Determination of Added Lysine in Fortified Wheat and Bulgur[1]

J. W. FINLEY, R. E. FERREL, and D. A. FELLERS, Western Regional Research Laboratory, Albany, California 94710

ABSTRACT

A colorimetric procedure for determination of added lysine-hydrochloride in wheat and bulgur is described. The method is based on quantitative conversion of the α-amino group of free lysine to a copper complex, followed by reaction with 1-fluoro-2,4-dinitrobenzene. The solution is acidified with dilute hydrochloric acid and thrice extracted with diethyl ether; the aqueous solution is clarified by addition of ethanol and filtration. Absorbance of the resultant ϵ-dinitrophenyl lysine is measured at 390 nm.

The need for a simple and specific means for determining lysine added to wheat and wheat products is well recognized. Early chemical methods for determination of free lysine are tedious and indirect and lack needed sensitivity. Ion-exchange or gel-permeation chromatography is more specific and exact; however, both procedures are time-consuming and require exacting conditions and sophisticated equipment. Ferrel et al. (1) have reported a simpler technique for determining

[1]Contribution from Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Albany, Calif. 94710.

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lysine and methionine added to wheat. This ninhydrin method, however, is not specific for lysine since many amino-containing compounds produce similar color reactions. Consequently, a sample blank identically treated is required for each fortified sample to be tested if meaningful results are to be obtained.

Spies and Chambers (2) reported the formation of copper salts of amino acids with copper phosphate in a borate buffer. Porter and Sanger (3) and Selim (4) synthesized ϵ-dinitrophenyl lysine (ϵ-DNP-lysine) by using a copper complex as an intermediate. The ϵ-DNP-lysine was formed specifically and quantitatively, and all interfering chromophores were removed by extraction with diethyl ether.

The present method is a specific procedure for determining free lysine added to wheat and bulgur, based on blocking the α-carboxyl and α-amino groups of the free amino acids with cupric ions, leaving the ϵ-amino group of lysine free to react with the 1-fluoro-2,4-dinitrobenzene (FDNB). After acidification and extraction with diethyl ether, the only acid-soluble chromophore absorbing at 390 nm. is the ϵ-DNP-lysine.

MATERIALS AND METHODS

Materials

The lysine-fortified bulgur samples were obtained from commercial sources. Lysine-fortified atta-flour samples were prepared in the laboratory by carefully blending the calculated amount of dry lysine monohydrochloride (lysine · HCl) with measured portions of a single large lot of 91% extraction atta flour. The unfortified bulgurs were prepared in the laboratory by soaking wheat for 75 min. in water at 65°C. to yield about 45% moisture, draining and tempering 30 min. at 65°C., steaming 20 min. at atmospheric pressure (100°C.), and drying in an air-dryer at 80°C.

The control ϵ-DNP-lysine was prepared according to Porter (5) and crystallized from HCl solution. Carbon, hydrogen, and nitrogen analysis confirmed the composition.

Calculated as monohydrochloride:

$$C = 41.4\%; N = 16.1; H = 4.89.$$

Found:

$$C = 41.2\%; N = 16.0; H = 4.87.$$

Spectral data and all absorbance data reported were obtained on a Beckman DK-2 spectrophotometer. Comparable results were obtained on a Coleman Universal Model 14 spectrophotometer. Moisture was determined by a vacuum-oven method (6). For purposes of comparison, free lysine was determined by the ninhydrin method of Ferrel et al. (1) and on the short column of a Phoenix Model *K* 8000-B amino acid analyzer by the Spackman (7) method. Samples for the short-column analysis were prepared by extraction with 0.1N HCl diluting to a known volume and filtering prior to freeze-drying. The freeze-dried material was then taken up in pH 2.2 buffer, centrifuged at 10,000 r.p.m. for 30 min., and filtered through Celite before being placed on the column.

Proposed Method

Reagents: Copper Chloride Solution. Twenty-eight grams of anhydrous cupric chloride is dissolved and made to 1 liter with distilled water.

Trisodium Phosphate Solution. Anhydrous trisodium phosphate (68.5 g.) is dissolved and made to 1 liter with distilled water.

Borate Buffer (pH 9.1 to 9.2). Sodium tetraborate decahydrate (54.64 g.) is dissolved and diluted to 4 liters with distilled water.

Copper Phosphate Suspension. Two hundred milliliters of copper chloride solution is slowly added to 400 ml. of trisodium phosphate solution with constant mixing. The suspension is transferred to centrifuge tubes and centrifuged for 5 min. at 2,000 r.p.m. The precipitate is washed three times by resuspending in borate buffer and centrifuging. The final precipitate is suspended in borate buffer and diluted to 1 liter with buffer.

10% Alanine Solution. Ten grams of alanine is dissolved and made to 100 ml. with distilled water.

FDNB Solution. Ten milliliters of FDNB is made to 100 ml. with methanol.

Lysine·HCl Standard Solution. A stock solution is prepared dissolving 0.4000 g. lysine·HCl in water and diluting to 100 ml. (4.0 mg. per ml.). A working standard containing 200 γ per ml. is made by diluting 5 ml. of stock solution to 100 ml. with water.

Method:

1. The sample is ground to pass a 40-mesh screen.
2. One (1.0000) gram of the sample is weighed into a 100-ml. volumetric flask. A 1,000-γ lysine·HCl standard (5 ml. of working standard) and a reagent blank are run with each set of samples.
3. Twenty-five milliliters of copper phosphate suspension is added, followed by 1.0 ml. of 10% alanine.
4. The mixture is shaken for 15 min. either on a mechanical shaker or with frequent shaking by hand, to assure complete dispersion of the sample.
5. Five-tenths milliliter of 10% FDNB solution is pipetted into the flask and the flask is placed in a boiling-water bath for 15 min.
6. The flask is removed from the water bath and 25 ml. of 1.0N HCl is added immediately, with shaking, to disperse and acidify all portions.
7. The flask is then cooled in a water bath to room temperature and brought to volume with distilled water.
8. Approximately 40 ml. of the suspension is centrifuged.
9. The supernatant is extracted three times with 25 ml. of diethyl ether, discarding the ether.
10. The aqueous phase is placed in running hot (about 65°C.) tap water, or water bath at 65°C., for 15 min. to remove residual ether.
11. A 10-ml. aliquot is mixed with 10 ml. of 95% ethanol and filtered through highly retentive filter paper (S&S 576 or S&S 602).
12. The absorbance is read at 390 nm., setting the instrument to zero with the reagent blank.
13. Calculation:

$$\% \text{ lysine·HCl} = \frac{\frac{\text{Sample absorb}}{\text{STD absorb}} \times \text{mg. lysine·HCl in standard} \times 100}{\text{Sample weight (mg.)}}$$

% added lysine·HCl = % lysine·HCl in fortified sample − % lysine in sample blank.

RESULTS AND DISCUSSION

Spies and Chambers (2) and Selim (4) report the complexing of copper by the α-carboxyl and α-amino groups of lysine to be rapid and quantitative in the presence or absence of other amino acids. Selim (4) also reports that the complex reacts quantitatively with FDNB to produce ε-DNP-lysine. At the much lower concentrations and higher temperatures present in our work, we were unable to obtain complete reaction with lysine·HCl, as shown in Table I. By increasing the total amino acid concentration through the addition of a neutral amino acid such as alanine (glycine was equally effective), quantitative reaction was achieved. It is interesting to note that even where reaction was incomplete the response to increasing lysine·HCl concentration was nearly linear.

A number of observations suggest that the copper-lysine complex has limited solubility, but that in the presence of alanine a more soluble mixed alanine-lysine-copper complex is formed, which is necessary for the formation of the ε-DNP-lysine. These observations were:

1. When lysine·HCl and alanine are reacted separately with copper phosphate, excess copper phosphate filtered out, and the filtrates combined and reacted with FDNB, only 40 to 50% of the theoretical ε-DNP-derivative is formed. If the two amino acid-copper complexes are formed separately, combined, then filtered to remove excess copper phosphate, and reacted with FDNB, 100% of the theoretical ε-DNP-lysine is formed.

2. When lysine·HCl and alanine solutions are reacted separately with only theoretical amounts of copper phosphate required to form the $Cu(AA)_2$ complex, the copper-lysine solution shows appreciable turbidity, whereas the copper-alanine solution is clear. When the two solutions are combined, the turbidity is lost.

3. Lysine·HCl was reacted with copper phosphate and centrifuged; the supernatant and precipitate were separated, and lysine was determined on the precipitate by the following method: Copper phosphate suspension was similarly separated and the supernatant combined with the precipitate from the lysine-copper phosphate mixture; alanine was added to this combination, the FDNB reaction carried out, and lysine content determined colorimetrically. It was apparent that most of the lysine had been in the precipitate of the lysine-copper phosphate mixture.

To eliminate the possibility of adsorption on excess copper phosphate, the following experiment was conducted. Stoichiometric amounts of copper phosphate and lysine were reacted to yield $Cu(AA)_2$, followed by reaction with FDNB

TABLE I. EFFECT OF VARIOUS AMOUNTS OF ALANINE ON FDNB REACTION WITH LYSINE·HCl

γ Lysine Reacted	mg. Alanine Added				
	0	25	50	100	200
	% measured as ε-DNP using HCl				
500	11.2	67.2	87.2	100.8	101.2
1,000	12.3	64.4	85.8	97.0	98.0
2,000	19.7	59.2	83.6	99.2	99.4
Atta wheat plus					
1,000	30.4	66.2	85.2	99.0	100.0
2,000	41.4	64.0	85.6	105.0	100.0

without adding alanine. The results were the same as when excess copper phosphate was added, indicating that the poor recovery in the absence of alanine was not due to adsorption on the excess copper phosphate.

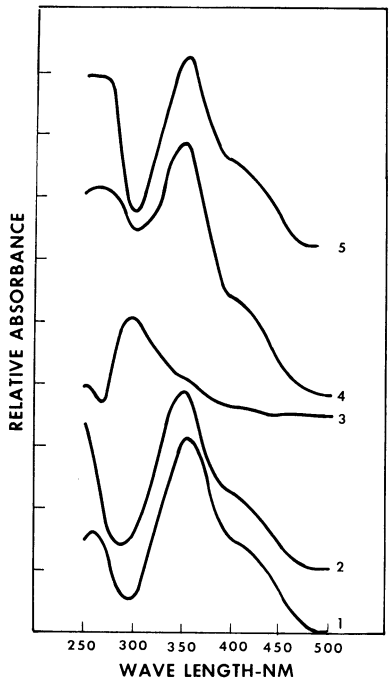
Analysis of lysine·HCl-fortified atta flours showed the same response to added alanine as did lysine·HCl standard solutions (Table I).

The quantity of FDNB also exerts controlling influence on the extent of reaction, as shown in Table II. The amount producing 100% recovery is 15 mg. for all standard lysine solutions, and 20 mg. for all fortified atta flours, regardless of the amount of lysine·HCl present. This suggests a concentration relationship, since the above figures represent 15 to 20 times the stoichiometric requirements. Excess amounts do not adversely affect the reaction, as shown by the data. We therefore recommend 50 mg. in our technique to allow for variations in the purity of the FDNB and estimates in lysine·HCl content. Previous investigators have carried out the FDNB reaction at temperatures ranging from ambient to 40°C., requiring 1 to 2 hr. for complete reaction. We have been able to shorten the time to 15 min. by reacting at 100°C.

Evidence that ε-DNP-lysine is the product formed under the conditions of reaction of this procedure is shown in Fig. 1. A lysine·HCl standard carried through the proposed method yields a derivative (Fig. 1, curve 2) with an absorption spectrum essentially identical to the spectrum obtained on the standard ε-DNP-lysine (Fig. 1, curve 1) prepared according to Porter (5). An unfortified atta blank (curve 3) demonstrates no appreciable absorption in the range of interest but it does have a maximum at 300 nm. similar to that of *O*-DNP-tyrosine. Lysine·HCl-fortified atta shows the typical ε-DNP-lysine absorption spectrum (curve 4). If fortified atta is read against the atta blank (curve 5), a spectrum identical to that of the standard ε-DNP-lysine is obtained. Whereas the absorption maximum of the ε-DNP-lysine is at 360 nm., we have chosen to read at 390 nm. so that simpler visual-range spectrophotometers can be used. Also, the extraction of

TABLE II. THE EFFECT OF VARIABLE AMOUNTS OF FDNB ADDED FOR REACTION WITH LYSINE·HCl

mg. FDNB Added	γ Lysine·HCl Reacted		
	500	1,000	2,000
	% measured as ε-DNP Lysine·HCl		
2	4.98	9.92	20.0
10	60.80	63.6	64.5
12	86.4	83.6	75.5
15	100.0	100.0	100.1
20	101.0	100.0	99.0
50	100.8	97.2	98.1
100	100.4	98.8	98.5
		Atta Flour	
10		58.0	47.0
12		67.9	55.2
15		87.5	72.0
20		100.4	100.0
50		98.6	100.0
100		100.4	99.0



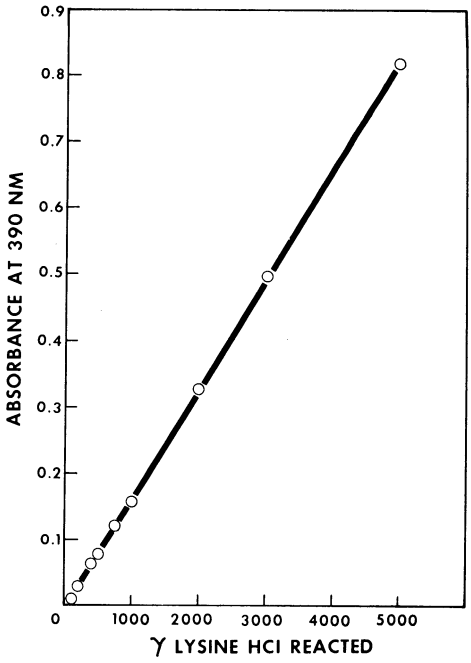


Fig. 1 (left). Absorption spectra for materials carried through the proposed reaction compared to authentic ε-DNP lysine·HCl: curve 1) authentic ε-DNP lysine·HCl; 2) lysine·HCl solution; 3) atta-flour blank; 4) lysine·HCl-fortified atta flour; 5) lysine-fortified atta flour run against atta-flour blank.

Fig. 2 (right). Standard curve for lysine·HCl carried through the proposed method (1-cm. light path).

the acid solution with ether removes all neutral and acid DNP-amino acid derivatives and breakdown products of the FDNB that would otherwise interfere at 390 nm. (4). The extraction leaves only *O*-DNP-tyrosine which has an absorption maximum at around 300 nm. and does not interfere at 390 nm., according to Selim (4) and Porter (5).

The effective range of the method is from 0.02 to 0.5% lysine·HCl (200 to 5,000 γ), as shown in Fig. 2. The practical upper limit may vary somewhat with different instruments and differing light paths. In these cases, when analyzing samples with higher levels of fortification, smaller starting samples can be used or the reaction mixture can be diluted to a greater volume.

For maximum utility the ideal method would require no sample blank. From a practical standpoint, the ability to make a standard correction for the blank or to use the raw material (raw wheat) would also be of great value. In the ninhydrin method of Ferrel, the use of raw wheat as a blank rather than an unfortified bulgur blank yields results which are inconsistent with the FDNB or ion-exchange method. Such results are owing to the fact that there are often significant losses in apparent lysine as determined by the ninhydrin method when bulgur is made from wheat.

Therefore the use of a wheat blank can lead to erroneously low values of added lysine. Since the FDNB method is specific for lysine this problem does not exist, as is shown by the data in Table III. The blanks for wheat and for bulgur made from it are equal, indicating that the raw material can be used as a blank for a processed product. The fact that blanks for wheat of different types and from widely ranging sources are also in close agreement suggests that a standard blank correction could be used in routine control or monitoring operations.

A comparison of results obtained from the proposed method with those obtained by ion-exchange chromatography is given in Table IV. Results on all the bulgurs compare very favorably with those obtained by the more complex ion-exchange method.

TABLE III. COMPARISON OF SAMPLE BLANKS FOR VARIOUS WHEATS AND BULGURS MADE FROM THEM

Wheat Type	% Lysine•HCl-Wheat	% Lysine•HCl-Bulgur
Hard white	0.019	0.020
White club	0.019	0.019
Soft white	0.020	0.020
Hard red spring	0.019	0.020
European soft red[a]	0.018	0.019
European soft red[a]	0.020	0.020
European soft red[a]	0.017	0.019
European soft red[a]	0.017	0.019

[a]Each sample was from a different country.

TABLE IV. COMPARISON OF VARIOUS METHODS TO DETERMINE ADDED LYSINE•HCl IN WHEAT AND BULGUR

Sample[a]	% Lysine•HCl	
	Proposed method	Ion-exchange
1	0.095 ±0.005[b]	0.091
2	0.076 ±0.007	0.072
3	0.084 ±0.004	0.081
4	0.067 ±0.007	0.063
5	0.080 ±0.001	0.082
6	0.080 ±0.005	0.079
7	0.091 ±0.004	0.088
8	0.093 ±0.011	0.096
9	0.071 ±0.005	0.073

[a]Samples are commercially prepared lysine•HCl-fortified bulgur. The raw wheat from which they were made was used as blanks in all analyses.

[b]Values are averages of six determinations and deviations are at the 95% confidence level.

CONCLUSION

Quantitative results for added lysine in wheat and bulgur products can be obtained by the method described. It offers the advantages of being rapid and accurate, requiring only a wheat blank or a correction factor for routine work and relatively simple equipment.

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