Application of the 2,4,6-Trinitrobenzene-1-Sulfonic Acid (TNBS) Method for Determination of Available Lysine in Maize Seed

I. U. OBI

Department of Crop Science, University of Nigeria, Nsukka, Nigeria

Received September 16, 1980

This paper reports on a modified procedure for determination of lysine in maize seeds. The modified procedure was economical, accurate and fast, and has been used to determine the available lysine in large numbers of maize grains. Protein sufficiently representative of total protein was extracted from samples ground in a water-cooled grinder. It was proposed that for samples with more than 15% protein the volume of NaOH–ETOH extractant be doubled. For maize samples with 15~30% oil content, 20 ml of acetone was used for fat extraction, as opposed to 15 ml of acetone used for samples with less than 15% oil content. The sub-sampling variation was not significant for the modified 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) procedure. The correlation coefficient between lysine values determined by the amino acid analyser and the TNBS method for 90 opaque-2 samples was high ($r=0.806$). The coefficient of variation for TNBS was 6.5% against 12.6% for the microbiological assay (MBA), indicating that more variation was associated with the MBA than with the TNBS method.

It has been shown that the poor nutritive value of maize (*Zea mays* L.) grains is due to low contents of lysine and tryptophan in the main protein component, Zein.[3] The discovery of an opaque-2 mutant gene by Singleton and Jones has changed the picture of the nutritive quality of certain strains of maize protein.[4] Nutritional studies showed that opaque-2 maize protein was superior to normal maize and was comparable to milk.[5~8]

Many institutions and industries are now involved in breeding programmes aimed at incorporating the opaque-2 gene into existing maize varieties, as well as investigating the agronomic characteristics of the newly-converted lines.[9~12]

A maize breeder or geneticist interested in increasing the levels of lysine and tryptophan in maize varieties is often faced with the problem of lack of a fast, economical and accurate method for the analysis of the limiting amino acids—lysine and tryptophan. The 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) colorimetric method previously developed[1,2] for the determination of lysine in foodstuffs is not fast enough to handle fairly large numbers of samples and at the same time is not economical enough to meet the plant breeder's needs. The objective of the studies presented here is to find a modified TNBS colorimetric method by which a large number of samples of maize grains can be analysed economically, accurately and rapidly. The lysine content in the protein from several varieties of maize was determined with the modified TNBS method and was compared with that from amino acid analysis (AAA)[13] and modified microbiological assay (MBA).[14]

MATERIALS AND METHODS

Materials. 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBS) and ε-TNP-L-lysine HCl·H_2O were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Maize types used were supplied by Dr. R. J. Lambert, Department of Agronomy, University of Illinois, Urbana Ill., U.S.A.

The standard curve. To 1.0 ml samples containing 0.02,

0.06, 0.10, 0.14, and 0.18 mg of ε-TNP-L-lysine was added 1.0 ml of 0.48 M $NaHCO_3$, pH 8.5, and 3 ml of concentrated hydrochloric acid. The tubes were autoclaved at approximately 120°C for 1 hr at $1.034 \sim 1.103 \times 10^5$ N/m^2 (15 ~ 16 psi), then cooled and diluted with 5 ml of distilled water. These solutions were mixed with a vortex mixer and filtered through Whatman No. 1 filter paper into 40 ~ 60 ml separatory funnels. The filtrates were extracted twice with 10 ml of ether (diethyl ether). The residual ether was removed by placing the tubes in a water bath at 40°C for 10 min. Absorbance of the aqueous solutions at 346 nm was measured with a Bausch & Lomb Spectronic 20. A linear standard line was obtained corresponding to the equation $y=bx$, where y is absorbance, x is ε-TNP-L-lysine (mg) and b is the slope of the line.

Sample preparation. Maize seeds were dried for 72 hr at 37.8°C. About 20 ~ 25 g of bulked seeds were ground in a water-cooled grinder. A Bauer Bros. water-cooled grinder, Springfield, Ohio, U.S.A., style 148, size 8, RPM 3600 was used. A portion of this sample (1.5 g) was defatted in 15 ml of acetone for 30 min. During this period each sample was mixed for two seconds every 10 min with the vortex mixer. The defatted samples were filtered on Whatman No. 1 filter paper and rinsed with 5 ~ 10 ml of acetone. The materials containing 15 ~ 30% oil were defatted with 20 ml of acetone. Samples were air dried and thoroughly mixed on filter papers. 500 mg of each sample were used for the lysine determinations.

To 500 mg of each sample in a plastic centrifuge tube was added 0.7 ml of 70% ethanol (v/v). Twenty plastic glass beads (0.9 cm in diameter) were added to each tube to increase the solubility of the protein. After the tubes were vortexed on the mixer for about 10 sec, 5 ml of 0.13 M NaOH was added and then mixed with the vortex mixer for 5 seconds. The tubes were closed with a rubber stopper and shaken with an automatic shaker (Eberbach 2-speed shaker) for 3 hr at 280 ~ 300 cycles per minute. Once every hour, the tubes were removed from the shaker and vortexed with the mixer to get the materials deposited on the side of the tubes back into the mixture. After shaking, the samples were centrifuged at 1,980 rpm for 4 min to obtain a clear supernatant (protein extract solution).

Lysine determinations. To 0.1 ml of the protein extract solution was added 1.0 ml 0.48 M $NaHCO_3$, pH 8.5. To the control sample (without protein) was added 0.1 ml of distilled water and 1.0 ml of $NaHCO_3$. The tubes were closed with a cork stopper and shaken for 10 min at 40°C. Then 1.0 ml of 2.4 mM TNBS was added to the tubes. The addition of $NaHCO_3$ to the protein extract from the normal maize samples produced a white precipitate which persisted after the addition of TNBS. This precipitate was not identified in these studies.

After adding the TNBS solution, the reaction mixture was incubated at 40°C in an incubator-type oven. After incubation for 2 hr, 3 ml of concentrated HCl were added to the tubes. The tubes were covered with ten-ml/vol glass vials and autoclaved at approximately 120°C for 1 hr at $1.034 \sim 1.103 \times 10^5$ N/m^2 (15 ~ 16 psi) to hydrolyse the TNP-protein into TNP-amino acids. The autoclaved tubes of the samples were cooled to room temperature and diluted with 5 ml of distilled water. The samples were then filtered through Whatman No. 1 filter paper into 40 ~ 60 ml separatory funnels. The filtrate was extracted twice with 10 ml of ether, to remove *N*-terminal amino acid or peptides, α-amino acids and picric acid produced during the hydrolysis of the TNP-protein. The picric acid could be produced from excess TNBS and α-TNP-amino acids.

After ether extraction, the tubes were put in a waterbath (40°C) for 10 min to remove any residual ether. The solutions were cooled to room temperature and the absorbance at 346 nm was measured against the control sample which was taken through the whole procedure. The ε-TNP-L-lysine content of the samples was obtained from the standard curve and converted to the lysine equivalent. Lysine contents are expressed as weight percent of lysine per dry weight of sample or lysine weight (g) per 100 g protein (L/P).

The amino acid analysis and microbiological assay of lysine. The lysine content of the materials used in these studies was also determined by two independent methods. A phoenix amino acid analyser, using the method of Moore *et al.*,[13] and microbiological assay using *Leuconostoc mesenteriodes*[14] were used. The amino acid analyser and microbiological assay lysine values were supplied by Dr. C M. Wilson and Dr. R. J. Lambert of the Department of Agronomy, University of Illinois, Urbana, Ill., U.S.A. The results were compared with those from the modified TNBS method.

Protein determination. Protein was determined by the standard Kjeldahl procedures (Kj · $N \times 6.25$).

Statistical analysis. An analysis of variance using a nested classification and Student's paired *t*-test were used to measure the importance of the fineness of sample grind and sub-sampling.

A split-plot experiment was used to measure the accuracy of the MBA and TNBS methods for the lysine determinations of floury-2 maize samples. The variables, per cent dry weight of lysine and L/P ratios, were used in the analysis of variance. The accuracy of the two methods (MBA and TNBS) was estimated by the error variance and coefficient of variation (c.v.). Using the AAA lysine values as a standard, linear correlation coefficients were determined for AAA and MBA, AAA and TNBS, MBA and TNBS methods using L/P ratios and percent dry weight of lysine of 90 opaque-2 single cross, F_2 samples. The correlation coefficient results were used to compare the MBA and TNBS methods for lysine determination. Linear correlation coefficients between MBA and TNBS

were calculated using per cent dry weight of lysine and L/P ratios of the maize samples with varying protein content.

RESULTS AND DISCUSSION

Sample size and fat extraction

A 20~25 g sample of whole kernels was ground in a water-cooled grinder. A 1.5 g portion of the ground material was found to be an adequate sample.

Initial fat extraction increases the ease of protein extraction at a latter stage. In addition it eliminates the possibility of contaminating the protein with fat which might interfere with the TNBS-protein reaction. A 30 min time interval was adequate to extract fat from the samples.

Fineness of the ground sample

Fineness of the ground sample is important for the TNBS method of lysine determination of maize seed protein. It increases the ease of both fat and protein extractions. A water-cooled grinder was used to achieve some degree of fineness. To investigate the importance of particle size, samples were ground in (a) a water-cooled grinder only and (b) a water-cooled grinder plus 3 min grinding in a microball mill. The mean L/P ratios of the two grinding methods were 3.39 and 3.53, respectively, and were not significantly different. However, the lysine values obtained from the samples ground in a water-cooled grinder plus 3 min grinding in a micro-ball mill had a higher standard deviation (S.D. = 0.964), compared to S.D. = 0.424 associated with the lysine values of samples ground in a water-cooled grinder only. Consequently, the water-cooled grinder procedure was adopted.

Protein extraction

The protein extraction technique of Subramanian, *et al.*[2] was tedious, slow and unable to accommodate large numbers of samples, and did not extract all the protein. As a result, the Vortex-Genie mixer was replaced by an automatic shaker. Twenty-nine different samples of F_2, opaque-2 seeds, single crosses, in three replicates, were used for the protein extraction studies. Protein was extracted for 2, 3 and 4 hr and the content of available lysine determined by the TNBS method.

The percentage protein in the extracts for 2, 3, and 4 hr shaking time was estimated by the Kjeldahl procedure. The results show that more protein solubilized with time, 90.7% for 2 hr, 92.8% for 3 hr and 94.2% for 4 hr. This relationship did not hold for the content of available lysine in the protein extract. A small difference was observed in the mean L/P ratios for the 4 hr shaking period. It was anticipated that the mean L/P ratio would increase with the time of protein extraction and eventually level off. Statistical analysis using Student's *t*-test showed the mean L/P ratio for 4 hr was significantly different from the mean L/P ratios at 2 and 3 hr. However, the mean L/P ratios of these samples analysed by the AAA was 4.64. This was identical to the mean L/P ratio for TNBS at a 3 hr protein extraction time. The MBA method gave a mean L/P ratio of 4.10. Consequently, a 3 hr protein extraction time was chosen.

Using the above protein extraction procedure, it was possible to extract protein from more than 100 different samples in a 3 hr period compared to $33\frac{1}{2}$ hr for protein extraction from 100 samples using a single Vortex or $6\frac{2}{3}$ hr using a more expensive 4-tube model Vortex mixer.

Sub-sampling

Twenty-nine different samples of F_2 seeds from 7 single crosses were used. Each sample was assayed for lysine in duplicate. A paired *t*-test of equal means showed that the mean L/P ratios of the duplicate, 4.63 and 4.65 were not significantly different.

Comparison of the modified TNBS method with the AAA and MBA methods

Having established the sample size, sample grinding, fat and protein extraction time period for the TNBS lysine assay method, samples were taken of opaque-2, floury-2 and normal maize seeds with varying protein levels. The

lysine determinations with AAA and MBA were compared with those from the modified TNBS method to estimate the accuracy of the TNBS and MBA methods, and also, to investigate the possibility of replacing the MBA method routinely used in this laboratory for lysine assays in a maize breeding programme with the TNBS colorimetric method.

Opaque-2 lysine determination

Six different opaque-2 single cross samples with two replications (90 total), were assayed for lysine, 30 samples were analysed per assay, using both MBA and TNBS methods. With the TNBS method it took 3 individual assays to complete the analysis of the 90 opaque-2 samples. The MBA method took 4 individual assays to complete a lysine determination of the 90 samples, due to reassay of the lost samples during hydrolysis or evaporation of the filtrate from protein hydrolyzate. The correlation coefficient was calculated according to individual assays by MBA and TNBS methods for L/P ratios and percent dry weight of lysine. The results are presented in Table I. Correlation coefficients (Table II) between AAA and MBA [r(AAA)(MBA)] and between MBA and TNBS [r(MBA)(TNBS)], vary more

Table I. Percent Dry Weight of Lysine, L/P Ratios of 30 Single Cross, F_2, Opaque-2 Maize Samples as Determined by the AAA, MBA and TNBS Methods

Pedigree	% Dry wt of lysine			L/P ratios		
	AAA	MBA	TNBS	AAA	MBA	TNBS
R802 × R803	.443	.387	.435	4.40	3.84	4.33
Oh43 × W64a	.438	.411	.465	4.20	3.94	4.46
R802 × R803	.438	.453	.491	4.26	4.21	4.57
Oh43 × W64a	.531	.362	.551	5.12	3.49	5.30
R801 × Oh7N	.516	.443	.507	4.40	4.73	5.41
R801 × R802	.531	.507	.527	4.64	4.43	4.60
R802 × Oh43	.464	.325	.435	4.82	3.38	4.52
R802 × Oh7N	.448	.488	.435	4.40	4.79	4.27
R801 × Oh7N	.594	.476	.575	5.31	4.26	5.13
R801 × R802	.568	.574	.583	4.41	4.46	4.52
Oh43 × W64a	.422	.331	.404	4.17	3.27	4.00
R802 × Oh7N	.469	.497	.491	4.58	4.85	4.79
R802 × R803	.490	.457	.465	4.69	4.38	4.46
R801 × R802	.547	.517	.515	4.66	4.40	4.79
R802 × Oh43	.443	.401	.412	4.89	4.42	4.54
R801 × Oh7N	.531	.456	.551	5.24	4.50	5.44
R802 × Oh7N	.438	.483	.471	4.52	4.98	4.88
Oh43 × W64a	.500	.375	.480	4.70	3.53	4.51
R802 × R803	.490	.419	.513	4.17	3.56	4.53
R802 × Oh43	.495	.474	.459	4.71	4.51	4.38
R801 × Oh7N	.578	.573	.630	4.40	4.37	4.80
R802 × R803	.542	.494	.523	4.38	3.99	4.23
Oh43 × W64a	.548	.354	.471	4.12	3.18	4.23
R802 × Oh7N	.453	.488	.475	4.34	4.68	4.55
R801 × R802	.589	.528	.559	5.04	4.51	4.78
R801 × Oh7N	.557	.521	.571	5.21	4.87	5.39
R802 × Oh43	.432	.337	.447	3.93	3.33	4.06
R802 × Oh7N	.458	.498	.492	4.76	5.18	5.11
R802 × R803	.448	.362	.463	4.10	3.31	4.23
R802 × Oh43	.505	.410	.471	4.70	3.82	4.40
Mean	.498	.447	.496	4.43	4.23	4.32
S.E.	.009	.013	.009	.163	.170	.169

TABLE II. CORRELATION COEFFICIENTS BETWEEN AAA AND MBA, AAA AND TNBS, MBA AND TNBS METHODS FOR L/P RATIOS AND PERCENT DRY WEIGHT OF LYSINE, ACCORDING TO INDIVIDUAL ASSAYS OF SIX SINGLE CROSS, F_2, OPAQUE-2 MAIZE SAMPLES (90 TOTAL)

		Individual assays				P.C.C.
		1	2	3	4	
r(AAA)(MBA)	A	0.0045NS	0.459*	0.784**	0.317NS	0.602**
	B	0.1086NS	0.666**	0.821**	0.592**	0.680**
r(AAA)(TNBS)	A	0.808**	0.818**	0.823**	—	0.806**
	B	0.822**	0.900**	0.881**	—	0.857**
r(MBA)(TNBS)	A	0.0999NS	0.527**	0.785*	0.240NS	0.596**
	B	0.3637*	0.681**	0.830**	0.465*	0.671**

P.C.C. = pooled correlation coefficients; NS = Not significant; *,** = Significant at 5% and 1% level, respectively; A = Correlation coefficient for L/P ratios; B = Correlation coefficient for percent dry weight of lysine; r = Correlation coefficient.

TABLE III. ANALYSIS OF VARIANCE OF L/P RATIOS OF 102 FLOURY-2 HALF-SIB FAMILIES OF MAIZE SAMPLES FOR MBA AND TNBS METHODS

Source	df	Mean squares	
		MBA	TNBS
Reps	1	1.74728*	0.37770*
Blocks	2	3.15817	1.88535
Blocks × reps	2	1.31049*	0.6372*
Treatment in blocks	48	0.12366	0.09269
Reps × treat. in blocks = error	48	0.17488	0.06770
Total	101	—	—
Means		3.31	3.98
C.V.		12.6%	6.5%

* Significant at 5% level.

TABLE IV. ANALYSIS OF VARIANCE OF PERCENT DRY WEIGHT OF LYSINE FOR 102 FLOURY-2 HALF-SIB FAMILIES OF MAIZE SAMPLES FOR MBA AND TNBS METHODS

Source	df	Mean squares	
		MBA	TNBS
Reps	1	0.02026*	0.00495*
Blocks	2	0.04341	0.01443
Blocks × reps	2	0.01228*	0.00734
Treatment in blocks	48	0.00148	0.00130*
Reps × treat. in blocks = error	48	0.00213	0.00059
Total	101	—	—
Means		0.3464	0.4159
C.V.		13.3%	5.8%

* Significant at 5% level.

with individual assays than the [r(AAA)(TNBS)]. In addition, the two methods, MBA and TNBS, had more variation associated with the L/P ratios than with the percent dry weight of lysine.

Floury-2 lysine values

Only the MBA and TNBS methods were used to assay the Floury-2 maize samples for lysine. One hundred and two samples were analysed. The lysine determinations expressed in L/P ratios and percent dry weight of lysine were used to determine the amount or level of variation in the two methods—MBA and TNBS. The results of the analysis of variance of the L/P ratios and percent dry weight of lysine are shown in Tables III and IV, respectively. The results in Table III show that the TNBS method had an error mean square of 0.0677 and a coefficient of variation (c.v.) of 6.5% compared to an error mean square of 0.1749 and a c.v. of 12.6% for the MBA method. Also, the mean L/P ratios of the two methods, MBA and TNBS, of 3.31 and 3.98, respectively were found to be significantly different. The results in Table IV show a

reduction in the error mean square (0.00059) and a c.v. (5.8%) for the TNBS method compared to an error mean square of 0.00213 and a c.v. of 13.3% for the MBA method. Based on these results the TNBS gave a more accurate estimate of lysine in floury-2 maize samples than the MBA method.

Acknowledgments. The author is indebted to Professors R. J. Lambert, D. E. Alexander and J. W. Dudley and other members of the maize breeding and genetics laboratories of the Department of Agronomy, University of Illinois, at Urbana-Champaign, Illinois 61801, U.S.A., for their financial assistance and materials that made this work possible.

REFERENCES

1) M. L. Kakade and I. E. Liener, *Anal. Biochem.*, **27**, 273 (1969).
2) S. S. Subramanian, R. Jambunathan, J. M. Concon and E. T. Mertz, Federation Proceedings (FASEB) 29, March 1970.
3) J. M. Concon, Proc. High Lysine Corn Conf., Corn Ind. Res. Found. Publication, U.S.A., 1966, p. 67.
4) R. A. Emmerson, C. W. Beadle and A. C. Fraser, *Cornell Univ. Agr. Expt. Sta. Memo*, No. 180 (1935).
5) E. T. Mertz, Proc. High Lysine Corn Conf., Corn Ind. Res. Found. Publication, U.S.A., 1966, p.12.
6) E. T. Mertz, L. S. Bates and O. E. Nelson, *Science*, **145**, 279 (1964).
7) R. Bressani, Proc. High Lysine Corn Conf., Corn Inc. Res. Found. Publication, U.S.A., 1966, p. 34.
8) R. A. Pickett, Proc. High Lysine Corn Conf., Corn Ind. Res. Found. Publication, U.S.A., 1966 p. 19.
9) D. E. Alexander, Proc. High Lysine Corn Conf., Corn Ind. Res. Found. Publication, U.S.A., 1966, p. 143.
10) R. J. Lambert, D. E. Alexander and J. W. Dudley, *Crop Sci.*, **9**, 242 (1969).
11) D. E. Alexander, R. J. Lambert and J. W. Dudley, International Atomic Energy Publ. Vienna, STI/Pub, 1969, p. 1212.
12) E. J. Wellhausen, Proc. High Lysine Corn Conf., Corn Ind. Res. Found. Publication, U.S.A., 1966, p. 148.
13) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
14) W. A. Feist and R. J. Lambert, M. S. Thesis, Univ of Illinois, Urbana-Champaign, Ill., U.S.A., 1969.