Studies on Methods for Amino Acid Analysis of Wheat Products[1]

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ABSTRACT

Hydrolysis conditions for amino acid analysis of wheat and wheat bran have been reinvestigated. The majority of the amino acids are completely liberated from wheat proteins by acid hydrolysis and are stable under the usual hydrolysis conditions. Serine and threonine are readily liberated by acid hydrolysis but are moderately unstable under these conditions. Correction factors have been developed so that these amino acids can be determined on the same hydrolysate as the stable amino acids. Valine and isoleucine are stable to acid but are released slowly. Correction factors have been developed so that these amino acids too can be determined on the same hydrolysate as those listed previously. Cystine and methionine are so unstable during acid hydrolysis that oxidative derivatives must be made to obtain reliable results. Tryptophan is determined after Ba $(OH)_2$ hydrolysis. Tyrosine is sensitive to trace amounts of oxygen and requires careful degassing prior to hydrolysis. Amino acid analyses on several series of wheats and millfeeds are presented.

The development of computerized formulation of feeds and the need for supplying nutritionally balanced foods to a growing population have increased the need for more precise and reliable data on the amino acid composition of wheat products. Many analyses have been carried out on whole wheat and on wheat millfeeds, and many conflicting results have been recorded in the literature. Part of the variability in results is due to variation in the ratio of albumins and globulins to the gluten proteins, which is related to total nitrogen level in wheat. Comparison of data on wheats of similar type and nitrogen content suggests that differences in analytical procedure may well be responsible for a substantial proportion of the variability in results.

In the analytical procedure the hydrolysis step is probably the major cause for variability. This subject has recently been reviewed by Hill (1). The present study was undertaken to investigate four facts which must be taken into account if low results for various amino acids are to be avoided: 1) Some amino acids are largely destroyed by hydrochloric acid and must therefore be converted to stable derivatives (e.g., cystine and methionine), or be released by alkaline hydrolysis (e.g., tryptophan). 2) Certain amino acids are destroyed to a moderate degree by acid but can be estimated by correction factors based on kinetic studies (e.g., threonine and serine). 3) Some amino acids are liberated slowly by acid hydrolysis and can be estimated by application of correction factors based on kinetic studies or can be determined after separate long-time hydrolysis procedures (e.g., valine and isoleucine). 4) One amino acid, tyrosine, is susceptible to oxidation by

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traces of residual oxygen present during ordinary hydrolysis and requires careful degassing of the acid mixture.

Hill (1) has pointed out that hydrolysis of each protein appears to be an individual problem and fixed correction factors cannot be employed generally. In our investigations we have derived time-of-hydrolysis correction factors for several of the amino acids. The present paper reports studies on whole wheat and bran and includes analytical data on a series of millfeed samples.

MATERIALS AND METHODS

Wheat and Millfeed Samples

The wheat used in the kinetic study was a sample of HRS wheat obtained from Fisher Flouring Mills. The bran used in the kinetic study was obtained from The Pillsbury Co. and was derived from a commercial blend of Midwestern soft wheats. Samples of wheat, straight-grade flour, bran, shorts, red dog, and germ from a milling of Kansas HRW wheat (12% protein) were obtained through the courtesy of E. Farrell of Kansas State University and the Millfeed Research Committee of the Millers' National Federation. They are part of a series of carefully prepared samples collected by the latter group for exhaustive compositional study.

Procedures

The sample of wheat or wheat product is ground in a ball mill to a fine, homogeneous powder. Hydrolysis procedure A, used in our early work, is as follows: 40 mg. of milled sample (dry weight basis) is placed in the hydrolysis tube made from a standard-taper 18/9 ball joint lengthened to 23 cm. and bottom-sealed. Ten milliliters of 6*N* HCl is added and the air space above the liquid flushed with high-purity nitrogen gas. An 18/9 socket cap is doubly clamped to the silicone-greased ball joint. The tube is heated in an oven at 110° ± 1°C. for 24 hr., or 24, 48, 72, 96, 120, and 144 hr. in the kinetic studies. After cooling to room temperature, the mixture is filtered through a 0.8-μ millipore filter into a 500-ml. round-bottomed flask. The tube, precipitate, and funnel are washed with a total of 30–40 ml. of deionized distilled water. The filtrate and washings are concentrated to dryness in a flask on a rotary evaporator at 40°–50°C. A 10-ml. portion of distilled water is added, the flask is swirled, and the contents are again evaporated to dryness. A second 10 ml. of water is added and the contents re-evaporated for complete removal of free HCl. The residue is dissolved in a known, appropriate amount of pH 2.2 diluting buffer to give an approximate concentration of 5 mg. of nitrogen (based on analysis of original sample) per 20 ml. of buffer.

Hydrolysis procedure B, developed to eliminate the variability in tyrosine results obtained by procedure A, is as follows: 40 mg. of milled sample (dry-weight basis) is placed in the hydrolysis tube (a 30-ml. Kjeldahl flask with a constriction in the neck). Ten milliliters of 6*N* HCl is added and the flask is attached to a high-vacuum line. The bottom of the flask is immersed in liquid nitrogen. When the contents are frozen, vacuum is applied

until the pressure is reduced to 10 μ. The tube is isolated from the vacuum system and the contents are thawed, refrozen, and re-evacuated to 10 μ. This procedure is repeated once again, and then the evacuated flask is sealed at the constriction. After the flask comes to room temperature it is placed in an oven at 110° ± 1°C. for 24 hr., or for the required periods of time in the kinetic studies. After cooling to room temperature, the flask is opened at the top and the mixture is prepared for analysis as described in procedure A.

Amino acid analysis is carried out by the Spackman, Stein, and Moore (2) protein hydrolysate method, with a modified Phoenix automatic amino acid analyzer with spherical resins and high-pressure columns (Beckman) to shorten analysis time. The chromatographic conditions are 1) basic column, 0.9-cm. diam. packed to a resin height of 9 cm.; 2) neutral and acidic column, 0.9 cm. packed to a resin height of 55 cm.; 3) flow rates, 60 ml./hr. for buffers, 30 ml./hr. for ninhydrin; 4) operating back pressures, 180 p.s.i. for neutral and acidic column, 90 p.s.i. for basic column; 5) analysis time, 3 hr., 10 min. for neutral and acidic amino acids, 90 min. for basic amino acids; 6) chart speed, 6 in./hr.; 7) printing interval, 2 sec.; 8) standard, 0.5 μM/ml. amino acid mixture (Beckman). A 1-ml. aliquot is used on both the basic and the neutral and acidic columns of the amino acid analyzer.

Cystine and Methionine

The procedure of Moore (3) is used except that the reaction vessel is a 30-ml. Kjeldahl flask fitted with an 18/9 ball joint. The degassing, vacuum-sealing procedure as used in procedure B showed no advantage over nitrogen flushing in this work, so the latter simpler procedure was adopted.

Tryptophan

A separate basic hydrolysis of the protein is necessary. Barium hydroxide is used for this purpose and starch (4) is added to the hydrolysis mixture. The hydrolysis container is a 120-ml. wide-mouthed polypropylene bottle with a Teflon-coated magnetic stirring bar placed inside. In the hydrolysis bottle 300 mg. of wheat, 5 g. $Ba(OH)_2 \cdot 8H_2O$, 400 mg. starch, and 8 ml. of water are added and mixed. An air condenser is attached and the container placed in an oil bath at 100°C. for 16 hr. At the end of this time the mixture is allowed to cool to room temperature. The air condenser and the walls of the container are washed down with a little water and the mixture, while being stirred rapidly, is neutralized to pH 7 with 1N H_2SO_4 with an automatic titrator (Radiometer). The mixture is transferred quantitatively to a TC graduated cylinder, diluted to 100 ml. with water, thoroughly mixed, poured into a centrifuge bottle, and centrifuged for 2 hr. at 9,400 r.p.m. A 50-ml aliquot of the supernatant is pipetted into a 500-ml. round-bottomed flask and dried at 40°–50°C. with a rotary concentrator. Five milliliters of pH 2.2 sample diluting buffer is added to the dry residue and swirled thoroughly around the walls of the flask. The insolubles are removed by centrifuging for 2 hr. at 17,000 r.p.m.

A 1.0-ml. aliquot of the clear buffer mixture is analyzed for tryptophan

on the basic column of the amino acid analyzer. Operating conditions are the same as for the basic amino acids except that the column temperature is raised to 65°C. to effect a good separation of tryptophan.

RESULTS AND DISCUSSION

Unless otherwise noted, the results reported for amino acids other than cystine, methionine, and tryptophan are those obtained under hydrolysis procedure B. All figures cited are averages of duplicate determinations.

Most amino acids showed no significant increase or decrease after 24 hr. of hydrolysis. This is illustrated by Fig. 1, showing the release pattern

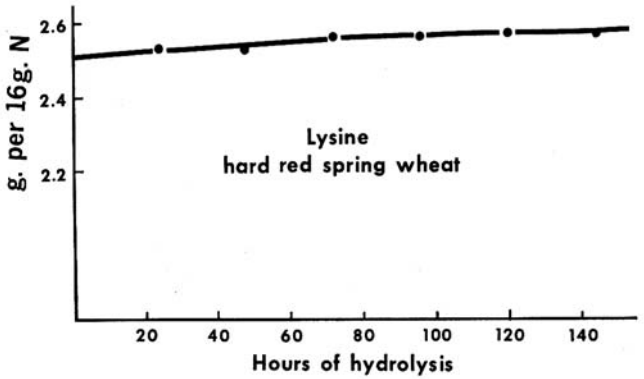

Fig. 1. Effect of hydrolysis time on a "normal" amino acid: lysine in HRS wheat. Each point represents the average of duplicate runs.

TABLE I

AMINO ACIDS READILY RELEASED AND STABLE TO ACID HYDROLYSIS

Amino Acid	HRS Wheat		Soft Wheat Bran	
	Procedure A	Procedure B	Procedure A	Procedure B
	g./16 g. N	*g./16 g. N*	*g./16 g. N*	*g./16 g. N*
Alanine	3.43	3.38	4.75	4.72
Arginine	4.21	4.45	6.04	6.59
Aspartic acid	4.96	4.80	6.69	6.76
Glutamic acid	30.6	30.4	19.0	19.9
Glycine	4.01	3.96	5.56	5.57
Histidine	2.19	2.14	2.62	2.59
Leucine	6.61	6.52	6.09	6.12
Lysine	2.64	2.53	3.70	3.65
Phenylalanine	4.66	4.61	3.97	3.95
Proline	9.94	9.52	6.33	5.95
Tyrosine	2.09	2.95	2.16	2.71

of a typical stable amino acid, lysine, in wheat. Table I contains wheat and bran data for this class of amino acids from both hydrolysis procedures A and B. The tyrosine figures show such large errors for procedure A that we conclude this method is not satisfactory. It has been pointed out that tyrosine is subject to destruction when carbohydrates are present in the sample (5). Tyrosine losses also occur if the hydrolyzing acid contains traces of either chlorine or bromine. Moreover, these losses are enhanced if minor amounts of air or any other oxidizing agents are present (1). Appar-

ently, oxidation was responsible for the low results we obtained from procedure A, since the more thorough deoxygenation carried out in procedure B increased values by about 25%.

Among the amino acids which do not fall in the category "acid-stable and readily released" are serine, threonine, isoleucine, and valine. To determine these amino acids correctly with the same 24-hr. hydrolysate, it was necessary to develop kinetic correction factors to compensate for their loss or slow release. An additional study of correction factors for the nongrain commodities of alfalfa, safflower kernel and hull, and castor meals had been carried out. The correction factors for specific labile or slowly released amino acids vary only slightly from commodity to commodity; hence, average factors are used in our laboratory for crude food or feed analysis (Table II). These factors should not be applied to single isolated proteins, since

TABLE II
AMINO ACIDS REQUIRING CORRECTION FACTORS

AMINO ACID	CORRECTION FACTOR	HRS WHEAT	SOFT WHEAT BRAN
		g./16 g. N	*g./16 g. N*
Cystine[a]	1.091	2.28	2.44
Isoleucine	1.078	3.83	3.45
Methionine[a]	1.034	1.82	1.63
Serine	1.082	4.69	4.70
Threonine	1.036	2.83	3.32
Tryptophan[b]	.....	1.19	1.74
Valine	1.081	4.69	5.15

[a] Determined as cysteic acid and methionine sulfone; correction factor based on recovery experiments. Results are reproducible to ± 3%.
[b] $Ba(OH)_2$ hydrolysis, minimum values. No correction factor applied because of erratic recoveries.

time-of-hydrolysis studies on pure proteins have shown that unique correction factors are necessary for the individual proteins studied (1,6). The

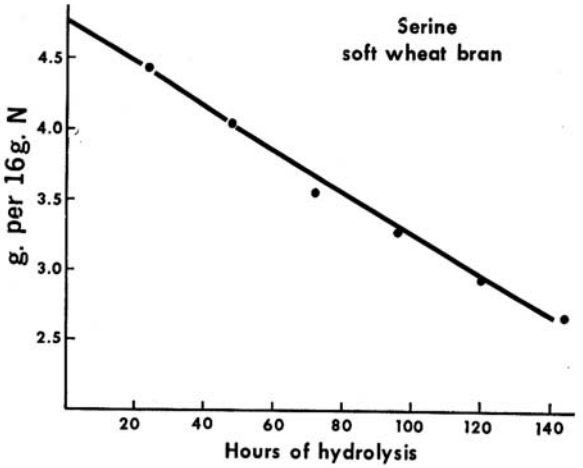

Fig. 2. Correction factor derivation for a moderately labile component: serine in soft wheat bran.

uniformity we observe is probably due to the fact that the crude materials contain mixtures of many proteins.

The amino acids which are destroyed to a moderate degree are serine and threonine. Figure 2 shows development of a correction factor for serine based on the rate of loss during hydrolysis of wheat bran. The data are fitted by least squares and extrapolated back to "zero time." The ratio of the zero-time value to the 24-hr. value is the correction factor. Correction factors used and corrected data for serine and threonine are shown in Table II.

Valine and isoleucine are stable in acid but released slowly during hydrolysis. Figure 3 shows a typical rate-of-release curve (isoleucine in

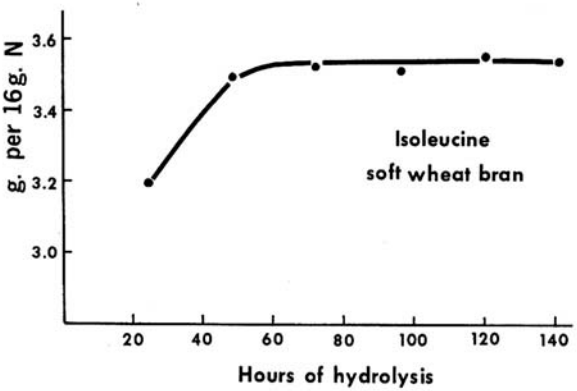

Fig. 3. Correction factor derivation for a slowly released component: isoleucine in soft wheat bran.

wheat bran). Values do not reach a plateau until after 72 hr. of hydrolysis. The correction factors for valine and isoleucine in Table II were derived by fitting curves to the data from the six commodities studied and determining the ratio of the plateau value to the 24-hr. value.

We noted that a very slight positive slope is shown for lysine (see Fig. 1) and a slight negative slope for arginine. It has been found by temperature-programmed, high-resolution runs that small amounts of ornithine are present, undoubtedly derived from arginine during the hydrolysis and therefore included in the lysine peak of the accelerated chromatographic system used. The correction factor was less than 1%, so it was considered unimportant.

A final class was found to include those amino acids which show major losses during acid hydrolysis; namely, cystine, methionine, and tryptophan. The sulfur-containing amino acids must be determined by the method of Moore (3), which involves oxidation of the intact protein with performic acid. The cysteic acid and methionine sulfone produced are stable to the subsequent acid hydrolysis. The correction factors shown in Table II are based on recovery experiments. Trytophan analyses are run by the use of the automatic amino acid analyzer after $Ba(OH)_2$ hydrolysis. Recoveries of added tryptophan have been erratic and drastically affected by hydrolysis

time. A 16-hr. hydrolysis period at 100°C. has yielded maximum values and recoveries (Fig. 4). Further work is in progress. However, the values shown in Table II may be considered to be reliable as minimum values.

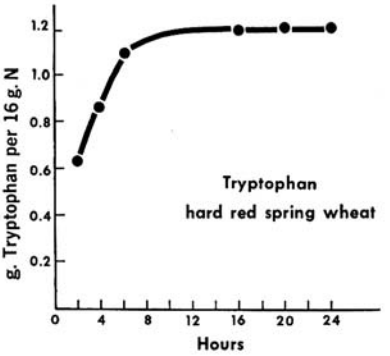

Fig. 4. Effect of time of hydrolysis on apparent tryptophan content of HRS wheat.

In addition to the hydrolysate-chromatographic analysis described above for the common 18 amino acids, the 24-hr. wheat sample was analyzed for unknown constituents by the technique developed for physiological fluids (2). This basic column analysis presented four additional peaks tentatively identified (by retention time) as gamma-amino butyric acid, ornithine (7), ethanolamine, and S-methyl methionine (8). Analysis for neutral and acidic ninhydrin-positive compounds showed ten additional compounds present in the hydrolysate, two of which appear to be cysteic acid and levulinic acid. Of the remaining eight, four have the high 440/570 ratios characteristic of some nonnitrogenous compounds.

A neutral and acidic analysis of the whole-bran 24-hr. acid hydrolysis sample showed 12 peaks in addition to the common amino acids; cysteic acid, levulinic acid, and beta-alanine were among them. The peaks showing a high 440/570 ratio were the same as appeared in the wheat analysis. Though there are a significant number of additional ninhydrin-positive components in wheat and bran, their total quantity is very small. Recovery of nitrogen by hydrolysate chromatography of the wheat and wheat bran in the form of amino acids and ammonia amounted to 96 and 85%, respectively. The additional ninhydrin-positive compounds account for very little of the unrecovered nitrogen.

Results on Midwestern and Pacific Northwest wheats and their corresponding millfeeds by the described methods are shown in Table III. For comparison, some literature figures are included (9,10,11); they were selected because they are quite recent and represent both microbiological and chromatographic methods of analysis. From this comparison, we conclude that essentially all of the previously published results on cystine and methionine are too low, undoubtedly because of oxidative losses during hydrolysis. Our results on valine and isoleucine tend to be higher than most reported results, although it appears that the hydrolysis conditions used by Hepburn *et al.* (12),

TABLE III

AMINO ACID COMPOSITION OF HARD RED WHEAT AND WHEAT PRODUCTS
(g. amino acid/16 g. N)

	WHOLE WHEAT				FLOUR		RED DOG		
	WRRL[a]	Hepburn *et al.*[b]	Lyman *et al.*[c]	Simmonds[d]	WRRL	Hepburn *et al.*	WRRL	Hepburn *et al.*	Lyman *et al.*
Nitrogen (dry basis) %	2.42	2.64	2.56	2.74	2.31	3.02	2.62	3.34	2.28
Recovery of N as amino acids and ammonia, %	96	....	....	....	92	....	98	....	....
Lysine	2.61	2.67	2.71	2.86	1.88	1.94	3.14	4.13	2.18
Histidine	2.29	2.12	2.55	2.31	2.04	2.06	2.38	2.22	2.04
Ammonia	3.92	....	....	....	4.18	....	3.82	....	....
Arginine	4.74	4.71	5.06	4.77	3.58	3.87	5.56	6.84	3.87
Aspartic acid	5.06	4.85	....	4.89	3.76	3.86	5.60	6.76	....
Threonine	2.98	2.76	3.03	2.53	2.67	2.73	3.18	3.11	2.81
Serine	4.90	5.22	....	4.16	4.96	5.32	5.18	4.85	....
Glutamic acid	30.8	29.3	....	23.27	34.4	34.7	29.2	17.9	....
Proline	9.46	9.94	....	....	12.7	11.75	9.19	6.30	....
Glycine	4.03	3.94	....	3.74	3.32	3.25	4.44	4.98	....
Alanine	3.49	3.37	....	3.27	2.77	2.75	4.08	4.70	....
Cystine	2.31	1.80	....	....	2.42	1.85	2.49	1.40	....
Valine	4.79	4.69	4.46	3.66	4.40	4.32	5.01	4.91	4.43
Methionine	1.70	1.74	1.32	....	1.86	1.71	1.96	1.70	1.55
Isoleucine	3.89	3.78	4.50	3.4	3.86	4.02	3.98	3.42	4.15
Leucine	6.79	6.52	6.48	6.05	6.75	6.59	6.92	5.77	7.17
Tyrosine	3.10	3.19	3.24	2.60	2.95	3.35	3.16	2.85	3.28
Phenylalanine	4.64	4.43	4.92	3.61	4.74	5.04	4.65	3.55	4.64
Tryptophan	....	1.13	1.53	....	....	1.01	....	1.25	1.55

(Continued on next page)

namely, 125°C. for 24 hr., are vigorous enough to liberate these two resistant amino acids. We checked the effects of going from 110° to 130°C. for 24 hr. on the recovery of the various amino acids, and found that recovery of valine and isoleucine was complete at the higher temperature. However, about 3% of the arginine was converted to ornithine, which increased the apparent lysine figure by 3% under the accelerated system. Threonine and serine losses of 26 and 47%, respectively, were encountered at the same time. This would necessitate much larger correction factors for these two amino acids; hence we prefer to use the 110° ± 1°C., 24-hr. system with the correction factors already described.

Comparison of the whole wheats shown in Tables I, II, and III and many others indicate that, with the exception of the shifts in composition which are correlated with nitrogen content, the major variations in reported values in the literature are due to variations in methods of analysis.

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TABLE III *(continued)*
(g. amino acid/16 g. N)

	Shorts			Germ			Bran		
	WRRL[a]	Hepburn *et al.*[b]	Lyman *et al.*[c]	WRRL	Hepburn *et al.*	Lyman et al.	WRRL	Hepburn *et al.*	Lyman *et al.*
Nitrogen (dry basis),%	3.28	3.33	2.69	4.52	4.63	4.51	2.88	2.95	2.33
Recovery of N as amino acids and ammonia, %	86	....	....	88	....	....	85	....	....
Lysine	4.79	4.18	4.04	5.25	5.28	6.55	3.69	3.77	4.39
Histidine	2.48	2.20	2.85	2.38	2.26	2.28	2.64	2.22	3.02
Ammonia	2.56	....	....	2.46	....	....	2.35	....	....
Arginine	6.84	6.85	6.35	7.36	6.88	7.41	6.84	6.60	7.48
Aspartic acid	7.27	6.95	....	7.67	7.48	....	6.96	6.64	....
Threonine	3.54	3.03	3.50	3.74	3.42	3.95	3.26	2.86	3.57
Serine	4.53	4.69	....	4.24	4.60	....	4.49	4.58	....
Glutamic acid	16.7	16.6	....	15.3	14.0	....	17.5	16.2	....
Proline	5.34	6.03	....	4.71	5.03	....	5.45	6.11	....
Glycine	5.34	5.33	....	5.47	5.22	....	5.66	5.12	....
Alanine	5.24	4.74	....	5.68	5.23	....	4.72	4.65	....
Cystine	2.11	1.36	....	1.66	1.04	....	2.16	1.52	....
Valine	5.12	4.84	5.16	5.25	4.90	5.02	5.02	4.69	5.56
Methionine	1.76	1.62	1.78	2.26	1.91	1.64	1.49	1.48	1.65
Isoleucine	3.51	3.31	3.74	3.58	3.48	3.45	3.32	3.29	3.70
Leucine	6.11	5.64	6.11	6.12	5.75	5.95	5.87	5.51	6.31
Tyrosine	2.67	2.82	2.83	2.72	2.85	2.95	2.82	2.82	3.25
Phenylalanine	3.88	3.44	3.86	3.67	3.38	3.96	4.12	3.58	3.77
Tryptophan	....	1.29	1.60	....	0.98	1.07	....	1.58	2.81

[a] Kansas HRW wheat (12% protein) or products therefrom.
[b] Blend of HRS and winter wheats. Microbiological method. (See ref. 9.)
[c] Average of five values for hard red wheat; for tyrosine average of four wheats. Microbiological method. (See ref. 10.)
[d] Simmonds' wheat sample No. 4. (See ref. 11.)

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