Direct Estimation of Lysine in Corn Meals by the Ninhydrin Color Reaction

Alfred C. Beckwith,* Jerrold W. Paulis, and Joseph S. Wall

The colored derivative produced by the reaction of ninhydrin with α- and ϵ-amino groups on amino acids, peptides, and proteins is the basis for a rapid spectrophotometric estimation of lysine in corn meals. If the meal is first extracted with trichloroacetic acid to remove free amino acids, the amount of colored ninhydrin derivative produced in a dimethyl sulfoxide solvent system can be used as a quantitative determination of lysine content to within the uncertainty found by amino acid analyses for lysine. The method has advantages of simplicity, rapidity, and economy, requires but small samples of meal (10–20 mg), and is highly useful in the screening of corn in breeding programs for high-lysine grains.

Since the discovery that *opaque-2* (o_2) and *floury-2* (fl_2) mutants of corn have grains high in lysine (Mertz *et al.*, 1964; Nelson *et al.*, 1965), extensive breeding programs have been underway to develop suitable high-lysine hybrids. The recent discovery of modifier genes has permitted development of high-lysine corns having vitreous kernels resembling normal dent corn in contrast to the floury characteristics of regular o_2 and fl_2 mutants (Bauman and Aycock, 1970). Breeding programs for high-lysine corn, especially of the vitreous type, have been handicapped by the lack of a simple rapid assay method for lysine content.

The use of colorimetric and enzymatic methods, gas and ion-exchange chromatography, and methods of analysis for different proteins has been reviewed by Paulis *et al.* (1974a,b) and Concon (1972). However, each of these methods for evaluating lysine content lacks either simplicity or accuracy.

Recently, Mertz *et al.* (1975) used the ninhydrin reagent upon extracts of individual kernels of several cereal grains to detect differences in free amino acid levels. Since high-lysine lines contain much higher levels of free amino acids (Mertz *et al.*, 1975), use of the ninhydrin color reaction permitted them to distinguish normal and high-lysine corns. Prior to the report of their findings, work at this laboratory established that the ninhydrin reaction of total amino groups in the kernel can be correlated with the lysine content of corn. However, if the free amino acids are removed from the meal of corn or other grains, the ninhydrin reagent can be used for a quantitative estimate of lysine content in protein in the grain samples. The production of color by ninhydrin reaction with α- and ϵ-amino groups in the grain proteins is facilitated by use of special solvent systems. The reaction is easy to perform and requires only small quantities of sample (10–20 mg).

MATERIALS AND METHODS

Corn Preparation and Analyses. Corn samples consisted of o_2, fl_2, and normal varieties obtained from several commercial hybrid seed producers and state experiment stations. The corn was ground by means of a Udy cyclone hammer mill to 60 mesh and defatted as described by Paulis *et al.* (1974a). Lysine was determined by automatic amino acid analyses on acid hydrolysates and total nitrogen by the Kjeldahl procedure on the corn meal (Paulis *et al.*, 1974a).

Of samples selected for this investigation, o_2 samples averaged with standard deviation 4.29 ± 0.28 g of lysine per 16 g of total nitrogen; the corresponding value for fl_2 corns was 3.47 ± 0.30; and for normals, 2.75 ± 0.34.

Chemicals. Ninhydrin, stannous chloride, dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), and metabisulfite were obtained commercially and were of the highest quality grade available.

Ninhydrin Reagent Solution. The reagent solution was prepared by dissolving 3.00 g of ninhydrin and 38 mg of stannous chloride in 100.0 ml of a solvent system consisting of DMSO, water, and 4 *M* acetic acid–acetate buffer (pH 5.5) (2:1:1 v/v) and was stored in light-proof glass-stoppered bottles. The reagent solution was prepared just before use.

Reaction of Ninhydrin with Corn Meals. For routine approximate estimation of lysine levels, the ninhydrin reaction can be conducted on the entire ground grain. But for quantitative estimate of lysine content, the meals are first extracted with TCA (10% w/v) to remove free amino acids. From 10 to 20 mg of meal is placed in Pyrex serological tubes (12 ml capacity) fitted with Teflon-lined screw caps. After 2.0 ml of TCA solution is added to the tube and it is capped tightly, the meal is agitated vigorously with extractant for 1 min on a vortex-type mixer. The suspensions are then centrifuged at 900–1000*g* for 10 min and the supernatant is removed by decantation.

To the tube containing corn meal or TCA extracted residue is added 0.2 ml of 0.125 *M* sodium metabisulfite and 2.3 ml of DMSO–water (4:1, v/v). The contents are mixed as described above to disrupt disulfide structuring in the corn protein and to disperse the meal in this solvent. After 1.0 ml of ninhydrin reagent is added the suspensions are heated 30 min in a boiling water bath and then cooled rapidly by partial immersion in cold water. Control color blanks contain DMSO–water and ninhydrin reagent only. For reasons given later, metabisulfite is omitted from blanks. The highly colored reaction mixtures are then diluted with 5.0 ml of DMSO–water solvent and the tubes again centrifuged at 900–1000*g*. The colored supernatants are decanted into commercially available colorimeter tubes (19 × 150 mm) and diluted with 5.0 ml of DMSO solvent. The absorbance at 580 nm is measured with any suitable spectrophotometer. If needed, the solutions are diluted further with DMSO–water (4:1, v/v) to place the absorbance value in the most sensitive range of the spectrophotometer.

Corn samples of known lysine and total nitrogen content are used as standard samples to estimate a linear relationship between 580-nm absorbance and lysine content. Preferably, at least two corn meal samples of varying lysine level from each corn variety should be used to obtain this relationship. The values obtained with these standards can be expressed on a weight of meal basis or on 16 g of total nitrogen basis. Plots or a linear equation describing these results are then used to estimate the lysine content in unknowns given determined absorbance value.

RESULTS

The total nitrogen contents, lysine contents, and absorbance values obtained in this study are summarized in

Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604.

Table I. Total Nitrogen Contents, Lysine Contents, and 580-nm Absorbance Values for Corn Meals

Type[a]	% total nitrogen (defatted meal)	Lysine content		Absorbance at 580 nm/g of meal[c]	
		mg/g of meal	g/16 g of N	Before TCA[b] ext.	After TCA ext.
o_2	1.69	5.03	4.68	158.0	34.7
o_2	1.38	5.17	4.14	90.2	35.2
o_2	1.52	4.06	3.91	98.4	
o_2	1.54	5.00	4.44	78.3	34.4
o_2	1.43	4.99	4.44	90.3	
o_2	1.62	4.48	3.92	79.9	34.9
o_2	1.30	3.77	4.47	70.1	26.1
o_2	1.80	4.89	3.95	83.2	34.1
fl_2	1.65	3.43	3.05	59.8	24.9
fl_2	2.03	5.37	3.91	116.0	37.0
fl_2	1.81	4.12	3.42	68.6	
fl_2	1.65	3.71	3.35	86.6	
fl_2	2.10	4.80	3.32	90.8	
Normal	1.43	2.46	2.66	39.2	18.7
Normal	1.48	2.69	2.71	52.9	
Normal	1.72	3.18	3.05	51.5	
Normal	1.70	3.08	2.72	50.9	
Normal	1.48	3.25	3.19	57.9	
Normal	1.28	3.00	2.82	49.9	22.0
Normal	4.18	5.58	2.11	100.0	45.6

[a] o_2 = *opaque-2*; fl_2 = *floury-2*. [b] TCA = trichloroacetic acid. [c] Absorbance values for 13.5 ml of solution.

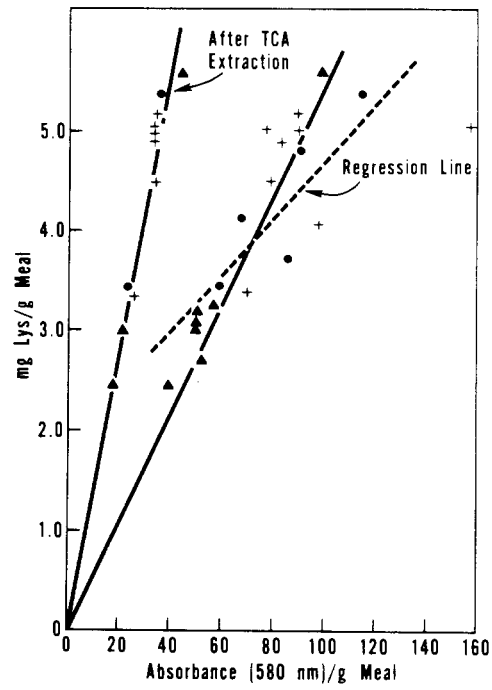

Figure 1. Relation between corn meal lysine content (milligrams/gram of meal) to ninhydrin absorbance at 580 nm for 13.5 ml of solution per g of meal before and after extraction with trichloroacetic acid: (+) *opaque-2* corn varieties; (●) *floury-2* varieties; and (▲) normal meals.

Table I. Figure 1 shows the relationship between milligrams of lysine per gram of meal and absorbance of solution at 580 nm per g of defatted meal both before and after removal of free amino acids. Only average values are presented in Table I and Figure 1. Lysine contents (milligrams of Lys/gram of meal) had relative standard deviations ranging from 1.2 to 15% while absorbance values at 580 nm were reproducible to within 0.9 ± 0.4 unit/g of meal.

For samples subjected to ninhydrin reaction without prior TCA extraction, the data were analyzed with a linear regression analysis to generate the dashed line in Figure 1. This dashed line has the following equation and parameters

$$L = 1.93 + 0.0277A \qquad (1)$$
$$S = \pm 0.61,\ S_I = \pm 0.42,\ S_S = \pm 0.0051$$

where L equals the milligrams of lysine per gram of meal; A is the absorbance at 580 nm per g of meal before TCA extraction; S is the standard deviation of points from the line; S_I is the uncertainty in the ordinate intercept; and S_S is the uncertainty in the slope of the line. The correlation coefficient for the line is 0.79.

The regression line has an undesirable feature in that the extrapolated line does not cross the A axis at zero or a positive number when L is equal to zero. Even though the lysine content might be negligible, α-amino groups should produce a residual ninhydrin color. Perhaps a straight line is a poor model for the data. Because of this objectional feature for the regression line, the solid line in Figure 1 was passed through the same data but was constrained to pass through the orgin also, in order to compare error estimates for different lines. The slope of this line was determined by averaging individual values of L/A from Table I to yield an average value and standard deviation of 0.0543 ± 0.0018 for the slope. The standard deviation for a single L/A value was 0.0081 which yields a relative deviation of 15%. This error estimate falls within the previously stated uncertainty range in lysine content. Therefore, without removing the free amino acids, the ninhydrin color reaction can be used to give a first approximation to the lysine content of different corn varieties.

The results obtained after extraction with TCA were also subjected to a linear regression analysis. On the basis of a student t test at a 95% confidence limit, the analysis showed that the ordinate intercept in Figure 1 was not significantly different from zero. Therefore, the slope of the solid line through these data was again evaluated by averaging individual ratios of L/A from Table I as before to yield an average slope equal to 0.139. Here the standard deviation for a single L/A value is 0.008 or only 6% of the average which is well within the known error in lysine content. Even though fewer data were used to estimate this latter straight line, the samples selected contained those showing the greatest deviations from the dashed regression line in Figure 1 prior to TCA extraction. The results after TCA extraction expressed on a 16 g of total nitrogen basis appear in Figure 2. The regression line in Figure 2 has the equation

$$\text{g of Lys/16 g of N} = -0.0643 + 0.133 \times 10^{-3}(A_{TCA}/16 \text{ g of N}) \qquad (2)$$
$$S = \pm 0.333,\ S_I = \pm 0.496,\ S_S = \pm 0.0168 \times 10^{-3}$$

where S, S_I, and S_S are defined as before. The correlation coefficient for the line is 0.90. On the basis of the ordinate intercept and the standard error in this value, the line again passes essentially through the origin in Figure 2. A line passing through the origin would have a slope of 0.130 ± 0.011 × 10^{-3} which is very close to the value found for the line on a weight basis (Figure 1) divided by 1000.

DISCUSSION

The TCA extractions removed an average of 60 ± 7% of the amino groups contributing to the 580-nm absorbance obtained by reacting the whole corn meals with ninhydrin.

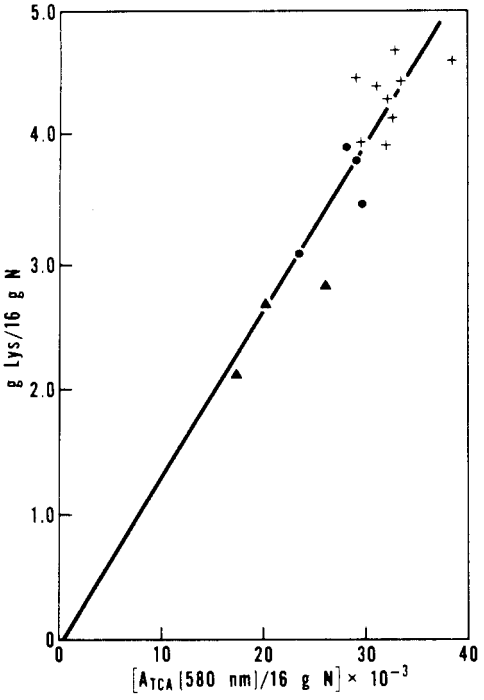

Figure 2. Relation between corn meal lysine content (g/16 g of nitrogen) to ninhydrin absorbance for 13.5 ml of solution at 580 nm per 16 g of nitrogen after extraction with trichloroacetic acid: (+) *opaque-2* corn varieties; (●) *floury-2* varieties; and (▲) normal meals.

Thus the difference between the solid lines in Figure 1 should be roughly proportional to the free amino acid content in the meals. In general, the o_2 varieties have the largest content of free amino acids and the normal varieties have the least. The last entry in Table I, however, is an exception to this relationship. This normal variety sample had a high total nitrogen content as well as a large amount of lysine on a per gram of meal basis. On the basis of the results in Figure 1, this sample also had a high level of free amino acids. Therefore, the level of free amino acids in the meals may be correlated with the lysine content on a weight basis. However, a high level of free amino acids in a meal does not necessarily indicate that the g of lysine per 16 g of total nitrogen is high, as can be seen by comparing results in columns 3 and 4 of Table I and Figure 2. In Figure 2 it is to be noted that points are grouped along the line more in accordance with corn type than in Figure 1.

The ninhydrin color yield from the TCA extracted corn meals is chiefly the result of two variables: (1) the amount of α-amino nitrogens on protein chains and (2) the amount of lysine ϵ-amino groups in the protein molecules. The contribution to total color yield for each type of amino group has been discussed by Slobodian *et al.* (1962). But from Figures 1 and 2, the average mean contribution of α-amino group to total ninhydrin color from corn meals is quite small after TCA extraction since the solid straight lines in these figures pass very close to or through the origin.

Ninhydrin in DMSO was used to follow changes in α- and ϵ-amino nitrogen content on proteins and polypeptides several years ago by workers at this laboratory (Krull *et al.*, 1961; Beckwith *et al.*, 1963; Friedman and Wall, 1964). Moore (1968) recommended DMSO as a component of a general solvent system. Whereas the reagent solution of Moore is probably more stable than the one employed here, stannous chloride and sodium acetate buffers were readily available and are used routinely here for automated amino acid analyses. Upon standing, a precipitate develops in the reagent solution described here and was, therefore, prepared fresh before each test.

Bisulfite was added primarily to break disulfide bonds in corn glutelin protein to yield smaller protein particles. But in preliminary investigations with highly pigmented whole grain sorghum flour, bisulfite also markedly reduced the color intensity of DMSO-soluble pigments. Unfortunately, bisulfite added to standard norleucine solutions inhibited ninhydrin color development at very low amino acid concentration, but the level of bisulfite employed did not appear to interfere with color development from corn meals. Standard amino acid solutions are really not essential to the method presented here. As mentioned earlier, grain samples well characterized as to total nitrogen and lysine content can serve to make quantitative absorbance comparison on a day-to-day basis. The need to remove fats and oils from cereal meals for routine use of this method is probably not essential for ninhydrin color development. For a nondefatted o_2, fl_2, and normal corn sample as well as a whole sorghum meal, 580-nm absorbance values per g of meal were about the same as values listed in Table I for defatted samples of the same lysine contents. But lysine content determinations by automatic amino acid analysis were made only on defatted meals.

The ninhydrin procedure for determination of lysine in TCA extracted corn meals certainly meets the requirements of corn breeding programs where simplicity and rapidity are essential. The accuracy of the method is well within the variability of lysine determination by amino acid analysis. The method required only about 3 hr to make duplicate analysis for lysine content on 20–25 samples.

LITERATURE CITED

Bauman, L. F., Aycock, H., *Proc. Annu. Corn-Sorghum Res. Conf., 25th*, 139 (1970).
Beckwith, A. C., Wall, J. S., Dimler, R. J., *Arch. Biochem. Biophys.* **103**, 319 (1963).
Concon, J. M., "Determination of Lysine in Cereal Proteins in Symposium: Seed Proteins," Inglett, G. E., Ed., Avi Publishing Co., Westport, Conn., 1972, p 292.
Friedman, M., Wall, J. S., *J. Amer. Chem. Soc.* **86**, 3735 (1964).
Krull, L. H., Beckwith, A. C., Wall, J. S., Dimler, R. J., Abstracts, 140th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1961, p 320.
Mertz, E. T., Bates, L. S., Nelson, O. E., *Science* **145**, 279 (1964).
Mertz, E. T., Misra, P. S., Jambunathan, R., *Cereal Chem.*, in press (1975).
Moore, S., *J. Biol. Chem.* **243**, 6281 (1968).
Nelson, O. E., Mertz, E. T., Bates, L. S., *Science* **150**, 1469 (1965).
Paulis, J. W., Wall, J. S., Kwolek, W. F., *J. Agr. Food. Chem.* **22**, 313 (1974a).
Paulis, J. W., Wall, J. S., Kwolek, W. F., *J. Agr. Food. Chem.* **22**, 318 (1974b).
Slobodian, E., Mechanic, G., Levy, M., *Science* **135**, 441 (1962).

Received for review June 13, 1974. Accepted October 21, 1974. Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.