Determination of Free Lysine and Methionine in Amino Acid-Fortified Wheat[1]

R. E. FERREL, D. A. FELLERS, and A. D. SHEPHERD[2], Western Regional Research Laboratory, Albany, California

ABSTRACT

Nutritional enhancement of cereal proteins by fortifying with limiting amino acids appears to be both simple and practical. A rapid and simple method for determining lysine and methionine added to wheat is reported. Finely ground fortified material is extracted with dilute hydrochloric acid. Methionine content of an aliquot of the extract is determined colorimetrically with nitroferricyanide, and total ninhydrin reactants are determined colorimetrically on a second aliquot. Lysine content is calculated from the total ninhydrin color and the methionine content. The effects of pH on the ninhydrin color and of added glycine on reducing interference in the nitroferricyanide method are discussed.

Lysine is the first-limiting amino acid in most cereal grains; sulfur amino acids (methionine and cystine) are estimated to be limiting in the total diet in many protein-deficient areas of the world (1). Recent developments in commercial production of lysine and methionine have stimulated renewed interest in their use to enhance protein quality of cereal-based diets. Graham et al. (2) have reported an infusion technique for preparing kernels of high-level lysine-fortified wheat. Unreported work by these authors indicates that methionine-fortified wheat can be prepared by similar techniques. These concentrated materials can then be blended with unfortified wheat to yield desired fortification levels.

For commercial application of these promising techniques, simple, reliable methods for monitoring fortification levels must be available. Sophisticated, automated techniques for determining all amino acids are well known but are too time-consuming and expensive for routine use.

Simple colorimetric methods, specific for certain amino acids, include Sanger's (3) method of using the yellow color developed when dinitrofluorobenzene reacts with the epsilon-amino group of lysine as a specific measure of this amino acid. While this method has been adapted to measure "available" lysine in proteins (4), the multistep procedure is too laborious and time-consuming for convenient use in monitoring the production of amino acid-infused grains. McCarthy and Sullivan (5) and Hess and Sullivan (6) reported a simple colorimetric method specific for methionine in protein hydrolysates that was based on its reaction with nitroferricyanide. The reaction of ninhydrin with the alpha-amino group of most amino acids to yield a blue compound has long been known and utilized for the determination of individual amino acids (7).

We have developed a procedure, based on the latter two techniques, for determining the added lysine and methionine in fortified wheat, whether present singly or together.

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[2]Present address: East African Industrial Research Organization, Nairobi, Kenya.

MATERIALS AND METHODS

Because lysine is usually available as the monohydrochloride (lysine•HCl), all values in this paper are reported on that basis. The L-lysine•HCl and DL-methionine were commercial-grade materials purchased in bulk quantities. Both amino acids assayed 99+% pure when compared with chemically pure products by the proposed ninhydrin method. All other chemicals used were C.P. or reagent grade.

Samples for lysine•HCl recovery experiments were prepared by slowly adding an aqueous solution of lysine•HCl to ground whole wheat while it was mixed rapidly. The moist fortified sample was dried to about 10% moisture in a forced-draft oven at 55° to 60°C. air temperature, thoroughly mixed, and reground to pass a 40-mesh screen. Samples for methionine recovery were prepared similarly, except that methionine was added in 0.1N hydrochloric acid solution. Samples containing both amino acids were prepared by blending the required proportions of the two individually fortified samples.

Stock solutions of lysine•HCl and methionine containing 5 mg. per ml. were stored under refrigeration, and working standards were prepared by dilution, as required.

The ninhydrin reagent was prepared by dissolving 0.500 g. ninhydrin (1,2,3-triketohydrindene) and 26.5 g. sodium chloride in about 70 ml. water plus 10 ml. 0.100N sodium hydroxide and diluting to 100 ml. This reagent is stable for several weeks at 4°C.

Moisture was determined by a 100°C. vacuum-oven method (8). Colorimetric measurements were made in a Coleman Universal Spectrophotometer, Model 14, using matched square cuvets with a 13-mm. light path.

Proposed Method

1. Weigh a sample 0.500 to 5.000 g., depending on expected amino acid content, ground to pass a 40-mesh sieve, into a 100-ml. volumetric flask. Weigh a similar-sized sample of unfortified material at the same time to be run in parallel as a sample blank to correct for naturally occurring ninhydrin- or nitroferricyanide-reacting materials.
2. Add 50 ml. of 0.1N hydrochloric acid with care to assure uniform sample dispersion, and extract 15 min. with mechanical shaking.
3. Dilute to volume with water and filter through a moderately fast paper (S&S 597 or equivalent).
4. Dilute an aliquot of filtrate, if necessary, to give a solution containing 50 to 300 γ per ml. total amino acids.
5. Transfer 1 ml. of this solution to a 25-ml. volumetric flask. Add 10 ml. 50% (v./v.) glycerol, 2 ml. pH 6.0 M phosphate buffer, and 1 ml. ninhydrin reagent. Prepare also a reagent blank and individual lysine•HCl and methionine standards (50 to 300 γ).
6. Heat in boiling-water bath 30 min., then transfer to cold-water bath and cool to room temperature.
7. Make to volume with water and after 15 min. read absorbance at 570 mμ, setting instrument to zero absorbance with the reagent blank. Correct absorbance of sample by subtracting absorbance of sample blank.

8. Transfer a 10-ml. aliquot of the original filtrate (step 3), diluted so as to contain 50 to 300 γ per ml. methionine to a 50-ml. Erlenmeyer flask. Prepare also a reagent blank and a methionine standard.

9. Add 4 ml. of 5N sodium hydroxide, 2 ml. 1% aqueous glycine solution, and 2 ml. 1% aqueous sodium nitroferricyanide solution, swirling to mix after each addition.

10. Heat 10 min. in a 40°C. water bath, then cool 5 min. in an ice-water bath.

11. Add 5 ml. 1:1 hydrochloric acid (v./v.) with swirling and cool 2 to 3 min. longer. Filter through a retentive filter (S&S 602 or equivalent) to remove precipitated protein.

12. Read absorbance at 510 mμ, setting the instrument to 0 with the reagent blank. Correct sample absorbance by subtracting absorbance of sample blank.

Relative concentrations of the two amino acids can be calculated in the following manner.

Calculate methionine concentration from the results obtained in the nitroferricyanide determination (steps 8 through 12) using eq. 1.

$$\text{Methionine concentration} = \frac{\text{corrected sample absorbance} \times \text{std. concn.}}{\text{std. absorbance}} \qquad (1)$$

By substituting this value (adjusted if necessary to the same dilution as the ninhydrin sample) in eq. 2, the portion of the total absorbance due to methionine in the ninhydrin reaction can be calculated, using the concentration and absorbance values for the methionine standard determined in the ninhydrin method (steps 1 through 7).

$$\text{Methionine absorbance} = \frac{\text{methionine concn.} \times \text{std. absorbance}}{\text{std. concn.}} \qquad (2)$$

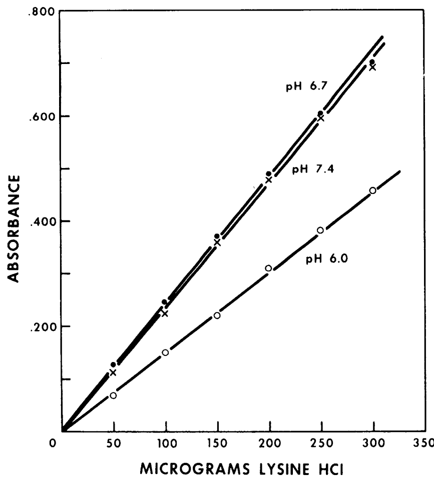
The methionine absorbance so calculated, when subtracted from the total absorbance determined in the ninhydrin method, yields the portion of this absorbance due to lysine•HCl (eq. 3).

$$\text{Lysine•HCl absorbance} = \text{corrected total absorbance} - \text{methionine absorbance} \qquad (3)$$

The lysine•HCl concentration can then be calculated (eq. 4) using this value and the concentration and absorbance values of the lysine•HCl standard in the ninhydrin method.

$$\text{Lysine•HCl concn.} = \frac{\text{lysine•HCl absorbance} \times \text{std. concn.}}{\text{std. absorbance}} \qquad (4)$$

When either amino acid occurs alone, it can best be determined by the ninhydrin method (steps 1 through 7), calculating the concentration with the use of eq. 4. Concentrations can, of course, be determined from a standard curve, but inclusion of a standard in each set of samples serves as a check on reagent stability and is generally considered the more precise technique.



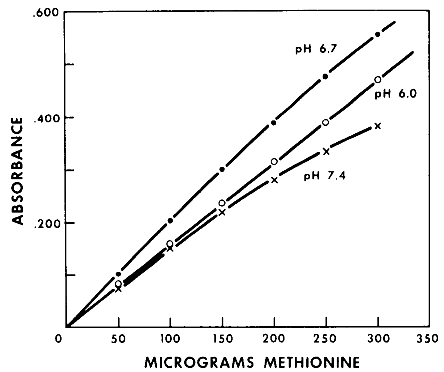


Fig. 1 (left). Effect of pH on ninhydrin reaction with lysine hydrochloride.

Fig. 2 (right). Effect of pH on ninhydrin reaction with methionine.

RESULTS AND DISCUSSION

It is possible that salt linkages or other weak bonds may exist between added amino acids and wheat components to interfere with ready extraction. Dilute acid as an extraction medium breaks such bonds. Experiments confirmed that 15-min. shaking with 0.1N HCl was sufficient to extract all the lysine•HCl or methionine infused into wheat kernels by the technique of Graham (2).

Lee and Takahashi (9) investigated the effect of several organic solvents on color intensity developed in the ninhydrin-amino acid reaction. They found 50 to 55% glycerol most satisfactory.Our investigations showed that dilution of the color, developed in 50% glycerol, with either water or ethanol yielded reproducible color. Dilution with ethanol (approximately 14:11, ninhydrin reaction mixture:ethanol) yielded a solution of 25% greater absorbance than did the same reaction mixture diluted with water. While dilution with either water or ethanol was satisfactory, for convenience dilution with water was chosen for use in this work. The resultant color was stable 1 hr., as evidenced by not more than 3% loss in absorbance.

The same authors (9) reported optimum color development at pH 6.2, but noted some variation dependent upon the individual amino compounds. Our studies showed a wider range of pH response. Data in Fig. 1 indicate that the pH optimum for lysine•HCl is between pH 6.7 and 7.4. Curves in Fig. 2 indicate that pH 6.7 is also near optimum for color development with methionine. Unfortunately, at this pH value as well as at values of 5.0, 7.4, and 7.8 the response to changes in methionine concentration is curvilinear. Of the pH values tested, only 6.0 yielded straight-line functions with both amino acids.

TABLE I. RECOVERY OF LYSINE HYDROCHLORIDE AND METHIONINE FROM FORTIFIED WHEAT BY THE NINHYDRIN METHOD[a]

Added	Lysine•HCl		Methionine	
	Found	Recovered	Found	Recovered
%	%	%	%	%
0.11	0.14 ± 0.01[b]	124	0.15 ± 0.01[b]	136
0.57	0.58 ± 0.02	102	0.62 ± 0.01	109
1.13	1.10 ± 0.02	97	1.12 ± 0.02	99
5.40	5.30 ± 0.05	98	5.42 ± 0.04	100
10.25	10.24 ± 0.05	100	10.27 ± 0.12	100

[a]All data on moisture-free basis.

[b]Standard error.

When applied to wheat fortified with lysine•HCl or methionine singly, the ninhydrin method appears to be applicable over a wide range of fortification levels, as indicated by data shown in Table I.

Comparison of results obtained by the ninhydrin method with those obtained by column chromatography were only partially satisfactory (Table II). Chromatographic results on lysine•HCl-fortified samples (after acid hydrolysis) agree reasonably well with both ninhydrin results and the amount added. Methionine values obtained by the chromatographic method (after performic acid oxidation and acid hydrolysis) are somewhat low, when compared with the other two values.

In the nitroferricyanide method for methionine determination, Csonka and Denton (10) claimed that the use of glycine to reduce interference of other amino acids is not necessary and that its use reduces the sensitivity of the method. We found (Fig. 3) that glycine did reduce the sensitivity of the method, but that it also tended to minimize the effect of interfering material extracted from the wheat.

The nitroferricyanide method was also applicable over a wide range of fortification levels, as evidenced by data in Table III. The developed color was

TABLE II. COMPARISON OF NINHYDRIN AND COLUMN CHROMATOGRAPHIC ANALYSIS OF FORTIFIED SAMPLES[a]

Added	Found			
	Lysine•HCl		Methionine	
	Ninhydrin	Column[b]	Ninhydrin	Column[b]
%	%	%	%	%
1.13	1.10	1.09	1.12	0.65
5.40	5.30	5.58	5.42	4.44
10.25	10.24	10.75	10.27	9.77

[a]All data on moisture-free basis.

[b]Corrected for content of an unfortified blank.

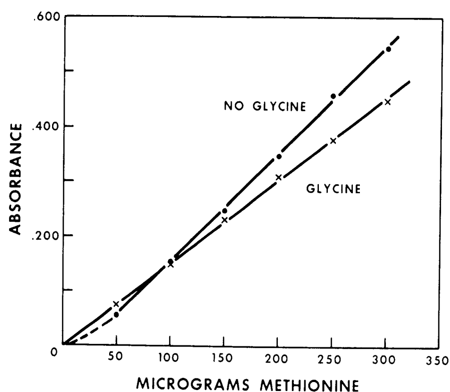


Fig. 3. Effect of glycine on nitroferricyanide reaction with methionine.

stable for at least 1 hr. at room temperature, showing not more than a 4% reduction in absorbance in that time.

When the proposed methods were applied to wheat samples fortified with both lysine•HCl and methionine, recoveries were similar to those obtained on singly fortified samples (Table IV).

TABLE III. RECOVERY OF METHIONINE FROM FORTIFIED WHEAT BY THE NITROFERRICYANIDE METHOD[a]

Added %	Found %	Recovered %
0.11	0.10 ± 0.01[b]	91
0.57	0.51 ± 0.01	90
1.13	1.03 ± 0.03	91
5.40	5.07 ± 0.07	94
10.25	9.65 ± 0.07	94

[a]All data on moisture-free basis.

[b]Standard error.

TABLE IV. RECOVERY OF LYSINE HYDROCHLORIDE AND METHIONINE FROM WHEAT FORTIFIED WITH BOTH AMINO ACIDS[a]

Added		Found		Recovered	
Lysine•HCl %	Methionine %	Lysine•HCl %	Methionine %	Lysine•HCl %	Methionine %
0.12	0.12	0.14 ± 0.02[b]	0.11 ± 0.01[b]	117	91
0.57	0.56	0.61 ± 0.02	0.51 ± 0.02	107	90
1.12	0.57	1.14 ± 0.02	0.52 ± 0.01	102	91
5.23	2.59	5.23 ± 0.21	2.50 ± 0.02	100	97
5.18	5.14	5.22 ± 0.21	4.85 ± 0.07	101	94

[a]All data on moisture-free basis.

[b]Standard error.

In summary, the proposed method offers a simple, reliable procedure utilizing equipment available in most laboratories for determining the content of methionine and lysine•HCl present singly or together in fortified wheat.

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