AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY

1. SUBJECT CLASSIFICATION

A. PRIMARY: Agriculture

B. SECONDARY: Cereal Crops

2. TITLE AND SUBTITLE

Simple chemical and biological methods used at Purdue University to evaluate cereals for protein quality

3. AUTHOR(S)

Mertz, E.T.; Jambunathan, R.; Misra, P.S.

4. DOCUMENT DATE	5. NUMBER OF PAGES	6. ARC NUMBER
1975	25 p.	ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS

Agronomy Department, Purdue University, Lafayette, Indiana 47907

8. SUPPLEMENTARY NOTES (Sponsoring Organization, Publishers, Availability)

Published in International programs in agricultural station Bul. No. 70

9. ABSTRACT

A lack of the amino acid lycine in cereals of economic importance makes them inferior to other high protein foods. The recent discovery that cereal proteins can be improved through genetic manipulation has made determination of the protein quality of these grains essential. This report details the simple methods by which even the most elementary equipped laboratory can produce reliable data for a plant-breeding program for protein qualtiy. Chemical reagents and procedures are described for nitrogen content analysis, tryptophan determination in maize, lysine levels in non-pigmented cereals, lysine screening using a ninhydrin reagent, and dye binding for high lysine cereals. Procedures for biological assay, based on the diets of young rats who receive all their protein from cereals, is also provided. Both the ninhydrin and dye binding tests are recommended for mass screening of large populations.

10. CONTROL NUMBER

PN-AAB-490

11. PRICE OF DOCUMENT

12. DESCRIPTORS

13. PROJECT NUMBER

14. CONTRACT NUMBER

CSD-2809 Res.

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

SIMPLE CHEMICAL AND BIOLOGICAL METHODS USED AT PURDUE UNIVERSITY TO EVALUATE CEREALS FOR protein quality

Edwin T. Mertz, Department of Biochemistry, Purdue University
R. Jambunathan, ICRISAT, Hyderabad, India
Prem S. Misra, Department of Biochemistry, Purdue University

Supported by Agency for International Development Contract CSD/2809, "Inheritance and Improvement of Protein Quality and Content in Maize," and Contract CSD/1175, "Inheritance and Improvement of Protein Quality and Content in Sorghum."

CONTENTS

	Page
INTRODUCTION	3
SAMPLE PREPARATION	4
NITROGEN DETERMINATION (PROTEIN)	4
Reagents	
Procedure	
NINHYDRIN TEST	6
Mass Screening	
Ninhydrin Reagent	
Floury Endosperm Cereals	
Non-Destructive Method for Single Seeds	
Destructive Method	
Vitreous Endosperm Cereals	
Non-Destructive Method for Single Seeds	
Confirmatory Ninhydrin Test	
Reagents	
Procedure	
TRYPTOPHAN IN MAIZE	9
Sample Preparation for Maize Endosperm Analysis	
Reagents	
Procedure	
LYSINE IN NON-PIGMENTED CEREALS	11
Reagents	
Copper Phosphate Reagent	
Pyridine Reagent	
Amino Acid Mixture	
Papain Solution	
Procedure	
DYE BINDING MASS SCREENING TEST	14
Reagent	
Procedure	
BIOLOGICAL ASSAY IN RATS	15
Rat Feeding Tests	
SUMMARY AND CONCLUSIONS	21

INTRODUCTION

The different cereals of economic importance, i.e., rice, wheat, maize, sorghum, barley, oats, and millets, all have one characteristic in common - they are low in the essential amino acid, lysine. Primarily because of the lack of adequate lysine, cereal proteins are inferior nutritionally to the proteins found in milk, meat and eggs.

With the discovery by Mertz, Bates and Nelson[1*], that the maize mutant opaque-2 had nearly twice as much lysine as ordinary maize, scientists realized that cereal grain proteins could be improved in quality by genetic manipulation. The recent discovery by Ingversen and co-workers in Denmark[2] and by Axtell and co-workers[3] in the United States of high lysine mutants of barley and sorghum, respectively, raises the hope that all cereals of economic importance may eventually be improved in protein quality by genetic selection.

The protein quality of a cereal grain can be determined only by chemical or biological methods. In a complementary report[4], workers at Purdue discuss the status of development of maize with improved protein quality, and briefly review the chemical and biological methods available to the plant breeder to evaluate protein quality. In this report we have outlined in detail, the simple methods used at Purdue that are suitable for a laboratory severely limited in equipment and trained personnel. We would expect such a laboratory to request standard check samples of normal and mutant cereal grains with known contents of protein, lysine and/or tryptophan from a protein laboratory in an international research center such as CIMMYT[5]. Using such standards, the simple methods described herein can be calibrated to give reliable data for a plant breeding program for protein quality where laboratory facilities are limited.

---

*Literature references are indicated by superscript numbers, equipment references by numbers in parenthesis.

SAMPLE PREPARATION

For all of the tests described below, except the split seed ninhydrin test, the grain sample must be ground to a fine powder. If available, use 10-30 grams of a representative sample. Where quantities of kernels are limited, as little as 500 mg of sample will be sufficient for a single determination with each of the various methods.

The sample is first ground in a Wiley mill to pass a sieve of 90 mesh (1) (or an ordinary hand grinder (2)). The ground material is placed in cellulose thimbles and defatted with hexane overnight in a Soxhlet apparatus (3). The sample is defatted to improve storage qualities and precision of analysis. The defatted sample is allowed to air dry and then ground to a fine powder in a small ball mill (4).

NITROGEN DETERMINATION (PROTEIN)

The most reliable method for nitrogen is the well-known micro-Kjeldahl method. It is used in a variety of forms. The simplest is the Association of Official Analytical Chemist (A.O.A.C.) method[6] which involves digestion-oxidation of the sample with sulfuric acid and steam distilling the ammonia formed at high pH into a boric acid solution. The ammonium borate formed is then titrated with standard hydrochloric acid. The percentage of protein is obtained by multiplying per cent nitrogen by the factor 6.25 (maize, millet and sorghum), 5.95 (rice), and 5.83 (barley, oats, rye and wheat)[7]. The reagents and procedure are described below.

Reagents

Sulphuric acid -- Sp. gr. 1.85, N-free.

Catalyst mixture -- 190 g of K_2SO_4 and 4 g HgO.

Sodium hydroxide -- sodium thiosulfate solution. Dissolve 800 g of

NaOH in 1500 ml of cold distilled water, adding small increments of the alkali at a time with constant stirring. Dissolve 100 g $Na_2S_2O_3 \cdot 5H_2O$ in distilled water (use 200 ml or less). Mix the two solutions and dilute to 2 liters.

Indicator dye -- Mix one part of 0.2% methyl red in ethanol with 5 parts of 0.2% bromocresol green in ethanol.

Standard hydrochloric acid -- 0.02N.

Boric acid -- 4% solution.

Procedure

Accurately weigh (5) an amount (40-50 mg) of sample and place in a 30 ml Kjeldahl digestion flask. Add 2 g of catalyst mixture and 2 ml of sulphuric acid.

Gently heat the flask on a rotary digestor (6) until the frothing ceases. Raise the temperature to boiling and boil for 1 hour after the contents of the flask are clear.

Cool, and add 8 ml distilled water.

Transfer the digest to a steam distillation apparatus (7) via the sample funnel and rinse the flask 5-6 times with 1-2 ml portions of distilled water.

Place a 50 ml Erlenmeyer flask containing 5 ml of boric acid and 4 drops of mixed indicator dye beneath the condenser, with the delivery tip immersed in the solution.

Add 15 ml of the sodium hydroxide-sodium thiosulphate solution to the digest, rinse the sample funnel with water and start steam distillation.

Collect distillate for 4-5 minutes, then rinse the condenser tip with water and titrate the distillate plus rinsing with standard hydrochloric

acid in a microburette (8) to a grey or blue end-point.

Run a blank containing the same quantities of all reagents but without sample for every set of nitrogen determinations. Also run a check sample of cereal grain of known protein content.

Calculate the percentage of nitrogen.

$$\% \text{ N} = \frac{(\text{ml HCl} - \text{ml blank}) \times \text{normality} \times 14.007 \times 100}{\text{mg sample}}$$

NINHYDRIN TEST

Mass Screening Test

Mertz, et al.[8] have shown that high lysine mutants of maize, sorghum, and barley give a more intense blue color with ninhydrin than their normal counterparts because of higher levels of free amino acids. Mass screening of floury endosperm types can be made rapidly with this method. Mass screening of vitreous endosperm types is slower because of lack of penetration of the ninhydrin reagent into the exposed surface of the endosperm. With vitreous endosperm the kernels can be ground before testing. The procedures are described here.

Ninhydrin Reagent

Grind a mixture containing 16 parts (by weight) of ninhydrin (practical), 58 parts of sodium citrate and 26 parts of citric acid to a fine powder with a mortar and pestle. Store the mixture in a brown glass bottle and protect from moisture. Under dry conditions in darkness the mixture does not deteriorate.

Floury Endosperm Cereals

Non-Destructive Method for Single Seeds. Expose the endosperm near the crown by rubbing the crown on sandpaper. Mount seed in modeling clay. Apply a 3% ninhydrin reagent solution (300 mg in 10 ml of water) to the exposed

endosperm. Allow to stand 5 minutes, then warm the surface with a source of hot air (i.e. hair dryer) or a heat radiating source. Treat a control normal kernel and an opaque-2 kernel (if available) the same way and note the differences in color intensity. The high lysine kernel should have a much darker blue color (see color plate for maize in reference 4).

Destructive Method. Place 5 kernels of maize, 15 kernels of sorghum, or 10 kernels of barley (or other cereal grain) in a 15 x 150 mm Pyrex test tube, after splitting the kernels lengthwise. Cover with 10 ml of 3% ninhydrin reagent solution and heat to boiling. Allow tubes to stand 5 minutes and compare with similarly treated normal kernels (see reference 8 for photograph of relative color intensities). If the colors are too intense, dilute all samples equally with water, if too faint, increase the number of kernels per tube.

Vitreous Endosperm Cereals

Non-Destructive Method for Single Seeds. From vitreous seed a maximum amount of endosperm is removed without damage to the embryo. The endosperm portion is weighed roughly on a balance (5), and placed in an 8 x 75 mm Pyrex test tube. Approximately the same weight of endosperm from a normal (and opaque-2 kernel if available) is placed in a separate test tube. The endosperms are ground to a powder in a small mortar and pestle and returned to the test tubes. One ml of 3% ninhydrin reagent is added, the tube is heated to boiling, then removed from the heat. The color develops within 5 minutes.

Confirmatory Ninhydrin Test

If the color differences between a possible high lysine cereal and its

normal counterpart are not marked enough to classify the former or confirmation of the visual test is desired, a confirmatory ninhydrin test can be performed[8].

Reagents

In order to prepare the ninhydrin reagent for testing cereals, two basic reagents are first prepared.

Dissolve 400 mg of reagent grade stannous chloride ($SnCl_2.2H_2O$) in 250 ml of sodium citrate buffer (4.3 g citric acid and 8.7 g sodium citrate diluted to 250 ml with water after adjusting the pH to 5.0 with sodium hydroxide using a pH meter (9)).

Dissolve 10 g ninhydrin (practical) in 250 ml of methyl cellosolve.

Mix the above two reagents in equal volumes. Filter if necessary and store in a dark bottle away from light. Fresh reagent should be made at least twice a week for routine analysis. Avoid contact with skin.

N-propyl alcohol solution, 50% aqueous solution (v/v).

Procedure (Corn as an example)

Suspend 100 mg of sample (defatted ground endosperm or whole kernel) in 10 ml distilled water in a 125 ml Erlenmeyer flask.

Shake at room temperature for 20 minutes (10).

Centrifuge (11) and filter the supernatant if necessary.

Place 0.10, 0.15 and 0.20 ml of the corn supernate in separate 15 x 150 mm Pyrex test tubes and dilute to 0.5 ml with distilled water.

Add 1.5 ml of ninhydrin reagent and mix well.

Heat the the tubes in a boiling water bath for 20 minutes. Cool to room temperature.

Add 8 ml of 50% N-propyl alcohol and mix vigorously on a Vortex mixer (12).

Read the absorbance in a Spectronic 20 spectrophotometer (13) at 570 nm.

Preparation of standard curve: Place at least 4 increments of L-leucine solution (0.5 μm/ml) in separate test tubes as described above for the corn supernates and dilute to 0.5 ml with distilled water, and carry the tubes through the color reaction. Plot micromoles of leucine on the X-axis and absorbance on the Y-axis.

Plot the absorbance of the corn samples on the standard curve and read the leucine equivalents. Calculate the leucine equivalents per 100 mg of protein (from micro-Kjeldahl value) for each corn sample.

TRYPTOPHAN IN MAIZE

Maize is the only cereal grain with a major deficiency in the essential amino acid, tryptophan. Lysine and tryptophan are about equally limiting in the maize endosperm, where they are consistently present in a ratio of approximately 4 parts of lysine to 1 part of tryptophan. Tryptophan can therefore be used as an indicator of the protein quality of maize. When the tryptophan level of the endosperm increases, an increase equal to approximately four times the tryptophan increase will be observed in lysine[9]. A breeding program for improved protein quality in maize has been carried on successfully at CIMMYT using the following tryptophan assay on maize endosperm[10]. The most promising selections are usually analyzed for lysine to confirm the presence of acceptable levels of this amino acid.

Sample Preparation for Maize Endosperm Analysis

Endosperm is used for tryptophan analysis because the embryos of normal and mutant maize kernels contain the same levels of tryptophan. This tends

to reduce the differences between normal and mutant whole kernels. Also, removal of pericarp reduces the chance of undesirable pigments interfering with the colorimetric determination. To prepare a sample of maize endosperm for analysis, proceed as follows:

1. Use a random sample of 10 seeds from one ear.

2. Wash off pesticide if seeds have been treated.

3. Soak seeds in distilled water for about 30 minutes. Peel off the pericarp with a scalpel and remove the germ. The remaining endosperm tissue is air dried overnight, ground and defatted as described under sample preparation.

For non-destructive single kernel analysis, seeds are washed to eliminate pesticide and then air dried. A small portion of endosperm from different sites of the kernel are drilled out with an electric drill using a 1/16 inch bit. The endosperm grindings from one kernel are combined, air dried and ground to a fine powder.

Reagents

The color reagent for tryptophan contains glyoxylic acid produced by mixing reagents A and B below.

Reagent A: 270 mg of $FeCl_3.6H_2O$ are dissolved in 0.5 ml of distilled water and the solution diluted to 1 liter with glacial acetic acid containing 2% acetic anhydride[11].

Reagent B: 30N sulfuric acid

Mix equal volumes of the two reagents A and B about one hour before use (Reagent C).

Papain solution: Papain-technical powder is dissolved in 0.1N sodium acetate buffer at pH 7.0 to a final concantration of 4 mg/ml. Prepare the enzyme solution daily.

Procedure

1. Place 100 mg of endosperm sample into a 20 x 65 mm screw cap vial (3 dram glass vial with cap). Add 5 ml of papain solution. Cap the vials and mix well. Incubate in a 65°C oven (14) overnight. Include a blank (5 ml of papain solution) with every group of samples.

2. Remove digest from oven, shake and allow to cool to room temperature. Centrifuge and decant the clear supernate digest.

3. Pipette 1 ml of the clear digest into a test tube. Add 4 ml of reagent C ($FeCl_3$-acetic-sulfuric). Mix using a Vortex mixer and allow the color to develop at 65°C for 15 minutes; then cool the tubes to room temperature.

4. Place tubes in a Spectronic 20 and read the absorbance at 545 nm.

5. Using the absorbance values obtained with pure L-tryptophan prepare a standard curve in the range of 0-40 mg of tryptophan per ml. Use 1 ml portions of tryptophan solutions with 4 ml of reagent C (step 3).

6. The tryptophan content of the maize samples can be read from the standard curve. The values should be calculated on the basis of grams of tryptophan per 100 grams of protein (micro-Kjeldahl).

LYSINE IN NON- PIGMENTED CEREALS

The ideal simple method for direct determination of lysine in cereals is yet to be found. All methods have disadvantages. The method developed by Tsai and coworkers[12] as modified by Villegas[10] has been the most

satisfactory to date. However, in some cases the lysine values by this method tend to be higher than those obtained by ion exchange. Fortunately, both the normal and the high lysine cereal samples are higher in lysine so that the relative difference between the two is similar to that observed by ion exchange. This emphasizes the necessity of using standard check samples of the normal and high lysine cereal in order to calibrate the method. In addition, the method works only on non-pigmented cereals. For example, high tannin[13] sorghums give unusually low lysine values. The reagents and method are described below.

Reagents

Copper Phosphate Reagent. This reagent is a suspension of copper phosphate in borate buffer.

Borate buffer (0.05 M, pH 9.0).

Copper phosphate suspension:

Solution (a): 2.8 g $CuCl_2 \cdot 2H_2O$ in 100 ml distilled water.

Solution (b): 13.6 g. $Na_3PO_4 \cdot 12\ H_2O$ in 200 ml distilled water. Pour solution (a) into (b) with swirling, centrifuge and discard the supernate. Resuspend the pellet three times in 15 ml of borate buffer and centrifuge after each suspension. After the third washing, resuspend the pellet in 80 ml of borate buffer. The reagent must be prepared fresh about every seven days.

Pyridine Reagent. Just prior to use, prepare a 3% solution of 2-chloro-3, 5 dinitropyridine in methanol.

Amino Acid Mixture. Simulated corn protein pattern for preparation of standard curve (Villegas modification[10] of Tsai method[12]). Prepare a synthetic acid mixture by grinding together in a mortar the following

quantities of pure amino acids:

(In milligrams): alanine, 30, arginine, 50, aspartic acid, 60, cystine, 20, glutamic acid, 300, glycine, 40, histidine, 30, isoleucine, 30, leucine, 80, methionine, 30, phenylalanine, 40, proline, 80, serine, 50, threonine, 30, tyrosine, 30, and valine, 40. Dissolve 100 mg of this mixture in 10 ml of carbonate buffer, (0.05 M, pH 9.0).

Papain Solution. Papain-technical powder is dissolved in 0.1N sodium acetate buffer at pH 7.0 to a final concentration of 4 mg/ml. Prepare the enzyme solution daily.

Procedure

1. Place 100 mg of the finely ground, defatted endosperm or whole kernel sample into a screw cap glass vial, add 5 ml of papain solution and follow instructions as given for tryptophan analysis to prepare papain digest.

2. Pipette 1 ml of the supernate digest into a 15 ml centrifuge tube and add 0.5 ml of carbonate buffer and 0.5 ml of copper phosphate suspension.

3. Shake the mixture for 5 minutes (Vortex mixer) and centrifuge.

4. Pipette 1 ml of the supernate into a 15 x 150 mm Pyrex test tube, add 0.1 of the pyridine reagent and mix well (Vortex mixer). Cap the tubes with rubber stoppers and shake for 2 hours at room temperature (10).

5. Add 5 ml of 1.2 N HCl and mix well (Vortex mixer).

6. Add 5 ml of ethyl acetate, invert capped tubes at least 10 times, then remove the top layer (ethyl acetate) by inserting a 5 3/4 inch disposable pipet (15) or equivalent connected to a water aspirator. This step should be repeated 3 times.

7. Determine the absorbance of the aqueous phase in a Spectronic 20

at 390 nm. Relate absorbance of cereal samples to the corresponding lysine value on the standard curve (step 8) and report as lysine in grams per 100 grams protein (micro-Kjeldahl).

8. Preparation of standard curve: Dissolve 62.5 mg of pure lysine mono-hydrochloride in 50 ml of carbonate buffer (1,000 μg lysine/ml). Prepare solutions to give the following concentrations of lysine: 0, 200, 400, 600, 800, and 1,000 μg lysine/ml. Mix 1 ml of these 4 solutions with 4 ml of papain solution to give 0, 40, 80, 120, 160, and 200 μg lysine/ml. Pipette 1 ml of each of the 6 standards into separate 15 ml centrifuge tubes. Add 0.5 ml of the amino acid mixture, and 0.5 ml of the copper phosphate suspension and do steps 3 to 7.

DYE BINDING MASS SCREENING TEST

The dye binding method was used successfully to identify a high lysine barley in the world barley collection[14]. It has been used for years as a rapid method to determine protein in wheat and other grains. Udy (16) markets a complete dye binding kit.

In order to use the dye binding method to screen for high lysine cereals, both the dye binding capacity value and the total protein value (micro-Kjeldahl) are needed. This is because the acidic dye binds to the basic amino acids arginine, histidine and lysine in the cereal proteins. In high lysine mutants, arginine and lysine are elevated and this raises the dye binding value. Simple increase in total protein also raises the value, so the dye binding value must be divided by the protein value in order to eliminate the effect of protein changes. Higher than normal dye binding capacity <u>per unit of protein</u> identifies high lysine mutants.

For a large scale screening program, the Udy kit is recommended. If funds are not available for this kit, a method using standard laboratory equipment and the Spectronic 20 is recommended. The latter method is described below.

Reagents

Buffered dye solution -- Dissolve 2 g of acilane orange G, 15.84 g citric acid, 2.98 g of $Na_2HPO_4 \cdot 2H_2O$ and 0.3 g of thymol in one liter of distilled or deionized water at 80°C. The buffered dye solution contains 0.65 absorption units per ml at 580 nm (Spectronic 20).

Procedure

To 200 mg of ground defatted whole kernel add 15 ml of buffered dye solution (an excess of dye), and agitate until the dye has become equilibrated with the reactive groups of the cereal sample. This requires 5 minutes with a blender (17) or homogenizer and probably longer with a standard shaker. Filter or centrifuge the suspension, dilute the filtrate 200-fold with water, and read at 480 nm against the original buffered dye solution also diluted 200-fold with water. The difference in absorption units per unit volume between the sample and the original dye solution gives the dye binding capacity of the sample. When the value is divided by the total protein content (micro-Kjeldahl) of the sample, one obtains the dye binding capacity per unit of protein.

BIOLOGICAL ASSAY IN RATS

Several methods are available for the determination of the protein quality of cereals with rats. The majority are based on the biological response of young weanling rats to a complete diet in which the cereal

grain supplies all of the protein. The response of the rat to the cereal protein can be measured in several ways, for example, weight gain, weight gain per unit of feed consumed (feed efficiency ratio or FER), weight gain per unit of protein consumed (protein efficiency ration or PER), gain in carcass protein per unit of protein consumed (net protein utilization or NPU) or rate of body weight change of animal fed various levels of the cereal protein compared with that obtained with animals fed a reference protein such as ANRC casein (relative protein value or RPV).

At Purdue maize and sorghum proteins have been evaluated using the PER method. The PER values obtained with opaque-2 maize in rats are approximately 90% of the values obtained on casein, the major protein in milk. Since workers in Guatemala[15] and in India[16] found opaque-2 maize to be 90 per cent as effective as milk protein for the preschool child, the PER method in rats can be considered to accurately predict the protein value of this improved cereal grain for humans.

We present the details of a typical bioassay of different cereal grains as carried out in our laboratory using the PER method.

The samples assayed are described in Table 1. All samples were analyzed in duplicate for total nitrogen (micro-Kjeldahl), moisture, fat, fiber and ash based on standard AOAC methods[6]. On the basis of these analyses, the total fat content of the diet was set at 5%, total mineral plus ash content was set at 5%, no corrections were made for moisture, and the fiber content was adjusted to a minimum of 2% by adding cellu-flour. With these parameters fixed, the floury-2 sample established the minimum protein level at which all samples would be fed: a level of 8.8% protein

(9.3 x 94.6) slightly below the 10% protein level usually used in PER determinations. Table 2 shows the final composition of the diets used in the feeding tests. All cereals were ground in a burr mill.

Table I

Identification and Proximate Analysis of Cereal Samples

Sample	Identification	Protein	Moisture	Fat	Fiber	Ash
		%	%	%	%	%
CM-4857	CIMMYT Tuxpeno, opaque-2 maize (soft endosperm)	9.7	8.0	4.4	2.5	1.8
CM-4858	CIMMYT Tuxpeno, normal maize (hard endosperm)	9.6	7.9	4.2	2.3	1.4
OH43 x B14	Purdue, near-isogenic floury-2 version of OH43 x B14	9.3	8.5	4.2	2.4	1.7
IS 2319	Purdue low pigment sorghum	11.7	6.7	4.3	2.0	1.7
CM 4859	CIMMYT 7 Cerros, wheat	11.1	5.9	1.5	2.9	1.6
CT 4861	CIMMYT TM-132, triticale	14.6	6.4	1.6	2.6	1.9
ANRC casein	Animal Nutrition Research Council reference casein[a]	91.0	4.8	-	-	2.3

[a]Sheffield Chemical Co., Union, N. J.

Table 2

Composition of Diets Containing 8.8% Protein

Diet	Protein Source		Cottonseed Oil	Mineral Mix[a)]	Vitamin Mix[b]	Cornstarch
		%	%	%	%	%
1	CM-4857	90.8	1.0	3.4	1.0	3.8
2	CM-4858	91.7	1.2	3.7	1.0	2.4
3	OH43 x B14 (floury-2)	94.6	1.0	3.4	1.0	0.0
4	IS 2319[c)]	75.2	1.8	3.7	1.0	17.8
5	CM-4859	79.3	3.8	3.7	1.0	12.2
6	CT-4861[c)]	60.2	4.0	3.9	1.0	30.4
7	ANRC Casein[c)]	9.7	5.0	4.8	1.0	77.4

[a)] Hawk-Oser salt mixture No. 3, Nutritional Biochemicals Corp., Cleveland, Ohio.

[b)] Vitamin Fortification Mixture for the rat, Cat. No. 40060, General Biochemicals Corp., Chagrin Falls, Ohio.

[c)] Add 2% cellulose (Cellu Flour or equivalent) for casein and 0.5% for sorghum and triticales samples.

Rat Feeding Tests

Ten male weanling rats (Wistar Strain) with an average initial weight of about 50 g were used for each cereal. The rats were housed in individual wire mesh cages and supplied with ground feed and water *ad libitum*. Special feed cups (18) were used which minimized the scattering of food and permitted accurate recording of the feed consumed. Each rat was weighed at weekly intervals for 28 days and an accurate record of food consumption was made.

Table 3 presents the summary results for the 28-day period. Using the PER for ANRC casein as the standard, Table 3 shows that the protein quality of the various cereals ranged from a low of 38 per cent of casein for sorghum, to a high of 87 per cent of casein for opaque-2 maize in the 28-day feeding trial. At the end of the first seven days the calculated per cent of casein PER was (columns 2-7), respectively, 83, 49, 53, 39, 57 and 65 indicating the same rank ordering (except for wheat) of cereal protein values as that obtained in 28 days. Wheat and corn were 47% as effective as casein in 14 days. Average amount of cereal grain consumed by the ten rats in 7 days was (in grams) 530 (opaque-2 maize), 410 (normal and floury-2 maize), 300 (sorghum), 332 (wheat) and 264 (triticale). In the 7-day test, normal and floury-2 maize differed significantly at the 1% level from opaque-2 maize. Triticale differed significantly at the 5% level from sorghum; however, wheat and sorghum showed no difference.

In the 28 day test, the same differences seen at 7 days were also observed at the 1% level for all cereals; however, wheat now differed significantly from sorghum. The 7-day test could thus be considered satisfactory for screening in breeding programs where quantities of grain for biological testing are very limited. Similar results have been obtained at Purdue using 6 male weanling rats for 14 days instead of 10 animals for 7 days. However, when adequate amounts of the cereal to be evaluated are available the 28-day AOAC feeding test utilizing 10 rats is considered to be the standard and should be used for all critical materials.

Table 3

Protein Efficiency Ratio (PER) Values of Cereals and Casein[1]

Items Compared	CM-4856 Opaque-2 Maize	CM-4858 Normal Maize	OH43 x B14 Floury-2 Maize	IS 2319 Sorghum	CM 4859 Wheat	CT 4861 Triticale	ANRC Casein
Average initial weight (g)	49.5	49.6	49.5	50.6	50.6	50.4	49.5(50.6)*
Average weight gained (g)	87.1	32.6	36.9	19.6	27.0	37.8	85.1(89.6)*
Average total feed consumed(g)	340.2	214.5	227.3	171.6	193.9	213.3	290.2(314.2)*
PER (g gain)/g protein	2.90	1.72	1.83	1.28	1.57	2.01	3.34(3.32)*
Per cent of casein PER	87	51	55	38	47	60	

[1]Duration of experiment = 28 days. Ten male rats in each group.
*Casein control for sorghum, wheat and triticale in parenthesis. These three were studied in a separate feeding test with their own casein control.

SUMMARY AND CONCLUSIONS

We have been continuously evaluating simple methods for protein, lysine and tryptophan in cereals and consider those described in this circular to be the best available at the present time. From time to time new assay methods are proposed and as better methods are developed they will replace those described. Both the ninhydrin and the dye binding tests are recommended for mass screening of large populations. Neither method measures lysine alone. The former is more rapid because it does not require the determination of total protein. The former measures total free amino acids, the latter total basic amino acids. Once promising selections are identified, estimation of their lysine content can be made using the micro-Kjeldahl method combined with the colorimetric tryptophan test (maize endosperm) or with the colorimetric pyridine test for lysine (other non-pigmented cereals). Those samples showing the best protein quality and quantity using these simple chemical tests can then be finally evaluated biologically using the PER method with male weanling rats. Other methods for bioassay are being developed, but none has been so thoroughly tested and used.

Using the simple methods described in this circular, it should be possible to provide a chemical and biological index on all newly improved crop varieties at the time they are registered for commercial production, a necessity as nutritive values become as important as the capacity to yield.

REFERENCES

1. Mertz, E. T., Bates, L. S. and Nelson, O. E., Science 145, 297 (1964).

2. Ingversen, J., Koie, B. and Doll, H., Experimentia 29, 1151 (1973). See also reference 14 and Tallberg, A., Hereditas 75, 195 (1973).

3. Singh, R. and Axtell, J. D., Crop. Sci. 13, 535 (1973).

4. Bauman, L. F., Mertz, E. T., Glover, D. V. and Crane, P. L., Progress in Developing Maize with Improved Protein Quality. Purdue University Research Bulletin 914, Purdue Agricultural Experiment Station, Lafayette, IN (October, 1974).

5. Protein Quality Laboratory, International Maize and Wheat Improvement Center, Londres, 40, Mexico, 6, D.F.

6. Official Methods of Analysis of the Association of Official Analytical Chemists. 11th Ed. 800 (1970).

7. Amino-Acid Content of Food and Biological Data on Protein. FAO, p 36, (1970). UNIPUB, Inc., 650 First Avenue, P. O. Box 433, N. Y., N.Y. 10016.

8. Mertz, E. T., Misra, P. S. and Jambunathan, R., Cereal Chem. 51, 305 (1974).

9. Hernandez, H. and Bates, L. S., A Modified Method for Rapid Tryptophan Analysis of Maize. Res. Bull. No. 13 (1969). CIMMYT, Londres, 40, Mexico 6, D.F.

10. Villegas, E. and Mertz, E. T., Chemical Screening Methods for Maize Protein Quality at CIMMYT. Res. Bull. No. 20 (1971) CIMMYT, Londres 40, Mexico 6, D.F.

11. Dalby, A. and Tsai, C. Y., Acetic Anhydride Requirements in the Colorimetric Determination of Tryptophan. Anal. Biochem. 63, 283 (1975).

12. Tsai, C. Y., Hansel, L. W. and Nelson, O. E. Cereal Chem. 49, 572 (1972).

13. Jambunathan, R. and Mertz, E. T. J. Agr. Food Chem. 21, 692 (1973).

14. Munck. L., Hereditas 72, 1 (1972).

15. Bressani, R. Protein Quality of Opaque-2 Maize in Children. Proc. High Lysine Corn Conf. p. 34 (1966). Corn Refiners Assoc., 1001 Connecticut Ave., N. W., Washington, D.C. 20036.

16. Reddy, V. and Gupta, C. P. Am. J. Clin. Nutrition 27, 122 (1974).

EQUIPMENT*

1. Wiley mill. Catalog No. 8670. Scientific Products Co., 1210 Waukegan Rd., McGaw Park, IL 60085. Price $362.00.
2. Hand grinder. Arthur H. Thomas Co., Mill, hand grinder. Cat. 3371-H10. Price $20.00.
3. Soxhlet apparatus. Scientific Products Co.
   a. Extraction apparatus - Cat. E6250-3. Price $44.34.
   b. Thimbles Cat. E6480-8. Price $ 4.50.
   c. Heating mantle Cat. H1640-3. Price $26.15.
   d. Autotransformer Cat. E2101-1. Price $34.00
4. Small ball mill. Wig-L-Bug Amalgamator. Crecent Dental Mfg. Co., 7950 W. 75th St., Lyons, IL 60534. Price $ 101.00.
5. Semi-micro balance. Scientific Products Co., 1210 Waukegan, Rd., McGaw Park, IL 60085. Ainsworth Type 28N Balance; Semi-Micro, Catalog No. B1498-2. Price $ 595.00.
6. Rotary digestor. (microkjeldahl). Rotary kjeldahl (digestion apparatus (12 place)). Cat. 4-1859. American Instrument Co., Inc., 8030 Georgia Ave., Silver Springs, MD. Price $ 275.00.
7. Steam distillation apparatus (microkjeldahl). Arthur H. Thomas Co. Cat. 7053-J10. Price $ 288.00.
8. Microburette. Arthur H. Thomas Co., Cat. 1993-C20, Price $ 62.00.
9. pH Meter. Fisher Scientific Co., Catalog 16-637-10. Price $235.00.
10. Shaking apparatus. Scientific Products Co., Shaker, Eberbach, Cat. S-1100. Price $ 290.00. Carrier utility, Eberbach, Cat. S-1101-2. Price $ 28.00.
11. Centrifuge. Scientific Products Co., Centrifuge, Dynac, Adams, Cat. 1416-6. Price $292.00.

12. Vortex mixer. Scientific Products Co. Mixer, Vortex Cat. S-8223 Price $65.00.

13. Spectronic 20 spectrophometer. Fisher Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219. Spectrophotometer, Spectronic 20. Cat. 7-143. Price $425.00. Infrared tube and filter. Cat. 16-637-10. Price $12.00.

14. Oven. Scientific Products Co. Single wall transite oven. Cat. N-86288. Price $125.00.

15. Disposable pipet. Scientific Products Co., Dispo pipets. Cat. P5205-1. Price $3.50 for 1 gross.

16. Udy dye binding kit. Udy Analyzer Co., P. O. Box 148, Boulder, Colorado 80302. Protein Analyzer, Model S. Cat. S-1000. Price $1,445.00.

17. Blender. Scientific Products Co., Blender, Waring. Cat. S-8350-2. Price $37.95.

18. Special feed cups. Wahmann Mfg. Co., P. O. Box 6883, Baltimore, MD 21204. Food Device, Cat. 7010, LC-306A. 3 1/8" inside diameter, 1 5/8" deep. Price $2.25 ea.

---

*Current prices may be higher than those quoted. Mention of firm names or trade products does not constitute endorsement by Purdue University over others of a similar nature not mentioned.

It is the policy of the Agricultural Experiment Station of Purdue University that all persons shall have equal opportunity and access to its programs and facilities without regard to race, religion, color, sex or natural origin.