

Chemical Methods Used at CIMMYT for Determining Protein Quality in Cereal Grains

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Contents

Page

1	Introduction
3	Sample Preparation for Endosperm Analysis
5	Micro-Kjeldahl Method for Determining Nitrogen Content
7	Nitrogen Determination with the Technicon Autoanalyzer
13	Tryptophan Determination
17	Lysine Determination
21	Dye Binding Capacity Method for Estimating Protein Quality in Maize, Barley and Triticale
24	Zein Determination in Maize
27	Ninhydrin Test to Determine Free Amino Acids in Floury, Opaque-2 Maize
29	Use of the Amino Acid Analyzer
33	Protein Fractionation

Introduction

This publication describes the methodology used in CIMMYT's Protein Quality Laboratory during the development of high protein quality maize (maize with the opaque-2 gene) and in selection for barley and triticale lines of superior nutritional quality. Among the techniques described are: a) rapid initial screening tests (for tryptophan in maize endosperm, for dye binding capacity—DBC—in barley and triticale, and for total nitrogen content); b) specific assays for the protein fraction, zein, in maize and for the presence of the opaque-2 gene in flourey maize (ninhydrin test), and c) more complicated analyses such as the colorimetric test for lysine, the fractionation of maize endosperm protein, and complete aminograms with the amino acid autoanalyzer.

Sample Preparation for Endosperm Analysis



Peeling off the pericarp and removing the germ for endosperm sample.

To rapidly identify maize materials with improved protein quality, it is recommended that the endosperm be analyzed. Maize endosperm protein is generally deficient in the amino acids lysine and tryptophan, while the germ has a relatively constant and well-balanced amino acid composition regardless of genetic background.

When the whole kernel is analyzed, the pericarp may contain pigments that interfere with colorimetric determinations. Therefore, if whole kernel analysis is desirable, other types of analyses must be made, such as that of dye binding capacity (DBC), to obtain the quality index (QI) of the protein.

For the Evaluation of Genetic Families of Maize

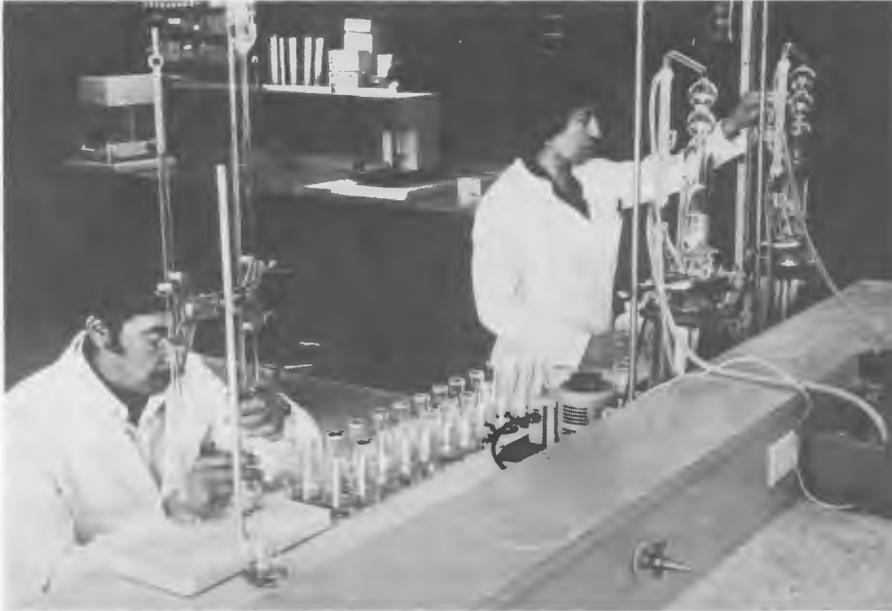
1. Take a random sample of 10-20 seeds as representative of each ear.
2. If the seeds have been treated, wash off pesticide with distilled water.

3. For endosperm analysis, soak seeds in distilled water for 20-30 minutes. Peel off the pericarp and remove the germ with tweezers and scalpel. Air dry the remaining endosperm material overnight.
4. Grind each sample, whether of endosperm or whole kernel, at the 0.5 mm setting of a cyclone mill.
5. Defat ground samples with hexane in a Soxhlet-type continuous extractor for four hours. Air dry.

For Small Grain Cereals

If the samples have been treated, wash off pesticide with distilled water and air dry. Grind as for maize.

Micro-Kjeldahl Method for Determining Nitrogen Content



Steam distillation and titration of ammonia.

Nitrogen content can be estimated by the micro-Kjeldahl procedure¹ and the percentage of protein calculated, using the factor 6.25 for maize and 5.83 for barley and triticale.

Equipment

1. Analytical balance.
2. 30-ml digestion flasks.
3. Micro-Kjeldahl digesting unit.
4. Micro-Kjeldahl distilling unit.
5. Autotransformer, variable 0 to 140 volts, Powerstat.*
6. 125-ml Erlenmeyer flasks.
7. 5-ml microburet.
8. Glassware (pipets, beakers, etc.).

* The mention of a specific commercial brand or product does not imply its backing by CIMMYT to the exclusion of others that may be appropriate.

Reagents

1. Concentrated sulfuric acid (98%) Sp.gr.1.84, N-free.
2. Catalyst mixture: 99 g K₂SO₄, 4.1 g HgO and 0.8 g CuSO₄. Or catalyst tablets (7.5 mg Se + 1.5 g K₂SO₄).
3. Sodium hydroxide - sodium thiosulfate solution. Dissolve 50 g NaOH and 5 g Na₂S₂O₃.5H₂O in distilled water and dilute to 100 ml.
4. 4% boric acid solution.
5. Methyl red - bromocresol green indicator solution (one part 0.2% methyl red in ethanol with five parts 0.2% bromocresol green in ethanol).
6. 0.02 N hydrochloric acid solution.
7. 30% hydrogen peroxide.

Procedure

1. Weigh 30-40 mg ground sample into digestion flask. Add 1 g catalyst powder mixture or one commercial catalyst tablet, 2 ml concentrated sulfuric acid and 2 ml 30% hydrogen peroxide.
2. Digest for 30 minutes, cool and add the minimum quantity of distilled water to dissolve solids. Let cool at room temperature.
3. Transfer digest to distillation apparatus, making sure none remains in the flask by rinsing it five or six times with 1-2 ml portions of distilled water.
4. Place a 125-ml Erlenmeyer flask with 6 ml boric acid solution and 3 drops indicator solution under a condenser whose tip extends below the surface of the solution.
5. Add 8 ml sodium hydroxide - sodium thiosulfate solution to still and steam distill until about 50 ml distillate collects.
6. Titrate to gray end point or first appearance of violet.
7. Make blank determination, using same quantity reagents and same digestion, distillation and titration process as for sample determination.
8. Calculate percentage of nitrogen:

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

$$\% \text{ Protein} = \% \text{ N} \times \text{corresponding conversion factor}$$

References

1. A.O.A.C. Official methods of analysis of the association of official agricultural chemists. 13th ed. P. 858. 1980.

Nitrogen Determination with the Technicon Autoanalyzer

The quantitative determination of total nitrogen in cereal grains involves the digestion of the organic matter to convert the nitrogen to the ammonium form for subsequent colorimetric analysis. The amount of ammonia can be determined colorimetrically due to the formation of a blue phenol organic compound that results from the alkaline reaction of ammonium acid sulfate with phenol and hypochlorite.

Equipment

1. Analytical balance.
2. 75-ml 17 x 150 mm digestion tubes.
3. 2-ml automatic syringe.
4. Digestion racks for 40 tubes.
5. Digestion blocks for 40 tubes.
6. Voltage regulator for maintaining digestion blocks at a constant temperature of $370 \pm 2^{\circ}\text{C}$.
7. Vortex mixer.
8. Autoanalyzer consisting of the following modular components:
 - a) Sampler. The sampler consists of an automatic pipet that suction aliquots of sample at regular intervals and a disk for forty vials for digested solution. Samples can be analyzed at the rate of 50 per hour.
 - b) Proportioning Pump. The pump, which is the basis of the entire autoanalyzer system, consists of a series of bars which roll at uniform velocity, furnishing a pumping action for seven flow-rated tubes. The tubes are of plastic with the ends colormarked to indicate their internal diameter (in inches) and use:

1)	Purple	Drainage (0.110 Internal Diameter)	White
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For washing the automatic pipet:

2)	Green	Washing solution (0.073 ID)	Green
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For the reaction chamber:

3)	Green	Sodium hypochlorite (0.073 ID)	Green
4)	Yellow	Dilute sodium phenate (0.056 ID)	Yellow
5)	Blue	Sodium hydroxide-EDTA (0.065 ID)	Blue
6)	Yellow	Sample (0.056 ID)	Yellow
7)	Black	Air (0.090 ID)	Purple

←—————
Flow

- c) Reaction Chamber. Tubes number 3 to 7 go to the reaction chamber which a thermostat (connected to the voltage regulator) keeps at a constant temperature of $38 \pm 1^{\circ}\text{C}$. The chamber has been adapted to the needs of the laboratory with a cover composed of two aluminum pans and, on the inside, three 10-watt bulbs, glass tubing of various lengths and the thermostat.
- d) Colorimeter consisting of a continuous flow system and two red filters of 630 nanometers (nm).
- e) Recorder which receives the information from the colorimeter and registers the percent of transmission of the samples.

All of the above modules are connected to a voltage regulator which is connected to a line of alternating current regulated to 110-115 volts.

Reagents

1. Commercial catalyst tablets (7.5 mg Se + 1.5 g K_2SO_4).
2. Concentrated sulfuric acid (98%).
3. Alkaline phenol (concentrated sodium phenate):
 - a) 88% phenol: 243 g phenol dissolved in 33 ml distilled water, with heat and magnetic mixing.
Note: Caution is recommended when handling phenol which is hygroscopic, toxic and causes burns on contact with the skin.
 - b) 40% sodium hydroxide solution (200 g sodium hydroxide in 500 ml distilled water).
 - c) Using a separatory funnel, slowly add liquefied phenol to the sodium hydroxide solution in a beaker in ice water, with constant magnetic mixing.
Note: Store in dark polyethylene container.
4. Dilute sodium phenate: 1:1 solution of concentrated sodium phenate and distilled water (prepared the day of use and placed in a dark polyethylene container).
5. Sodium hydroxide - EDTA solution (200 g sodium hydroxide and 30 g EDTA in 1 l distilled water).
6. Sodium hypochlorite, 13% in distilled water. Commercial bleach is used but, as the strength may vary, it is necessary to test the concentration of each container.
7. Organic detergent solution, 50% in distilled water.
8. Washing solution: 1.5 N sulfuric acid and 0.2% Brij solution (125 ml concentrated sulfuric acid and 6 ml organic detergent, 50% in 3 l distilled water).

9. Ammonium sulfate stock solution, concentration 1 mg N per ml (molecular weight of ammonium sulfate 132.14).
10. Reagent blank. In each of two Kjeldahl flasks place seven catalyst tablets, 17 ml concentrated sulfuric acid and 83 ml distilled water. Digest approximately 30 minutes, cool to room temperature and make up to 1 l using the solution from both flasks and distilled water.
11. Prepare the calibration curve in the following manner:

(NH ₄) ₂ SO ₄ stock solution (1 mg N per ml)		N concentration (µg per ml)
0.0 ml	dilute to 250 ml with reagent blank	0
0.2 ml	dilute to 100 ml with reagent blank	2
0.4 ml	dilute to 100 ml with reagent blank	4
0.6 ml	dilute to 100 ml with reagent blank	6
0.8 ml	dilute to 100 ml with reagent blank	8
1.0 ml	dilute to 100 ml with reagent blank	10
1.2 ml	dilute to 100 ml with reagent blank	12
1.4 ml	dilute to 100 ml with reagent blank	14

Procedure

Digestion of the sample:

1. Weigh sample in an aluminum recipient and transfer it to the digestion tube. The amount of sample will vary according to its nitrogen content:

Maize endosperm	40-45 mg
Maize - whole kernel	30-35 mg
Wheat	30-35 mg
Barley	30-35 mg
Triticale	30-35 mg
2. Add a catalyst tablet and 2.5 ml concentrated sulfuric acid, making sure all the material is scraped down into the digestion tube.
3. Digest for 60 minutes at 370°C, with the digestion block inside an extractor hood. Digestion time can be reduced to 30 minutes by adding 2 ml 30% H₂O₂.
4. Dilute the digested sample to 75 ml with distilled water, mixing to obtain a homogeneous solution. Place an aliquot of the solution in a test tube for colorimetric nitrogen determination.

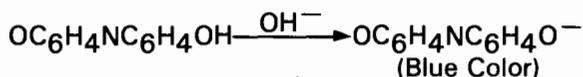
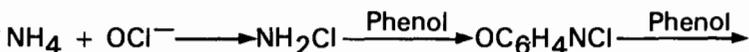
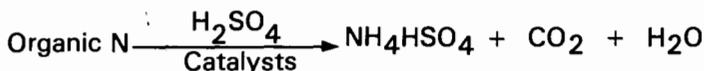
Operation of the autoanalyzer:

1. Start the analyzer one hour before use by turning on the reaction chamber, the colorimeter and the instrument switch of the recorder.
2. It is recommended that the sodium phenate solution be prepared daily and that the sodium hypochlorite and sodium hydroxide - EDTA solutions be filtered.
3. Thirty minutes after turning on the analyzer, place tubes in the sampler, add reagents and turn on the proportioning pump to eliminate any air in the tubes and to establish a continuous flow in the system.
4. Twenty minutes after the pump begins to work, turn on the chart drive switch of the recorder. It must be set at zero and at $99.5 \pm 0.5\%$ of the percent of transmission shown by the colorimeter.
5. Connect the sampler to start the analysis.



Modular components of the autoanalyzer working in series.

The entire process can be shown by the following reactions:



Calculations

To obtain the percentage of nitrogen in the sample, the transmittance reading of the sample is subtracted from that of the blank and the difference multiplied by the factor obtained by the calibration curve. The resulting figure is divided by the weight of the sample in mg. Each sample is analyzed in duplicate with a maximum acceptable difference of $\pm 0.08\%$ nitrogen.

This can be shown in the following equation:

$$\%N = \frac{F(B - S)}{\text{mg of sample}}$$

$$F = \text{Factor} = (\text{Place on the curve}) (\text{Dilution}) \frac{\text{Base of plot}}{\text{Height of plot}}$$

B = Percent of transmittance of blank

S = Percent of transmittance of sample

To calculate the percentage of protein from the percentage of nitrogen the following factors are used:

Maize and sorghum	6.25
Wheat, barley and triticale	5.83

References

1. Ferrari, A., E. Catanzaro and F. Russo-Alesi. Nitrogen analysis by a continuous digestion system. *Annals of the New York Academy of Sciences* 130:602-620. 1965.
2. Hofstader, R.A. Application of an autoanalyzer to the automation of some microchemical determinations. *Microchemical Journal* 10:444-455. 1966.

Tryptophan Determination



Pipeting an aliquot of hydrolysate for colorimetric determination of tryptophan.

Lysine and tryptophan are two essential amino acids which limit the nutritional value of maize endosperm protein. Because of the relationship between these amino acids in the protein of the opaque-2 maize endosperm (approximately 4 to 1),¹ tryptophan may be used as a single parameter for the evaluation of the nutritional quality of the protein.

Various analytical methods for determining tryptophan content—in the areas of ion exchange chromatography, spectrophotometry and

microbiology—have been extensively studied. They have been found to be complicated and laborious and therefore unsuitable for screening large numbers of samples.

For several years the CIMMYT laboratory has effectively utilized, for its simplicity and reproducibility, the Opienska-Blauth *et al* colorimetric method² modified by Hernandez and Bates.¹ It is based on the Hopkins-Cole reaction in which 1 molecule glyoxylic acid and 2 molecules tryptophan form a colored compound with a maximum absorption at 560 nm. Using this method, it is possible for two people to analyze up to 160 duplicate samples a day, thus satisfying the needs of the maize breeding program.

Equipment

1. Analytical balance.
2. Incubator oven.
3. Vortex mixer.
4. 5-ml automatic syringes.
5. Racks for 40 tubes.
6. 1 and 3-ml volumetric pipets.
7. 1-l volumetric flasks.
8. 1-l Erlenmeyer flasks.
9. 13 x 100 mm test tubes with screw caps.
10. 16 x 150 mm test tubes.
11. Calibrated colorimetric tubes.
12. Spectrophotometer or colorimeter.
13. pH meter.
14. 1-l graduated cylinders.
15. Centrifuge.

Reagents

1. 270 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolved in 0.5 ml distilled water and diluted to 1 l with glacial acetic acid (Reagent A). Each bottle of acetic acid must be tested for color development in the presence of tryptophan, as some aldehyde-free acetic acid does not produce enough glyoxylic acid to react with tryptophan to produce the colored compound. This problem has sometimes been solved by adding 2 to 4% acetic anhydride; higher concentrations of the anhydride can also inhibit color development.³

2. 30 N sulfuric acid (Reagent B). Prepare a volume-to-volume mixture of reagents A and B about an hour prior to use (Reagent C). This solution will contain glyoxylic acid which is an impurity of acetic acid and is also formed on mixing acetic acid containing ferric chloride with sulfuric acid. This glyoxylic acid in the presence of tryptophan (indole group) produces the color.
3. Papain solution: commercial grade enzyme dissolved in 0.1 N sodium acetate buffer (4 mg per ml), pH 7.0. This solution should be prepared just minutes before use.
4. Standard tryptophan solution of 100 μg per ml (for preparation of the standard curve).

Procedure

1. Weigh 80-85 mg of finely ground, defatted maize endosperm sample into 13 x 100 mm test tubes with screw caps, and add 3 ml papain solution. Carefully shake tubes, making sure the sample is thoroughly wetted. Include two tubes with just papain solution for use as blanks throughout the procedure. Also, in each rack of 40 tubes, include two check samples with a known quantity of tryptophan.
2. Place the samples in an incubator oven at $63 \pm 2^\circ\text{C}$ for 16 hours (overnight).
3. Remove the hydrolysates from the oven, shake, let cool to room temperature ($21\text{-}25^\circ\text{C}$) and centrifuge at 2,500 rpm for 5 minutes.
4. Pipet 1 ml hydrolysate into test tubes containing 4 ml Reagent C. Shake vigorously and incubate at $63 \pm 2^\circ\text{C}$ for 15 minutes for maximum color development.
5. Cool and transfer to calibrated tubes. Read the optical density of the solutions in a photocolrimeter at 560 nm.
6. Prepare a standard curve for tryptophan with a range of concentration of 0 to 35 μg per ml.
7. Calculate the tryptophan content of the sample from the standard curve and report as grams per 100 g protein. The protein percent of each maize sample is obtained by multiplying the previously figured percent of nitrogen by the factor 6.25.

References

1. Hernandez, H. H., and L.S. Bates. A modified method for rapid tryptophan analysis in maize. CIMMYT Research Bulletin no. 13. May, 1969.
2. Opienska-Blauth, J., M. Charenzinsky and H. Berbec. A new rapid method of determining tryptophan. Rep. Analytical Biochemistry 6:69. 1963.
3. Dalby, A., C.Y. Tsai. Acetic anhydride requirement in the colorimetric determination of tryptophan. Anal. Biochem. 63:283-285. 1975.

Lysine Determination



Ethyl acetate extraction of the compounds that interfere with determination of ϵ -dinitroimidazole lysine.

A limiting factor in the determination of lysine in cereal grains breeding programs has been a lack of reliable techniques that are rapid, reproducible and of moderate cost. After weighing the advantages and disadvantages of various colorimetric methods, the CIMMYT laboratory adopted the method designed by Tsai *et al* ¹ and modified by Villegas ² to determine the lysine content in maize. Using that method, up to 60 duplicate samples can be analyzed by two people each day.

This method utilizes the compound 2-chloro-3, 5-dinitropyridine which reacts with the ϵ -amino group in lysine after having blocked with copper the α -groups of the amino acids and peptides of low molecular weight that are present in the hydrolyzed protein. The ϵ -dinitroimidazole lysine formed is water soluble but insoluble in ethyl acetate, thus permitting that other compounds formed in the reaction be eliminated with that solvent, along with the excess 2-chloro-3, 5-dinitropyridine. The absorbancy of the aqueous solution of ϵ -dinitroimidazole lysine is read in a photocolormeter at 390 nm.

Lysine determination is performed only when materials have been previously selected colorimetrically as having high tryptophan values, or when protein quality index ($QI = DBC \div \% \text{ Protein}$) has been indirectly determined for samples of whole kernel maize. This, like other colorimetric methods, can be used successfully only with nonpigmented maize.

Equipment

1. Analytical balance.
2. Incubator oven.
3. Vortex mixer.
4. Centrifuge.
5. pH meter.
6. Colorimeter.
7. Racks for 40 tubes.
8. Calibrated colorimeter tubes.
9. 13 x 100 mm test tubes with screw caps.
10. 16 x 150 mm test tubes.
11. 50-ml automatic syringe with polyethylene tube.
12. Glassware (pipets, beakers, etc.)

Reagents

1. Papain solution: 4 mg papain per ml 0.03 M phosphate buffer, pH 7.4.
2. 0.05 M carbonate buffer, pH 9.0.
3. 0.05 M borate buffer, pH 9.0.
4. Copper phosphate suspension:
Dissolve 2.8 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml distilled water (Reagent A).
Dissolve 13.6 g $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ in 200 ml distilled water (Reagent B).
Mix reagents A and B; centrifuge at 2,000 rpm for 5 minutes and discard the supernatant. Resuspend the precipitate 3 times in 15 ml borate buffer and then suspend the final precipitate in 80 ml borate buffer. This reagent can be used for one week.
5. 1.2 N HCl solution.

6. Amino acid mixture:
- | | | | |
|------------|-------|---------------|--------|
| Cystine | 20 mg | Phenylalanine | 40 mg |
| Methionine | 20 mg | Valine | 40 mg |
| Histidine | 30 mg | Arginine | 50 mg |
| Alanine | 30 mg | Serine | 50 mg |
| Isoleucine | 30 mg | Aspartic acid | 60 mg |
| Threonine | 30 mg | Glutamic acid | 300 mg |
| Tyrosine | 30 mg | Leucine | 80 mg |
| Glycine | 40 mg | Proline | 80 mg |
- Dissolve 100-mg samples of the mixture in 10 ml carbonate buffer.
7. 2-chloro-3, 5-dinitropyridine solution at 3% in methanol (prepared just prior to use).

Procedure

1. Weigh 100 mg finely ground, defatted sample into test tubes and add 5 ml papain solution. Make sure sample is thoroughly wetted; shake tubes at least twice in the first hour of incubation. Include blanks of just papain solution.
2. Incubate at $63 \pm 2^{\circ}\text{C}$ for 16 hours. Shake, cool to room temperature and centrifuge at 2,500 rpm for 5 minutes. (Aliquots of this hydrolysate can also be used for tryptophan determination.)
3. Pipet 1-ml aliquots of the supernatant into centrifuge tubes and add 0.5 ml carbonate buffer and 0.5 ml copper phosphate suspension.
4. Shake the mixture for five minutes and centrifuge.
5. Pipet 1-ml aliquots of the supernatant into large test tubes and add 0.1 ml 2-chloro-3, 5-dinitropyridine. Shake well.
6. Allow the mixture to stand at room temperature for two hours, shaking every 30 minutes.
7. Add 5 ml 1.2 N HCl to each test tube and shake well.
8. Add 5 ml ethyl acetate and mix well, inverting the capped tubes at least ten times; extract the upper phase, using a syringe adapted with a polyethylene tube. This step must be performed three times.
9. Transfer the aqueous phase to calibrated tubes and read in the photocolormeter at 390 nm, adjusted with the blanks.
10. Calculate lysine content of samples by comparing with a standard curve. Report on a protein basis.

Standard Curve

Prepare a standard curve with a range of 0 to 200 μg lysine per ml. Stock solution of lysine: 62.5 mg lysine-monohydrochloride in 20 ml carbonate buffer (2500 μg lysine per ml). Dilute the stock solution of lysine to 0, 250, 500, 750 and 1000 μg lysine per ml.

To 1 ml of each of the solutions, add 4 ml papain solution (5 mg papain per ml phosphate buffer). Pipet 1 ml of each solution into centrifuge tubes and add 0.5 ml amino acid mixture and 0.5 ml copper phosphate suspension. Continue with step d) of Procedure.

References

1. Tsai, C.Y., L.W. Hansel and O.E. Nelson. A colorimetric method of screening maize seeds for lysine content. *Cereal Chemistry* 49:572-579. 1972.
2. Villegas, E., and E.T. Mertz. Screening techniques used at CIMMYT for protein quality maize. Technical Bulletin No. 20. International Maize and Wheat Improvement Center, Mexico. 1970.

Dye Binding Capacity Method for Estimating Protein Quality in Maize, Barley and Triticale



Determination of percent transmission in the evaluation of protein quality index.

This method is commonly used to determine protein content in natural, unaltered commodities such as cereals, oilseeds and milk. It is based on the fact that there is a constant percentage of available cationic groups in the basic amino acids arginine, histidine and lysine and in the end groups of the protein chains. It cannot be used to determine protein content in materials in which the content of available cationic groups has been altered by processing or by genetic mutations (such as in cereal grains whose lysine content has been raised through mutation).

In the laboratory, dye binding capacity (DBC), complemented by Kjeldahl nitrogen determination, is used to obtain the protein quality index ($QI = DBC + \% \text{ Protein}$) in samples of maize, barley and triticale. There is a significant correlation between QI and basic amino acid content, permitting the identification of grains with high lysine content.

Equipment

1. Analytical balance with 1 mg sensitivity.
2. Cyclone-type mill with 0.5 mm screen.
3. Horizontal shaker with capacity of two racks for 40 tubes.
4. 5-ml automatic pipet.
5. 10 x 130 mm culture tubes with screw caps.
6. Centrifuge with metal shields for 64 tubes.
7. Udy Model 101 Colorimeter or spectrophotometer with 0.1 cm cell and 475 nm color filter.

Reagents

Dye solution (quantities necessary for 1 l reagent):

- 2 g acilane orange GH (Bayer Co. 76113220).
- 15.84 g citric acid.
- 2.38 g anhydrous Na_2HPO_4 .
- 2 g oxalic acid.
- 0.3 g thymol.

Among the dyes that can be used are Acid Orange 10, Acid Orange 12 and Acid Orange C.I. 15970. In an acid medium they react with the basic amino acids of protein—arginine, histidine and lysine.

Preparation of the dye solution:

1. Oxalic-citric acid solution (Reagent A). Dissolve 237.6 g citric acid ($\text{C}_6\text{H}_8\text{O}_7$) in approximately 500 ml distilled water in a 2-l volumetric flask. Dissolve 30 g oxalic acid in the solution and dilute to 2 l with distilled water.
2. Acilane sodium phosphate solution (Reagent B).
 - a) Dissolve 44.7 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in approximately 500 ml hot distilled water.
 - b) Dissolve 30 g acilane orange (previously dried for two hours at 120°C) in the above phosphate solution in a water bath at approximately 80°C .
 - c) Add 4.5 g thymol (previously pulverized in a glass mortar) and dilute to 2 l with distilled water.
3. Mix reagents A and B and dilute to 15 l with distilled water. Shake vigorously and let settle 24 hours. The dye solution must have a pH of 2.3.

Reference dye solution:

Dilute 50 ml dye solution in 50 ml distilled water in a volumetric flask.

Procedure

1. Weigh 100 mg ground sample into culture tubes.
2. Add 5 ml acilane dye buffer solution, making sure that sample and dye are completely mixed.
3. Place racks in horizontal position in the shaker and shake at 22-23°C for exactly 60 minutes, the racks being inverted after 30 minutes.
4. Centrifuge the sample dye buffer solution at 5,000 rpm for 15-20 minutes. If a centrifuge is not available, filter through inert material as recommended by Udy.²
5. Read the percent transmission on the Udy colorimeter, warmed for two hours and calibrated with the reference solution to scale reading of 42.
6. Divide transmission percent reading (DBC value) by the Kjedahl percentage of protein in each sample to get the protein quality index (QI); this has a significant correlation with the content of the basic amino acids arginine, histidine and lysine.¹

References

1. Mossberg, R. New Approaches to breeding for improved plant protein. International Atomic Energy Agency, Vienna. 1969.
2. Udy, Doyle C. Improved dye method for estimating protein. Journal American Oil Chemists' Society 48 no. 1:29A-33A. 1971.

Zein Determination in Maize

Zein or prolamine is the alcohol-soluble protein fraction that composes 50-60% of the endosperm protein of normal maize. Its nutritional value is low because the two essential amino acids tryptophan and lysine are not present in the molecule. This protein fraction is reduced to 20-35% when the opaque-2 gene is incorporated into normal maize; thus, there is a significant negative correlation between the content of zein and that of lysine in maize endosperm.^{1,2}

Zein determination is carried out in CIMMYT's Protein Quality Laboratory when the colorimetric test for tryptophan is not reliable. This is the case with colored maize with a high content of pigments or with double mutant sugary-2/opaque-2 (su2o2) materials in which high sugar content interferes with the final color of the reaction. It also occurs when acetic acid of the necessary quality (a low content of aldehydes and high glyoxylic acid) is not available for tryptophan determination. To indirectly determine lysine content using this procedure, it is necessary to obtain the statistically correlated calibration between lysine determined by ion exchange chromatography and quantity of zein.

A rapid turbidimetric zein method was designed in Illinois, USA, for determining lysine content in maize through the extraction of zein with 70% ethanol and sodium acetate, followed by precipitation by 1% NaCl.² In Germany a similar method³ was designed in which zein was extracted with anhydrous butanol, and determination made from the soluble nitrogen in the extract and residual nitrogen in the extracted maize sample. Both methods show good results in maize protein quality evaluation and can be recommended when tryptophan cannot be estimated. In the CIMMYT laboratory, the Paulis-Wall method² is used, especially for evaluating samples of whole kernel maize.

Equipment

1. Horizontal reciprocal shaker.
2. 14 x 150 mm culture tubes with screw caps.
3. Colorimeter.
4. 10-ml pipets or automatic syringe.
5. Calibrated colorimeter tubes.
6. 13 x 100 mm test tubes.

7. Cyclone mill with 0.5 mm screen.
8. Analytical balance.
9. Centrifuge for 64 13 x 100 mm tubes.
10. Racks for 40 tubes.

Reagents

1. 70% ethyl alcohol solution with 0.5% anhydrous sodium acetate:
5 g anhydrous sodium acetate in 1 l 70% ethyl alcohol.
2. 1% sodium chloride: 10 g NaCl in 1 l distilled water.

Procedure

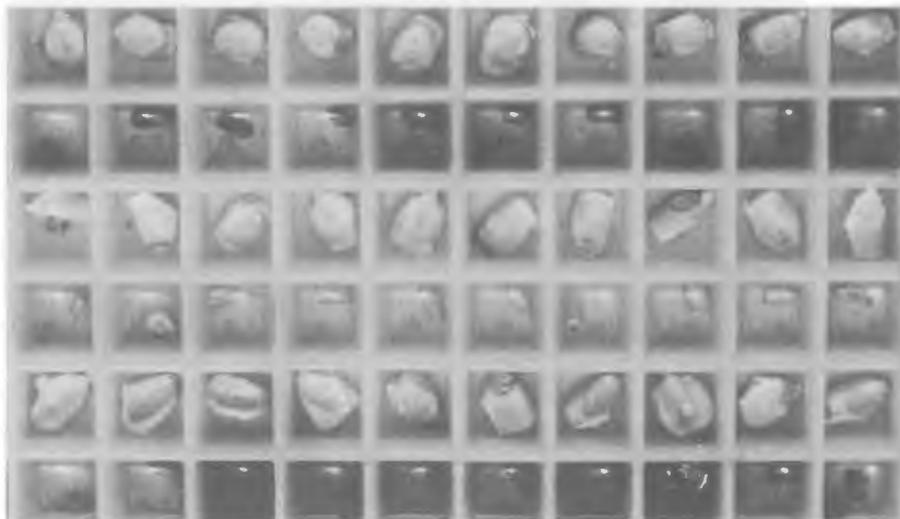
1. Weigh 100 mg finely ground, nondefatted sample into culture tubes with Teflon-lined screw caps.
2. To each tube add 10 ml 70% ethanol solution containing 0.5% sodium acetate. Shake well so that samples are completely wetted.
3. Cap tubes, first with a piece of polyethylene and then with the screw caps.
4. Include two maize samples as checks—one normal and one opaque-2.
5. Shake for one hour, inverting the position of the racks after 30 minutes.
6. Transfer to centrifuge tubes and centrifuge at 2,500 rpm for 10 minutes.
7. Using a volumetric pipet, put 2-ml aliquots in calibrated colorimetric tubes.
8. Blow 6 ml 1% NaCl into the solution with a volumetric or automatic pipet so that the mixture is completely homogeneous.
9. Leave the tubes at room temperature for exactly one hour.
10. Eliminate air bubbles in the tubes and read the absorbance in the colorimeter at 590 nm.
11. Select those samples with lowest optical densities. They will have the lowest content of zein and highest levels of lysine.

References

1. Frömberg, H.K., W. Christ and W.G. Pollmer. A rapid method based on butanol extraction for selecting high lysine maize (*Zea mays* L.). *Crop Science* 11:567-569. 1971.

2. Paulis, J.W., J.S. Wall and W.F. Kwolek. A rapid turbidimetric analysis for zein in corn and its correlation with lysine content. J. Agr. Food Chem. 22 no. 2:313-317. 1974.
3. Pollmer, W.G., and H.K. Frömberg. Estimating lysine content of maize (*Zea mays* L.) by means of alcoholic extraction. Z. Pflanzenzüchtg. 69:50-57. 1973.

Ninhydrin Test to Determine Free Amino Acids in Floury, Opaque-2 Maize



Kernels bearing the opaque-2 gene develop an intense purple color in the presence of ninhydrin.

In 1974, researchers at Purdue University developed a rapid colorimetric test for selecting mutant lines of maize, sorghum and barley with high lysine content.¹ This test is based on the reaction of ninhydrin with the free amino acids found in high quantity in grains of superior protein quality. The results of the test are affected by the hardness of the endosperm of the materials; it can be used satisfactorily only with those with soft endosperm for maximum ninhydrin penetration.

In CIMMYT's Protein Quality Laboratory the test is used qualitatively on the endosperm of floury maize from the highlands of Peru, Bolivia, Colombia and Ecuador. The opaque-2 gene has been incorporated into the materials as part of those countries' national maize improvement programs. The procedure is nondestructive in that the remainder of each test kernel selected for positive ninhydrin reaction can be planted to preserve quality protein genetic material.

Equipment

1. Wooden cutting board
2. Dissection kit.
3. 5-ml pipets.
4. Plastic mini ice cube trays.

Reagents

Ninhydrin solution with stannous chloride:

1. Dissolve 400 mg stannous chloride ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) in 250 ml sodium citrate buffer solution: 4.3 g citric acid and 8.7 g sodium citrate diluted to 250 ml with distilled water and adjusted to a pH of 5.0 (Reagent A).
2. Dissolve 10 g ninhydrin in 250 ml Methyl Cellosolve (Reagent B).
3. Mix reagents A and B. This solution can be kept under refrigeration for five days.

Procedure

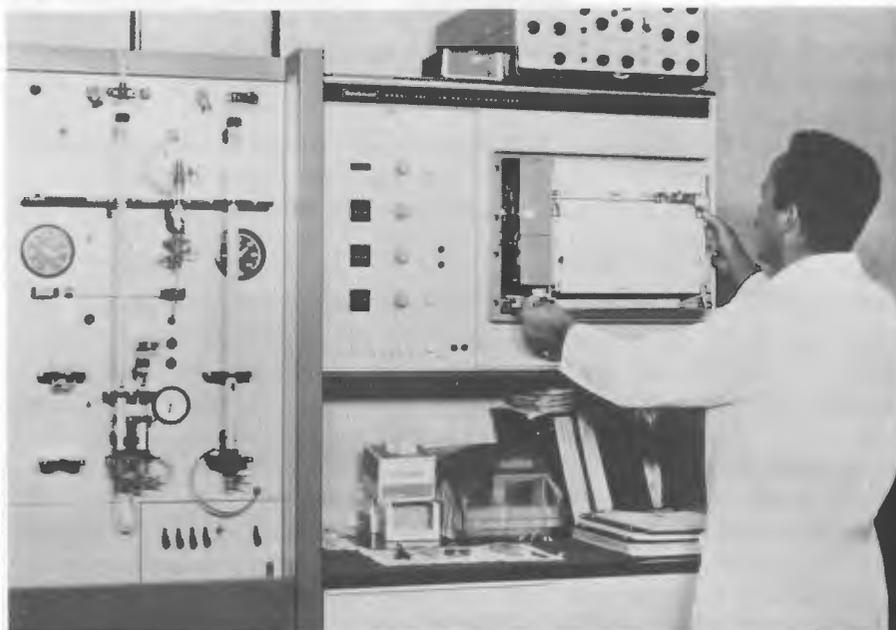
1. Cut a section of endosperm from each kernel to be tested (without damaging the germ) and place it in the plastic tray. Place the rest of the kernel containing the germ beside it for later identification.
2. Similarly place check samples (one opaque and one normal) in the tray for comparison.
3. Completely cover each endosperm section with 0.5 ml ninhydrin solution.
4. Let react for 30 minutes.
5. Select those kernels showing a positive reaction (intense purple color) as compared to the opaque-2 check sample.

This test has been useful for the preliminary selection for high protein quality in flourey maize with the opaque-2 gene. Quantitative lysine or tryptophan analyses can then be carried out on the progenies of the selected kernels.

References

1. Mertz, E.T. A rapid ninhydrin color test for screening high lysine mutants of maize, sorghum, barley, and other cereal grains. *Cereal Chemistry* 51:304-307. 1974.

Use of the Amino Acid Analyzer



Amino acid determination by ion exchange chromatography.

Complete amino acid analysis (aminograms) are made only on those genetic materials which have been selected in the maize, wheat, barley and triticale breeding programs for their high tryptophan and/or lysine content. Although a number of methods for separating and quantifying amino acids in protein are described in literature, the one most recommended for accuracy and reproducibility is the one based on ion exchange chromatography. The use of this test has now been greatly facilitated with the amino acid analyzer.⁴

To quantify the various amino acids, it is necessary to totally hydrolyze the protein of the sample to prepare a hydrolysate with an adequate amino acid concentration and pH level and to separate the components on ion exchange columns containing appropriate resins. The hydrolysis of the sample is usually done with 6 N HCl at a temperature of $100 \pm 2^\circ\text{C}$ for 24 hours.

Organic acids such as p-toluenesulfonic acid (p-TSA) have also been used successfully as hydrolytic agents.¹ This type of hydrolysis has a time saving advantage (approximately 3 hours) since evaporation of residual

chloride is not necessary as it is after hydrochloric acid hydrolysis. However, information on the amount of each amino acid recovered through the use of organic acids is still incomplete; therefore, caution is advised when using them in complete amino acid analysis.

In the CIMMYT laboratory, 6 N HCl is used for hydrolysis in complete amino acid analysis of protein. In the case of lysine determination, hydrolysis with 3 N p-TSA has been found to be satisfactory and the resulting time saving a great advantage.

The separation of the amino acids liberated by hydrolysis is carried out using two types of columns adapted to the Beckman Model 120C Analyzer. In the case of the separation of the basic amino acids arginine, histidine and lysine, the short ion exchange column (5 cm) is used with Aminex A-5 type resin (Biorad Co.) and a pH 5.2 buffer solution. To separate the acidic and neutral amino acids, a longer column of 50 cm is used with Aminex A-4 type resin and two different buffer solutions—one of pH 3.25 and the other pH 4.25. The quantitative estimation of the separated amino acids is made with an electronic integrater connected to the amino acid analyzer or by manually calculating the area of the curve for each component. It is necessary to run an analysis on a standard amino acid solution of known concentration each day for comparison.

Equipment

1. Analytical balance.
2. 20 x 70 and 20 x 95 mm vials with Teflon-lined screw caps.
3. Graduated conical centrifuge tubes with screw caps.
4. Heating unit with temperature control.
5. Beckman Amino Acid Analyzer, Model 120C, with long and short glass columns and necessary resins.
6. Roto evaporator with vacuum pump.
7. Glassware (pipets, beakers, round bottomed flasks, etc.).
8. pH test strips with multiple indicators.
9. pH meter with expanded scale.

Reagents

1. 6 N hydrochloric acid.
2. 3 M p-toluenesulfonic acid.
3. Sodium citrate.
4. Citric acid.

5. Sodium hydroxide.
6. Caproic acid.
7. Stannous chloride.
8. 2% ninhydrin solution.
9. Sodium acetate.
10. Organic detergent.
11. Nitrogen gas.
12. Methyl Cellosolve.
13. Acetic acid.
14. 25% thiodiglycol.
15. Neutralizing solution: 60 g NaOH and 4.9 g sodium citrate made up to a final volume of 250 ml.
16. Sodium citrate buffer solutions with pH of 2.2, 3.25, 4.25 and 5.20.²

The specific reagents for each amino acid separation must be prepared as per instructions in the Beckman 120C Analyzer manual² or that of the corresponding equipment used.

Procedure

Basic amino acid separation (by hydrolysis with p-TSA):

1. Weigh 25 mg finely ground, defatted sample into graduated conical glass tubes.
2. Add 3 ml 3 M p-TSA, making sure sample is completely wetted. Bubble nitrogen and cap tightly.
3. Incubate at 95-100°C for 26 hours. Cool to room temperature.
4. Add 1.4 to 1.7 ml neutralizing solution. Shake well and make sure the pH is between 1.5 and 2.0.
5. Dilute to 10 ml with buffer solution of pH 2.2.
6. Filter through a millipore, or centrifuge at 9,000 rpm.
7. Inject 0.5-ml aliquots into the short column of the amino acid analyzer. Elute with pH 5.20 buffer solution.
8. A standard basic amino acid solution of known concentration (0.2 μ moles per ml) must be analyzed the same day.

Complete aminogram:

1. Weigh 25 mg finely ground, defatted samples into vials with Teflon-lined screw caps.
2. Add 5 ml 6 N HCl, making sure that sample is completely wetted.
3. Flush out air with nitrogen and cap tightly.
4. Place in the heating unit at 100 + 3°C for 24 hours.

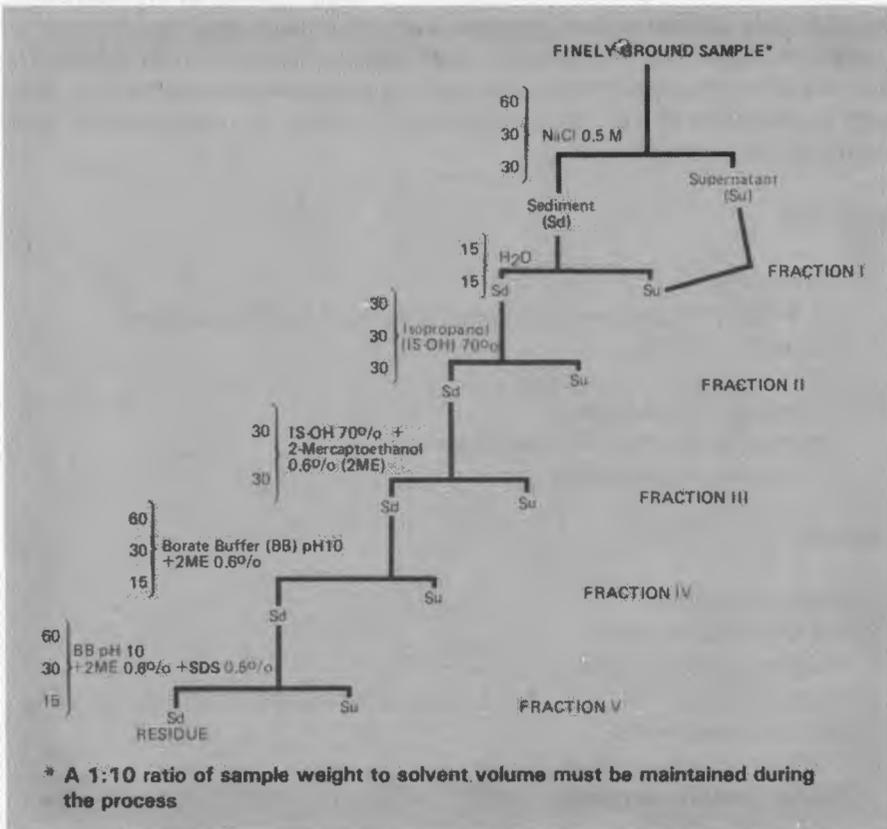
5. Filter sample through a millipore, rinsing each test tube with distilled water.
6. Evaporate in a vacuum three times, each time rinsing the round bottomed flasks with distilled water.
7. Dissolve the residue in 5-10 ml buffer solution (depending on the % protein of the sample) at pH 2.2.
8. Inject one aliquot in the short column of the analyzer for basic amino acid separation. Elute with the 5.2 pH buffer solution. After this separation, inject another aliquot of acid and neutral amino acids in the long column. Elute with buffer solutions of pH 3.25 and 4.25 successively at intervals indicated in the analyzer manual. Standards of known concentration (0.2 μ M/ml), prepared with commercial preparations, must be analyzed daily in each column. It is necessary to determine the percent of recovery of an internal standard (norleucine) included in the hydrolyzed samples.

Note: It is important to note that the amino acids cysteine, cystine and methionine are partially destroyed in hydrolysis by HCl and p-TSA and that tryptophan is completely destroyed by 6 N HCl. For analysis of the sulfur amino acids, a previous oxidation must be carried out with performic acid³ for the recovery of cysteic acid and methionine sulfone in the hydrolysate. In the case of opaque-2 maize, tryptophan is determined by the Hernandez and Bates colorimetric method.

References

1. Liu, T.Y., and Y.H. Chang. Hydrolysis of proteins with p-toluenesulfonic acid. Determination of Tryptophan. *Journal of Biological Chemistry* 246:2842-2848. 1971.
2. Manual for the amino acid analyzer, Beckman Model 120C. Spinco Division, Beckman Apparatus. Stanford Industrial Park, Palo Alto, California. 1965.
3. Moore, S. On the determination of cystine as cysteic acid. *Journal of Biological Chemistry* 238:235-237. 1963.
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Protein Fractionation



Protein fractionation diagram (Landry-Moreaux, 1970).

The increase in lysine and tryptophan content in the endosperm protein of opaque-2 maize is the result of the distribution of the various protein fractions, a different distribution than that found in normal maize. It is important to understand these differences and how they occur through cycles of selection for maize of high protein quality.

On incorporating the opaque-2 gene (as well as other mutant genes such as flourey-2, opaque-7 and brittle-2) in maize, the content of prolamine-zein, deficient in lysine and tryptophan, is reduced. This results in an increase in other fractions such as albumins, globulins and glutelins, fractions that are not deficient in lysine and tryptophan. It is possible to quantify the changes in the various protein fractions through the process of fractionation.

Material has been published on several protein fractionation methods. The CIMMYT laboratory utilizes the Landry-Moureaux method¹ that consists of the sequential extraction of each protein fraction, utilizing various solvents and extraction times. Recoveries for each protein fraction are made on the basis of the content of soluble nitrogen as determined by the micro-Kjeldahl method.

Equipment

1. Centrifuge.
2. 60-ml polypropylene centrifuge tubes with rubber stoppers.
3. Horizontal shaker.
4. Glass rods with rubber tips.
5. 125-ml plastic bottles.
6. Equipment for micro-Kjeldahl method.
7. Pipet safety manipulator.

Reagents

1. Sodium chloride.
2. 99.6% isopropanol.
3. 95% mercaptoethanol.
4. Sodium borate, pH 10 (18.3 ml 0.2 M NaOH + 50 ml .025 M sodium tetraborate).
5. Sodium dodecyl sulfate (SDS).
6. Micro-Kjeldahl reagents.

Procedure

1. Weigh 2 g finely ground, defatted samples of maize endosperm into 60-ml plastic centrifuge tubes. Prepare each sample in duplicate.
2. Extract each fraction in a horizontal shaker at low velocity at room temperature, following the fractionation diagram.
3. After each centrifugation, mix the sediment with the corresponding solution with a glass rod, making sure the sample is completely wetted by the solvent.

Note: It is necessary to work under an extraction hood and with adequate ventilation when using solvents containing mercaptoethanol.

4. Keep protein fractions to be analyzed under refrigeration.
5. Determine the nitrogen content of each fraction using the micro-Kjeldahl method, calculating the percentage of each fraction according to the total nitrogen content of the original sample.

References

1. Landry, J., and Th. Moureaux. Heterogeneity of the glutelins of the grain of corn: selective extraction and composition in amino acids of the three isolated fractions. *Bull. Soc. Chem. Bio.* 52:1021-1037. 1970.

Correct citation: Villegas, E., E. Ortega and R. Bauer. Chemical Methods Used at CIMMYT for Determining Protein Quality in Cereal Grains. Londres 40, Apdo. Postal 6-641, 06600 Mexico, D.F. Mexico. 1984.

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