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soybean hulls and cereal brans, 9–11 mol % of the sugar was ribose.

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An Evaluation of Three Methods for the Selection of High Lysine Genotypes of Maize

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The prolamin turbidity test, the ninhydrin color test, and a method based on butanol extraction for the measurement of zein have been evaluated for the selection of high lysine maize genotypes derived from the mutant *opaque-2*. For preliminary screening of large populations the ninhydrin color test and the prolamin turbidity test were sufficiently sensitive to differentiate between high lysine and normal genotypes. In addition these were rapid and simple techniques whereby one person could screen about 100 samples/day. However, for further selection within a high lysine population using the method based on butanol extraction a good correlation was obtained between the protein content after butanol extraction and lysine expressed as a proportion of grain dry weight. The output of this method is 60 samples per person per day.

A simple and rapid technique for the selection of "modified endosperm" high lysine genotypes is required in maize breeding programs using the *opaque-2* mutant. Quite frequently breeders are now able to grow two generations per year by using sites in both the southern and northern hemispheres. Consequently the period available for lysine selection prior to resowing may be only a few weeks. In addition, such preliminary screening is often carried out in field stations where laboratory facilities are limited, so that screening methods should not be dependent upon complex autoanalysis equipment.

A number of such techniques are currently in use in maize breeding programs. These include: measurement of basic amino acids by dye binding capacity (Fornasari et al., 1975), measurement of tryptophan by glyoxylic acid reagent method and subsequent correlation with lysine content (Hernandez and Bates, 1969), and measurement of lysine content by reaction with 2-chloro-3,5-dinitro-pyridine (Villegas and Mertz, 1971).

Two of the methods investigated in this study are based on the negative correlation between the content of zein and lysine in maize derived from the *opaque-2* mutant (Salamini and Baldi, 1969). Frömberg et al. (1971) found that after butanol extraction of zein from 48 selfed lines of "modified endosperm" *opaque-2* maize the ratio of butanol-insoluble nitrogen to total kernel nitrogen was closely correlated ($r = 0.817$) with lysine content of the protein (g of lysine/100 g of protein) and this was designated the "nitrogen–butanol–nitrogen" (NBN) method. In addition, the content of insoluble nitrogen was closely correlated ($r = 0.847$) with the lysine content on a whole kernel basis (g of lysine/100 g of dry matter) and this was designated the BN method. The second method studied here, based on estimation of zein, involves extraction with 70% (v/v) ethanol, followed by addition of a salt solution to the ethanolic extract and measurement of the resulting turbidity. This technique has been developed for maize (Paulis et al., 1974a,b) and barley (Rhodes, 1975) and in this paper is referred to as the prolamin turbidity test.

The third method studied here depends upon the three–four-fold increase in free amino acids found in the grains of *opaque-2* maize (Mertz et al., 1974). The presence

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Table I. Estimation of the Lysine Content in the Dry Matter from the Prolamin Turbidity Test, the Ninhydrin Color Test, and the BN Method[a]

	correlation coefficient (r)			standard error about the regression		
	HL + N	HL	HL + HP	HL + N	HL	HL + HP
prolamin turbidity test						
absolute absorbance	−0.593	0.073	−0.815	0.0465	0.0409	0.0912
absorbance/gram of protein	−0.738	−0.310	−0.869	0.0390	0.0389	0.0780
ninhydrin color test						
absolute absorbance	0.836	0.729	0.883	0.0317	0.0280	0.0741
absorbance/gram of protein	0.841	0.684	0.855	0.0313	0.0300	0.0816
BN method						
protein content after butanol extraction	0.905	0.800	0.969	0.0245	0.0246	0.0389
significance points	21 df	18 df	18 df			
$P = 0.05$	0.43	0.44	0.44			
$P = 0.01$	0.54	0.56	0.56			

[a] Ranges of lysine content (g/100 g of dry matter): series HL + N, 0.29–0.49; series HL, 0.35–0.49; series HL + HP, 0.34–0.83.

of abnormally high levels of free amino acids may therefore be regarded as indicative of above average levels of lysine. Ninhydrin reagent reacts with the α-amino groups of free amino acids to form a colored complex, the intensity of which is proportional to the concentration of total free amino acids. This reaction is the basis of the technique referred to as the ninhydrin color test.

A critical assessment of these techniques has been made concerning their use for selection of: (i) modified high lysine genotypes from a population containing normal genotypes, (ii) genotypes with different degrees of kernel modification and lysine content from within a high lysine population, and (iii) genotypes possessing both high lysine and high protein content. In addition the merits of these methods for the selection for both lysine content expressed on a dry matter basis and that expressed on a protein basis are discussed.

MATERIALS AND METHODS

Plant Material. Material which had been analyzed for lysine and protein contents was used in these experiments and consisted of (i) high lysine–normal protein maize lines from a diallel which had endosperm structures varying from completely opaque to almost completely translucent. This series also contained three normal lysine controls and is referred to as HL + N, (ii) the same high lysine lines as in i but with the omission of the controls and referred to as HL, (iii) high protein maize lines with a wide range of lysine contents, HL + HP.

Butanol Extraction. This was carried out according to Frömberg et al. (1971) with the modification described by Pollmer and Frömberg (1973).

Nitrogen Analyses. Double nitrogen determinations before and after butanol extraction were performed, on samples dried to constant weight at 60 °C under reduced pressure, with an automated nitrogen analyser (Azotomat Mikro Rapid N; W. C. Heraeus GmbH). The ratio of the nitrogen content after extraction to that before extraction is an estimate of the lysine content of the protein.

Ninhydrin Color Test. Samples of the milled whole grain (50 mg) were placed into centrifuge tubes and 10 mL of ninhydrin reagent added. The reagent was prepared daily from 4.8 g of ninhydrin, 17.4 g of trisodium citrate, and 7.8 g of citric acid made up to 1 L with distilled water and stored in a darkened bottle. After homogenization of the samples on a vortex mixer, the tubes were placed in a boiling water bath for 5 min, then immediately removed from the water bath, homogenized, and allowed to stand for an additional 5 min. At the end of this period the samples were centrifuged at 5000 rpm for 5 min and the absorbance of the resulting supernatant solution was measured on a Zeiss PM2 DL spectrophotometer at 570

nm, using the ninhydrin reagent as blank.

Prolamin Turbidity Test. A 40-mg sample of each milled maize, which had previously been dried to constant weight at 60 °C under reduced pressure, was placed into a centrifuge tube and 3 mL of a 70% (v/v) solution of ethanol added. The samples were homogenized on a vortex mixer and the tubes stoppered and placed in a water bath at 60 °C for 1 h. The tubes were then removed from the bath and the samples rehomogenized and centrifuged at 5000 rpm for 5 min. A 2-mL aliquot of the resulting supernatant was pipetted into a fresh tube and 6 mL of a 0.2 M solution of sodium chloride added to it. Each tube was then stoppered and inverted at least twice to ensure thorough mixing of the contents. The turbidity of the resulting solutions was measured after 30 min on the spectrophotometer at 590 nm against a blank prepared from 2 mL of 70% ethanol plus 6 mL of 0.2 M sodium chloride solution.

Amino Acid Analysis for Lysine Content. All samples were analyzed for lysine content on a Beckman Unichrom amino acid analyzer according to Frömberg et al. (1971).

RESULTS

Estimation of Lysine Content in Total Dry Matter (Table I). The lysine content measured by amino acid analyses ranged from 0.29–0.49 g/100 g of dry matter in series HL + N; 0.35–0.49 g/100 g of dry matter in series HL and 0.34–0.83 g/100 g of dry matter in series HL + HP. Data from all three screening methods gave significant correlation coefficients with lysine in the total dry matter for HL + N; the negative correlations for the prolamin turbidity test indicated the negative relationship between zein and lysine content. The correlation coefficient for the turbidity test was increased considerably, from −0.593 to −0.738, when the absorbance was expressed per unit weight of protein, thus removing the effect of protein content on the estimation. The highest correlation coefficient 0.905 was obtained between lysine (g/100 g of dry matter) and the insoluble nitrogen after butanol extraction in the BN method.

In series HL which did not contain the three normal lysine controls, the correlation of lysine content in the dry matter with turbidity was not significant but that with the ninhydrin color test was still highly significant. For the BN method lysine content was highly correlated with the protein content after butanol extraction ($r = 0.800$).

The accuracy of an estimation of lysine content would be directly related to the standard error about the regression (SD). From these calculated variations in Table I the standard error about the regression was between 0.0245 and 0.0465 for all the methods in the series HL + N and

Table II. Estimation of the Lysine Content in the Protein from the Prolamin Turbidity Test, the Ninhydrin Color Test, and the NBN Method[a]

	correlation coefficient (r)			standard error about the regression		
	HL + N	HL	HL + HP	HL + N	HL	HL + HP
prolamin turbidity test						
absolute absorbance	-0.776	-0.207	-0.930	0.307	0.251	0.314
absorbance/gram of protein	-0.816	-0.345	-0.927	0.281	0.240	0.321
ninhydrin color test						
absolute absorbance	0.672	0.378	0.823	0.360	0.237	0.487
absorbance/gram of protein	0.730	0.387	0.850	0.333	0.236	0.452
NBN method						
proportion of insoluble nitrogen of total nitrogen	0.863	0.328	0.961	0.246	0.242	0.238
significance points	21 df	18 df	18 df			
$P = 0.05$	0.43	0.44	0.44			
$P = 0.01$	0.54	0.56	0.56			

[a] Ranges of lysine content (g/100 g of protein): series HL + N, 2.64-4.22; series HL, 3.47-4.22; series HL + HP, 1.96-4.50.
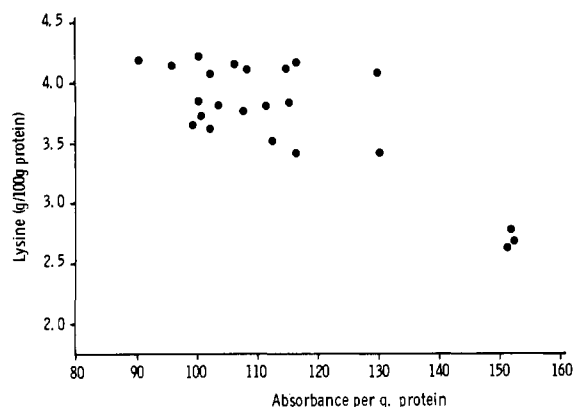

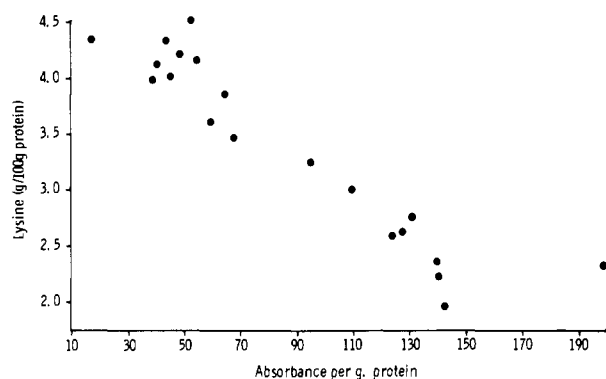Figure 1. Prolamin absorbance per gram of protein series HL + N.

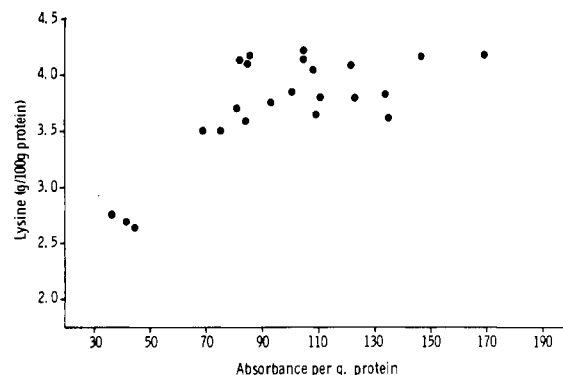Figure 2. Prolamin absorbance per gram of protein series HL + HP.

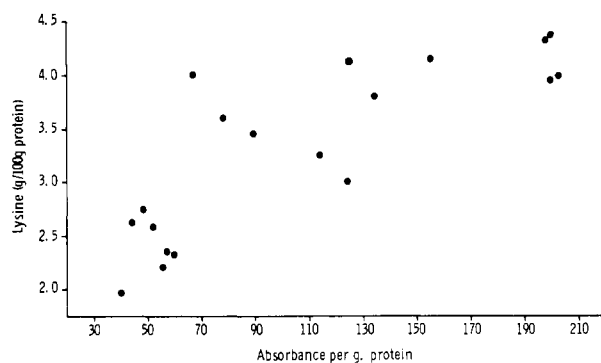Figure 3. Ninhydrin color absorbance per gram of protein series HL + N.

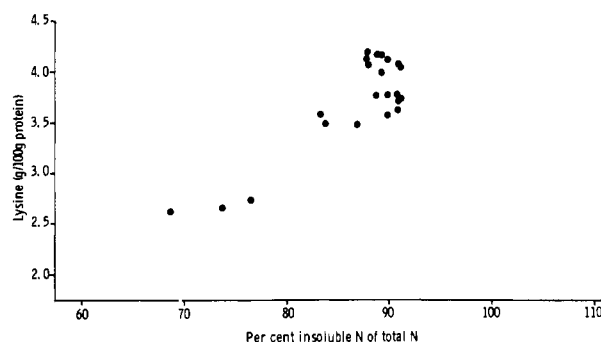Figure 4. Ninhydrin color absorbance per gram of protein series HL + HP.

Figure 5. NBN method, percent insoluble nitrogen of total nitrogen series HL + N.

HL but in the series HL + HP the standard error was from 0.0741 to 0.0912 using the prolamin turbidity and ninhydrin tests probably due to the much greater range of lysine and protein contents in this series. However, the standard error using the BN method was 0.0389, which was comparable to the standard errors obtained in series HL + N and HL.

Estimation of the Lysine Content of the Protein (Table II). The lysine content (g/100 g of protein), measured by amino acid analysis, ranged from 2.64 to 4.22 in series HL + N, 3.47 to 4.22 for series HL, and from 1.96 to 4.50 for series HL + HP. Significant correlation coefficients ($P = 0.01$) were obtained for the data from all three methods in series HL + N, although those for the prolamin turbidity test and the ninhydrin color test were improved further by expressing the absorbances on a unit protein basis (Figures 1 and 3). (Figures 1-6 show the relationship

between the lysine content in the protein and the prolamin turbidity test, ninhydrin color test, and the NBN method in series HL + N and HL + HP.) However, when the three

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Table III. Relationships between Crude Protein Content and Measurements from the Prolamin Turbidity Test, Ninhydrin Color Test, BN, and NBN Methods

|	correlation coefficient (r) with protein content		
series	HL + N	HL	HL + HP
prolamin turbidity test			
absolute absorbance	0.300	0.347	0.069
absorbance/gram of protein	0.016	−0.153	−0.132
ninhydrin color test			
absolute absorbance	0.568	0.816	0.365
absorbance/gram of protein	0.444	0.730	0.232
BN and NBN methods			
protein content after butanol extraction (BN)	0.502	0.902	0.489
proportion of insoluble nitrogen of total nitrogen (NBN)	−0.189	−0.158	0.034
g of lysine/100 g of dry matter	0.397	0.760	0.411
g of lysine/100 g of protein	0.015	0.265	0.092
significance points	21 df	18 df	18 df
	0.43 (P = 0.05)	0.44 (P = 0.05)	0.44 (P = 0.05)
	0.54 (P = 0.01)	0.56 (P = 0.01)	0.56 (P = 0.01)
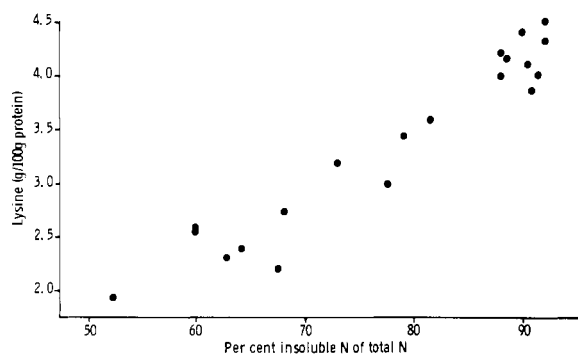
Figure 6. NBN method, percent insoluble nitrogen of total nitrogen series HL + HP.

control samples were omitted, i.e., in series HL, none of the methods gave a significant correlation coefficient. In series HL + HP, where there was a large range of lysine and protein contents all three methods gave highly significant correlation coefficients (Figures 2, 4, and 6). The standard errors about the regression were from 0.236 to 0.360 for all the methods in all three series, except using the ninhydrin color test in series HL + HP where the absolute absorbance and the absorbance per gram of protein were 0.487 and 0.452, respectively.

DISCUSSION

Pollmer et al. (1971) found that the ratio of insoluble nitrogen after butanol extraction to total nitrogen was closely correlated with the lysine content of the protein (NBN method), whereas the protein content of the residue after butanol extraction was highly correlated with the lysine content on a whole kernel basis (BN method). These results have generally been confirmed in this study and can be explained on the one hand by the high correlation between the protein content after butanol extraction and the grain protein content and on the other hand by the lack of correlation between the ratio of insoluble nitrogen to total nitrogen and crude protein content (Table III). Similarly, absolute absorbance in the prolamin turbidity test correlates better with lysine content expressed as a proportion of the protein than with the lysine content in the dry matter. However, when the turbidity is expressed per unit of protein the correlation with lysine in the dry matter is improved because of compensation for the effect of protein variation on the amount of estimated prolamin (Paulis et al., 1974a,b).

When considering the suitability of the three methods for the selection of high lysine lines from normal lines, series HL + N and HL + HP can be considered together because both contained normal and high lysine lines. It

can be seen from the data presented that there is little to choose between the three methods in their ability to differentiate these lines either on the basis of the lysine content in the protein or in the total dry matter. This is particularly so if the absorbances for the prolamin turbidity test and the ninhydrin color test are expressed on a unit protein basis.

However, for the detection of differences within a high lysine population there are distinct differences between the methods. It would appear that the prolamin turbidity test is not sufficiently sensitive. Evidently this method has limitations in distinguishing the small differences in lysine within a high lysine population although it readily distinguishes between high lysine and normal lines. Paulis and Wall (1975) found that extraction of the alcohol-soluble glutelin fraction together with prolamin by the addition of 0.1 M 2-mercaptoethanol to the extraction resulted in a better correlation for both maize and barley between turbidity and lysine content of protein. It is possible that greater accuracy could have been achieved in the present study by incorporating this modification. On the other hand, the ninhydrin color test distinguishes between these lines for the lysine content in the dry matter. This can be attributed mainly to the previously mentioned strong positive correlation of the absorbance of the ninhydrin color with crude protein content (Table III).

The high correlation coefficient between the protein content after butanol extraction and the lysine content in the dry matter suggests that the BN technique could be used to differentiate between high lysine lines. However, using the NBN method, the ratio of insoluble nitrogen to total nitrogen was not a sufficiently sensitive index to select for lysine content as a proportion of the protein in this high lysine population.

In conclusion, the numbers of samples that one person could analyze in 1 day by the different methods of lysine estimation have been calculated: lysine estimation on a Beckman "Unichrom" amino acid analyzer, 12 samples/day; BN or NBN method, 60 samples/day; prolamin turbidity test, 100 samples/day; ninhydrin color test, 120 samples/day. Thus, for example, screening of a segregating population where very large numbers were involved, the prolamin turbidity test would be preferred because of its simplicity of use and greater throughput of samples. In this case, selection can be for lysine on either a protein or a total dry matter basis. This method is comparable in speed and accuracy to selection by dye binding capacity (Fornasari et al., 1975) or the measurement of tryptophan and subsequent correlation with lysine content (Hernandez and Bates, 1969) mentioned earlier. For more detailed differentiation between the selected high lysine lines, the

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protein content after butanol extraction by the BN method would appear to offer a more sensitive selection criteria on the basis of the lysine content in the dry matter. This method offers greater accuracy than either the ninhydrin color test or the prolamin turbidity test but has a lower throughput of samples; being comparable in this respect to the method of Villegas and Mertz (1971) using 2-chloro-3,5-dinitropyridine.

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Tannin Content as a Function of Grain Maturity and Drying Conditions in Several Varieties of *Sorghum bicolor* (L.) Moench

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Twelve varieties of sorghum grain were assayed for tannin content at various stages of maturity. For varieties which were found to contain tannin, maximum values per seed were obtained between 25 and 40 days after half-anthesis. Wide variability in apparent loss of tannin as the grains matured was found between varieties, with tannin contents of mature seed ranging from 3 to 93% of the maximum found in the immature seed. We suggest that varieties with maximum decreases in tannin *may* be similar to low tannin varieties in the nutritional quality of the mature grain, yet provide bird resistance during immature stages. Drying immature grain at room temperature after boiling for 3 min or freezing caused a drastic reduction in apparent tannin content over untreated controls, but these treatments had little effect on nutritional quality of the grain.

In many geographic areas otherwise well suited to growing grain sorghum, the loss of grain from depredation by birds can be substantial. Damage to the crop caused by birds in Louisiana was reported to be more serious than losses caused by insects (Tipton et al., 1970), with near total grain loss when sorghum was first commercially grown in that state. Harris (1969) observed 50% losses in Georgia and had reports of total destruction of some plantings. The problem is especially acute in parts of Africa where several billion queleas (red-beaked weaving sparrow, *Quelea quelea*) range over 20% of the continent in migrating flocks (De Grazio and Besser, 1974).

Significant protection of sorghum grain can be achieved by taking advantage of an aversion by birds for varieties containing tannin. A high negative correlation has been reported between tannin content of sorghum grain and damage by birds (Tipton et al., 1970; McMillian et al., 1972). Because of this "bird-resistance" as well as improved resistance to weathering (Harris and Burns, 1973) and preharvest germination (Harris and Burns, 1970) conferred by tannin, sorghum varieties which contain relatively high amounts of tannin are grown in many regions around the world.

The agronomic advantages of high-tannin sorghum are counterbalanced by corresponding antinutritional effects of the tannin. Tannin in animal diets causes a reduction in weight gain per unit of feed consumed and, under some conditions, in rate of growth, as well as other problems (Price and Butler, 1979). Producers whose environmental conditions enable them to grow sorghum that does not contain tannin thus gain an advantage in the export market over those who are limited to producing high tannin varieties.

Much of the bird damage occurs to the grain before maturity. For example, in the milk stage, birds were observed to crush the grain without plucking it from the head, ingesting the juice (Tipton et al., 1970). Perhaps varieties can be found which contain substantial amounts of tannin in the immature grain but in which all or part of the tannin is converted to nontoxic forms by the time the grain matures. If so, protection from birds would be provided during much of the growing season, with the nutritional problems of feeding mature, high-tannin grain reduced or eliminated. Such "disappearance" of tannin is common in many fruits upon ripening (Goldstein and Swain, 1963).

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