Rapid Determination of Tryptophan in Beans (*Phaseolus vulgaris*) by the Acid Ninhydrin Method

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The rapid and simple acid ninhydrin method (Gaitonde, M. K., Dovey, T., *Biochem. J.* **117**, 907 (1970)) was adapted for the colorimetric determination of tryptophan in protein extracts of beans. Total extraction of the seed nitrogen is possible by a simple extraction procedure using 0.2% NaOH. A sample blank is necessary to compensate for nonspecific absorption due mainly to pigments, but the extracts are otherwise free of interfering factors. Tryptophan values obtained compare favorably with literature values provided a simple correction for tyrosine is made. The method was shown to be applicable to protein extracts of maize and wheat. The rapidity, simplicity, and accuracy of the method suggest it should be useful for both routine analysis and screening purposes.

The determination of tryptophan in food crops is especially important since it is often one of the limiting amino acids (Kaul, 1973). However, existing methods of tryptophan analysis in proteins are generally complex, time consuming, and sometimes of doubtful accuracy (Friedman and Finley, 1971). Tryptophan methods may be divided into two categories: those involving hydrolysis of the protein before tryptophan assay and those involving the intact protein. For the analysis of tryptophan in foodstuffs the former method has been preferred, despite the problematic and tedious special hydrolysis procedures necessary to release it from proteins. Intact protein methods have not proven popular for foodstuffs because of the insolubility of some proteins and the production of undesirable colors by the nonprotein fraction of the food (Miller, 1967). Furthermore, inaccuracies inherent in these methods can lead to values that are too high even with purified proteins (Spies, 1967).

More recently, Gaitonde and Dovey (1970) published a new method for the determination of tryptophan in the intact protein. This method is different in principle from previous ones, depending on the reaction of tryptophan with ninhydrin under special acid conditions. Gaitonde and Dovey demonstrated the accuracy of their method using certain pure proteins, although a simple, generally small, correction for tyrosine interference is necessary (Zahnley and Davis, 1973). The simplicity and rapidity of the method prompted us to investigate its usefulness for the routine analysis of tryptophan in certain food crops. In this paper, we have attempted to show that the method is applicable to crude protein extracts of beans and other seeds.

EXPERIMENTAL SECTION

The three varieties of Brazilian beans (*Phaseolus vulgaris* L.) used in this study were: Goiano Precoce (brown seed coat), 1868 Sacaven 1061 (black), and EEP 25-623 (white). These are referred to in the paper by their seed coat color.

The beans were ground to a fine powder in a ball mill (Mixer/Mill 8000, Spex Industries, Metuchen, N.J.). The meal was partially defatted by suspending in 20 vol of acetone and stirred occasionally for 0.5 hr. After filtering, the powder was left to air dry. For some studies the seed coats were removed prior to grinding. For this purpose the seeds were soaked for 5–10 min in water at 60° to facilitate the removal of the seed coat.

Protein Extracts of Beans. Portions (100 mg) of the meal were extracted with 5.0 ml of 0.2% NaOH in a centrifuge tube for 0.5 hr, occasionally stirring and homogenizing with a glass rod. After centrifugation, the residue was extracted with a further 5.0 ml of 0.2% NaOH and the supernatants combined. In routine determinations, 0.5-ml aliquots were taken for tryptophan assay.

In some experiments a portion of the extract was mixed with an equal volume of 10% Cl_3CCOOH to precipitate protein. After standing overnight at 4° the precipitates were collected by centrifugation, washed with 5% Cl_3CCOOH, and redissolved in the original volume of 0.5% NaOH.

Fractionation of Bean Proteins. Bean meal (1 g) (Goiano Precoce) was extracted four times with 10 ml of 1 *M* NaCl in 0.05 *M* potassium phosphate buffer (pH 7) at 0° with occasional mixing for 0.5 hr. After centrifugation a portion of the combined supernatants was dialyzed against several changes of distilled water to separate globulins and albumins. The residue was extracted twice with 10 ml of 0.2% NaOH at room temperature to form the glutelin fraction.

Protein Extracts of Maize. The procedure for the extraction of protein from maize (*Zea mays* L.) was based on that of Subramanian et al. (1970). Whole grains were ground in a Wiley mill to pass a 20 mesh screen. The meal was partially defatted with acetone, as described for beans, and grinding finished off with a mortar and pestle. Meal (500 mg) was extracted in a centrifuge tube with 1.0 ml of 70% ethanol containing 2-mercaptoethanol for 0.5 hr at room temperature. The mixture was occasionally stirred and homogenized with a glass rod. NaOH (5.0 ml; 0.5%) was then added and extraction continued for another hour. After centrifugation, a clear supernatant was collected and 0.15 ml taken for tryptophan assay.

Protein Extracts of Wheat. The procedure for the extraction of protein from wheat (*Triticum aestivum* L.) was similar to that of maize except that the volumes of the solvents in relation to meal weight were doubled, and 2-mercaptoethanol was omitted from the ethanol solution since it was found to offer no advantage in the case of wheat. Furthermore, the grains were ground in a ball mill. Aliquots (0.2 ml) were taken for tryptophan assay.

Tryptophan Determinations. The acid ninhydrin method of Gaitonde and Dovey (1970) was followed, using reagent b (250 mg of ninhydrin dissolved in 10 ml of formic acid–hydrochloric acid, 3:2, v/v) for the determination of tryptophan in proteins. Readings were made against a reagent blank in a Beckman DBG spectrophotometer at 390 nm using cuvettes of 1-cm light path.

Sample blanks contained a similar aliquot of protein extract together with reagent b without ninhydrin. These were read against a reagent blank containing ninhydrin-free reagent b.

After subtracting the absorbance value of the sample blank from the test value, the tryptophan content was read

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Table I. Tryptophan Determination in Protein Extracts of Beans with and without the Seed Coat

Seed coat color	± seed coat	Absorbance, 390 nm A[a]	B[a]	A–B	Trp g/100 g of protein[b,c]	Lysozyme recov., %[d]
White	+	0.350	0.038	0.312	1.20	96
	−	0.395	0.040	0.355	1.27	100
Black	+	0.450	0.120	0.330	1.40	96
	−	0.380	0.053	0.327	1.42	97.5
Brown	+	0.525	0.200	0.325	1.26	98
	−	0.420	0.053	0.367	1.28	97.5

[a] A = reagent + ninhydrin; B = reagent − ninhydrin (sample blank). [b] Corrected for tyrosine interference (Zahnley and Davis, 1973). [c] Literature values: (i) 1.0–1.38, range of values for 12 Brazilian varieties of *Phaseolus vulgaris* by de Morães and Angelucci (1971) using Miller's (1967) method; (ii) 1.01, value given by FAO (1970) for *Phaseolus vulgaris* using a microbiological method. [d] Protein-bound tryptophan in the form of lysozyme recovered after spiking with 100 μg of lysozyme.

Table II. Tryptophan Determination in Protein Extracts of Beans after Precipitation with Cl_3CCOOH

Seed coat color	± seed coat	Absorbance, 390 nm A[a]	B[a]	A–B	% of total Trp in ppt.[b]	Lysozyme recov., %[c]
White	+	0.310	0.010	0.300	96	96
	−	0.355	0.010	0.345	97	97.5
Black	+	0.370	0.050	0.320	97	100.5
	−	0.330	0.010	0.320	98	95
Brown	+	0.405	0.090	0.315	97	99.5
	−	0.365	0.010	0.355	97	98.5

[a] A = reagent + ninhydrin; B = reagent − ninhydrin (sample blank). [b] [A − B (this table)]/[A − B (Table I)] × 100. [c] Protein-bound tryptophan in the form of lysozyme recovered after spiking with 100 μg of lysozyme.

Table III. Contribution of Individual Protein Fractions to the Total Tryptophan Content of the Bean Extract

Fraction	Trp content, % of whole extract
Albumin	13.4
Globulin	62.2
Glutelin	23.8
Sum total	99.4

off a standard curve. Lysozyme (grade I from egg white; Sigma Chemical Co., St. Louis, Mo.) was used to construct the standard curve, assuming a tryptophan content of 43.2 μmol (8.8 mg)/100 mg of lysozyme, as given by the method of Gaitonde and Dovey (1970). Tryptophan values obtained from this graph may then be corrected for tyrosine interference, applying the formula of Zahnley and Davis (1973).

Nitrogen Determinations. Nitrogen was determined by the Kjeldahl method (Johnson and Ulrich, 1959). The factors 6.25 (for beans and maize) and 5.7 (for wheat) were used to convert nitrogen into protein.

RESULTS AND DISCUSSION

Extraction of Proteins from Beans. The use of 0.2% NaOH permits the extraction of 97.7% (SE ±0.9) of the nitrogen in the bean meal, and such protein extracts were used for tryptophan assay. Higher concentrations of NaOH, e.g. 0.5%, could not be used with finely powdered meal because of the formation of gels during extraction.

Contribution of Pigments to the Absorbance. Since the extracts were brown in color, due to the extraction of pigments, our main concern was over the possible interference by pigments in the tryptophan determinations. With this in mind, three varieties of beans were selected to represent the different pigmentations commonly found, that is, white, brown, and black. The contribution of pigments to the absorbance in the tryptophan assays was estimated by running sample blanks (see Experimental Section). As may be seen in Table I, the contribution of the blank may be considerable, but does not depend entirely on the quantity of pigment present. For example, white beans also produce a significant blank even though their extracts are almost colorless.

When the blank values are subtracted, the tryptophan levels obtained fall in the range of expected values. However, in order to check the validity of the sample blank, a comparison was made with extracts of the same three bean varieties but with the seed coat removed prior to grinding. Since pigments are localized in the seed coat, the extracts are almost colorless for all three varieties. As may be seen in Table I, the removal of the seed coat of the pigmented seed greatly reduces the contribution of the sample blank to the absorbance. In the case of the white seeds, removal of the seed coat makes little difference to the sample blank reading. The tryptophan values given by seed with and without the seed coat are in reasonable agreement. This suggests that the subtraction of the high blank values in the case of colored extracts is valid.

Investigation of Interfering Factors in the Extract. In order to check for other possible interfering factors in the crude protein extracts, these were partially purified by precipitation of the protein with Cl_3CCOOH, and tryptophan was determined in the precipitates. An average of 97 ± 1% of the tryptophan was recovered in the precipitates (Table II). Thus, virtually all the tryptophan color given by the crude extracts is produced by this fraction. It is noteworthy that the contribution of the sample blank is almost zero in the case of samples without the seed coat, whereas for those with the seed coat it was reduced only by little more than half. This was due to the adsorption of pigment by the protein precipitate which was not removed on washing with Cl_3CCOOH.

As a further test for the presence of interfering factors

Table IV. Tryptophan Determination in Protein Extracts of Wheat and Maize

	% protein in grain	% of total N extracted	Lysozyme recov., %[a]	Absorbance, 390 nm			Trp g/100 g of protein[c]	Lit. values		
				A[b]	B[b]	A–B		*d*	*e*	*f*
Maize, normal (cateto sulino)	10.7	91.5	97	0.245	0.040	0.205	0.65	0.67	0.67	0.70
Maize, opaque-2 (opaco amarelo)	10.3	98.5	95	0.390	0.053	0.337	1.18		1.1	
Wheat (IAS-54)	13.0	92.0	101	0.343	0.043	0.300	1.28	1.2	1.2	1.2

[a] Protein-bound tryptophan in the form of lysozyme recovered after spiking with 100 μg of lysozyme. [b] A = reagent + ninhydrin; B = reagent − ninhydrin (sample blank). [c] Corrected for tyrosine interference (Zahnley and Davis, 1973). [d] Miller (1967); value for wheat recalculated using the factor N × 5.7. [e] Report on Maize and Wheat Improvement (1972). [f] FAO (1970).

the extracts were spiked with lysozyme (a pure protein with a high tryptophan content). As may be seen in the appropriate columns of Tables I and II, recoveries of added protein-bound tryptophan (lysozyme) are high (95–100.5%). Similar high recoveries were obtained with both crude extracts (pigmented or not) and Cl_3CCOOH precipitates, suggesting that there is negligible interference in the tryptophan–ninhydrin reaction.

Other data which suggest the absence of interfering factors are that the sum of the tryptophan contents found for each of the various protein fractions is about equal to the tryptophan content of the crude extract (Table III). Thus, isolated bean proteins give the same amount of tryptophan color as in the crude extracts.

Meal Quantity and Absorbance. The relationship between absorbance and the quantity of meal taken for tryptophan assay was investigated using the brown seeds, both with and without the seed coat. The extracts were assayed before and after precipitation with Cl_3CCOOH. As shown in Figure 1, in all cases but one the absorbance (difference values) was practically linear over the range studied (25–250 mg of meal). In the case of crude extracts of seed with the seed coat, the response was linear only up to 100 mg of meal, thereafter falling off. This fall-off was not due to a less efficient extraction of protein at higher quantities of meal, since different amounts of the 250-mg extract produced a similar curve (data not shown). Moreover, the corresponding Cl_3CCOOH precipitate curve was linear. When absorbance values are plotted without subtracting the sample blank, a linear response is then obtained over the whole range (see Figure 1). It appears that above 100 mg of meal the high contribution of the sample blank to the absorbance is causing some problem. We would suggest then that extracts producing sample blanks with absorbance values above 0.20 be diluted before assay.

Application of the Method to Maize and Wheat. It was of interest to investigate the applicability of the method to other food crops. In Table IV typical data are presented for wheat and normal and opaque-2 maize. The tryptophan values obtained compare favorably with literature values. The high recoveries of protein-bound tryptophan (lysozyme) added to the maize and wheat extracts suggest that negligible amounts of interfering factors are present.

A different extraction procedure was necessary to solubilize the cereal proteins in a single solution. By using a combination of 70% ethanol and 0.5% NaOH over 90% of the grain nitrogen may be solubilized. In the case of maize it was necessary to include 2-mercaptoethanol to improve the extraction of proteins and this did not interfere in the tryptophan assay. However, by carefully controlling the conditions of extraction (e.g., particle size, etc.) near 100% solubilization of nitrogen is possible for maize (Concon, 1973) without the use of 2-mercaptoethanol. Such attention to the conditions of extraction should allow the total extraction of protein in a single solution for any seed.

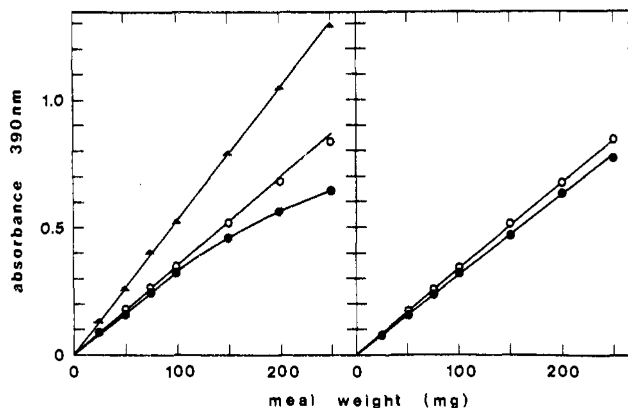

Figure 1. Relationship between absorbance and quantity of bean meal taken for tryptophan assay: (A, left) crude extracts; (B, right) Cl_3CCOOH precipitates of crude extracts; (●) with seed coat; (O) without seed coat; (▲) with seed coat (sample blank not subtracted).

General Considerations. The rapidity and simplicity of the acid ninhydrin method suggest it should be useful for screening purposes. A mass screening method for tryptophan is particularly useful for maize. Since tryptophan was found to be highly correlated with lysine, the first limiting amino acid, Hernández and Bates (1969) suggested that tryptophan analysis alone is adequate for protein quality evaluation in maize. For screening purposes, where rapidity is more important than accuracy, the sample blank of the present method could be omitted in initial surveys. The contribution of the sample blank to the absorbance is generally low (see Table IV), though higher values may be found with certain pigmented varieties of maize.

The accuracy of the acid ninhydrin method was originally demonstrated with certain pure proteins (Gaitonde and Dovey, 1970; Zahnley and Davis, 1973). The objective of the various tests carried out in this paper was to evaluate the accuracy of the method for crude protein extracts of beans. The use of a sample blank adequately compensates for nonspecific absorption, and the crude protein extracts appear to be free of any other interfering factors. Furthermore, tryptophan values obtained for beans, wheat, and maize fall close to literature values obtained by methods different in principle from the present one. At least the method appears to be no less accurate for these food crops than currently accepted methods. Being much simpler and more rapid, the acid ninhydrin method is an attractive alternative for the routine analysis or screening of tryptophan in such crops.

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Chemical Examination of Seleniferous Cabbage *Brassica oleracea capitata*

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The water–methanol soluble extract of seleniferous cabbage leaves was separated into several ninhydrin-positive spots or bands using thin-layer chromatography (TLC). The chromatographic behavior, ninhydrin color, and $R_f \times 150$ values of the radioactive spots were compared with TLC spots of chromatographically pure known sulfur or selenium compounds. Radioactive band eluates were co-spotted on TLC plates with selected sulfur or selenium compounds, and the plates subjected to one- or two-dimensional development with different solvent systems for identification purposes. Evidence obtained strongly suggests the presence of these ^{75}Se-containing soluble compounds in seleniferous cabbage: *Se*-methylselenomethionine, selenocystathionine, *Se*-methylselenocysteine selenoxide, selenohomocystine, *Se*-methylselenocysteine, and selenomethionine. Evidence indicating the presence of selenopeptides, selenoproteins, and substituted selenium containing cysteine or cystine type compounds was obtained. The presence or absence of selenocystine in the cabbage extract could not be definitely established.

Cabbage (*Brassica oleracea capitata*) is a common family garden and commercially grown member of the Cruciferae (Mustard) family and is capable of absorbing, metabolizing, and storing in its tissues relatively large quantities of selenium. The available selenium present either as selenate, selenite, or organic compounds is readily absorbed and metabolized. This vegetable is grown in many areas and is consumed by large numbers of people. A strong possibility exists that humans consuming seleniferous cabbage may suffer harmful effects. Sulfur compounds present in cabbage have been extensively studied and many have been identified. The organic selenium containing compounds present in cabbage are of interest but have not been extensively investigated. Knowledge of the relative amounts and chemical identity of the selenium compounds present in cabbage will assist in an understanding of the metabolism of selenium by members of the *Brassica* genus. This information will assist in predicting the harmful effects of ingestion of seleniferous Cruciferae.

MATERIALS AND METHODS

Cabbage plants were grown from seed, in the greenhouse, on black loam soil with selenium added periodically as a dilute acid solution of $H_2{}^{75}SeO_3$. The plants were approximately 2 months of age when green leaves were removed for fractionation. The leaves were rinsed in distilled water, dried, and dipped successively in five portions of hexane to remove epicuticular wax. The hexane portions were combined and these as well as other extracts were reduced in volume in a rotary evaporator utilizing low temperature and reduced pressure. The hexane rinsed leaves were reduced to a finely divided slurry and successively extracted with Bligh and Dyer (1959) reagent until the residue was free of green color and the extract contained little ^{75}Se. The extracts were composited in a separatory funnel, additional water and chloroform were added, and the extract was partitioned into chloroform and water–methanol soluble phases. The amount of radioactivity in the fresh leaves, concentrated rinses or extracts, and the insoluble residues was measured.

Chromatographically pure known sulfur and selenium compounds were obtained from commercial sources or interested scientists and were chromatographed on the same plates as were the extract fractions or were co-spotted with each other or plant fractions. Bands or spots were identified by detection reagents such as ninhydrin, naphthoresorcinol, and sulfuric acid and were removed from the plates and activity measurements made. Selected active bands were removed and eluted, and the eluates chromatographed alone or co-spotted with known compounds. Using the $R_f \times 150$ values, detection reagents, band intensity, and other chromatographic characteristics a tentative identification of the selenium compound or compounds present in that particular band was made.

RESULTS AND DISCUSSION

A summary of solvent fractionation of several samples of cabbage leaves and the ^{75}Se content and percentage of radioactivity present in the components is found in Table I. The amount of radioactivity, 0.14%, present in the hexane wash of the leaf surfaces, soluble epicuticular wax, was less than expected and apparently was a result of nonstress greenhouse conditions. No efforts were made to fractionate the hexane soluble material.

The green chloroform extract contained chlorophyll, lipids, and other compounds and represented 0.38% of the total ^{75}Se. Limited TLC indicated numerous spots or bands and two carried measurable ^{75}Se.

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