Automated Determination of Tryptophan in Legumes and Cereals

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An automated procedure for tryptophan determination in full-fat legumes and cereals was developed that included a modification of the *p*-dimethylaminobenzaldehyde (DAB) reaction. Minimal sample preparation was required. Samples were either hydrolyzed in 5 N KOH for 3 h for accurate determination or homogenized for 30 s for direct screening (25 samples/h). Reactants entered the manifold as follows: 0.16 ml/min sample (0.7–2 mg of protein/ml, 1 M KCl, pH 6 ± 0.5), 0.43 ml/min DAB reagent (0.154 M *p*-dimethylaminobenzaldehyde, 3 N HCl, 14 N H_2SO_4), and 0.32 ml/min dioxane reagent (*p*-dioxane–*n*-butyric acid–water, 2:2:1) 60 s later. Color absorbance (570 nm) was read 100 s after addition of dioxane. Values (coefficient of variation 5–10%) for casein, peanut flour, whole peanuts, soybean, wheat, oats, and sorghum agreed closely with those reported in the literature.

The importance of tryptophan in the nutritional quality of food protein has brought a need to analyze large numbers of samples in new-seed breeding programs (Villegas, 1975). Modified versions of the Opienska-Blauth et al. method (1963) are gaining rapid acceptance (Villegas, 1975; Dalby and Tsai, 1975; Concon, 1975), but the time required for sample preparation considerably limits the rate of analysis. Moreover, these procedures have not been extended to oilseeds or legumes where the presence of fats introduces further complications. Automation of the reaction of tryptophan with ether glyoxylic acid, acetic anhydride, or *p*-dimethylaminobenzaldehyde (DAB) has not been practical in the past partly because of the long reaction times required and partly because of the involved sample preparation and hydrolysis procedures.

This report shows that it is possible to modify the classical DAB–tryptophan reaction of Spies and Chamber (1948), making it suitable for the automated analysis of full-fat oilseeds or legumes as well as cereals. For accurate determinations, samples are first subjected to a 3-h alkaline hydrolysis. For the screening of crude seed homogenates, samples can be analyzed directly without hydrolysis for an estimate of their relative tryptophan content.

MATERIALS AND METHODS

Reagents. (A) *DAB Reagent.* A solution was prepared by dissolving 11.50 g of a reagent grade *p*-dimethylaminobenzaldehyde in 500 ml of warm 27.7 N H_2SO_4 (70 v/v %). Five hundred milliliters of 6 N HCl was slowly stirred into the above solution under a fume hood and with ice cooling. This reagent generated considerable amounts of HCl gas during the addition of 6 N HCl, and its final appearance varied from light brown to pink. After cooling, the DAB reagent was stored in amber acid bottles at room temperature and was stable for at least 3 months. Residual HCl gas evolution near the instrument was controlled by keeping the reservoir closed on the analyzer bench (a polyethylene tube was lowered into the reservoir through a hole on the cap and aluminum foil was wrapped around the neck and the tube during operation).

(B) *Dioxane Reagent.* The oxidizing reagent was a mixture of *p*-dioxane (Fisher Scientific, Matheson Coleman and Bell, or Baker Analyzed Reagents), *n*-butyric acid (Eastman Organic Chemicals), and water in a 2:2:1 ratio. This mixture was prepared daily. Although the age of the dioxane lot was usually unimportant, occasional departure from Beer's law was observed with lots that were several years old. Reservoirs for the oxidizing reagent also had screw caps with a drilled hole slightly larger then the diameter of the suction tube.

(C) *Tryptophan Standards.* A stock solution containing 500 μg of L-tryptophan/ml in 0.10 M NaOH was used to prepare working standards of 10, 20, 30, and 40 μg/ml in 1 M KCl.

(D) *Other Solutions.* Solutions of 5 N KOH and 5 N HCl were delivered by 10-ml capacity Dispensettes (Brinkmann Instruments).

Sample Preparation. (A) *With Hydrolysis.* Particle size of the milled material was not a critical factor for the accurate measurement of tryptophan in hydrolyzed samples except for soybean, which required prior homogenization with a Polytron (a combination of mechanical shear and ultrasonic disruption). It is recommended, however, that cereals and peanuts be milled to 40 and 10 mesh, respectively. Six milliliters of 5 N KOH was added to samples containing 20–60 mg of protein in 10-ml screw cap, polypropylene tubes (Oak Ridge type, Dynalab Co.), flushed with nitrogen gas for about 10 s, and tightly capped. The tubes were autoclaved (122 ± 2 °C) for 1, 2, 3, 6, and 10 h. After cooling, the hydrolysates were transferred quantitatively to 150-ml beakers with two 9-ml washings and neutralized with 6 ml of 5 N HCl to pH 6 ± 0.5. Although the hydrolysate solutions were not necessarily clear, turbidity disappeared later with the addition of the oxidizing reagent. A brief centrifugation (bench type) step is recommended when particles tend to settle from some neutralized cereal hydrolysates.

(B) *Without Hydrolysis.* Hydrolysis was not performed in sample screening for relative tryptophan level. Instead, homogenization with a Polytron followed by filtration with glass wool and/or centrifugation was sufficient to provide a suspension free from fast-sedimenting particles. Routinely, 5- to 8-ml samples (in alkali or water) were homogenized with the Polytron (type PT 20, Brinkmann Instruments) in a 50-ml plastic graduated cylinder. The color absorption values did not increase significantly beyond 30 s of homogenization at medium speed. Both Polytron homogenization and alkaline hydrolysis were required for accurate measurements on soybean samples. Sorghum grain was milled to pass a 60-mesh screen; whole wheat flour and oatmeal were used as available commercially; soybeans and cured peanuts with testa (5–6% moisture) were milled to pass at least a 10-mesh screen. Removal of fat or extraction of the protein was unnecessary. With

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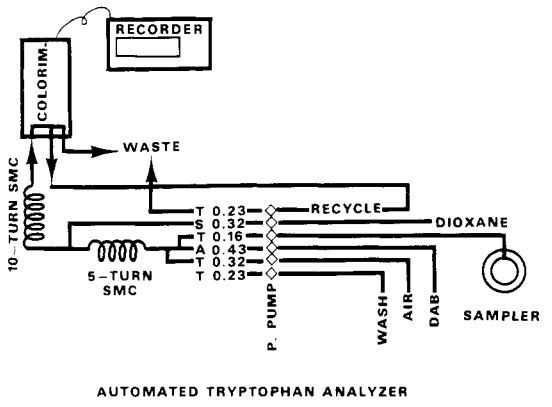

Figure 1. DAB reagent, sample, and air are mixed by an HO-type cactus and allowed to react for 55–60 s. Dioxane reagent is added by a D2-type cactus and the color read (570 nm) between 100 and 110 s later. Hydrolysates and sediment-free homogenates are sampled at a rate of 25 and 50/h, respectively: T, tygon; S, silicone; A, acidflex; SMC, single mixing coil (5 = turn part numbers, 10 = turn part number); p. pump, proportioning pump.

the unhydrolyzed samples, a stirring device was used on the sampler. A consistent, low background from light scattering of such samples was determined by exchanging the orders of addition of dioxane and the sample.

Equipment and Procedure. The system consisted of a Technicon (Technicon Corp., Tarrytown, N.Y.) Sampler II (cam wheel for 50, 2/1, samples/hour with water blanks placed between samples for accurate measurements); a proportioning pump I or II (the speed was 70% and equivalent to that of the newer models II and III); and a dual-channel colorimeter II (Standard Calibration Control set at 6.50 with full pen deflection of about 0.22 absorbance unit). A Heath-Schlumberger SR-255 B recorder (variable range adjusted to 10 mV and chart speed to 0.05 in./min) was incorporated in the above system as shown in Figure 1. Since the mixing coils served also as reaction coils, the lengths recommended herein were essential. Solvaflex pump tubing could not be used since it was readily attacked by the dioxane–butyric acid–water mixture, thus changing the flow rate of the oxidant. Consequently, silicone flow-rated tubing was essential for this reagent also. Other details of the manifold and reaction coils are included in Figure 1.

The sample (0.16 ml/min) was introduced and mixed with the DAB (0.43 ml/min) and air (0.32 ml/min). Reaction I, carried out for 55–60 s in a single-mixing coil, was followed by the addition of dioxane reagent (0.32 ml/min). Reaction II was then allowed to proceed for 100–110 s in another single-mixing coil. About 3 min elapsed from the time the sample was mixed with the DAB reagent until it reached the flowcell. The sampling rate was 25/h for hydrolysates and for sediment-free homogenates. As a precaution, the system was operated with water for a few minutes after any tubing was changed.

Peak heights were measured and corresponding concentrations were obtained from the standard curve of 10, 20, and 30 and 40 μg of tryptophan/ml run each day. Occasional washing of the system was necessary by pumping 0.2 N NaOH through the "air" line to remove the coating formed inside the flowcell.

RESULTS AND DISCUSSION

The color-forming reaction of tryptophan with DAB in acid was significantly accelerated by the use of hydrogen chloride and *p*-dioxane as follows:

$$\text{Trp} + \text{DAB} \xrightarrow[\text{HCl}]{\text{I}} \text{pink intermediate} \xrightarrow[\text{dioxane}]{\text{II}} \text{blue chromophore}$$

By such modification it was possible to automate the reaction and develop a general procedure for rapid analysis and screening of legumes and cereals.

Both the color intensity and the concentration range depended on the time the reaction was allowed to proceed (Figure 2). The short reaction times selected for the instrument were adequate for routine analysis of samples containing between 10 and 30 μg of Trp/ml (about 4 mg of legume/ml) given the high sensitivity of the colorimeter.

The time ratio for reactions I and II in the automated procedure was not necessarily the same as in the test tube because of the different mixing conditions. Nevertheless, the duration of each reaction, as given under Materials and Methods, was critical in order to maintain a linear response.

Effect of Hydrolysis on the Reaction. Unlike free tryptophan, protein-bound tryptophyl residues have been known to exhibit a certain hyperchromicity accompanied by a variable (usually red) shift rather characteristic for each protein (Spies, 1967; Horn and Jones, 1945). Figure 3 depicts the release of tryptophan from interfering neighboring residues by hydrolysis with 5 N KOH. After 3 h of hydrolysis at 121 °C, the tryptophan values became remarkably constant for the seed proteins when hydrolyzed in the presence of their natural carbohydrates. The apparent concentration of tryptophan in casein, however, declined continuously due to the expected loss of the indole ring in unprotected proteins (Spies, 1967). This protective effect of starch has been noted (Dreze, 1960; Howe et al., 1972) during a slower hydrolysis procedure with 5 N NaOH. A 3-h hydrolysis with 5 N KOH was sufficient to remove the different degrees of hyperchromicity. In whole wheat flour, hydrolysis apparently also mediated the complete exposure of the tryptophyl residues otherwise inaccessible to the reagent in the unhydrolyzed protein (Figure 3).

The present hydrolysis procedure required minimal preparation or handling of samples and was practical when large numbers of samples (100 or more at a time) were analyzed by the automated method. Recently, it was reported that prior hydrolysis of the protein was not necessary for the accurate determination of tryptophan by the glyoxylic acid reaction in a number of cereals (Concon, 1975). Circumventing hydrolysis by such a method, however, necessitates milling of the seed to an extremely fine powder followed by a protein-extraction step which, alone, may involve as much time and labor as the present alkaline hydrolysis procedure. Furthermore, oilseeds could not be prepared as suggested (Concon, 1975) without previous removal of the fat.

Automated screening of legumes or cereals is feasible by the present method since the tryptophan content can be rapidly estimated in small, unhydrolyzed samples. In such cases, full advantage can be taken of the hyperchromicity noted for some of the proteins by using less concentrated samples; 1–2 mg of seed material/ml was directly analyzed after a 30-s homogenization and a 30-s filtration step.

With the exception of the tannins present in bird-resistant varieties of sorghum, seed testa pigments of the seeds analyzed posed no difficulties after neutralization of the alkaline hydrolysates.

Hydrochloric Acid. The role of HCl in the reaction appeared to be that of a specific acid catalyst since the chromogenic response was dependent on the final acid

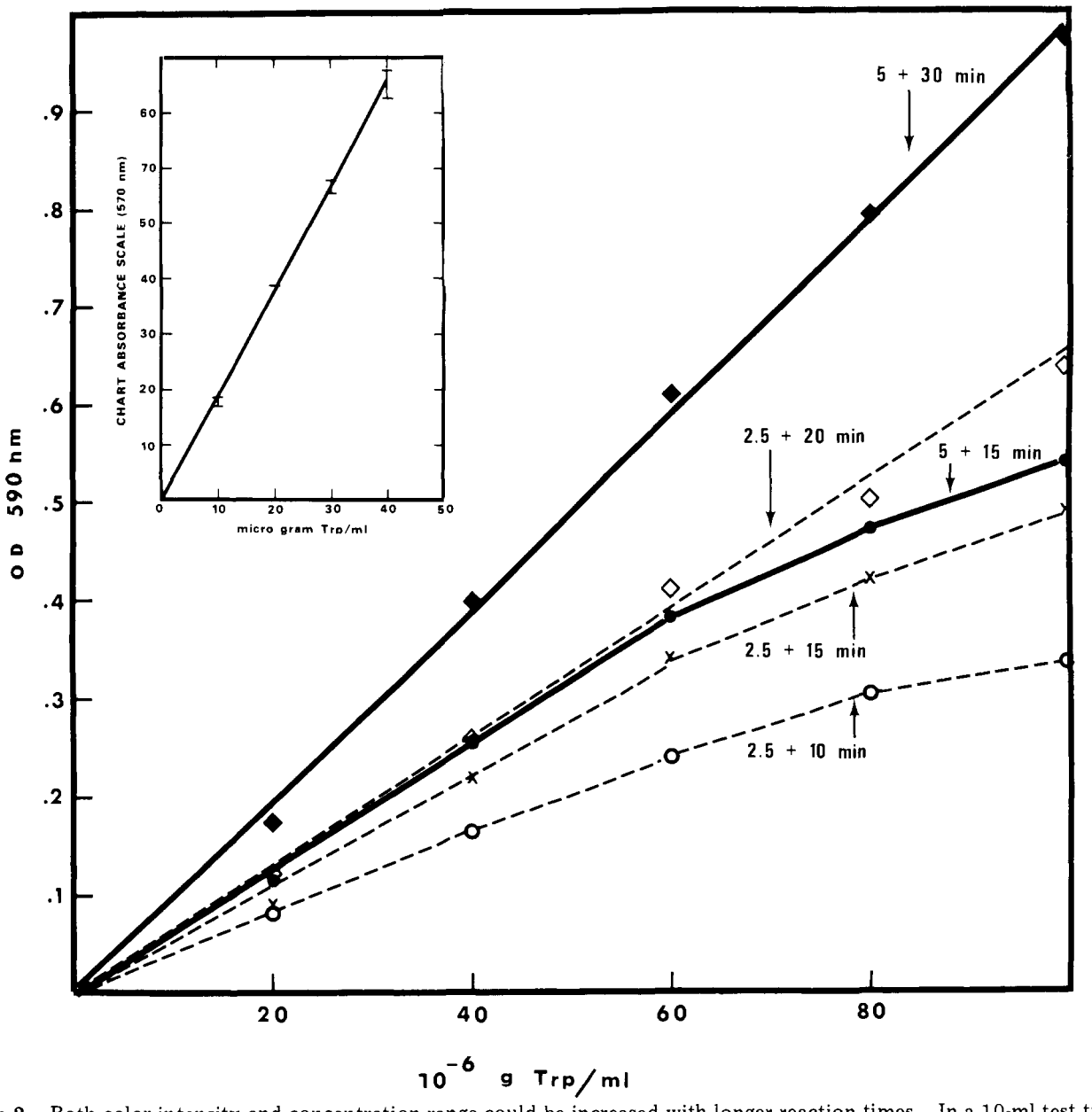

Figure 2. Both color intensity and concentration range could be increased with longer reaction times. In a 10-ml test tube, DAB reagent (0.9 ml) was added to the sample (0.3 ml), gently mixed, and allowed to react for either 2.5 or 5 min (reaction I). This was followed by addition of the dioxane reagent (0.7 ml), and gentle mixing (reaction II). Absorbance at 590 nm was measured exactly at the times indicated. The inset is a tryptophan standard curve obtained in the instrument at 6.50 sensitivity.

concentration (with maximum effect beginning at about 3 N HCl), and other acids tested either inhibited or did not affect the reaction.

Hydrochloric acid has been previously used to accelerate the condensation of tryptophan with DAB (May and Rose, 1922) but the innovation was not successfully utilized because the color faded with the addition of hydrogen peroxide (Sullivan and Hess, 1944). Later, the prolonged evolution of HCl fumes in the original procedure was found objectional (Spies and Chamber, 1948) and, most importantly, substitution of hydrogen peroxide by sodium nitrite gave a nonlinear response with tryptophan concentration (Matheson, 1974).

***p*-Dioxane.** The use of *p*-dioxane in reaction II had the advantage over $NaNO_2$ of providing a linear response for predefined concentration intervals while aiding sample solubility. Whether reaction II proceeds as a consequence of the peroxides present in *p*-dioxane was not determined. Dioxane supplied by four different manufacturers gave consistent results, and a reasonable aging of the reagent had no noticeable effect on the reaction. In fact, two out of three bottles stored for about 5 years gave satisfactory results while the third one produced a large positive deviation from Beer's law.

While hydrogen chloride catalyzes the overall reaction, it also promotes the decay of the final chromophore at a rate dependent on the type of reagent used in reaction II. If $NaNO_2$ is used, for example, the decay rate (independent of the chromophore concentration) is high enough to make Beer's law valid within very small concentration ranges only. Use of *p*-dioxane, however, allowed measurement by the automated method within wider and more practical concentration ranges.

The blue chromophore was formed rapidly also when a freshly prepared saturated solution of *N*-bromosuccinimide (NBS) was added instead of *p*-dioxane. This new system could be adaptable to a manual procedure if the color is stabilized by making the NBS solution 20%

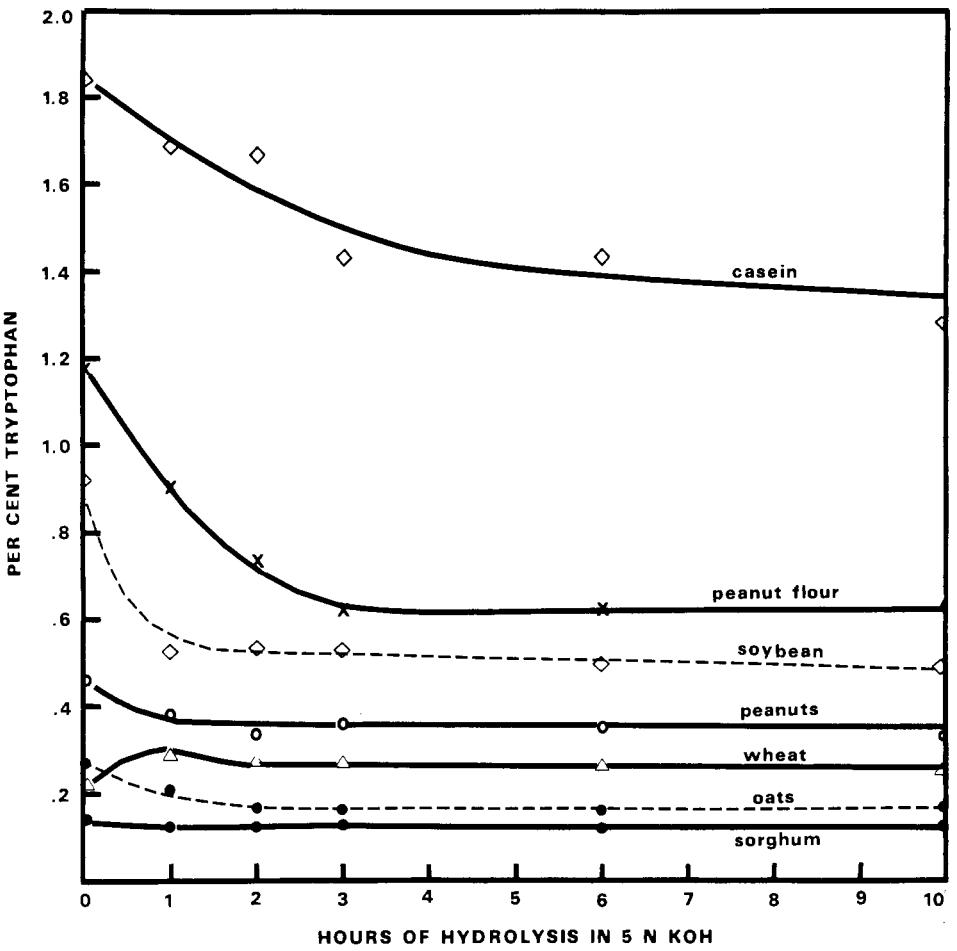

Figure 3. Determination of minimum hydrolysis time for the removal of hyperchromicity in several seed materials and casein. After 3 h of hydrolysis at 122 ± 2 °C in 5 N KOH, the tryptophan levels of most seeds remained remarkably constant in the absence of protective additives. The amount of hyperchromicity appeared to be rather characteristic for each seed. No hyperchromicity was observed in the wheat proteins until partial hydrolysis made tryptophyl residues fully accessible (1 h).

in butyric acid, and the sensitivity increased by using less 6 N HCl in the DAB reagent.

n-**Butyric Acid.** Addition of butyric acid to the system is unnecessary for the reaction and it could be deleted where fat solubilization is not needed. Use of butyric acid is valuable particularly in the screening of unhydrolyzed oilseeds.

Precision and Accuracy. The total experimental error introduced by the present hydrolysis and analysis procedures gave an average coefficient of variation of 5.9%, as determined from the duplicate experiments of Table I. For a concentration range of 0–40 μg of tryptophan/ml, most accurate results were obtained for values between 10 and 30 μg/ml. Although standard curves were reproducible from day to day, duplicate or multiple sets of standards were run daily with every group of samples. As in the classical method of Spies and Chambers (1948), this modified reaction was specific for tryptophan and some indole derivatives not substituted at the 1 or 2 position (Gruen and Rivett, 1971). The other protein amino acids did not interfere. The presence of KCl in the hydrolysates did not interfere and had a stabilizing effect on the color. Concentrations of KCl higher than 1 M, however, induced precipitation of the DAB.

Table I compares the tryptophan values obtained by this method with those found in the literature for casein, soybean, peanuts, oats, wheat, and sorghum. In evaluating the method for seeds or other products not shown in this table, a hydrolysis curve (such as in Figure 3) is recom-

Table I. Tryptophan Content of Casein, Some Legumes, and Some Cereals as Determined by the Automated Method[a]

Material	Total nitrogen, %	Tryptophan, % This method (mean ± S.D.)	Tryptophan, % Lit.
Casein	14.05	1.36 ± 0.11	1.30 (5)
Peanut flour (Florunner)	9.16	0.60 ± 0.07	
Soybean, cotyledon[b] (Davis)	3.12	0.52 ± 0.02	0.53 (14)
Whole peanut meal (commercial cultivars)[c]	4.98	0.31 ± 0.02	0.30 (14)
Whole wheat flour (commercial)	2.43	0.22 ± 0.02	0.21 (8)
Oatmeal (commercial)	2.49	0.16 ± 0.01	0.17 (14)
Sorghum[b] (DeKalb E-59)	3.70	0.13 ± 0.00	0.12 (14)

[a] Except for casein and whole peanut meal, all samples were homogenized in a Polytron prior to the 3-h alkaline hydrolysis. The average of at least two replicates. For comparison, average values of many samples from the literature are provided. [b] Samples supplied by Dr. H. B. Harris, Department of Agronomy, University of Georgia. [c] The average of 40 varieties, each in duplicate. The percent recovery of added tryptophan was 98 ± 4 after a 3-h hydrolysis. All figures are uncorrected in view of the constancy of the tryptophan levels after 3 h of hydrolysis and the uncertainty involved in taking free tryptophan as representative of protein tryptophan.

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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