength of 660.4 nm the absorbance change is designated $\Delta A^{660.4}$ and may be calculated as follows: $\Delta A^{660.4} = A_u^{660.4} - A_c^{660.4}$, where the symbols A_u and A_c represent absorbances at the specified peak point of the unconverted and converted samples, respectively, that is before and after acidification. Similarly, the absorbance change at the chlorophyll b absorption peak (642.0 nm) may be indicated by $\Delta A^{642.0} = A_u^{642.0} - A_c^{642.0}$. The absorbances may be expressed in terms of the concentrations of each of the absorbing species:

$$A^{\lambda} = \sum_{i=1}^{n} \epsilon_i^{\lambda} b c_i$$

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Table I. Absorptivities (Micromoles^{-1} Centimeter^2) of Chlorophylls, Pheophytins, and Copper(II) 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 Cu(II)Py_a, 649.8 nm	Max Cu(II)Py_b, 628.2 nm
C_a	90.3	13.2				
C_b	3.86	59.0				
Py_a	37.0	5.21	55.9	17.0	9.67	4.31
Py_b	23.5	10.4	7.24	36.7	28.2	3.02
$CuPy_a$			17.5	59.6	67.9	14.4
$CuPy_b$			1.30	3.43	5.58	49.8

[a] Symbols used are: C_a = chlorophyll a; C_b = chlorophyll b; Py_a = pheophytin a; Py_b = pheophytin b; Cu(II)Py_a = copper(II) pheophytin a; Cu(II)Py_b = copper(II) pheophytin b.

A^λ is the absorbance at the specified wavelength, λ; ϵ_i^λ is the absorptivity in micromoles^{-1} centimeter^2 for component i at λ; b is the cell thickness, which is 1 cm in all cases; c_i is the millimoles/liter concentration of the component i; and n is the number of components. Substituting for the absorbances of the unconverted and converted samples, an expression for $\Delta A^{660.4}$ and $\Delta A^{642.0}$ may be obtained in terms of the concentrations of chlorophylls a and b. Using the absorptivities in Table I, the following equations were derived for the spectrophotometric estimation of all pigments:

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

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

where the concentrations are expressed in micromoles/liter. [Symbols used are: C_a = chlorophyll a; C_b = chlorophyll b; Py_a = pheophytin a; Py_b = pheophytin b; $CuPy_a$ = copper(II) pheophytin a; $CuPy_b$ = copper(II) pheophytin b; Py_at = Py_a + C_a (Py_at = original Py_a + Py_a formed from C_a upon acidification); Py_bt = Py_b + C_b (Py_bt = original Py_b + Py_b formed from C_b upon acidification).]

The copper pheophytins and the pheophytins were not changed by the acidification treatments followed in this study. Therefore, the concentrations of copper(II) pheophytins, total pheophytins, and free pheophytins in mixtures were estimated following solution acidification by eq 3 through 8 as shown. These equations were subjected to experimental verification and modification, as discussed later. All concentrations are expressed as micromoles/liter.

$$[CuPy_a] = 45.6A^{649.8} - 2.75A^{628.2} + 3.10A^{667.2} - 35.4A^{654.4} \quad (3)$$

$$[CuPy_b] = -8.46A^{649.8} + 20.7A^{628.2} - 1.69A^{667.2} + 5.13A^{654.4} \quad (4)$$

$$[Py_at] = -4.89A^{649.8} + 0.0549A^{628.2} + 18.7A^{667.2} + 0.0575A^{654.4} \quad (5)$$

$$[Py_bt] = -71.0A^{649.8} + 2.51A^{628.2} - 13.5A^{667.2} + 84.3A^{654.4} \quad (6)$$

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

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

In the fluorometric studies it was observed that the copper pheophytins are nonfluorescent. Also, evidence was obtained that the copper pheophytins do not interfere with the fluorometric estimation of the chlorophylls and pheophytins in mixtures with the copper pheophytins.

As shown in Table I the absorptivities of $CuPy_a$ and Py_b at the Py_b absorption peak are 59.6 and 36.7 μmol^{-1} cm^2, respectively. Since the absorptivity for $CuPy_a$ is larger than that for Py_b it appeared that considerable error might be encountered in the spectrophotometric estimates of Py_bt in the presence of $CuPy_a$. To avoid this possible error, an alternative method was investigated. By this procedure the $CuPy_a$, $CuPy_b$, and Py_at concentrations were estimated from the absorbances at their red λ_{max} and the concentration of Py_b which was estimated by the fluorometric method of White et al. (1972). The alternative is referred to as the combined spectrophotometric–fluorometric method. Equations 9–11 were derived for the necessary calculations.

$$[CuPy_a] = 15.8A^{649.8} - 1.70A^{628.2} - 2.59A^{667.2} - 0.420[Py_bt] \quad (9)$$

$$[CuPy_b] = -4.14A^{649.8} + 20.6A^{628.2} - 0.870A^{667.2} + 0.0609[Py_bt] \quad (10)$$

$$[Py_at] = -4.84A^{649.8} + 0.530A^{628.2} + 18.72A^{667.2} - 0.0007[Py_bt] \quad (11)$$

Table II. Estimation of Copper Pheophytins by Spectrophotometry and by a Combination of Spectrophotometry and Fluorometry[a]

Pigment and instrumental procedure[b]		a (intercept)[c]	b (slope)[c]	R^2	Pigment concn range[d]
$CuPy_a$	(S)	0.019NS	0.954**	0.990	1.7-5.8
	(S-F_b)	−0.210*	1.016**	0.996	1.7-5.8
$CuPy_b$	(S)	0.033NS	1.026**	0.992	0.8-2.0
	(S-F_b)	0.038NS	1.005**	0.994	0.8-2.0
C_a	(S)	−0.064NS	0.977**	0.999	0.0-6.3
C_b	(S)	0.071*	1.019**	0.997	0.0-1.9
Py_at	(S)	0.174NS	0.980**	0.999	0.0-10.8
	(S-F_b)	0.266*	0.976**	0.998	0.0-10.8
Py_bt	(S)	0.422**	1.024**	0.987	0.0-3.6
	(S-F_b)	−0.008NS	1.025**	0.998	0.0-3.6
Py_a	(S)	0.296**	0.969**	0.998	0.0-10.0
	(S-F_b)	0.336**	0.966**	0.998	0.0-10.0
Py_b	(S)	0.396**	0.996**	0.998	0.0-3.4
	(S-F_b)	−0.068NS	1.044**	0.996	0.0-3.4

[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 11 Y-X pairs. [b] S = spectrophotometric with eq 1-8; S-F_b = combined spectrophotometric-fluorometric with eq 9, 10, and 11, $CuPy_a$, $CuPy_b$, and Py_at estimated spectrophotometrically, Py_bt estimated fluorometrically. [c] NS indicates nonsignificance; * indicates significance at the 0.05 level; and ** indicates significance at the 0.01 level. [d] Pigment concentration expressed in micromoles per liter.

Presented in Table II are the statistics from the regression analyses of the results of the estimation of diethyl ether solution mixtures of the copper(II) pheophytins and related pigments by the spectrophotometric and the combined spectrophotometric–fluorometric procedures mentioned above. Regression analyses were run on the

amount of pigment recovered (Y) upon the amount added (X). The model tested was fitted using 11 mixtures of the six pigments which were present in varying amounts over the concentration ranges of each as listed. The data in Table II indicated that by spectrophotometric estimation the recoveries for $CuPy_a$, $CuPy_b$, C_a, C_b, and Py_at were from 95 to 103% at the pigment concentration ranges investigated. Py_bt recovery was erroneously high as estimated by spectrophotometry (S). At predicted Py_bt concentrations of 4.0 and 1.0 μmol/l. the Py_bt(S) recoveries based on eq 6 would be 113 and 144%, respectively. Because the slope for Py_bt by spectrophotometry was 1.024 the over-recovery observed was primarily due to the bias introduced by the intercept or a value, 0.422.

The deviations of observed from predicted Y values were calculated for all responses (S data) to check assumptions of their independence. For C_b, $CuPy_b$, Py_at, and Py_a there was evidence that these residuals are correlated. This would suggest using caution in extrapolation of the curves, in making tests of significance, and in calculating confidence limits for relevent regression parameters. The other variables did not appear to have this autocorrelation problem.

The procedure designated combined spectrophotometric–fluorometric (S–F_b) was proposed to avoid the possible error mentioned above. Shown in Table II is the regression for Py_bt(S–F_b), which was estimated fluorometrically with an a value of −0.008 and a b value of 1.025. These regression values are considered to be justification for replacement of eq 6 with an adjusted equation (eq 12), as listed:

$$[Py_bt(adj)] = -71.0A^{649.8} + 2.51A^{628.2} - 13.5A^{667.2} + 84.3A^{654.4} - 0.422 \quad (12)$$

Substituting eq 12 for eq 6 provides Py_bt recoveries of 103% in the study reported.

The calculation of Py_b in mixtures from spectrophotometric data has been indicated in eq 8. Attention is called to the importance of using the adjusted value for Py_bt obtained by eq 12. When this value is used, the recovery of Py_b by spectrophotometry will approximate 100%. Equation 8 is replaced by eq 13.

$$[Py_b(adj)] = [Py_bt(adj)] - [C_b] \quad (13)$$

The spectrophotometric recovery of Py_a was nearly 10% high over the range of pigment concentration. A small over-recovery of Py_a was expected due to the slight under-recovery of C_a.

The described spectrophotometric procedure has been shown to be effective for the estimation of all components of mixtures of copper(II) pheophytins, chlorophylls, and pheophytins in diethyl ether solution, providing correction is made as suggested for an observed large over-recovery of total pheophytin b (Py_bt). The adjusted spectrophotometric procedure is based on the use of eq 1, 2, 3, 4, 5, 7, 12, and 13 for pigment calculations. This spectrophotometric procedure is far simpler than the combined spectrophotometric–fluorometric procedure discussed and is recommended for analysis of the pigment mixtures in diethyl ether.

In this investigation the pigments estimated were phytyl esters, namely copper pheophytins, free pheophytins, and chlorophylls. Estimation of the corresponding phytol-free pigments, copper pheophorbides, free pheophorbides, and chlorophyllides could be conducted according to the scheme presented by White et al. (1963). Reports indicating that the spectral curves of copper pheophorbides are identical with those of the corresponding copper pheophytins have been published by Schanderl et al. (1965) and Jones et al. (1968). These findings were in keeping with those of White et al. (1963) who observed that the coefficients of the two pheophytins did not differ materially from those of the two pheophorbides.

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