Table III. Summary of Assay Results for Vitamin B_6 in Breakfast Cereal Samples as Determined by Microbiological and LC Methods[a]

	microbiological calculation employed[b]		
cereal	Y intercept	mean	LC[c]
Post Toasties	48.6	35.6 ± 10.9	44.2 ± 0.8
Super Sugar Crisp	52.9	43.5 ± 6.8	41.8 ± 1.0
Fruity Pebbles	92.8	62.5 ± 13.1	64.9 ± 4.9
Cocoa Pebbles	70.7	52.8 ± 8.1	52.7 ± 1.4
Rice Krispies	31.6	32.1 ± 6.6	35.5 ± 1.6

[a] Data represent micrograms of vitamin B_6/gram of cereal. Mean ± standard deviation. [b] Methods used in microbiological assay quantification: "Y intercept" = Y intercept of linear regression curve of micrograms/gram (x) vs. milliliters of extract/tube (Y); "mean" = conventional method based on the mean of the observed responses. [c] LC = high-performance liquid chromatography.

such as *Tetrahymena pyriformis* and *Kloekera brevis* should be investigated.

The results of this study indicate that the reverse-phase LC procedure provides a rapid, precise, and accurate technique for the determination of vitamin B_6 in fortified cereals. The problems encountered in the direct microbiological assay for total vitamin B_6 in these products, in addition to the cumbersome nature and poor precision of the method, represent serious analytical limitations. Research is presently in progress concerning the application of this basic HPLC procedure to the determination of the naturally occurring B_6 vitamers in more complex food matrices.

ACKNOWLEDGMENT

The technical assistance of Doris Manley and the statistical consultation of John Cornell, University of Florida, Statistics Department, are gratefully acknowledged.

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Received for review August 28, 1979. Accepted December 13, 1979. Florida Agricultural Experiment Station Journal Series No. 1909. This research was supported by funds from the Florida Agricultural Experiment Station.

Automated Procedure for Routine Analysis of Tryptophan in Cereal and Legume Food Samples

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Tryptophan is one of the most important amino acids. Quantitative determination of tryptophan is still difficult. The various methods of quantitative analysis published so far and currently used are not well adapted to routine determination of tryptophan in vegetable food samples. The method described here is based on a colorimetry principle used for tryptophan analysis in pure proteins. The ground flour samples (40 μm) of cereal and leguminous plants are hydrolyzed with barium hydroxide and are analyzed by a two-step automatic colorimetry process with *p*-dimethylaminobenzaldehyde and sodium nitrite. Interferences from the various seed components and especially from pigments absorbing at 590 nm have been eliminated. The hydrolysis conditions have been optimized in order to allow routine analysis of samples for our breeding programs. The procedure permits the analysis of more than 50 samples/day and per person. The whole process has a reproducibility of 4%. The sensitivity of this method enables quantification of very small amounts of tryptophan (less than 10 μg/mL) with accuracy.

The knowledge of the essential amino acid composition and especially the amount of tryptophan is necessary to determine the protein quality of leguminous or cereal seeds (Mitchell and Block, 1946; Oser, 1964). Following the procedure of Moore and Stein (1951), acid hydrolysis with HCl 6 N is used to release most of the amino acids which can then be quantified by ion-exchange liquid chromatography. Under such conditions, tryptophan is destroyed and cannot be directly determined in acid hydrolysates just

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0021-8561/80/1428-0489$01.00/0

like any other amino acid. Nevertheless protective agents such as p-toluene, sulfonic acid (Liu and Chang, 1971), thioglycolic acid (Gruen and Nicholls, 1972; Matsubara and Sasaki, 1969), sulfurous hydrogen, 3,3-indoylpropionic acid, and others reagents (Penke et al., 1974) have been offered by some authors to attenuate tryptophan degradation during classical acid hydrolysis. The addition of such components did not allow a quantitative recovery of tryptophan. In some cases, they interfere in the quantitative determination of other amino acids by liquid chromatography.

So far, these various substances have been more specifically used in the case of pure proteins for the determination of amino acids, including tryptophan, from the same acid hydrolysate. In the case of plant proteins, the other seed components seem to lower the protective effect of these additives whose concentration cannot be indefinitely increased.

Carbohydrates resulting from acid hydrolysis of starch into smaller polysaccharides and glucose may be responsible for the partial destruction of tryptophan. Nevertheless, the in situ reduction into sorbitol (Friedman and Findley, 1971; Finley et al., 1975) enables a quantitative determination of tryptophan by liquid chromatography. However, some other amino acids are more or less destroyed during this process and must be determined by separate analysis from a new hydrolysate.

The tryptophan analysis remains a compromise between its own determination and one of other amino acids. The protective agents are not always without any effect upon the other amino acids of proteins. The different procedures published cannot be easily applied to the analysis of all amino acids of proteins in plant food samples from the same acid hydrolysis.

Confronted with the problems set by the routine analysis of amino acids of plant proteins for our breeding programs of leguminous and cereal seeds, we looked for a simple, fast, and accurate method to carry out tryptophan determinations at a high speed. Other amino acids are measured by high-pressure liquid chromatography on a Durrum D 500 amino acid analyzer from an acid hydrolysate.

Automatic colorimetry may present these numerous advantages if the preparation of solutions of tryptophan, liberated from seed proteins, is adapted to the speed of analysis that can be reached with this method.

Tryptophan release can be achieved chemically or enzymatically. Pronase (Spies, 1967; Howe et al., 1972), pepsin, trypsin (Opienska-Blauth et al., 1963), and papain (Hernandez and Bates, 1969; Oste et al., 1976) have often been used to hydrolyze or to solubilize proteins. This step is time-consuming and the hydrolysis is not always complete. An enzymatic hydrolysis cannot be used easily for a routine analysis. Tryptophan is relatively stable in alkali solutions, so many authors have preferred the alkali extraction of proteins (Sodek et al., 1975; Kuiken et al., 1947) or the alkali hydrolysis to the enzymatic or acid hydrolysis. NaOH or $Ba(OH)_2$ are the most often used hydrolyzing agents (Spies, 1967; Howe et al., 1972; Matheson, 1974; Robin and Robin, 1971). Free tryptophan is then analyzed by colorimetry or by ion-exchange chromatography (Hugli and Moore, 1972; Devenyi et al., 1971). Liquid chromatography is indeed the best way for separation and determination of tryptophan; but this method is quite laborious to be used for a quantitative analysis achieved just for one amino acid. We therefore prefer to use a shorter procedure to determine tryptophan directly in the hydrolysis solution. This is a more convenient routine analysis. Direct ultraviolet determination of tryptophan is possible but tyrosine residues interfere in this case (Holiday, 1936; Bebeze and Schmid, 1957). Yet, the development of a colored reaction with tryptophan seems to be the method presenting the greatest number of advantages for automation. The p-dimethylaminobenzaldehyde colorimetry usually used for tryptophan analysis in pure proteins (Spies and Chambers, 1949; Graham et al., 1947; Miller, 1967; Westgarth and Williams, 1974) is considered to be best adapted to an automatic determination of tryptophan in cereal and leguminous seeds.

EXPERIMENTAL SECTION

Reagents and Standard Solutions. *Hydrolysis Agents.* Barium hydroxide octahydrate (Merck, ref. 1737) and sodium hydroxide (Merck, ref. 6462) were used for alkali hydrolysis.

Chemicals. All chemicals used for colorimetric analysis were of analytical grade. Solutions of p-dimethylaminobenzaldehyde (Merck, ref. 803057) and sodium nitrite (Merck, ref. 6544) were kept away from daylight.

Standards. Standard solutions of L-tryptophan (Calbiochem, ref. 6540) in deionized water ranging from 10 to 60 mg/L were used as references. These solutions were kept away from daylight and stored in a refrigerator when not used. These solutions were made each week.

Analytical Instrumentation. Colorimetric analysis was made with a second generation "autoanalyser Technicon" equipped with a sampler IV (came wheel for 30, 1/1), a proportioning pump at fixed speed, a light proof oil bath, a spectrophotometer with two cells (15-mm flowcell length) able to work in differential (full scale deflection of 0.5 OD), and a recorder (chart speed of 0.25 in./min).

Methods of Analysis. Hydrolysis of plant food samples was made in a 25-mL Pyrex tube (Sovirel, 20 × 200 mm) fitted with a screw cap and a Teflon air-tight gasket. To 500 mg of a finely ground leguminous or cereal flour (40 μm) containing 0.10–0.25% (w/w) of tryptophan (FAO, 1970), 10 mL of a hydrolyzing alkali solution (NaOH or $Ba(OH)_2$) was added (see Results and Discussion section for optimum concentration). Barium hydroxide powder which is slightly soluble in water at normal temperature was poured into Pyrex tubes and 10 mL of deionized water was then added. Pure proteins were treated in the same way, taking care that the amount of protein poured in the tube corresponded to a quantity of 0.5–2 mg of tryptophan.

Chemical hydrolysis took place overnight (15 h) at 110 °C. The hydrolysate was then neutralized with sulfuric acid (3.6 N).

Tryptophan determination was made directly on the hydrolysate solution by automatic colorimetry with p-dimethylaminobenzaldehyde (DMAB) and sodium nitrite at 590 nm.

RESULTS AND DISCUSSION

Hydrolyzing Agents. Enzymatic hydrolysis was not used for the reasons mentioned above. The chemical hydrolysis method was therefore preferred because it was more convenient and suitable to use for routine analysis. Sodium hydroxide and barium hydroxide were thus used in various concentrations to liberate tryptophan from proteins of leguminous seed flours (*Vigna*, *Phaseolus*, peanut, etc.) and of cereal flours (millet, sorghum, etc.).

When NaOH was used to hydrolyze flours, the formation of a gel due to the presence of starch made the neutralization of the hydrolysates difficult. Quantitative recovery of free tryptophan thus became a problem. So, a previous additional operation of starch solubilization was necessary when using NaOH (Hugli and Moore, 1972).

Table I. Influence of Hydrolysis Conditions for Tryptophan Determination[a]

	$Ba(OH)_2, 8H_2O$								NaOH			
	1 g/10 mL		2 g/20 mL		3 g/10 mL		4 g/10 mL		2.5 N		5 N	
samples	air	N_2	air	N_2	air	N_2	air	N_2	air	N_2	air	N_2
Sorghum vulgare	0.104[b]	0.111	0.101	0.105	0.093	0.099	0.087	0.095	0.069	0.071	0.066	
	0.107[c]	0.113	0.105	0.109	0.099	0.105	0.095	0.098				
Vigna unguiculata	0.231[b]	0.260	0.221	0.279	0.231	0.284	0.240	0.269				
% recov of L-Trp	85[b]	96	84	95	74	93	75	92	60	80	57	
after hydrolysis	87[c]	98	87	99	79	99	82	100	60	90	57	
(2 mg/10 mL)												
lysozyme	7.15[b]	7.10	7.55	7.58	6.88	7.00	6.71	6.77	5.86	5.94	6.35	6.67
	7.29[c]	7.24	7.86	7.88	7.33	7.44	7.23	7.38	5.86	5.94	6.35	6.67
casein				1.34[c]								

[a] Literature values (μg %): sorghum = 0.12% (FAO, 1970), *Vigna unguiculata* = 0.26% (FAO, 1970), lysozyme = 7–7.8% (Spies, 1967; Howe et al., 1972; Spies and Chambers, 1949), casein = 1.2–1.8% (Spies, 1967; Miller, 1967). [b] Values in this line are the corrected recovery percentage of pure L-tryptophan after hydrolysis. [c] Values in this line are the percent recovery of pure L-tryptophan after hydrolysis using the theoretical dilution factor of 5.

On the other hand, barium hydroxide at the concentration level of 2 g/10 mL enabled a complete tryptophan hydrolysis. The resulting colored solution was limpid and can be treated according to the subsequent steps. The hydrolysate was quantitatively transferred into a 50-mL flask and neutralized with 3.6 N sulfuric acid. The precipitate of barium sulfate settled at the bottom of the flask. It was then adjusted to a final volume of 50 mL with deionized water.

The volume of the precipitate was measured for each concentration of barium hydroxide used. This volume was taken into account when corresponding dilution factors were calculated. The results so obtained were expressed as the corrected percentage of tryptophan in the sample. The results presented in Table I show that tryptophan recovery is maximum when, all things being equal, the theoretical dilution factor is taken into consideration and not the true volume of the barium precipitate. Consequently this factor is constant with whatever barium hydroxide quantity is used for hydrolysis. It seems that barium sulfate absorbs a quantity of tryptophan in proportion to the volume occupied by the precipitate.

Use of the theoretical dilution factor ($d = 5$) permitted maximum recovery of tryptophan without being obliged to make extra operations such as separation by centrifugation and washing of the precipitate. These are time consuming and inconvenient for routine analysis.

Influence of Atmosphere. Tryptophan is rapidly degraded in the presence of oxygen. Protein hydrolysis must therefore be done in a medium free from oxidizing components. As early as 1949, Spies and Chambers recommended carrying out hydrolysis in a digester under hydrogen atmosphere. In alkali medium some authors have shown that the more inert the atmosphere is, the greater is the recovery of tryptrophan (Spies, 1967; Robin and Robin, 1971). Table I confirms these results. Losses of tryptophan from 10 to 20% were noted when barium hydrolysis was achieved under natural atmosphere. Recovery is improved when nitrogen atmosphere was used.

Nitrogen atmosphere was achieved by bubbling nitrogen for 5 min before hydrolysis into a Pyrex tube using a polyethylene capillary tube. This process has a dual result. It yielded an atmosphere free from an oxidant both in the solution and in the barium powder, the latter being completely dissolved at a concentration of 2 g/10 mL. Furthermore, it facilitated the mixing of flour samples with chemicals prior to hydrolysis, thus providing a homogeneous solution.

Effect of Temperature and Time of Hydrolysis. The effects of temperature and time of hydrolysis were studied on leguminous and cereal samples. Figure 1 shows

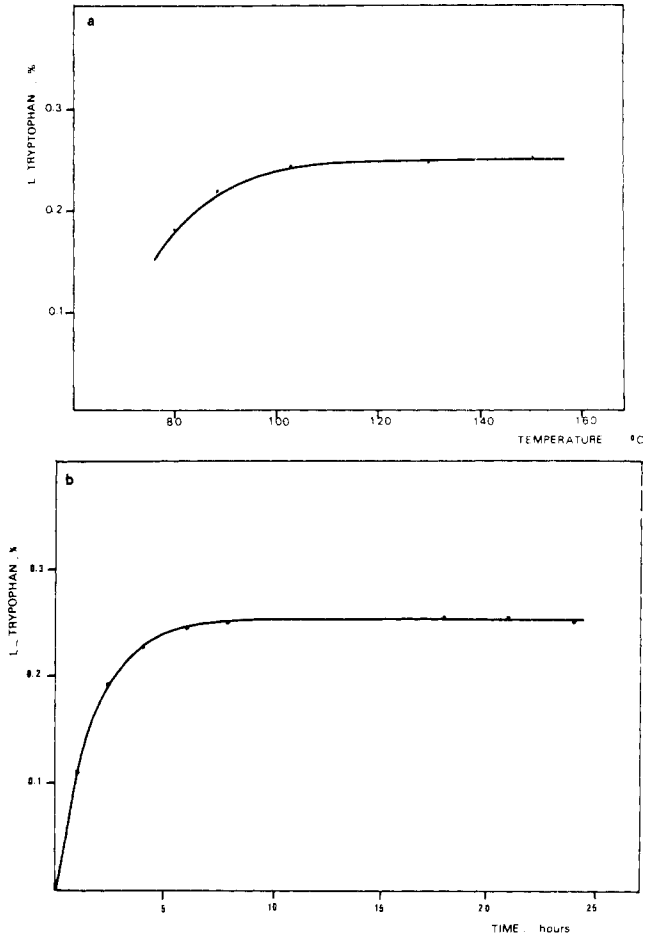

Figure 1. Optimization of hydrolysis conditions of tryptophan in *Vigna unguiculata* ($Ba(OH)_2 \cdot 8H_2O$, 2 g/10 mL): (a) constant hydrolysis time = 15 h, (b) constant hydrolysis temperature = 110 ± 1 °C.

that maximum liberation of tryptophan is reached after 8 h of hydrolysis at 110 °C. This temperature was chosen because it is the one used for normal acid protein hydrolysates which are also performed at 110 ± 1 °C in the same oven. For convenience, a 15-h period was chosen so that hydrolysis may take place overnight.

Automation of Colorimetry with *p*-Dimethylaminobenzaldehyde. Free tryptophan obtained by barium hydrolysis of food samples was determined by Erlich colorimetric reaction. Tryptophan residues reacted with *p*-dimethylaminobenzaldehyde (DMAB) in a sulfuric medium to give a chromogene which is oxidized in situ by

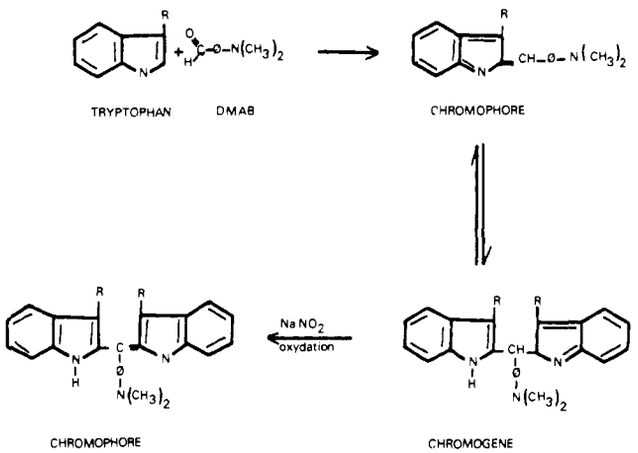

Figure 2. Chromophores formation in the colorimetric reaction of tryptophan with DMAB.

Table II. Comparison of the Composition of the Reactive Mixture in Manual and Automated Method for Tryptophan Determination

	colorimetry			
	manual		automatic	
products	mL	% total vol	mL/min	% total vol
H_2SO_4	8	79.2	1.84	65.45
DMAB	1	9.90	0.23	8.21
$NaNO_2$	0.2	0.99	0.10	11.43
L-Trp standards (10–60 mg/L)	1	9.90	0.42	14.90

sodium nitrite to give a chromophore; its maximum absorption in relation to the mixture (Trp + DMAB) used as a reference was about 590 nm (Figures 2 and 7).

The manual method used as a reference is the procedure that Spies and Chambers (1949) used for tryptophan determination in pure proteins. This procedure is described as follows: 8 mL of 23.5 N H_2SO_4 is pipetted into a 20 × 200 mm Pyrex tube. One milliliter of 30 g/L of DMAB–sulfuric acid solution (2 N) is added to 1 mL of a solution containing 200–600 μg of tryptophan. After the solution has been stirred thoroughly and allowed to react for 1 h in the dark, 100 μL of 0.045% aqueous nitrite sodium solution is added. This new mixture is left again in the dark for 45 min at room temperature in order that the color may develop. The optical density is read at 590 nm. The reference solution is treated in the same way except that sodium nitrite is omitted.

This procedure was automated on a segmented flow colorimetric chain equipped with two channels. According to the flow conditions inherent in the system used (internal diameter tubes = 1.7 mm), it is impossible to maintain such a long reaction time as the one proposed in the manual method (2 h). On the other hand, in order to avoid problems of pollution and contamination among samples, the output flow of the pumping tubes must be chosen according to two imperative requirements: (1) perfect hydraulics of the viscous and corrosive air-segmented flow and (2) an optimum proportion between the different reactants to get a maximum in the development of the color at 590 nm.

The volume composition of the reactive mixture found for the automatic colorimetry is given in Table II.

Optimization of Reagent Concentrations. The influence of reagent concentrations was studied with standard solutions of L-tryptophan ranging from 10 to 60 mg/L. The spectrophotometer operates at 0.7 OD full

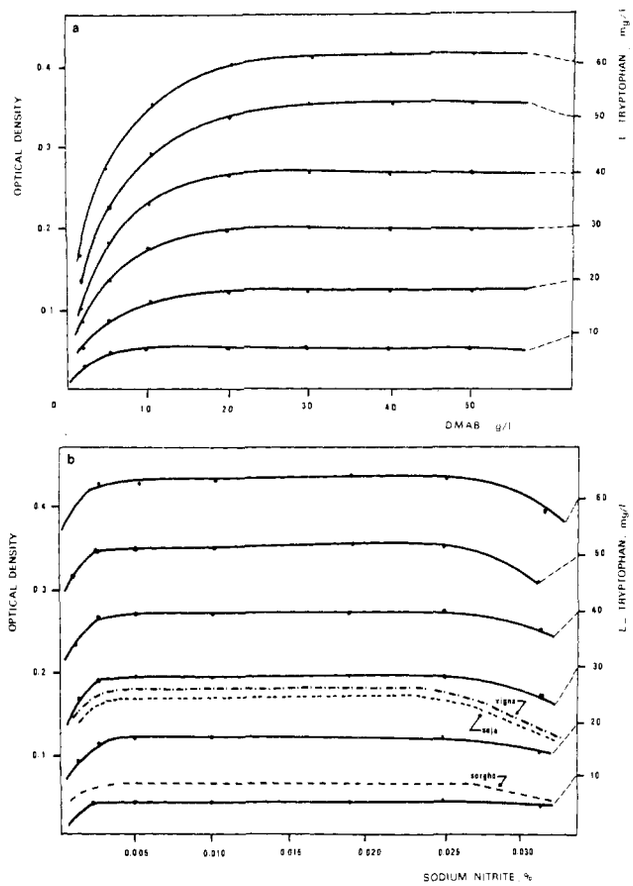

Figure 3. Effects of DMAB and $NaNO_2$ on the colorimetric reaction for tryptophan determination: (a) constant $NaNO_2$ concentration = 0.045% (stock solution), (b) constant DMAB concentration = 30 g/L (stock solution).

scale. Under these conditions about 500 mg of flour (cereal or leguminous seeds) can be used for hydrolysis.

The recorded peak heights corresponding to the tryptophan contained in the sample hydrolysis solutions are within the range of the peak heights corresponding to the two extreme standard solutions used (10 and 60 mg/L).

Figure 3 shows that maximum absorption is reached for the highest concentration of L-tryptophan (60 mg/L) when a 20 g/L DMAB stock solution was used at a constant sodium nitrite concentration identical with the one recommended by the Spies and Chambers method.

In the conditions of a DMAB pseudo-zero-order reaction, Figure 3b shows that the maximum blue color development is reached for concentrations of sodium nitrite above 0.003% (w/w). However, it has been noted that if the concentration of this sodium nitrite stock solution is over 0.025%, with all variables being constant, the colorimetric response is no longer linear with tryptophan concentrations.

For the fixed flow conditions listed in Table II, with stock solutions of 23.5 N H_2SO_4, DMAB (30 g/L), and $NaNO_2$ (100 mg/L), hydrolysate solutions of food samples gave a proper absorption (Figure 3b), allowing tryptophan to be determined with accuracy.

The color variation of the reaction medium is highly dependent on the sulfuric acid concentration. Below a concentration of 25 N in acid, the blue color intensity of the reaction decreased very quickly. Beyond this concentration value, the slopes of the curves, as shown in Figure 4, tend to decline. Moreover, with sulfuric acid concentrations beyond 25 N, the viscosity of the air-segmented flow increased rapidly and the hydraulics of the

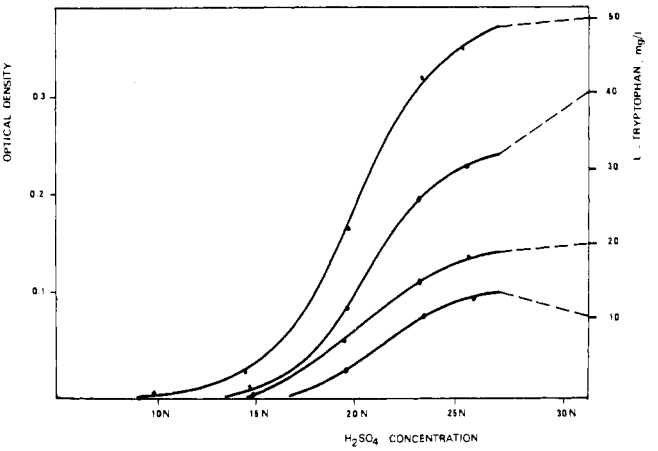

Figure 4. Effect of H_2SO_4 concentration on the coloration of the mixture (tryptophan + DMAB + $NaNO_2$).

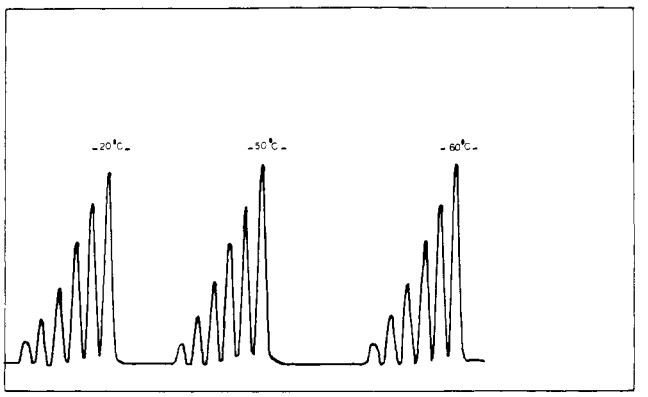

Figure 5. Effect of the reaction coil temperature on the colorimetric response of tryptophan determination; standards of L-tryptophan, 10–60 mg/L.

pumping system were not improved.

On the basis of these observations, the concentration of sulfuric acid recommended by Spies and Chambers (1949) appears to be an optimized value for the determination of tryptophan by the automatic method.

Temperature Effect on the Kinetics of the Colorimetric Reaction. The experimental conditions described above concerning the manual method need a 2-h reaction time between tryptophan and the various reactive agents used. In an automatic analysis with a continuous flow, it is very difficult to realize such a long reaction time without being inconvenienced by the pollution which contaminates each sample. This pollution is mainly due to the high viscosity of the concentrated sulfuric acid solution used.

In order to minimize this problem of diffusion, the length of the analytical circuit was shortened without reducing the maximum intensity of the color development, but while keeping a perfect hydraulic system. The colorimetric detection was carried out 12 min later after the sample was pumped in from the sampler.

The temperature rise of the reaction coil no longer affects the color intensity of the complex formed from standard tryptophan solutions as it is shown in Table III. Therefore a 12-min reaction time seems sufficient to reach the maximum formation of the colored complex.

The chemical reactions involved in tryptophan determination, in seed flours, is hardly affected by a change in temperature of the reaction medium. Even in a strong acid medium and in the presence of other components of leguminous seeds, especially sulfur amino acids, tryptophan does not seem to be destroyed by a rise in temperature (Figure 5). Consequently, the reaction may have already reached its maximum in these experimental conditions or because limiting factors of the kinetic reaction.

Consequences of a Two-Step Reaction on the Automation of Tryptophan Colorimetry. The colorimetric reaction of tryptophan according to the *p*-dimethylaminobenzaldehyde method (Figure 2) involves a two-step chemical reaction: (1) the condensation of 2 mol of tryptophan with 1 mol of DMAB in a concentrated sulfuric acid medium and (2) the oxidation of the resultant product.

The condensation product obtained from the reaction of tryptophan with DMAB and $NaNO_2$ gave two absorption maxima: the first one at 590 nm and the second one at 780 nm. The highest peak lies at about 780 nm. At this wavelength, absorption is indeed very much affected by the nature of the sample used, as shown in Figure 6. As a matter of fact, the complex DMAB + tryptophan gave different absorption values depending on whether the tryptophan solution analyzed is pure L-tryptophan or is obtained from hydrolysis of a seed flour. These variations are far less important when absorption was measured at 590 nm. In this case, the difference in absorption between (Trp + DMAB + $NaNO_2$) and the reference mixture (Trp + DMAB) used in the automatic analysis was the greatest. Moreover, the reference absorption was far less affected by the nature of the flour sample at 590 nm than it is at 780 nm.

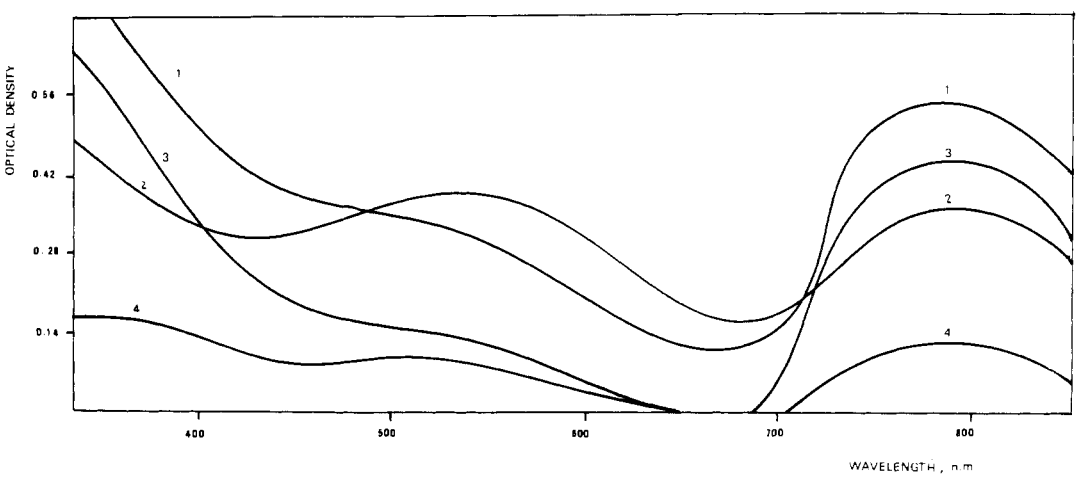

Figure 6. Comparison of absorption curves obtained with various reactive mixtures; effect of seed components on the coloration of the mixture: (1) H_2SO_4 + DMAB + $NaNO_2$ + *Phaseolus vulgaris* (Red Bean), (2) H_2SO_4 + DMAB + *Phaseolus vulgaris*, (3) H_2SO_4 + DMAB + $NaNO_2$ + L-tryptophan (40 mg/L), (4) H_2SO_4 + DMAB + L-tryptophan (40 mg/L).

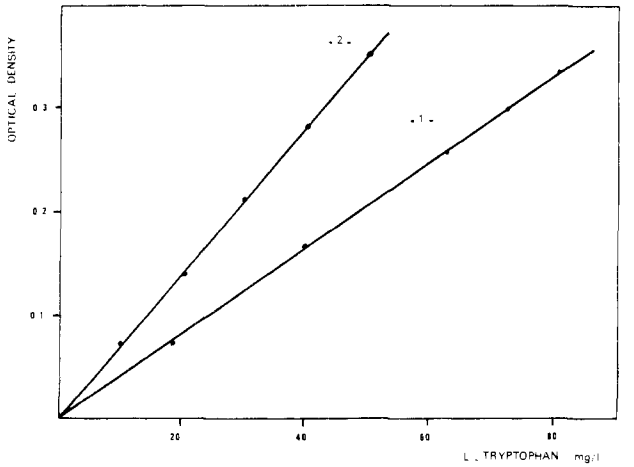

Figure 7. Variation of the optical density with L-tryptophan concentration according to the Beer-Lambert law: (1) manuel method, (2) automatic method.

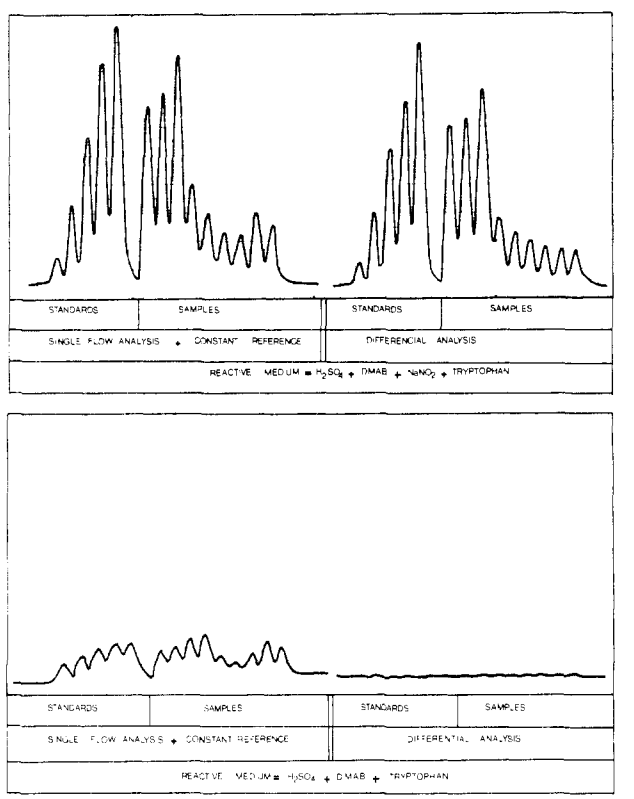

Figure 8. Interferences affecting tryptophan determination when using a two-step colorimetry; influence of origin of samples.

In order to measure only the absorption of the tryptophan complex, it is necessary to work with a differential procedure of analysis. In these conditions, the absorption due to the reaction between sample and DMAB at 590 nm no longer gives interferences. The reference for each sample is then made up by the mixture of all reactive components except sodium nitrite which is substituted by deionized water (Figure 8). The variations in color formation obtained are proportional to tryptophan concentrations, according to the Beer-Lambert law. The standard curve representing the variations of the optical density with tryptophan concentrations shows a higher slope than the one we get with the manual method (Figure 7), all things being equal except for the chemical concentrations and the continuous stirring provided by the automatic segmented flow device used in this method.

Table III. Effect of Reaction Coil Temperature in Tryptophan Determination of *Vigna unguiculata* Samples (Niebe)

reaction coil temp, °C	25	40	50
tryptophan, g/16 g of N	1.14	1.14	1.15

Table IV. Interferences of Legume Seed Pigments in Tryptophan Determination with the DMAB Method

	analysis methods			
samples	differential, g/16 g of N	single flow, g/16 g of N	SD, %	color of hydrolysis soln
Niébé 59.24	0.91	1.01	10	deep brown
Niébé 58.162	0.84	0.93	10	light
Niébé 67.61	1.04	1.09	5	light yellow
Niébé Katchaké	0.98	1.03	5	orange-yellow
Niébé local Dschang	0.77	0.89	14	deep brown
lysozyme	7.80	7.85	0.5	colorless

A tryptophan analysis according to the differential colorimetric method (Figure 8) further avoids some other interferences caused by seed components. The color of the hull, which is not removed before grinding, gave appreciable changes in the case of red beans (*Phaseolus vulgaris*) or colored coat seeds (Concon, 1975; Amaya et al., 1977). Other pigments less visible as anthocyanins can also interfere in the colorimetric determination of tryptophan (Hernandez and Bates, 1969; Sodek et al., 1975; Matheson, 1974).

Series of analysis was carried out with various lines of Niebe (*Vigna unguiculata*) of different colored hulls. The colors of the hydrolysis solutions differed from one sample to the other. Nevertheless this coloration intensity was not related to the differences in tryptophan contents determined with the single flow system (constant reference) and the differential one. On the other hand, for a pure protein such as lysozyme, the deviation observed between the two automatic methods is very small and negligible, as shown in Table IV.

Therefore, other interfering substances are present in the seeds. They can absorb at 590 nm and consequently give misleading results for tryptophan determinations with a single flow system. All specifications concerning the manifold used for automated colorimetry are given in Figure 9.

Tests of tryptophan recovery during a complete procedure (hydrolysis and automatic colorimetry) were achieved by using compounded flours from various samples of the same variety: Niebe (*Vigna unguiculata*) or sorghum or defatted peanuts. Large batches of these mixed flours of leguminous or cereal seeds are ordinarily used as reference samples in the routine analysis in our laboratory. So, the results reported in Table V are not obtained from pure line samples. Microbiological determinations of tryptophan were also made according to the method of Wright (1963).

Recovery percentages of pure tryptophan added in different amounts to samples of Niebe flours (expt: 7–13) are good if the amount of the supplement does not exceed the initial value of tryptophan contained in the sample.

Losses of added tryptophan are more important in the case of sorghum samples where additions of free tryptophan can exceed 300% of the initial amount of tryptophan contained in the sample. Nevertheless, determinations of tryptophan achieved on mixed flours of sorghum and defatted peanuts (expt 16–20) showed that the experimental values are consistent with the theoretical ones calculated from the percentage of tryptophan contained in each pure

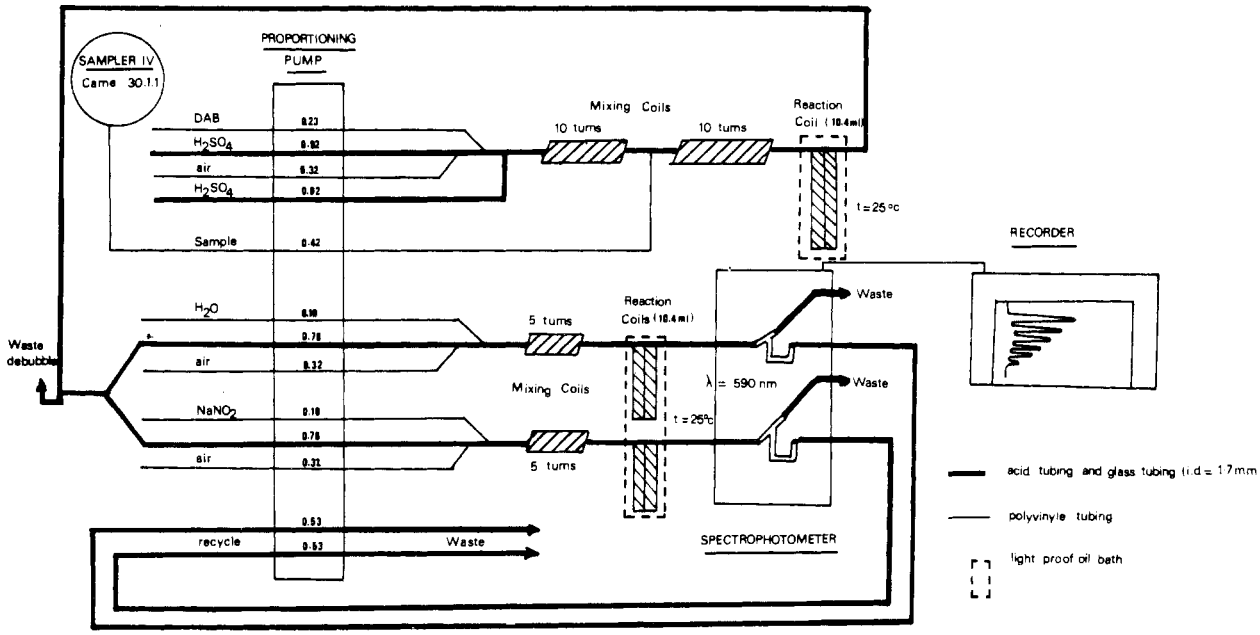

Figure 9. Manifold for automatic and differential determination of tryptophan in food samples.

Table V. Tryptophan Determination by Automated Colorimetric Method in Legume and Cereal Flours Mixed or Supplemented with Pure Free L-Tryptophan (OD = 0.7 Full Scale)

	hydrolyzed samples (mg of flour/10 mL of alkali soln)				quantity of Trp found after hydrolysis by automatic colorimetry			% Trp (g/100 g of flour)	
	Vigna (Niebe)	*Sorghum vulgare*	defatted peanut	L-Trp pure	exptl value, mg/10 mL	theoret value, mg/10 mL	% recov of Trp	chemical detn	microbiol detn according to Barton Wright (1963)
1	512.9				1.246			0.243	0.235
2	526.7				1.264			0.240	
3		537.9			0.626			0.123	0.122
4		529.4			0.636			0.120	
5			272.7		1.156			0.424	0.430
6			286.2		1.208			0.422	
7	591.3			0.10	1.522	1.528	99.6		
8	520.0			0.20	1.412	1.456	97		
9	517.5			0.30	1.488	1.550	96		
10	545.6			0.40	1.670	1.718	97.2		
11	532.5			0.50	1.741	1.786	97.5		
12	546.7			1.00	2.188	2.320	94.3		
13	495.5			1.75	2.629	2.954	89		
14		575.1		0.50	0.988	1.202	82.2		
15		494.7		1.00	1.300	1.599	81.3		
16		565.5		1.75	1.820	2.440	74.6		
17		321.1	35.2		0.534	0.544	98.1		
18		343.9	94.6		0.792	0.820	96.6		
19		322.8	142.5		0.975	0.997	97.8		
20		330.6	234.5		1.344	1.395	96.3		

sample (expt 1–6) and from the composition of the mixture of flours. On the basis of this observation, tryptophan added to a leguminous or cereal flour does not behave in the same way as added tryptophan obtained from a non-hydrolysate protein.

Consequently, the recovery corrections recommended by Miller (1967) and further by Westgarth and Williams (1974) are not taken into account in our calculations.

CONCLUSION

The method described above allows a precise determination of tryptophan, an essential amino acid in proteins of leguminous and cereal seeds, by automatic colorimetry with DMAB after barium hydrolysis. Routine analysis is also possible. The various operations of protein extraction recommended to avoid interferences due to presence of different seed components absorbing at 590 nm are no longer necessary. The analytical speed of 200–300 samples per week and per person can be reached easily. The coefficient of variation calculated from the various standard samples used during a 2-month routine analysis (50 standard samples) is about 4% (σ = 0.0091) for 500-mg samples of flour containing about 0.1–0.5% (w/w %) of tryptophan. The differential and automated method described above maintains the same characteristics of accuracy and reproducibility even when quantities of flour samples as small as 100 mg (0.2% tryptophan) are used.

The sensitivity of the automated method makes possible the determination of quantities of tryptophan as small as 5 μg/mL in the reactive medium with the same accuracy.

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Received for review January 25, 1979. Accepted November 13, 1979.

Extractability, Solubility, and Molecular Size Distribution of the Nitrogenous Constituents in Coastal Bermuda Grass Silage

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Ensiled Coastal Bermuda grass was extracted at pH 7.9 with 0 and 1% sodium dodecyl sulfate ($NaDodSO_4$), followed by filtration, centrifugation, and preparative chromatography on G-15 Sephadex. Two insolubles, R_I and R_{II}, and four soluble fractions, proteins (C1), polypeptides (C2), smaller polypeptides and amino acids (C3), and degradation products (C4), were obtained. At 0% $NaDodSO_4$, the percentage of total nitrogen (N_T) for R_I, R_{II}, C1, C2, C3, and C4 was 28.5, 3.41, 1.27, 22.1, 18.9, and 4.68, respectively. At 1% $NaDodSO_4$, R_I was 23.6 in N_T and C1 was 7.2; the rest were unchanged. Gel chromatography gave a broad molecular weight distribution [(1.0×10^6)-(2.5×10^3)] for C1, but a low average [$(4-8) \times 10^3$]. C2 and C3 had narrow molecular weight distributions (averages of 1800 and 950). Molecular weight averages were independent of percentage $NaDodSO_4$ in extractant (i.e., 0 and 1%) and of wavelength of detection (i.e., 254 and 206 nm). The chloroplastic proteins (i.e., most of the proteins in cut 1 from the $NaDodSO_4$ extraction) were insolubilized by lyophilization. Gel chromatography indicated that the chlorophyll-protein complex had a molecular weight in excess of 1 million.

Ensiling is a major method of preserving and storing fresh forage. Changes in the chemical composition of ensiled forages have been studied intensively over the last 30 years and are important from a nutritive standpoint. Recently, McDonald and Whittenbury (1973) reviewed thoroughly the research on the chemical changes which occur in silage.

The degradation of amino acids in silage has been well documented (e.g., Hughes, 1970; Macpherson and Violante, 1966a,b; Macpherson 1962). Perhaps the most comprehensive study of nitrogenous compounds in silage was that of Hughes (1970), who analyzed by groups the water-soluble proteins, peptides, amino acids, and amides and volatile amines in rye grass. Hughes found no water-soluble proteins in rye grass, but did not extract proteins from the silage with a buffered solution of about pH 8.0 or which also contains detergent. Such solutions will maximize protein removal from fresh grasses (Fishman and Burdick, 1977). Therefore, in this report, Coastal Bermuda grass silage was extracted with buffer and buffered detergent solutions with the intent of extracting maximum protein.

The data reported here should be useful for evaluating Bermuda grass silage as a potential source of protein concentrate. Furthermore, the data should provide basic information to those interested in the nitrogen and the actual protein content in grass silage since the molecular size distribution of nitrogenous constituents has been measured directly and quantitatively.

EXPERIMENTAL SECTION

Coastal Bermuda Grass. Coastal Bermuda grass (*Cynodon dactylon* [L.] Pers) was obtained from Coastal Farms, Inc., Estill, SC. It was harvested and fertilized as previously described (Fishman and Burdick, 1977). A

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