study whereas there were only 1.43 and 0.41 μg/g of fish after 24 h in the 144-UP and elimination studies. We believe that the differences in concentrations between the three studies are correlated with the differences in loading ratios and the rate at which the fish were removed from the treatment solutions.

Statham and Lech (1975) reported that [^{14}C]Bayer 2353 was taken up and metabolized by rainbow trout to a glucuronide. We found that after β-glucuronidase treatment of bass bile, the ether extracts contained a compound which had the same R_f as B73-mix and co-chromatographed with it. We were unable to perform spectral analyses because the quantity of the compound available for study was extremely limited, thus we cannot prove the presence of a glucuronide in bass bile, but presume this is the case, based on the work of Statham and Lech (1975).

We also attempted to quantify the amount of B73-mix in the fish muscle by gas chromatography. Although a precise method has not been developed, we ascertained that 60 to 80% of the radioactive materials found in muscle tissue consisted of B73-mix. This finding was confirmed by thin-layer chromatography. We did not analyze other tissues or organs because of the inadequacies of the method.

Our experiments show that B73-mix is taken up and distributed throughout the various organs and tissues of fish; that the bile contained the greatest concentration of B73-mix and that the highest concentration of B73-mix was found in fish from the experiment which had the lowest loading rate (24-UP).

LITERATURE CITED

Gonnert, R., *Pflanzenschutz-Nachr.* **15**, 4–24 (1962).
Rodgers, C., Stalling, D. L., *Weed Sci.* **20**, 101–105 (1972).
Schultz, D. P., *J. Agric. Food Chem.* **21**, 186–192 (1973).
Schultz, D. P., Harman, P. D., *J. Fish. Res. Board Can.* **33**, 1121–1129 (1976).
Statham, C. N., Lech, J. J., *J. Fish. Res. Board Can.* **32**, 515–522 (1975).
Statham, C. N., Melancon, M. J., Jr., Lech, J. J., *Science* **193**, 680–681 (1976).

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Nutritive Value of the Nitrogen-Fixing Aquatic Fern *Azolla filiculoides*

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Azolla filiculoides had a low nutritive value for growing rats when given as the sole source of protein in the diet. The addition of lysine, methionine, and histidine gave a marked increase in rat growth and PER. The high neutral detergent fiber of *Azolla* (39%) is a major limiting factor for the efficient utilization of *Azolla* as a protein source for simple-stomached animals. In vitro digestability data showed that *Azolla* was readily degraded by rumen microorganisms and indicated its possible usefulness as a protein source for ruminants.

Floating fresh water ferns of the genus *Azolla* have a wide geographic distribution (Ashton and Walmsley, 1976; Stewart and Pearson, 1970). Several species of *Azolla* are cultivated in Southeast Asia as a green manure to fertilize rice paddies and as food for animals (Moore, 1969). Current interest in *Azolla* arises from its symbiotic relationship with a nitrogen-fixing blue-green algae, genus *Anabaena*. The algae live in leaf cavities of *Azolla* and are capable of using their own photosynthetic energy to reduce atmospheric nitrogen and produce ammonia which can be used by the fern to meet all its nitrogen requirements (Ashton and Walmsley, 1976; Newton and Cavins, 1976; Marx, 1977). This attribute is of considerable potential importance to agricultural regions with inadequate energy for the production of synthetic nitrogen fertilizers (Marx, 1977). This symbiotic relationship also permits the fern to be relatively independent of fixed nitrogen in its environment. Thus *Azolla* tends to be high in nitrogen and is a potentially attractive source of protein for animal rations.

A tropical species of *Azolla* has reportedly been used as a feed for pigs and ducks in Indochina; for cattle, fish, and poultry in Vietnam; and for pigs in Singapore and Formosa (Moore, 1969). The North Vietnamese (Thuyet and Tuan, 1973) describe *Azolla* as an excellent substitute for green forage for cattle and suggest that it may replace 50% of the rice bran used as feed for pigs. They also reported that the crude protein of *Azolla* was 13% of dry matter and that lysine and tryptophan were low compared to rice protein. Fujiwara et al. (1947) reported 23.8% crude protein in *Azolla*. However, no substantiating reports of complete proximate analysis of *Azolla* could be found in the literature. Data from animal feeding trials in which *Azolla* was compared to common foodstuffs are also lacking. This information is requisite to the consideration of *Azolla* as a feedstuff, and so the present study was undertaken to determine the chemical composition of *Azolla*; compare the growth of weanling rats given protein from *Azolla* and casein; determine the effect of the fiber and mineral components in *Azolla* on its utilization by rats; determine the effect of supplementation of *Azolla* with lysine, methionine, and histidine in rat rations; and

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Table I. Composition of Diets (g/kg)

ingredient	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6
Azolla	500	500			200	
casein			112	112	200	200
amino acids[a]	6.7	7.78	3.1	3.1	3.1	3.1
sucrose	150	150	190	150	190	190
starch	183.3	182.22	564.9	345.9	276.9	373.4
corn oil	80	80	50	50	50	50
Solka-floc[b]				200[c]		80[d]
vitamin mix[e]	10	10	10	10	10	10
mineral mix[f]	50	50	50	50	50	50
additional minerals				59[g]		23.5[h]
carboxymethylcellulose	20	20	20	20	20	20

[a] See Table II for list of additional amino acids. [b] Trade name for purified cellulose. [c] Equal to the NDF for 500 g of *Azolla*. [d] Equal to the NDF for 200 g of *Azolla*. [e] Vitamins per kg diet: D-biotin, 2 mg; vitamin D_3 (dry; 400 000 IU/g), 3 mg; folic acid, 10 mg; thiamine·HCl, 15 mg; pyridoxine·HCl, 15 mg; riboflavin, 15 mg; menadione, 15 mg; vitamin A palmitate (dry; 250 000 IU/g), 40 mg; nicotinic acid, 50 mg; D-calcium pantothenate, 50 mg; DL-α-tocopherol (dry; 250 IU/g), 200 mg; vitamin B_{12}, 50 mg; choline chloride, 900 mg; inositol, 1,000 mg. [f] The salt mixture (Rogers and Harper, 1965) provided (as percent of the diet): Ca, 0.592; P, 0.394; K, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0049; Cu, 0.0019; Mn, 0.00195; Zn, 0.0004; I, 0.000019; Mo, 0.000005; Se, 0.0000025. [g] Equal to the Ca, Na, and K in 500 g of *Azolla*. [h] Equal to the Ca, Na, and K in 200 g of *Azolla*.

conduct a short-term toxicity trial. The results of these studies form the basis of this article.

METHODS AND PROCEDURES

Samples of *Azolla filiculoides* Lam. (Mason, 1969) were collected on November 3 and 5, 1974, from Los Gatos Creek in an urban area of Santa Clara County, California (latitude 37° 15′ 59″ North, longitude 121° 56′ 52″ West; USGS San Jose West 7.5′ quadrangle). Each sample was drained of free water, transported to the University of California at Davis, and stored at −4 °C until it was lyophilized, then ground in a Wiley mill to pass a 40-mesh screen.

Analytical Methods. Freeze-dried material was used in all analyses. Nitrogen was determined by the Kjeldahl method (AOAC, 1975a). Amino acids were determined by ion-exchange chromatography on 25-mg samples of *Azolla* hydrolyzed in 25 mL of 6 N HCl under vacuum at 121 °C for 26 h. The hydrolyzate was dried under vacuum at 50 °C and dissolved in 2 mL of citrate buffer at pH 2.2, and aliquots were used for analysis. The unhydrolyzed residue was negative for nitrogen by the micro-Kjeldahl method (AOAC, 1975b). Tryptophan was determined by ion-exchange chromatography after hydrolysis with barium hydroxide (Kohler and Palter, 1967).

Purines were determined by high-pressure liquid chromatography, using a cation exchange resin. For this analysis samples of 0.5 g of *Azolla* were homogenized in 9.5 mL of 1.2 N perchloric acid. The homogenates were placed in boiling water for 30 min and then filtered through a Millipore filter. The filtrates were analyzed for the purine bases, adenine, guanine, hypoxanthine, and xanthine, on the chromatograph using a nongradient elution with 0.5 N ammonium dihydrogen phosphate, pH 2.0, at a flow rate of 12 mL/h and a column pressure of 112.5 kg/cm^2 (Clifford and Story, 1976).

Sodium, potassium, calcium, iron, and copper were determined by atomic absorption spectrophotometry. *Azolla* samples were prepared by digestion in perchloric and nitric acids. Samples analyzed for phosphate by a colorimetric assay were prepared in the same manner (Krawl, 1966). Chlorine, manganese, cobalt, zinc, lead, molybdenum, and strontium were determined by X-ray fluorescence (Brady and Cahill, 1973). The sample target was prepared by spreading about 500 mg of ground lyophilized *Azolla* into a 1.25 in. die and pressing into a pellet with a 20-ton hydraulic press.

Azolla was analyzed for cellulose, lignin, acid detergent fiber (ADF), neutral detergent fiber (NDF), and ether extract by the Goering and Van Soest method (Goering and Van Soest, 1970). Ash was determined by heating samples for 12 h at 575 °C.

Table II. Sources of Protein in the Diets[a]

protein source	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6
	% protein in the diet					
Azolla	10.8	10.8			3.9	
casein			10.2	10.2	18.2	18.2
L-alanine	0.67					
L-lysine·HCl		0.35				
DL-methionine		0.23	0.31	0.31	0.31	0.31
L-histidine·HCl		0.09				
total protein	11.47	11.47	10.51	10.51	22.41	18.51

[a] Protein estimated as the sum of the amino acids and calculated on a dry weight basis.

Animals. Thirty female weanling rats (30 to 34 days old) of the Sprague-Dawley strain (82 to 117 g) were randomized into six groups, each of five rats, on a stratified body weight basis. Rats were housed individually in stainless steel cages with wire screen bottoms. They were given the experimental diets for 10 days. Body weights were recorded on days 1, 5, and 10. Urine and feces were collected daily from days 5 through 10 and stored at 0 °C pending analysis. Urine was collected in beakers containing sulfuric acid.

Diets. The composition of the experimental diets is shown in Tables I and II. Carboxymethylcellulose was added to all diets to aid their compression into pellets. *Azolla* was the sole protein source in diets 1 and 2, supplying 10.8% protein. At this level in the diet we calculated from the amino acid composition that *Azolla* would not provide the National Research Council (NRC) requirements for the growing rat for methionine, lysine, or histidine. In diet 2 these amino acids were supplemented to meet the NRC requirements for the growing rat (National Academy of Sciences, 1972). Alanine was added to diet 1 to make it isonitrogenous with diet 2. Diets 3 and 4 were relatively isonitrogenous to diets 1 and 2, with casein plus methionine as the source of amino acids. In diet 4 a portion of the sucrose and starch was replaced with cellulose and additional minerals to approximate the indigestible fiber (NDF) and major minerals (Ca, Na, and K) supplied by *Azolla* in diets 1 and 2. Diets 5 and 6 contained 18.2% crude protein supplied by casein plus methionine. Diet 5 contained 20% *Azolla* in order to test for the presence of a toxin or growth inhibitor in *Azolla*. Diet 6 contained 20% casein plus cellulose and additional minerals to approximate the indigestible fiber (NDF) and

Table III. Composition of *Azolla*

	% of dry matter[a]	Minerals[b,c]			
		mg/g		μg/g	
ash	15.54 ± 2.51	Na	15.25 ± 1.21	Cu	28.05 ± 2.89
acid detergent fiber (ADF)	26.58 ± 2.23	K	20.13 ± 1.44	Mn	771
neutral detergent fiber (NDF)	39.16 ± 1.56	P	4.87 ± 0.58	Co	4.6
		Ca	9.73 ± 0.26	Zn	54.7
cellulose	15.19 ± 1.35	Fe	1.00 ± 0.25	Pb	25.0
lignin	9.27 ± 1.19	Cl	10.7	Mo	7.0
nitrogen	4.47 ± 0.03			Sr	64.4
ether extract	5.05 ± 0.05				

[a] Values represent mean and standard deviation for three samples of freeze-dried *Azolla*. [b] Values with standard deviation represent mean-values for three samples of freeze-dried *Azolla* (92.27 ± 1.26% dry matter). [c] Values without standard deviation represent one determination by X-ray fluorescence.

Table IV. Amino Acid Composition of *Azolla*[a] Compared to Alfalfa,[b] Soybean,[c] and Corn[c]

	g/100 g of protein[d]				% dry matter			
amino acid	*Azolla*	alfalfa	soybean	corn	*Azolla*	alfalfa	soybean	corn
threonine	4.70	5.11	3.91	3.71	1.10	1.05	1.74	0.39
valine	6.75	6.91	4.88	4.94	1.58	1.42	2.17	0.52
methionine	1.88	1.85	1.28	2.00	0.44	0.38	0.57	0.21
isoleucine	5.38	5.64	4.61	3.80	1.26	1.16	2.05	0.40
leucine	9.05	8.95	7.88	12.83	2.12	1.84	3.51	1.35
phenylalanine	5.64	6.13	5.01	5.04	1.32	1.26	2.23	0.53
lysine	6.45	5.01	6.47	2.76	1.51	1.03	2.88	0.29
histidine	2.31	2.28	2.56	2.76	0.54	0.47	1.14	0.29
arginine	6.62	4.91	7.35	4.28	1.55	1.01	3.27	0.45
tryptophan	2.01	2.68	1.30	0.76	0.47	0.55	0.58	0.08
aspartic acid	9.39	11.67	11.86	6.46	2.20	2.40	5.28	0.68
glutamic acid	12.72	11.82	18.98	19.39	2.98	2.43	8.45	2.04
serine	4.10	5.01	5.19	5.13	0.96	1.03	2.31	0.54
proline	4.48	5.11	5.57	9.12	1.05	1.05	2.48	0.96
glycine	5.72	5.88	4.25	3.80	1.34	1.21	1.89	0.40
alanine	6.45	6.52	4.31	7.70	1.51	1.34	1.92	0.81
cystine	2.26	1.17	1.35	1.62	0.53	0.24	0.60	0.17
tyrosine	4.10	3.36	3.19	3.90	0.96	0.69	1.42	0.41
Met + Cys	4.14	3.02	2.63	3.52	0.97	0.62	1.17	0.37
Phe + Tyr	9.74	9.48	8.20	8.94	2.28	1.95	3.65	0.94
protein[d]					23.42	20.56	44.51	10.52

[a] Amino acid values for *Azolla* represent the mean of four amino acid analyses, except for tryptophan which represents one analysis. [b] Data determined for 22% protein grade alfalfa; American Dehydrators Association (1969). [c] FAO (1970). [d] Protein estimated as sum of amino acids.

major minerals supplied by the 20% *Azolla* in diet 5. All diets and water were provided ad libitum, and food intake was recorded on days 5 and 10.

Dietary Evaluation. Protein quality of the experimental diets was compared using the protein efficiency ratio (PER) (Hegsted, 1974). Dry matter digestibility and apparent nitrogen digestibility were measured directly from food intake and fecal output. The data from the feeding trials were analyzed statistically by Student's *t* test (Woolf, 1968).

RESULTS

Results of the proximate and mineral analysis of *Azolla filiculoides* are presented in Table III. The values for ADF and NDF were high. Alfalfa of a comparable protein content to *Azolla* has a similar ADF (27.4%), and an NDF of about half that of *Azolla* (20.2%) (American Dehydrators Association, 1969). The value for nitrogen was high, apparently due to the nitrogen fixing blue-green alga in the leaves of *Azolla*. Recovery of Kjeldahl nitrogen as amino acids, purines, and ammonia was 82.6%. Relative to alfalfa the major minerals were adequately supplied by *Azolla* (American Dehydrators Association, 1969), and the Ca/P ratio was 2.0. Some of the trace minerals were in higher concentration than in alfalfa: iron four times, copper three times, and manganese 22 times higher. The extent to which the mineral composition of *Azolla* was determined by the mineral concentration in the water was not investigated. Preliminary results, however, indicated that a 100-fold increase in phosphorus concentration in the water (0.1 to 10.0 ppm) produced a fivefold increase in *Azolla* phosphorus concentration on a dry matter basis. It was also observed that the nitrogen concentration of *Azolla*, grown in media containing 10 ppm phosphorus, could be increased to as much as 6%. Whether the additional nitrogen was in the form of protein was not determined.

The amino acid composition of *Azolla* is presented in Table IV; and for comparison the amino acid compositions of alfalfa, soybean, and corn are also listed. Recovery of Kjeldahl nitrogen as amino acids in *Azolla* was about 70%. The composition of essential amino acids in *Azolla* (g/100 g protein) compared well with the reference protein sources. Methionine was low, as is true for many leaf proteins, but the value for lysine was more than twice that of corn. On a percent of dry matter basis *Azolla* amino acids and total protein (23.42%) compared favorably with good quality alfalfa.

Results of purine analysis of *Azolla* are shown in Table V, along with the purine composition of selected legumes. Hypoxanthine and xanthine were not present in either *Azolla* or alfalfa, but both contained large amounts of adenine.

The data from the rat feeding trials are presented in Table VI. The *Azolla* diet (diet 1) did not support growth, either for the complete 10-day trial or for the last 5 days

Table V. Purines in *Azolla* Compared to Selected Legumes

	adenine, mg/100 g	guanine, mg/100 g	hypoxanthine, mg/100 g	xanthine, mg/100 g	total purines, mg/100 g	% nitrogen recovery as purines
Azolla[a]	240 ± 19	265 ± 14			492 ± 11	5.54 ± 0.34
lentils[b]	104	82	20	16	222	
blackeye peas[b]	77	80	32	41	230	
alfalfa[c]	374 ± 17	503 ± 12				

[a] Values represent mean and standard deviation for three samples of freeze-dried *Azolla*. [b] Clifford and Story (1976). [c] Clifford et al. (1975).

Table VI. Results of Animal Feeding Trial (Days 6-10)

diet	feed consumption,[a] g[e]	weight gain, g[e]	dry matter digestibility,[a] %	apparent nitrogen digestibility,[a] %	PER, gain/protein
1. *Azolla*	51.3 ± 3.4[o]	−1.4 ± 4.4[q]	71.0 ± 3.4	60.3 ± 4.2	*g*
2. *Azolla* + limiting aa[b]	72.8 ± 7.8[o,p]	19.2 ± 4.9[q,r]	67.2 ± 2.2[t,u]	56.3 ± 3.2[x,y]	2.51
3. casein	84.0 ± 7.3[p]	31.0 ± 4.4[r,s]	92.2 ± 0.8[t,v]	95.1 ± 1.7[x,z]	3.51
4. casein[c] + cellulose + minerals	89.8 ± 10.3	24.8 ± 3.5[s]	73.7 ± 1.0[u,v]	87.3 ± 0.8[y,z]	2.63
5. high casein + 20% *Azolla*	71.7 ± 11.6	28.8 ± 5.9	82.8 ± 1.7[w]		
6. high casein[d] + cellulose + minerals	74.1 ± 7.4	24.8 ± 4.3	87.2 ± 0.3[w]		

[a] Mean and standard deviation, on a dry weight basis. [b] Lysine, methionine, and histidine. [c] Cellulose and minerals added to approximate the amount of NDF and minerals (Ca, Na, K) provided by *Azolla* in diets 1 and 2. [d] Cellulose and minerals added to approximate the amount of NDF and minerals (Ca, Na, K) provided by *Azolla* in diet 5. [e] Mean weight per animal. [f] Paired means with the same superscript in the same column were compared using a two tailed "T" test and found to be different at the following levels of significance: p, r, s = $P < 0.05$; o, q, u, w = $P < 0.0005$; z = $P < 0.00005$; x = $P < 0.5 \times 10^{-6}$; t = $P < 0.1 \times 10^{-6}$; y = $P < 0.5 \times 10^{-7}$; v = $P < 0.5 \times 10^{-8}$. Diet groups 5 and 6 were compared only to each other. [g] PER cannot be calculated for a negative weight gain (Hegsted, 1974).

from which the data in Table VI were collected. The addition of lysine, methionine, and histidine (diet 2) resulted in an increased food intake that was accompanied by a marked increase in weight gain and PER values. Diet 3 represented the casein control to which the *Azolla* diets were compared. The casein based diet with added cellulose and minerals (diet 4) produced a significantly lower weight gain and PER than diet 3 which contained the same level of casein. Food intake and weight gain for diets 5 and 6 were not significantly different.

DISCUSSION

The results of the analysis of *Azolla*, and the growth data presented, indicate that *Azolla* has a low nutritive value when used as the sole source of protein for the growing rat and presumably other simple-stomached animals. Two factors contribute to its low nutritive value. The first is a deficiency of the essential amino acids methionine, lysine and histidine as evidenced by the low weight gain and PER observed in diet 1 compared to diet 2 (Table VI). At dietary levels of *Azolla* in diets 1 and 2 methionine was probably the first limiting amino acid, supplying 71.6% the requirment for the growing rat, followed by lysine at 76% and histidine at 81.8% of the calculated requirement.

The second factor is a high neutral detergent fiber (NDF = 39.16% of dry matter) and mineral content as evidenced by the depressed weight gain and PER in diets 4 vs. 3 (Table VI). As NDF represents the cell wall constituents of the fern which are not digested by the simple-stomached animal, this results in a significant reduction in digestible energy intake beyond that which a simple-stomached animal can compensate by increasing intake. The effect of the high NDF fraction was apparent in the low dry matter digestibility values of diets 1 and 2 in Table VI. Approximately 30% of the dietary dry matter was not digested.

The impact of the high NDF content of *Azolla* on weight gain (as evidenced by the decreased weight gain in diet 4) is also apparent in the decreased dry matter digestibility and apparent nitrogen digestibility values for diet 4 compared to diet 3 (Table VI). The fact that the dry matter digestibility values for diets 4 and 1 (the *Azolla* diet) and that the weight gains for diets 4 and 2 (the *Azolla* diet plus supplemental amino acids) are not significantly different (at the 0.05 level) supports the conclusion that the high NDF content of *Azolla* is a limiting factor responsible for the low weight gain observed when *Azolla* was supplemented with methionine, lysine, and histidine. The effect of *Azolla* NDF on nitrogen digestibility was not investigated beyond the observation that the addition of cellulose and minerals to diet 4 resulted in a significant reduction in apparent nitrogen digestibility compared to diet 3 (Table VI). Since the value of 87.3% is still very significantly greater than the 60.3 and 56.3% apparent nitrogen digestibility values for the *Azolla* diets, it appears that factors other than the mere presence of indigestible material are in part responsible for the low digestibility of *Azolla* nitrogen. One possibility is the entrapment of nitrogenous compounds in insoluble lignin or cellulose complexes that make up the cell wall constitutents of the fern.

A total of 5.5% of the *Azolla* nitrogen was present as purines resulting in a purine concentration of 492 mg/100 g of *Azolla*, equally distributed between adenine and guanine. This is a relatively high value, respresenting an adenine content of 0.24% of *Azolla* dry matter. Clifford et al. (1976) reported that increased dietary adenine is associated with elevated serum uric acid levels in normal and hyperuricemic humans. In another paper Clifford and Story (1976) showed that adenine, at 0.3% of the diet of growing rats, produced significantly decreased weight gain, as well as decreased renal function, resulting in greatly increased plasma urea levels. Although *Azolla* adenine represented only 0.12% of diets 1 and 2, Clifford and Story demonstrated that 0.1% dietary adenine for 14 days measurably increased the concentration of 2,8-dioxyadenine in the urine of rats. They suggested that this

increase was responsible for the decreased renal function that finally became evident at 0.3% dietary adenine. Dietary adenine in *Azolla* probably did not influence weight gain in this experiment, but the high level of adenine may represent a possible limitation to the use of *Azolla* for some species of animals.

As there were no significant differences with respect to weight gain or food intake of rats given diets 5 and 6, it appears unlikely that *Azolla* contains a toxic substance inhibiting growth in the short term.

The high NDF and possibly adenine of *Azolla* limit its usefulness as a food for simple-stomached animals. However, the in vitro dry matter digestion coefficent for *Azolla* using the two-stage Tilley and Terry method (1963) was 77%, indicating that the cell walls are readily digested by ruminants. This digestion coefficient is considerably greater than that of alfalfa which averaged 55.6%. Therefore, it would appear that ruminants may be the logical species which could effectively utilize both the high protein and energy of *Azolla*.

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LITERATURE CITED

American Dehydrators Association, "Dehydrated Alfalfa Assay Report", 3rd ed, Kansas City, Mo., 1969.
Association of Official Agricultural Chemists, "Official Methods of Analysis", 12th ed, Section 2.049, 1975a, p 15.
Association of Official Agricultural Chemists, "Official Methods of Analysis", 12th ed, section 47.021, 1975b, p 928.
Ashton, P. J., Walmsley, R. D., *Endeavour* **35**, 39 (1976).
Brady, F. P., Cahill, T. A., "Development of X-Ray Fluorescence Analysis and Application", a report to the National Science Foundation, Division of Advanced Technology Applications, Research Applied to National Needs, 1973.
Clifford, A. J., Reumallo, J. A., Young, V. R., Scrimshaw, N. S., *J.. Nutr.* **106**, 428 (1976).
Clifford, A. J., Story, D. L., *J. Nutr.* **106**, 435 (1976).
Clifford, A. J., Weir, W. C., Vasconcellos, J. A., Forman, L. P., Lumijarvi, D., "A Nutritional Evaluation of Leaf Protein Concentrates", for presentation at the 1975 Annual Meeting of the American Society of Agricultural Engineers, Paper No. 75-1059, ASAE, St. Joseph, Mich.
FAO, Policy and Food Science Service Nutrition Division, "Amino Acid Content of Foods", published by F.A.O., United Nations, Rome, 1970.
Fujiwara, A., Tsuboi, I., Yoshida, F., *Nogaku* 1, 361 (1947).
Goering, H. K., Van Soest, P. J., "Forage Fiber Analyses", Agriculture Handbook No. 379, Agricultural Research Service, U.S. Department of Agriculture, 1970.
Hegsted, D. M., "Assessment of Protein Quality", Improvement of Protein Nutrition, National Academy of Sciences, Washington, D.C., 1974.
Kohler, G. O., Palter, R., *Cereal Chem.* **44**, 512 (1967).
Krawl, M., *Clin. Chim. Acta* **13**, 442 (1966).
Marx, J. L., *Science* **196**, 638 (1977).
Mason, H. L., "A Flora of the Marshes of California", University of California Press, Berkeley, Calif., 1969.
Moore, A. W., *Bot. Rev.* **35**, 17 (1969).
National Academy of Sciences, "Nutrient Requirements of Laboratory Animals", No. 10, 2nd revised ed, Washington, D.C., 1972.
Newton, J. W., Cavins, J. F., *Plant Physiol.* **58**, 798 (1976).
Rogers, Q. R. Harper, A. E., *J. Nutr.* **87**, 267 (1965).
Stewart, W. D. P., Pearson, G. W., *Proc. R. Soc., B.* **175**, 293 (1970).
Thuyet, T. Q., Tuan, D. T., "*Azolla*: A Green Compost", Vietnamese Studies, Vol. 38, Agricultural Problems No. 4, Agronomical Data, Hanoi, 1973.
Tilley, J. M. A., Terry, R. A., *J. Br. Grassld. Soc.* **18**, 104 (1963).
Woolf, C. M., "Principles of Biometry", Van Nostrand, Princeton, N.J., 1968, pp 62–70.

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Methionine and Cystine Contents of Bean (*Phaseolus*) Seeds

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An improved microbiological procedure for determination of methionine and cystine in bean seeds was developed. Finely ground beans were hydrolyzed with 20% HCl by heating in the autoclave for 30 min at 121 °C. The hydrolysates were neutralized and assayed for methionine and cystine with *Leuconostoc mesenteroides*. Growth was measured by increased turbidity after 20 h incubation. Five hundred and forty-four bean samples were analyzed for crude protein (N × 6.25), methionine, and cystine. Methionine contents of air-dried whole seeds ranged between 0.16 and 0.33%, methionine plus cystine between 0.29 and 0.56%, and crude protein between 16.1 and 33.9%. Calculated on a basis of 16% nitrogen, methionine in bean proteins was between 0.51 and 1.24%, and methionine plus cystine was between 1.17 and 2.49%. Correlation coefficients between protein content of beans and methionine content of bean protein and between protein content of beans and methionine plus cystine content of bean protein were calculated for 198 samples of *Phaseolus vulgaris* from around the world. A correlation coefficient of −0.439 between protein and methionine was obtained, which is significant at the 1% level. A correlation of −0.531 between protein and methionine plus cystine was obtained.

Legume seeds are used extensively in the poorer nations of the world as a source of dietary protein because of a shortage of animal proteins and because legume seeds are a relatively rich source of protein compared to cereal grains. For example, dry edible beans (*Phaseolus vulgaris*) are used extensively in Mexico and in Central and South America. Legume seeds, however, are deficient in the sulfur-containing amino acids, methionine and cystine (Evans and Bandemer, 1967).

The objectives of the present investigation were to develop a rapid method for determination of methionine and cystine in small quantities of bean seeds and to analyze a broad range of bean seeds to identify any lines which

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