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CHEMICAL AND BIOLOGICAL METHODS FOR GRAIN AND FORAGE SORGHUM

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Finally, sincere gratitude is extended to Ardith Spies and Susan Stone for preparing this manuscript. Cover illustration was kindly supplied by Karen Hott.

References

Harris, Loren E., Nutrition Research Techniques for Domestic and Wild Animals, Vol. 1., Part 2 - Chemical and Biological Methods. 1408 Highland Drive, Logan, Utah 84321.

INTRODUCTION

This manuscript was compiled by Purdue University under the auspices of United States Agency for International Development Contract AID-ta-C-1212 entitled "Inheritance and Improvement of Protein Quality and Content in Sorghum bicolor (L.) Moench. It is intended to be a service to people interested in quality improvement in cereal grains and forages, but with limited access to technical information of this nature.

The physical, chemical, and biological methods of analysis of grain and forage contained herein are offered as guides. In some cases they will need to be modified to fit conditions of the laboratory or experiment.

Suggestions for any modifications or corrections are encouraged and welcomed.

Mention of firm names or trade products in this manual does not constitute endorsement by Purdue University over others of a similar nature not mentioned.

TABLE OF CONTENTS

SECTION I

CHEMICAL AND BIOLOGICAL METHODS FOR GRAIN SORGHUM

Introduction..... 1

Defatting of Ground Samples..... 3

Separation of Embryo and Endosperm..... 5

Extraction and Fractionation of Carbohydrates..... 7

Nelson's Test for Reducing Sugars (Glucose)..... 14

Modified Roe's Procedure for Fructose and Sucrose Determination .. 18

Oil Determination..... 21

Analysis for Carotenes and Xanthophylls in Dried Plant Material .. 25

Micro-Kjeldahl Determination of Total Nitrogen..... 32

Preparation of Deionized Water..... 38

Factor to Convert Percent N to Percent Protein for

Different Species and Plant Products..... 41

Dye Binding Procedure for Estimating Protein and Lysine..... 44

Rapid Ninhydrin Tests..... 55

Analytical Ninhydrin Test..... 58

Lysine in Non-Pigmented Cereals..... 62

Tryptophan Determination..... 67

Rapid Sectioning of Cereal Grain Endosperm for

Examination of Proteins..... 71

Protein Fractionation Principle..... 76

Gel Electrophoresis Using Dodecyl-Sulfate-Polyacrylamide..... 83

Tannin Analysis..... 96

Bleach Test to Identify Brown-Coated Sorghum Grain..... 106

Chemical Dehulling of Sorghum Grain..... 108

Diethyl Sulfate Induced Mutation Procedure..... 110

Biological Assay in Rats..... 112

TABLE OF CONTENTS

SECTION II

CHEMICAL AND BIOLOGICAL METHODS FOR FORAGE SORGHUM

Introduction..... 120

Weighing Samples by Difference for Chemical Analysis..... 122

Dry Matter and Partial Dry Matter..... 124

Determination of Dry Matter..... 131

Determination of Gross Energy..... 133

Determination of Ash..... 138

Determination of Crude Fiber..... 140

Determination of Ether Extract..... 145

Determination of Nitrogen Free Extract..... 148

Determination of Nitrogen and Crude Protein..... 149

Micro-Kjeldahl Determination of Total Nitrogen in Plant Material... 156

Determination of Exchangeable Ammonium by Steam-Distillation Method. 162

Rapid Field Screening Procedures for Nitrate Accumulation..... 164

Determination of Nitrates and Nitrites in Forage..... 168

Determination of Total Non-Structural Carbohydrates..... 174

Organic Matter..... 179

Determination of Cell Contents..... 180

Determination of Cell Walls (Neutral Detergent Fiber) and
Cell Contents..... 182

Determination of Acid Detergent Fiber..... 186

Determination of Acid Detergent Lignin..... 189

Determination of Permanganate Lignin, Cellulose and Silica (Insoluble Ash) 192
Preparation of Ash Solution for the Determination of Calcium, Magnesium, and Phosphorus 199
Determination of Calcium 201
Determination of Magnesium 205
Determination of Phosphorus 208
Trace Element Analysis 211
Determination of Cobalt 214
Determination of Manganese 219
Determination of Carotene in Feeds and Forages 222
Determination of Cyanide in Plants 226
Evaluation of Feeds 229
Supply House Addresses 233

SECTION I

CHEMICAL AND BIOLOGICAL METHODS FOR GRAIN SORGHUM

INTRODUCTION

The growing realization of the importance of cereal grains as a large and, for many millions, predominant part of the human diet in all parts of the world has led to renewed efforts not only to increase grain yield, but to improve a variety of parameters of grain quality relative to human nutritional requirements. Moreover, this expansion of efforts has not been limited to laboratory oriented scientists, such as nutritionists, biochemists, and physiologists, but has become an integral part of the goals of agricultural scientists involved in various applied areas, such as plant breeding.

It is primarily to the peculiar needs of the latter group, the cereal breeders, who must screen many hundreds of new genotypes for a wide variety of agronomic and quality criteria, that this compilation of physical and chemical grain analysis methods has been addressed. Obviously, many of the procedures found herein cannot be conducted in the field. However, the techniques selected have been simplified as much as possible to enable the breeder to extend, on his own, his capabilities for screening for at least some quality parameters with a minimal investment in laboratory equipment and expenditure of time.

More detailed analysis procedures have also been included (i.e., protein fractionation and gel electrophoresis) to aid the investigator who has access to a fairly sophisticated laboratory and to give a more comprehensive review of available techniques.

Occasionally, methods pertinent to cereal grain analysis will be found in the forage section and *vice versa* in order to avoid undue duplication.

DEFATTING OF GROUND SAMPLES

Principle

Certain procedures for analysis of sorghum grain require defatting of ground samples (i.e., protein fractionation, gel electrophoresis and amino acid analysis) prior to determination due to interference by fat soluble materials. This, however, is a simple routine operation which can be done simultaneously for many samples.

Equipment

a) Macro Soxhlet extractor

b) Heating mantle

c) Petroleum ether (Skelly Solve B or Pentane-Hexane)

d) Variable power transformer (rheostat)

e) Glass wool

f) Large and small extraction thimbles

Procedure

a) For extracting many samples fill small soxhlet extraction thimbles with ground sample and plug with glass wool. Place thimbles in a large soxhlet thimble.

b) If one large sample is to be defatted place directly in the large thimble and cover with glass wool.

c) Fill distilling flask (3000 ml round bottom) about half full with solvent.

d) Place extraction thimble in the top of the soxhlet extraction.

e) Attach condenser to extractor and adjust water flow through it.

f) Attach variable power transformer to heating mantle holding the distilling flask. Set to 50 - 60 C.

g) Reflux solvent 18 - 24 hours.

h) Remove thimble(s). Air dry at room temperature.

SEPARATION OF EMBRYO AND ENDOSPERM

Principle

The characterization of sorghum grain includes some analyses (i.e., protein fractionation and gel electrophoresis) which require separation of the endosperm from the germ. The following technique involves manual dissection of the seed using a scalpel.

Equipment

a) Petri dishes

b) Arrow-shaped dissecting scalpel

c) Distilled water

d) Paper towels

e) Razor blade

f) Forceps

Procedure

a) Soak seeds in distilled water 10-30 minutes. The exact time depends on the vitreousness of the endosperm and must be determined empirically for each line.

b) Blot seeds and place them on damp paper towels.

c) Grasp seed with forceps or between the fingers with the ventral side (showing the embryo) face up.

d) For seed with vitreous endosperm, insert the scalpel tip at the endosperm-embryo interface and cut along the entire embryo perimeter keeping the scalpel blade somewhat angled towards the embryo center. Then insert the scalpel at the top of the embryo at an oblique angle and pry up. The embryo should pop out intact.

e) For seeds with floury endosperm, cut seed in two, along its main axis with a razor blade such that the embryo is divided into equal parts. Using the scalpel tip, pry out the embryo halves which are now readily visible.

f) It is <u>important</u> that all of the embryo (which can be differentiated from the endosperm on the basis of color) is separated from the remainder of the seed because even a small amount of contamination can affect the results due to the high concentration of high quality protein and oil in the germ.

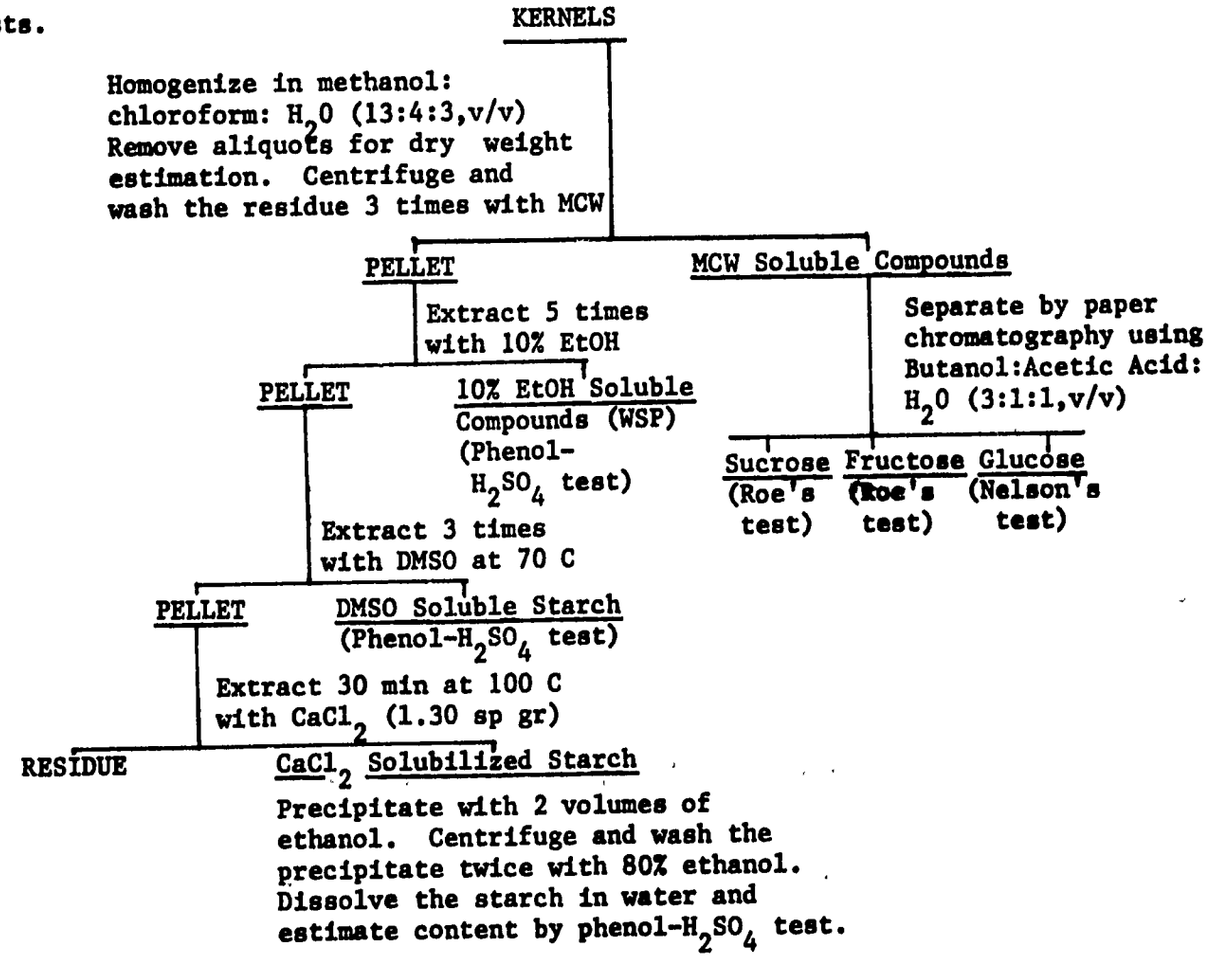
Reference

Jambunathan, R. and E. T. Mertz. 1973. Relationship between tannin levels, rat growth, and distribution of proteins in sorghum. J. Agr. Food Chem. 21:692-696.

EXTRACTION AND FRACTIONATION OF CARBOHYDRATES

Principle

The procedure of Shannon allows stepwise extraction, fractionation and quantitative measurement of glucose, fructose, sucrose, the water soluble polysaccharide fraction (WSP) and starch from a single sample. It was devised in order to study the carbohydrate composition of immature corn kernels, but can also be used for other seeds including immature and low tannin sorghum kernels. However, caution is advised in use of this procedure on sorghum seed which is highly pigmented or contains high levels of tannin, unless preliminary testing is done, because of the possibility of interference by these compounds with the fractionations and the quantitation tests.



Equipment

a) Virtis Model 45 homogenizer or equivalent

b) Centrifuge

c) 40 ml and 50 ml centrifuge tubes

d) 30 ml hypodermic syringe fitted with 3 in cannula

e) Flash evaporator and round bottom flasks

f) Controlled temperature water bath

g) Whatman 3 mm chromatography paper

h) Chromatography tank

i) Glass wool

j) Buchner funnel fitted with fiber glass filter

k) Beakers

l) 5 ml scored tubes

m) Template overlay for detecting chromatogram spots; consists of a 1/16 in plastic or plexiglass front with a 1 mm slit and a 1/4 in back. Chromatograms are sandwiched between templates and held in position with stainless steel clips.

n) 50 ml syringe for streaking chromatogram or equivalent

o) Magnetic stirrer and stirring bar

Reagents

a) Methanol: chloroform: water (13:4:3, v/v MCW)

b) Chloroform

c) Butanol: Acetic acid: water (3:1:1, v/v, BAW)

d) Ethanol (10% and 80% v/v solutions)

e) Dimethyl sulfoxide (90 and 95% v/v solutions, DMSO)

f) Calcium chloride (sp. grav. 1.30, pH 7.0 solution, $CaCl_2$)

g) Aniline - diphenylamine phosphate spray reagent

1) Prepare and store separately at 0 C:

- Diphenylamine 2% w/v in acetone
- Aniline 2% w/v in acetone
- 85% phosphoric acid

2) Combine immediately before use of a, b, and c 5:5:1 v/v.

3) After application to chromatograms, air dry and heat to 100 C to develop colors.

Procedure

a) Soluble sugar extraction.

1) Homogenize 10 g of frozen kernels (fresh wt) in 20 ml of a mixture of methanol: chloroform: water (13:4:3, v/v; MCW) for 1 min at two-thirds full speed in a Virtis Model 45 Homogenizer at 0 - 4 C.

2) Quantitatively transfer homogenate to a 50 ml centrifuge tube. Centrifuge 10 min at 1000 x g. Save the resulting supernatant.

3) Wash pellet residue three times with 25 ml portions of MCW by resuspension and centrifugation. Composite the original and MCW wash supernatants.

b) Purification and fractionation of alcohol soluble sugars.

1) Concentrate composited MCW extract in an air stream at 45 C to approximately 15 ml.

2) Transfer extract to 40 ml tapered glass centrifuge tubes. Add 10 ml chloroform, shake briefly, and separate the phases by centrifugation at 800 x g for 5 min. Remove chloroform layer to a beaker with the aid of a 30 ml hypodermic syringe fitted with a 3 in cannula. Wash aqueous layer again with 10 ml of chloroform.

3) Following removal of bulk of chloroform from the second wash, concentrate residual chloroform to bottom of tube by brief centrifugation.

4) Transfer aqueous layer to a round bottom and flash evaporator flask.

5) Wash residual chloroform phase twice with 10 ml water to remove water soluble components trapped in the chloroform.

6) Add aqueous layers to original aqueous phase and concentrate under vacuum on a flash-evaporator at 45 C.

7) Transfer to 5 ml scored tubes and bring to volume.

8) Store sugar concentrate in freezer.

c) Chromatography and quantification of sugars.

1) Apply 0.05 ml of sugar concentrate as a narrow streak along a line 10 cm from the upper edge of a strip of Whatman 3 mm paper 4 cm x 50 cm.

2) Develop descending chromatograms 48 hrs. in the monophasic solvent system butanol: acetic acid: water (3:1:1, v/v).

3) Locate sugar zones by spraying a 1 mm section of strip down the center of the paper (using a plastic template) with aniline-diphenylamine phosphate spray.

4) Cut out detected zones and elute (soak out or rinse out) with water.

5) Collect eluant in 5 ml scored tubes and bring to volume with water.

6) Estimate glucose concentration using Nelson's reducing sugar test (see page 14). Determine fructose and sucrose contents by Roe's resorcinal method (see page 18).

d) Water soluble polysaccharide.

1) Suspend MCW residue in 30 ml of 10% ethanol overnight in the cold.

2) Collect insoluble residue by centrifugation at 1000 x g for 10 min.

3) Decant supernatant through a thin pad of glass wool.

4) Wash residue 4 times with 25 ml portions of 10% ethanol by suspension and centrifugation as above.

5) The composited supernatant is brought to volume and the carbohydrate content estimated by Phenol-Sulfuric acid procedure (see page 177).

e) Starch extraction by Dimethyl Sulfoxide (DMSO).

1) Heat the 10% ethanol residue 2 hrs. at 70 C in 30 ml of 95% DMSO with continuous stirring.

2) Collect insoluble residue by centrifugation at 1000 x g for 10 min. at room temperature.

3) Re-extract residue twice, as above, using 20 ml portions of 90% DMSO (more extractions may be required for mature kernels).

4) Determine total carbohydrate content of DMSO extract by Phenol-Sulfuric acid method (see page 171) which measures the glucose equivalent of starch.

5) Correct for the approximate 10% difference in weight between glucose equivalents (by Phenol-Sulfuric acid test) and starch (due to glucosidic bond formation with release of water in starch) by multiplying mg glucose equivalents x 0.88.

f) Starch extraction by calcium chloride ($Ca\ Cl_2$).

1) Extract the DMSO residue in 30 ml of 1.30 specific gravity $CaCl_2$ solution pH 7.0 for 30 min. in a boiling water bath.

2) Filter through a Buchner funnel filled with fiber glass filter.

3) Precipitate a 25 ml aliquot of the $CaCl_2$ extract with 2 volumes of ethanol. Wash precipitated material twice with 80% ethanol to remove $CaCl_2$.

4) Redissolve the precipitate containing washed polysaccharides in water and estimate the carbohydrate content by the Phenol-Sulfuric acid procedure using the 0.88 correction factor.

5) The portion of carbohydrate in this fraction is very small relative to that in the DMSO extract and may be contaminated with non-starch polysaccharides such as hemicelluloses.

Reference

Shannon, J. C. 1968. A procedure for the extraction and fractionation of carbohydrates from immature *Zea mays* kernels. Research Bulletin No. 842, Purdue University, Agricultural Experiment Station.

NELSON'S TEST FOR REDUCING SUGARS (GLUCOSE)

Principle

This procedure is a modification of Somogyi's titrimetric method for use with a colorimeter and employs a copper and an arsenomolybdate reagent. In the presence of a reducing sugar such as glucose, the copper is oxidized, forming a colored complex with arsenomolybdate. The range of the test is 5 to 600 μg of sugar.

Equipment

a) Spectrophotometer or colorimeter with a No. 520 filter

b) Colorimeter tube or cuvette

c) Test tubes, tube closures (marbles), test tube rack

d) Boiling water bath

e) Pipettes

f) 100 ml volumetric flasks

g) Magnetic stirrer and stir bar

h) Vortex test tube mixer

Reagents

a) <u>Low alkalinity copper reagent</u>.

1) Dissolve 12 g of potassium sodium tartrate (KNa $C_4H_4O_6 \cdot 4H_2O$) and 24 g of sodium carbonate (Na_2CO_3, anhydrous) in 250 ml distilled water.

2) Dissolve 4.0 g of copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) in distilled water and add to 1) with stirring.

3) Dissolve 16 g of sodium bicarbonate ($NaHCO_3$) in 2) with stirring.

4) Dissolve 180 g of sodium sulfate Na_2SO_4 in 500 ml H_2O and boil to expell air.

5) Combine 3) and 4) and dilute to 1 liter with distilled water to make Reagent A.

6) Store in polyethylene bottle with polyethylene cap.

7) Let stand 1 week before using the clear supernatant solution.

b) Arsenomolybdate reagent. (Reagent B)

1) Dissolve 25 g ammonium molybdate tetrahydrate [$(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$] in 450 ml distilled water.

2) Add to the above solution 21 ml of concentrated sulfuric acid (H_2SO_4).

3) Dissolve 3.0 g disodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) in 25 ml distilled water.

4) Combine and mix the two solutions and store in a brown glass bottle for 24 hr. at 37 C before using.

5) Reagent should be yellow with no green tint.

c) Glucose standard.

1) Dissolve 100 mg glucose in 100 ml distilled water. This can be stored under refrigeration after adding a small

amount of powdered thymol.

2) From the stock solution, pipette 1-6 ml into 100 ml volumetric flasks and bring to volume with distilled water. This gives 10-60 μg/ml standards.

Procedure

a) Add 1 ml of low alkalinity copper reagent (Reagent A) to 1 ml aqueous sugar solution, having no more than 120 μg glucose (or equivalent) in 10 ml scored test tubes. Cover tubes with marbles.

b) Heat all samples, blanks, and standards for 10 minutes in rapidly boiling water bath.

c) Cool. Then add 0.8 ml of arsenomolybdate reagent (Reagent B). Stir each tube immediately after addition.

d) Bring each tube up to 10 ml with water. Stir again very well.

e) Allow tubes to stand 15 minutes prior to reading (no more than 40 minutes after addition of Reagent B). Stir tubes just prior to reading.

f) Read OD at 500 mμ on a spectrophotometer or using a No. 520 filter on a colorimeter against a blank treated as above, but containing water instead of sugar solution.

Precautions

a) Since arsenomolybdate reagent is easily contaminated by metal surfaces, avoid metal syringes, bottle caps, etc.

b) One ml sugar aliquots should contain less than 50% ethanol.

Reference

Hodge, J. E. and B. T. Hofreiter. 1962. Determination of reducing sugars and carbohydrates. Methods in Carbohydrate Chemistry, Vol. 1:380. Edited by R. L. Whistler and M. L. Wolfrom.

MODIFIED ROE'S PROCEDURE FOR FRUCTOSE AND SUCROSE DETERMINATION

Principle

This assay, first adapted for quantitative determination by Roe, is based upon the estimation of a chromophoric substance formed by the reaction of resorcinol and hydroxymethylfurfural. Although the test is not specific for fructose, its conditions have been refined to give maximum sensitivity for quantitative estimate of this sugar with minimal interference from glucose. The test does not distinguish between free fructose and fructose contained in sucrose.

Equipment

a) Spectrophotometer or colorimeter with No. 42 filter (400-465 mμ).

b) Automatic pipette (1.0 ml).

c) Vortex test tube mixer.

d) Test tubes, tube covers (marbles), tube rack.

e) Controlled temperature water bath (77 C).

f) Ice water bath.

Reagents

a) Concentrated HCl.

b) 0.05% resorcinol in absolute ethanol (0.05 g resorcinol in final volume of 100 ml ethanol). Store in glass stoppered brown bottle.

c) Fructose or sucrose standard - use 10, 25, 40, 50 μg/ml.

Procedure

a) Use samples with 0.02 to 0.25 micromoles of sugar. Bring all samples to 1.0 ml volume with water.

b) Add 1.0 ml of resorcinol with automatic pipette.

c) Add 3.0 ml of HCl with acid syringe (under hood).

d) Mix tubes thoroughly. Cover tubes with marbles.

e) Heat at 77 C for 8 minutes. Cool immediately at 0 C (ice water bath) approximately 5 minutes.

f) Read at 420 mμ after tubes are allowed to warm up approximately 10 minutes against a water blank treated as above.

Addendum

Estimation of sucrose content in the presence of free fructose is accomplished by initial destruction of free reducing sugar (fructose) by hot alkaline (KOH) treatment of the sugar solution.

a) Add 0.1 ml of 2 N potassium hydroxide (KOH) to a 1.0 ml aliquot of sugar solution.

b) Heat tubes in a boiling water bath 10 minutes. Then cool.

c) Proceed with modified Roe's test as above, using sucrose as a standard.

Precautions

Temperature conditions are critical. Exact length of heating must be observed, and cooling must be immediate to prevent interference by glucose.

Reference

Davis, Joan S., and J. E. Gander. 1967. A re-evaluation of the Roe procedure for determination of fructose. Anal. Biochem. 19:72-79.

OIL DETERMINATION

Principle

In this method, oil and other substances obtained by petroleum ether extraction are quantitated gravimetrically with correction for sample moisture. Determinations can be made on cottonseed meals, soybeans, peanuts, sunflower seeds, sorghum seed, flax seed and other oil crops.

Equipment

a) Butt type extraction apparatus, assembled as indicated in the illustration.

b) Filter paper 150 mm, (S and S no. 597, Whatman No. 2, Reeve-Angle No. 211, or equivalent).

c) Absorbent cotton, free of petroleum ether extract.

d) Porcelain mortar and pestle, the mortar must be at least 4 inches in diameter at the top. The pestle handle must be large enough to afford a firm hand grip. The inner surface of the mortar is kept rough by occasionally grinding sand in it.

e) Sieve, U.S. No. 20

f) Sieve, U.S. No. 30

g) Laboratory mill suitable for grinding the samples to a maximum particle size of the U.S. No. 20 sieve (Use No. 30 for linseed meal).

Procedure

a) Grind the 100 g portion from A.O.C.S. Official Method Ba 3-38, Section B, through a laboratory mill to a uniform fineness, ca. 20-mesh (about 30-mesh for linseed meal). Return immediately to an air-tight container. Oil and ground moisture are determined on this portion.

b) Weigh about 3 g of the ground sample into a filter paper folded in such a fashion as to prevent escape of the ground sample (see illustration). The second paper is left open at the top like a thimble. A piece of absorbent cotton may be placed in the top of the thimble to distribute the solvent as it drops on the sample.

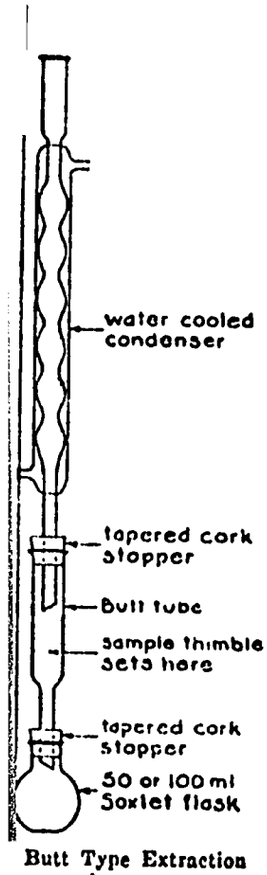
c) Place wrapped sample in the Butt extraction tube and assemble the apparatus.

d) Put about 25 ml of petroleum ether into the tared extraction flask before attaching to the tube.

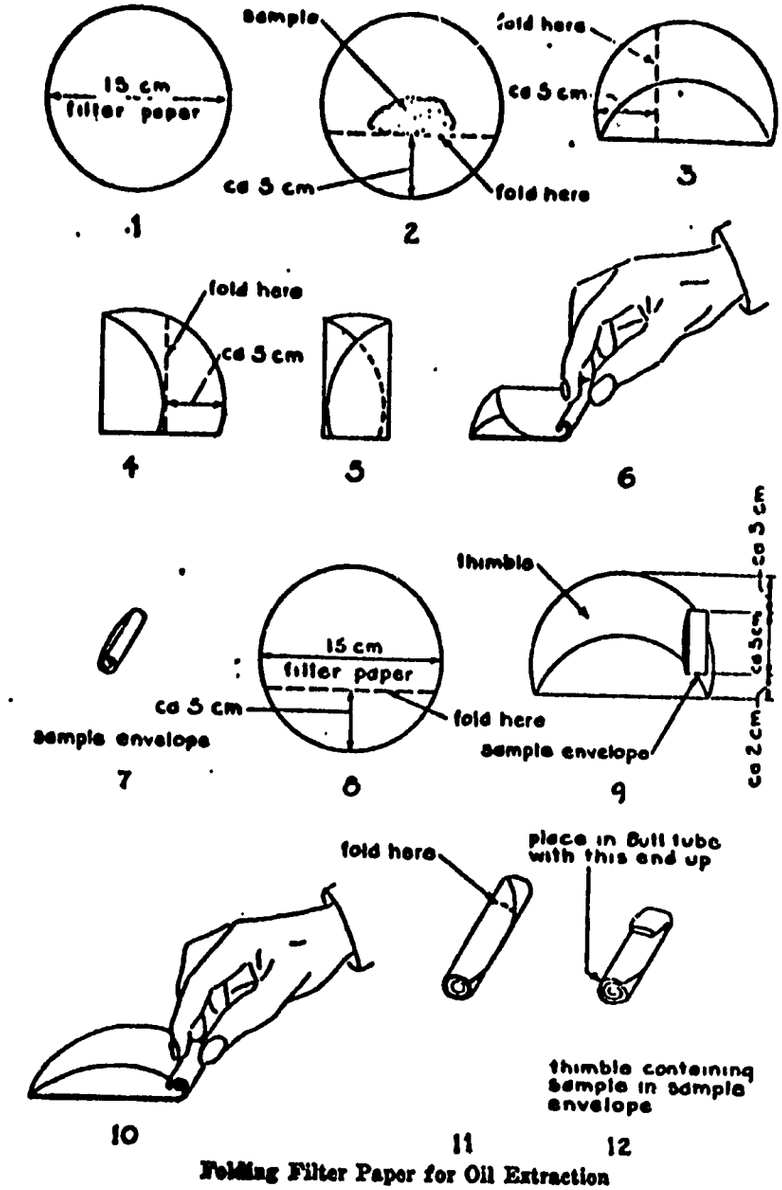
e) Heat on a water bath or electric hot place at such a rate that the solvent will drop from the condenser on the center of the thimble at the rate of about 150 drops per minute.

f) Keep the volume of solvent fairly constant by adding enough to make up for any that may be lost due to evaporation. Continue extraction for 3 hours.

g) Cool and disconnect the extraction flask. Evaporate the ether



Butt Type Extraction Apparatus



Folding Filter Paper for Oil Extraction

on a steam or water bath until no odor of ether remains. A gentle stream of clean, dry air may be used to facilitate removal of the solvent. Cool to room temperature, carefully remove any moisture or dirt from the outside of the flask and weigh. Repeat heating until constant weight is obtained.

h) Determine the moisture in the ground sample by taking 3 g (oven method).

Calculation

$$\text{Oil } \% = \frac{\text{weight of oil} \times 100}{\text{weight of sample}}$$

The percentage of oil may be calculated to any desired moisture basis with the following formula:

$$\text{Oil, desired moisture basis, } \% = \frac{F\ (100 - \%\ \text{moisture desired})}{100 - \%\ \text{moisture in sample analyzed}}$$

Note: F = % oil in sample analyzed.

References

A.O.C.S. Official Method Ba 3-38. Sampling and analysis of oilseed by-products. April 1954.

ANALYSIS FOR CAROTENES AND XANTHOPHYLLS IN DRIED PLANT MATERIAL

Principle

A method is presented for the analysis of carotenes, carotenols, and carotendiols in dried plant materials. The extraction procedure is applicable to both alfalfa meals and corn products, as well as yellow endosperm sorghum grain. Column chromatography on silica gel G. permits separation of carotenes from xanthophylls and subsequent fractionation of xanthophylls into monohydroxy (MHP) and dihydroxy pigments (DHP) which are free from polyoxy pigments. A procedure is proposed for calculation of a "DHP-equivalent" as a measure of pigmenting effectiveness for avian skin and yolks. The method appears to be applicable to mixed feeds. The need for an improved analytical procedure for the carotenes and for an acceptable procedure for xanthophylls in dried plant materials is widely recognized. Xanthophylls are of commercial interest primarily for their avian-pigmenting properties. However, methods which have been proposed for total xanthophylls are inadequate because monohydroxy pigments (MHP) are inferior to the dihydroxy pigments (DHP) and polyoxy pigments have little or no pigmenting ability. For maximal extraction of the carotenoid pigments, different solvent systems, depending on origin of product, have seemed necessary. Thus, a mixture of toluene, ethanol, ethyl acetate, and water (TEEW) has been used widely for corn products. Extraction of alfalfa meals with a hexane-acetone mixture, used in

the official method, seems to be improved by addition of alkali. If mixtures such as occur in poultry feeds are to be analyzed, a solvent system suitable for use with all components must be available.

In the present work, a mixture of hexane, acetone, ethanol, and toluene was a suitable extractant for corn products, alfalfa meals, and mixed feeds which contain these materials. The extracts have been chromatographed to obtain fractions of carotenes and the different classes of xanthophylls.

Equipment

a) Chromatographic tube - 12.5 mm id x 30 cm, pyrex, with bottom capillary tube 2 mm id x ca 10 cm to extend into neck of 25 ml vol. flask.

b) Vacuum filtration device - for collection of eluate in vol. flask (Fisher Scientific Company "Filtrator" or equivalent). Attach rubber stopper to column to fit device.

Reagents

a) Acetone - dry, alcohol-free. Distill over Zn (granular, ca 10 mesh).

b) Hexane - Com., Phillips Petroleum Company "High purity", or equivalent.

c) Extractant - Hexane-acetone-absolute alcohol-toluene.

d) Adsorbent I. Mix in mechanical blender one to two hours 1 + 1 (w/w) silica gel G. (according to Stahl, Brinkmann Instruments, Inc.) and diat. earth (Hyflo Super-Cel, Fisher Scientific Company).

e) Adsorbent II. Mix in mechanical blender one to two hours 1 + 1 (w/w) activated magnesia (Sea Sorb 43, Fisher Scientific Company) and diat. earth (Hyflo Super-Cel).

f) Methanolic potassium hydroxide - 40%. Dissolve 40 g KOH in MeOH, cook, and dilute to 100 ml with MeOH.

g) Sodium sulfate solution - 10%. Dissolve 10 g anhydr. Na_2SO_4 in 100 ml H_2O.

h) Eluants.

1) Carotenes - Hexane-acetone (96 + 4)

2) Monohydroxy pigments (MHP). Hexane-acetone (90 + 10).

3) Dihydroxy pigments (DHP). Hexane-acetone (80 + 20).

4) Total xanthophylls (TX). Hexane-acetone-MeOH (80 + 10 + 10)

i) 1) 1-(Phenylazo) - 2 -naphthol (C.I. Solvent Yellow 14; Sudan I) standard solutions.

2) Stock solution - 1.0 millimolar (mM). Recrystallize Standard (Aldrich Chemical Company or MC/B Manufacturing Chemists) from hot absolute alcohol. Dry crystals to constant weight in 70 C vacuum oven. Dissolve 0.1241 g in 500 ml acetone-isopropanol (4+1).

3) Working solution - 0.04 mM. Dilute 20 ml stock solution to 500 ml with acetone-isopropanol (1+1). Store in dark.

Preparation of Sample

Grind sample to pass No. 40 sieve. Accurately weigh sample (2 g corn gluten or alfalfa meal; 50 mg marigold meal; 4 g mixed feed) into 100 ml vol. flask. Pipet 30 ml extractant into flask, stopper, and swirl one minute. For low moisture samples, e.g., marigold meal, dehydrated alfalfa, or corn gluten (not air dried samples), also pipet 1 ml H_2O/2 g sample into flask, stopper, and swirl one minute. For high moisture (air dried) samples, omit addition of H_2O.

a) Hot Saponification - (for rapid extraction and for samples containing xanthophylls esters), pipet 2 ml (2 ml for 4 g samples of mixed feed) 40% methanolic KOH into flask, swirl one minute, and place flask in 56 C H_2O bath 20 minutes. Attach air condenser or cool neck of flask to prevent loss of solvent. Cool sample, and let stand in dark one hour. Pipet 30 ml hexane into flask, swirl one minute, dilute to volume with 10% Na_2SO_4 solution, and shake vigorously one minute. Let stand in dark one hour before chromatography. Upper phase is 50 ml.

b) Cold (overnight) Saponification - (For samples not containing xanthophyll esters). Let mixture stand in dark about 16 hours. Pipet 2 ml 40% methanolic KOH into flask and swirl one minute; let stand in dark one hour; then proceed with hexane addition as in a).

Chromatography

With column on Filtrator, place absorbent cotton or glass wool plug in bottom and add ca 12 cm layer Adsorbent 1. Apply full vacuum and add more adsorbent to give 7 cm layer. Use flat instrument such as inverted cork on glass rod to press and flatten surface of adsorbent. Place 2 cm layer anhyd. Na_2SO_4 above adsorbent and press firmly.

a) Total carotenes.

With 25 ml volumetric flask as receiver, pipet 5 ml (or 10 ml if low pigment) of upper phase onto column and adjust vacuum for flow of 2 or 3 drops/second. Needle valve in vacuum line helps control flow rate. Add carotene eluant as last of solution enters adsorbent and continue until carotene band is collected in flask. Keep adsorbent covered with solvent at all times. Release vacuum, place carotene solution in dark until it reaches room temperature and dilute to volume with carotene eluant. Invert flask several times to mix; then determine A immediately.

Xanthophylls remain on column. For separation of mono-

hydroxy from dihydroxy pigments (both free from epoxy and polyoxy pigments), or for total xanthophylls, proceed as in b) or c).

b) Separation of xanthophylls.

1) With 25 volumetric flask in Filtrator and vacuum applied to column let eluant level approach adsorbent surface; then immediately add MHP eluant. Band of monohydroxy pigments (zeinoxanthin, cryptoxanthin) and any persistent mono- or di-esters should move down column ahead of other bands. When elution of MHP band is complete, place flask in dark to attain room temperature before diluting to volume with MHP eluant and A determination.

2) Proceed as in 1) using DHP eluant to collect pigments of next band (lutein, zeaxanthin, and their isomers) in 25 (or 50) ml volumetric flask. Violaxanthin, neoxanthin, and other polyoxy pigments (POP) remain on column.

c) Total xanthophylls.

If value for total xanthophylls is desired, pipet fresh aliquot from upper phase of original extract onto 7 cm column of Adsorbent II, and elute carotenes with hexane-acetone-MeOH (80+10+10).

Determination

Measure A promptly to minimize isomerization and autoxidation losses. First check calibration of spectrophotometer by reading working standard solution at 1 nm intervals between 469 and 479 nm. If maximum value is not at 474 nm, recalibrate instrument. When instrument shows maximum A

at 474 nm and slit width is 0.03, working solution readings should be 0.561 (474) and 0.460 (436). Correct calculations from equations below, for instrument deviation factor. If instrument lacks controllable slit, estimate concentration by assuming that working standard solution of dye, (1) (2), has same A as 2.35 mg carotenes/L at 436 nm and 2.38 mg xanthophylls/L at 474 nm.

Determine A of carotene fraction at 436 nm and MHP and DHP fractions at 474 nm. For highest accuracy, control solution volumes to give A between 0.25 and 0.75.

Calculations

The following equations are applicable to A data obtained from calibrated spectrophotometers that operate with narrow slit width. Values 196 and 236 are a for trans-B-carotene and trans-lutein at prescribed wavelengths; b - cell length in cm; d - dilution factor = (g sample x ml extract on column)/(50 ml upper phase x ml final dilution); and f = instrument deviation factor = 0.460/observed A_{436} or 0.561/observed A_{474}. Carotene fraction concentration (mg/lb) = $(A_{436} \times 454 \times f)/(196 \times b \times d)$. MHP fraction concentration (mg/lb), or DHP fraction concentration (mg/lb), or total xanthophylls (mg/lb) = $(A_{474} \times 454 \times f)/(346 \times b \times d)$.

Reference

Quackenbush, F. W., M. A. Dyer, and R. L. Smallidge. 1970. Analysis for carotenes and xanthophylls in dried plant materials. Journal of the AOAC. Vol. 53, No. 1, 1970.

MICRO-KJELDAHL DETERMINATION OF TOTAL NITROGEN

Standard Method

Principle

Micro-Kjeldahl procedures have been widely used for total N analysis because of increased speed of analysis, decreased cost per sample, smaller sample size required, and the reduced volumes of corrosive acid and alkali that are handled by the analyst compared to the macro-Kjeldahl procedure.

The maximum number of analyses that can be performed daily is generally limited by the lack of digestion heaters due to the necessity of using them under a hood.

Equipment

a) Digestion heater rack capable of refluxing H_2SO_4

b) Steam distillation apparatus

c) Titration apparatus

Reagents

a) Catalyst: Mix (in a Waring blender) 190 g potassium sulfate (K_2SO_4) and 4 g mercuric oxide (HgO) under a hood. Caution: HgO is highly toxic.

b) Sulfuric Acid: Use concentrated reagent grade H_2SO_4.

c) Potassium sulfide - Sodium hydroxide:

1) Place a 1 liter beaker in a bucket of ice on a magnetic stirrer.

2) Add 500 ml distilled water and a stirring bar.

3) Add 40 g potassium sulfide (K_2S) and mix until dissolved.

4) Add 600 g sodium hydroxide (NaOH) pellets, about 50 g at a time. Mix each batch until completely dissolved.

5) Make up to 1 liter with distilled water.

6) When cool, store in a stoppered plastic bottle.

d) Boric Acid: Dissolve 160 g of H_3BO_3 in 1 liter of distilled water and bring up to 4 liters with distilled water.

e) Standard Hydrochloric Acid:

1) Add about 7 ml reagent grade concentrated HCl to 4 liters of distilled water in a ground glass stoppered bottle. Shake well.

2) Dry sodium carbonate Na_2CO_3 in an oven at least 2 hours. Cool in a desiccator.

3) Weigh out approximately 30 mg Na_2CO_3 and place in a 125 ml Erlenmeyer flask. Record exact weight.

4) Add 40 ml distilled water, 4 drops of indicator and titrate with the HCl solution to the apparent grey-pink end point.

5) Carefully boil the titrated solution and cool to room temperature.

6) If the blue color of the indicator returns, continue the titration until the end point is again reached.

7) Repeat boiling and titrating until the end point is stable.

8) Run a blank containing only water and indicator.

9) Calculation:

$$\text{Normality HCl} = \frac{\text{mg } Na_2CO_3}{[\text{ml HCl (Titrated)} - \text{ml HCl (blank)}] \times 52.997}$$

Averaged normality of three replicates will be about 0.02 N.

f) Indicator:

1) Weigh out 20 mg methyl red and 100 mg bromcresol green.

2) Add dyes to 60 ml of 70% ethyl alcohol.

3) Dissolve by mixing on a magnetic stirrer and store in a stoppered brown bottle.

Procedure

a) Weigh out 50 mg of ground sample into 30 ml Kjeldahl flasks.

b) Add 2.5 ml concentrated H_2SO_4 and 2 g catalyst.

c) Place flasks on preheated digestion heaters.

d) Rotate and shake flasks periodically during clearing to wash down all particles into the acid. Allow samples to digest 30 minutes after clearing.

e) Remove from heaters, and allow to cool 2 minutes. Add 8-10 ml distilled water. Reheat flasks if necessary to redissolve the flask contents.

f) Transfer digest to distillation apparatus. Rinse the flask 4 - 5 times with 1 - 2 ml por. 's of water.

g) Place a 125 ml Erlenmeyer receiver flask containing 5 ml of boric acid and 4 drops of indicator under the condenser with its tip extending below the surface of the solution.

h) Add 15 ml NaOH-K_2S solution and steam distill until 50 ml of distillate is collected.

i) Titrate distillate to a grey-pink end point with 0.02 N standardized HCl diluted five-fold.

j) Prepare blanks by digesting, distilling, and titrating 2.5 ml H_2SO_4 plus 2 g catalyst in the same manner as the samples.

Calculations

$$\% N = \frac{[(\text{ml HCl sample}) - (\text{ml HCl blank})] \times \text{normality of HCl} \times \frac{14.007}{5} \times 100}{\text{sample dry weight (mg)}}$$

% Protein = % N x conversion factor (page 38)

Reference

Assocation of Official Analytical Chemists. 1970. Methods of Analysis, 11th Edition. p. 858.

Modified Method

Principle

The modification of Concon and Soltess differs from the standard

micro-Kjeldahl procedure in that hydrogen peroxide is employed as an oxidant to destroy foam-forming materials and speed digestion. It has proved useful in our laboratory both for routine and foam-producing samples to reduce clearing time.

Equipment

As for standard method.

Reagents

As for standard method with addition of: Hydrogen peroxide (H_2O_2), 30% solution.

Procedure

As for standard method with substitution of steps c), d), and j) with:

c) Place flasks on preheated digestion heaters. Heat rapidly until a full bloom foam threatens to escape up the neck of the flask (about 15-30 sec.).

d) Remove from the heater and while still hot, slowly add 1 ml of 30% H_2O_2 down side of tube while rotating it. Mix well, while adding oxidant to hasten the decomposition of foam forming products. If foaming is still excessive, add an extra 0.5 - 1.0 ml H_2O_2. Continue heating an additional 5 min. after the digest has cleared.

j) Run a blank containing acid and catalyst with H_2O_2 added in same manner as above.

Precautions

a) Add H_2O_2 with extreme care, under a hood with hood sash partially closed, and with Kjeldahl flask mouth aimed away from the face due to rapid evolution of gas on addition.

b) Cool blanks several minutes before adding H_2O_2 since the oxidant vigorously decomposes at high temperatures in the absence of organic matter.

Reference

Concon, J. M. and Diane Soltess. 1973. Rapid micro-Kjeldahl digestion of cereal grains and other biological materials. Anal. Biochem. 53:35-41.

PREPARATION OF DEIONIZED WATER

Introduction

Distilled water is normally used in Kjeldahl nitrogen determinations in the steam generator, for dilution of samples after digestion, and in reagents. Unfortunately, distilled water frequently contains small amounts of dissolved ammonia which may contribute to high blanks. The following procedure describes a simple apparatus for removal of ammonia from water by passage through a bed of ion-exchange resin.

Equipment

a) One gallon water bottle fitted with a 2-hole rubber stopper.

b) Glass tubings (4mm ID) 6 and 7 cm long inserted through the 2-hole stopper such that the 1 cm difference in lengths extends outside of the bottle.

c) Glass column (2.5 cm ID) 30 cm long equipped with a stopcock at the tapered end.

d) Glass wool.

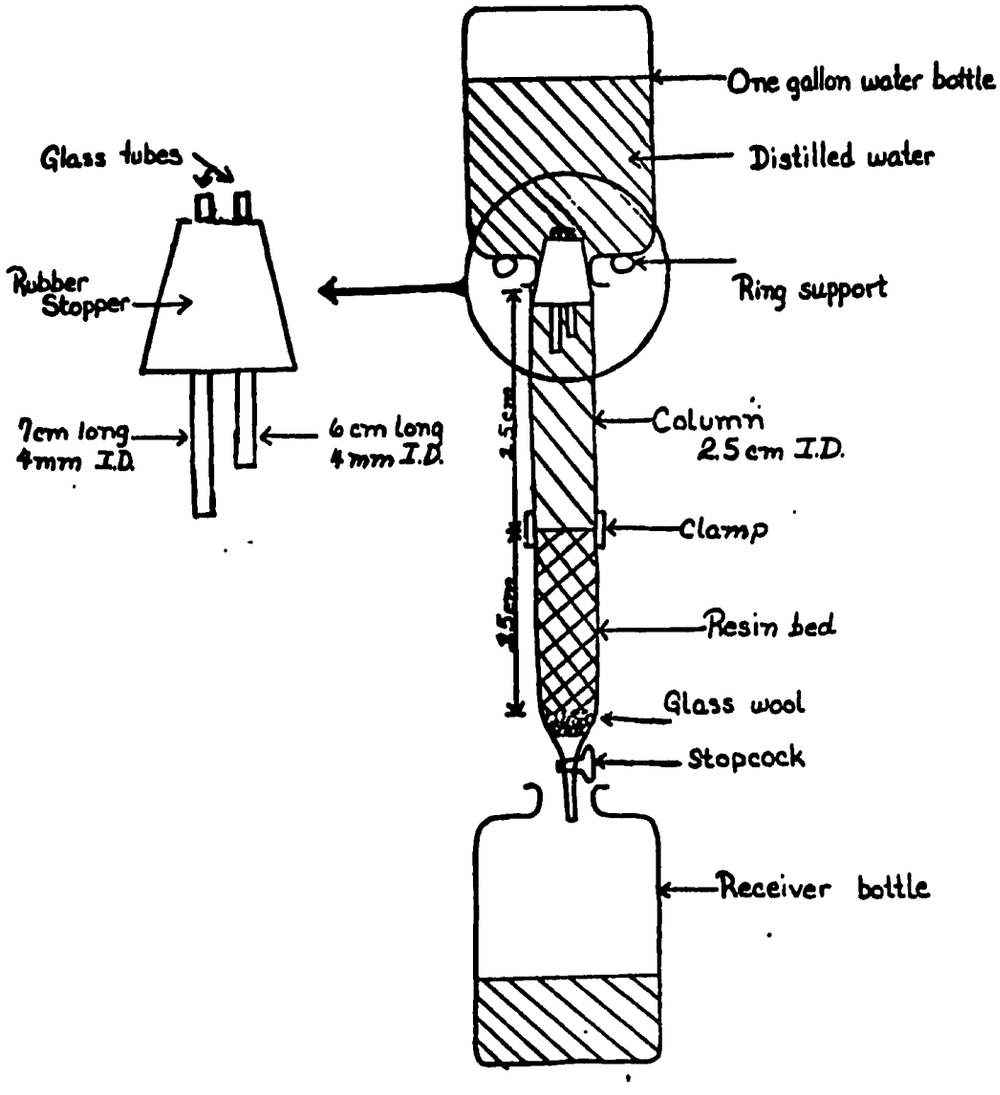
e) Strong acid cation exchange resin; e.g. Amberlite IR-120.

f) Two gallon receiver bottle.

Assembly

a) Fill one gallon water bottle with distilled water.

b) Insert 2-hole rubber stopper fitted with 6 and 7 cm glass



not drawn to scale

tubings into water bottle. (Water bottle should be supported in inverted position by a ring clamp or equivalent.)

c) Plug 30 cm column with glass wool and pack with a strong cation exchange resin to 2 heights of 15 cm.

d) Invert the one gallon water bottle onto the column and place the two gallon receiver bottle below column.

e) Control water flow rate with stopcock.

Note

a) A suitable apparatus can be assembled following the above procedure but any arrangement providing a steady flow of distilled water over the resin can be used.

b) Rate of flow of distilled water should be about 1 liter per minute. The above apparatus feeds water automatically at the rate determined by the setting of the stopcock.

c) The cation exchange resin should be flushed with hydrochloric acid once every two months.

Reference

Shuman Chemical Laboratory, Inc., Battle Ground, Indiana

FACTOR TO CONVERT PERCENT N TO PERCENT PROTEIN FOR DIFFERENT SPECIES AND PLANT PRODUCTS

Cereals:	Factor
Barley (*Hordeum vulgare*) whole seed	5.83
Buckwheat (*Fagopyrum sagittatum*) hulled, dark flour	6.25
Foxtail Millet (*Setaria italica*)	6.25
Maize (*Zea mays*) (grain or whole meal)	6.25
Millet (*Pennisetum* spp.) grain	6.25
Oats (*Avena sativa*) meal	5.83
Proso Millet (*Panicum miliaceum*) husked	6.25
Rice (*Oryza* spp.) brown or husked	5.95
Rye (*Secale cereals*) whole meal	5.83
Sorghum (*Sorghum* spp.)	6.25
Teff (*Eragrostis tef*) whole	6.25
Wheat (*Triticum* spp.) whole grain	5.83
" " germ	5.80
" " bran	6.31
" " flour 80-90% e.r.	5.70
" " flour 70-80% e.r.	5.70
" " flour 60-70% e.r.	5.70
" " parboiled (Bulgur)	5.83

	Factor
Legumes:	
Bean (*Phaseolus vulgaris*)	6.25
Broad Bean (*Vicia faba*)	6.25
Chickpea (*Cicer arietinum*)	6.25
Cowpea (*Vigna* spp.)	6.25
Groundnut (*Arachis hypogaea*)	5.46
Lentil (*Lens culinaris*)	6.25
Lima Bean (*Phaseolus lunatus*)	6.25
Lupine (*Lupinus* spp.)	6.25
Pea (*Pisum sativum*)	6.25
Pigeon Pea (*Cajanus cajan*)	6.25
Soybean (*Glycine max*) seed	5.71
Vetch (*Vicia sativa*)	6.25
Nuts and Seeds (generally)	5.30
Exeptions: Almond (*Prunus amygdolus*) dry	5.81
Colewort (*Crambe abysinica*)	6.25
Lotus, Egyptian (*Nymphaea lotus*)	6.25
Parinarium (*Parinari pumila*)	6.25
Pecan (*Carya illinoensis*)	6.25
Tropical Almond (*Terminalia catappa*)	6.25
Turnip seed (*Brassica rapa*)	6.25
Vegetables and forages	6.25
Fruits	6.25
Milk and Milk Products	6.38

	Factor
Fish and Fish Products	6.25
Yeast and Algae	6.25

Reference

Amino Acid Content of Foods and Biological Data on Proteins. By the Food Policy and Food Service, Nutrition Division, Food and Agriculture Organization of the United Nations. Rome, Italy. No. 24, 1970.

DYE BINDING PROCEDURE FOR ESTIMATING PROTEIN AND LYSINE

Principle

The dye binding procedure employs a monosulfonic azo dye, acid orange 12, to estimate protein, and, in conjunction with an independent protein determination, basic amino acids in grain and other natural products. The dye, which is used in a stabilizing pH 2.0 buffer system, reacts rapidly with basic groups of the protein which originate from the basic amino acids of histidine, arginine, and lysine. The reaction rate is limited by the exposure of each binding site. Protein bound dye is precipitated from solution, resulting in a decrease in absorbance which is measured colorimetrically and which is directly related to protein x basic amino acid concentration. Because levels of arginine and histidine vary little across lines within most cereal grains, the dye binding procedure has been widely used as a rapid screening method for lysine.

Equipment

General lab equipment

a) Clock timer

b) Small plastic funnels

c) Magnetic stirrer

d) Hot plates

e) 10 ml volumetric pipette

f) 200 ml, 1000 ml volumetric flask

g) 1 ml pipettes

h) 50 ml, 4000 ml beakers

Items which may be purchased from Tecator/Udy Inc., 1898 South Flat Iron Court, Boulder, Colorado 80301 include:

a) Balance (the Tecator/Udy torsion balance (S-1400) is adequate, but any balance sensitive to 1 mg will suffice and may be preferable.)

b) Reagent dye concentrate (SL-1213)

c) 20 L dispensing carboy (SL-1200)

d) Cuvet cleaner (SL-1520)

e) Reference dye concentrate (SL-1513)

f) Filter assembly (SL-1115)

g) Filter discs (200) (SL-1108)

h) Calibration test kit (SL-1511)

i) Colorimeter (Model 101) - A standard single beam colorimeter preferably with flow through cell, and short light patch cuvet will substitute, but this model is best for the money if you are assaying many samples.

j) 40 ml automatic pipettor (SL-1224) is recommended for dispensing carboy.

k) Feeler gauge for cleaning cuvet (SL-1125)

l) Spare parts: extra 4-way stopcocks (SL-1219)

extra photocells (SL-1110)

extra colorimeter lamps (SL-1101)

extra weighing pan (SL-1401)

Items which may be purchased elsewhere include:

a) Garver deluxe electric tester centrifuge, (Model 73 Z-278N-73 from Nasco Agricultural Scientific Products, Ft. Atkinson, Wisconsin 53538).

b) Eberbach variable speed table shaker (8287-E-15 from A. H. Thomas, Vine Street at Third, P.O. Box 779, Philadelphia, Pennsylvania 19105).

c) Plastic bottles (if the Garver tester centrifuge is used). The plastic bottles in which Tecator/Udy dyes come do not fit the centrifuge. One can order proper fitting bottles from Continental Glass Company, 4000 Meadow Drive, Indianapolis, Indiana 42605. (S-61264 - 2 oz. 18-410 polyethylene Boston Rounds)

d) Caps may be ordered from Bel Arts Products, Applied Coating, Inc., Pequawnock, New Jersey 07440. (F-12627-18 18 mm with polypropylene screw caps). Soak off inner cardboard liners prior to use.

It has been the experience of the Purdue Laboratory that 15-minute centrifugation in a Garver tester centrifuge removes protein particles from the supernatant equally as well as filtration through the Tecator/Udy apparatus, except for sugary lines of sorghum where the centrifugation procedure gives dye binding capacity readings of 2-4 units higher than the filtration procedure. The centrifugation procedure has the advantage of not requiring filter assembly and washing. However, one must obtain proper fitting 2 oz. plastic bottles if the Garver tester centrifuge is used.

Reagents

Reagent dye:

a) Unscrew the reagent dye dispenser carboy. Empty out the 40 ml delivery system. Wash out the dispenser carboy by several rinses of deionized water. Let dry.

b) Heat approximately 8 liters of distilled water in 4000 ml beakers on hot plates to 70 C.

c) Warm dye by placing in hot tap water for one hour or more. (Dye is in ½ gallon plastic jug.)

d) Pour 12 liters of warm water (8 liters hot + 4 liters at room temperature) into the 20 L calibrated mixing carboy.

e) Add dye concentrate being very careful not to spill any.

f) Rinse ½ gallon dye container three or four times into the carboy and bring volume to 20 liter mark.

g) Tap sides of carboy with rubber-tipped hammer to remove air bubbles. If necessary, refill to the mark.

h) Using a magnetic stirrer, mix contents 30 minutes, incubate one day and mix again 5 minutes.

Reference dye:

a) Warm reference dye about ½ hour in warm water.

b) Pour all the dye intoclean, dry 1 liter volumetric flask.

c) Rinse dye bottle three to four times into flask.

d) Complete dilution with warm distilled water (75 F) up to the calibration mark.

e) Tap sides of flask with rubber-tipped hammer to remove air bubbles. Refill to mark if necessary.

f) Invert flask about ten times to mix thoroughly.

Dye Standardization

Reagent dye: (to be done each time new dye is prepared)

a) Take a 10 ml aliquot of freshly prepared dye with a 10 ml volumetric pipette. Place in 25 ml beaker.

b) Dilute it exactly 1:1 with distilled water. Mix thoroughly.

c) Read on Tecator/Udy colorimeter previously standardized to 42.00.

d) If reading of 1:1 diluted dye is higher than 36.25:

1) Add dye concentrate from opened bottle reserved for

this purpose at the rate of no more than 1.0 ml per trial to the dye carboy.

2) Mix dye thoroughly (3 min.) on magnetic stirrer.

3) Withdraw 10 ml with volumetric pipette as above.

4) Dilute with 10 ml distilled water as above.

5) Read on Tecador/Udy colorimeter just previously standardized to 42.00.

6) Repeat process until colorimeter reading is 36.25, dye is now ready to use.

e) If reading of the 1:1 diluted dye is lower than 36.25:

1) Add distilled water at the rate of no more than 2.0 ml per trial to the carboy.

2) Stir three minutes.

3) Withdraw 10 ml aliquot, mix with 10 ml distilled water.

4) Read on just previously standardized Tecador/Udy colorimeter.

5) Repeat process until reading is 36.25.

Reference dye: (to be done each time new dye is prepared)

a) Standardize Tecator/Udy colorimeter at 42.00 with the standard reference dye found in the calibration kit (or reference dye labelled "60").

b) Remove 10 ml of newly prepared reference dye from the 1000 ml volumetric flask with a volumetric pipette and run through the colorimeter.

c) If reading is higher than 42.00 add no more than 1.0 ml of reference dye concentrate to the volumetric flask, shake thoroughly. Invert 10 times.

d) Remove 10 ml aliquot with volumetric pipette and read on Tecator/Udy colorimeter.

e) Repeat until reading is 42.00, standardizing with standard reference dye from calibration kit each time.

f) If reading is lower than 42.00 add no more than 0.2 ml distilled water to volumetric flask, shake thoroughly.

g) Remove 10 ml from volumetric flask. Read on colorimeter.

h) Repeat until reading is 42.00, standardizing with standard reference dye from calibration kit each time. Reference dye is now ready to use.

Calibration Instructions

a) Calibration with the standard dyes contained in the Tecator/Udy calibration kit should be done once a month and after changing the colorimeter lamp or photocell. Instructions are included with the kit.

b) Calibration of the 40 ml carboy dispenser:

   1) Dispense 5 aliquots (about 40 ml) of reagent dye into a clean dry 200 ml volumetric flask. Tap flask to break all bubbles.

   2) Take an additional 40 ml aliquot of dye from the dispenser into a 100 ml beaker.

3) If the 5 aliquot volume is less than 200 ml, add dye to bring the meniscus up to the graduation line. Record the volume of dye needed to do so as (-) volume.

4) If the 5 aliquot volume is more than 200 ml, remove dye to bring the meniscus down to the graduation line. Record the volume removed as (+) volume.

5) If the volume needed to bring level of dye to 200 ml line is greater than ± 0.1 ml then recalibration will be needed by moving the set screw.

Routine Dye Binding Procedure

a) Allow the colorimeter to warm up one hour before use.

b) Weigh 1 g ± 1 mg ground samples into 2 oz. plastic screw cap bottles or equivalent container.

c) Dispense 40 ml of reagent dye into each bottle from the dispense carboy by turning the knob clockwise at 30° angles.

d) Cap bottles making sure that they are screwed on tightly.

e) Place bottles in shaker with caps facing one direction. Secure them by wedging cards or paper in one end of shaker table.

f) Shake one hour at low speed.

<u>Sample screening by centrifugation</u>:

a) After shaking remove 20 bottles from the shaker rack and place in milk tester centrifuge.

b) Insure that centrifuge is balanced by putting bottles directly opposite each other in the rotor head.

c) Centrifuge 15 minutes. Do not brake when stopping centrifuge.

d) Remove bottles carefully and line them up in order to run on the colorimeter.

e) Place the remaining 20 bottles in the centrifuge and spin for 15 minutes.

f) Standardize colorimeter by pouring reference dye into cuvette. When the colorimeter needle stops moving adjust needle to 42.00 with black knob. The pinch clamp on flow through tubing must be removed to get flow started. Repeat the standardization every ten samples.

g) Take first bottle, unscrew cap while firmly holding the base on the counter top.

h) Carefully pour supernatant solution into cuvette, filling twice. On the second time when the needle is constant and the solution is dripping, record the percent transmission value.

<u>Sample screening by filtration:</u>

a) Insert a filter support into the cap of the filter assembly. Place one filter paper on it.

b) Screw filter cap (containing the support and filter paper) tightly onto the filter holder (half cut plastic bottle), otherwise dye will run out before reaching filter paper.

c) Make sure the filter paper is not torn.

d) Place a filter assembly in the cuvette. Pour half the contents of a bottle into the filter.

e) Make sure that the filter is dripping into the cuvette and that the sample is running through the cuvette base into the collection bottle. If not, blow on the cuvette rim to force the sample through it.

f) Allow time for the needle to stop completely, then read percent transmission value.

Interpretation of Data

The percent transmission value (UIR) obtained from the Tecator/Udy colorimeter can be converted to AO-12 dye concentration using semi-log paper by plotting on the log scale UIR values against dye concentration. Standard dye concentrations in the range of 0.350 - 0.850 mg/ml and UIR to dye concentration conversion tables may be obtained from the Tecator/Udy Company.

Mg dye bound/g sample = 40 (1.3000 mg/ml - sample dye concentration mg/ml.)

Because of the great variability of protein content in grain sorghum, meaningful screening for high lysine lines requires an independent protein determination in conjunction with dye binding analysis. In this manner individual samples which deviate a given amount from the regression line obtained by plotting UIR or mg dye bound/g sample or dye concentration values against percent protein for all samples can readily be identified as high lysine lines at any given protein level.

References

Udy, Doyle C. 1971. Improved dye method for estimating protein. J. of Amer. Oil Chemists, Vol. 48.

RAPID NINHYDRIN TESTS

Principle

Mertz et. al. 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. However, kernels with vitreous endosperm can be ground prior to testing.

Reagents

a) Grind a mixture containing 16 parts (by weight) of ninhydrin (practical grade), 58 parts of sodium citrate and 26 parts of citric acid to a fine powder with a mortar and pestle.

b) Store the mixture in a brown glass bottle and protect from moisture to avoid deterioration.

Procedures

a) <u>Floury endosperm cereals</u>:

1) Non-destructive method for single seeds:

a) Expose the endosperm near the crown or apex by rubbing with sandpaper.

b) Mount seed on modeling clay.

c) Apply a 3% ninhydrin reagent solution (300 mg in 10 ml water) to the exposed endosperm.

d) Allow to stand 5 minutes, then warm the surface with a source of hot air (i.e. hair dryer) or a heat radiating surface.

e) Treat a control normal kernel and a high lysine kernel (if available) the same way and note the difference in color intensity. The high lysine kernel should have a much darker blue color.

b) Destructive method:

1) 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.

2) Cover with 10 ml of 3% ninhydrin reagent solution and heat to boiling.

3) Allow tubes to stand 5 min. and compare with similarly treated normal kernels.

4) If colors are too intense, dilute all samples equally with water; if too faint, increase the number of kernels per tube.

c) Vitreous endosperm cereals:

1) Non-destructive method for single seeds:

a) Remove a maximum amount of endosperm from a vitreous seed without damaging the embryo.

b) Weigh endosperm portion roughly and place in an 8 x 75 mm Pyrex test tube.

c) Approximately the same weight of endosperm from a normal (and high lysine kernel if available) is placed in a separate test tube.

d) Endosperms are ground to a powder in a small mortar and pestle and returned to the test tubes.

e) One ml of 3% ninhydrin reagent solution is added, the tube heated to boiling and cooled.

f) Color develops within 5 minutes.

Precautions

a) Use only fresh seed for analysis. Slow proteolysis occurs in kernels during storage resulting in accumulation of free amino acids which obscure any differences between seeds.

Reference

Mertz, E. T., R. Jambunathan and S. Misra. 1975. Simple chemical and biological methods used at Purdue University to evaluate cereals for protein quality. Stat. Bull. No. 70, Biochemistry Department, Purdue University, W. Lafayette, Indiana 47907.

ANALYTICAL NINHYDRIN TEST

Principle

If the difference between the color of a possible high lysine cereal and its normal counterpart is not marked enough to classify the former or if confirmation of the visual test is desired, a confirmatory ninhydrin test can be performed.

Reagents

a) N-propyl alcohol (50% aqueous, V/V)

b) Reagent A:

   1) Dissolve 400 mg reagent grade stannous chloride ($SnCl_2 \cdot 2H_2O$) in 250 ml of sodium citrate buffer (4.3 g citric acid and 8.7 g sodium citrate diluted with water to 250 ml after adjusting to pH 5.0 with NaOH).

c) Reagent B:

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

   Mix Reagents A and B 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.

Equipment

a) 125 ml Erlenmeyer flasks

b) Shaker

c) Centrifuge

d) Pyrex test tubes (15 x 150 mm)

e) Boiling water bath

f) Vortex test tube mixer

g) Spectronic "20" spectrophotometer or equivalent

Procedure

(using corn as example)

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

b) Shake at room temperature for 20 minutes.

c) Centrifuge and filter the supernatant if necessary.

d) Place 0.10, 0.15 and 0.20 ml of corn supernatant in separate pyrex test tubes and dilute to 0.5 ml with distilled water.

e) Add 1.5 ml of ninhydrin reagent and mix well.

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

g) Add 8 ml 50% N-propyl alcohol and mix vigorously on a vortex mixer.

h) Read the absorbance on a spectronic 20 spectrophotometer at 470 mμ.

i) Preparation of standard curve:

1) Place at least 4 increments of L-leucine solution (0.5 micromole/ml) in separate test tubes, dilute to 0.5 ml with distilled water and carry tubes through the color reaction.

2) Plot micromoles of leucine on the X-axis and absorbance on the Y-axis.

j) Plot the absorbance of the corn samples on the standard curve and read the leucine equivalents.

k) Calculate the leucine equivalents per 100 mg of protein for each corn sample.

Calculations

To calculate μm leucine (reference amino acid)

<u>Stock leucine</u> 0.5 μm/ml

L-leucine → 65.59 mg/100 ml H_2O = 5 μm/1 ml

Dilute 1 ml of stock leucine to 10 ml = 0.5 μm/ml

ml	µm (conc.)	O.D. 570	Equivalence = $\frac{\text{O.D.}}{\text{conc.}}$
0.1	0.05	0.115	2.30
0.2	0.10	0.250	2.50
0.3	0.15	0.353	2.35
0.4	0.20	0.480	2.40
			ave. 2.39

Sample	ml	10 x mg	O.D. 570	O.D./E / µm leucine	µm leucine / 100 mg sample	µm leucine / 100 ml protein
Corn	0.1	1.0	0.03	0.0139	1.39	15.44
	0.15	1.5	0.045	0.021	1.39	15.44
	0.2	2.0	0.060	0.028	1.39	15.44
						ave. 15.44

Note: % protein of corn = 9.0

Example for calculation:

100 mg/10 ml or 1 mg/ml

$$\mu\text{m leucine} = \frac{\text{O.D. 570}}{E} = \frac{0.03}{2.39} = 0.0139$$

$$\frac{\mu\text{m leucine}}{100\text{ mg sample}} = 0.0139 \times 100 = 1.39$$

$$\frac{\mu\text{m leucine}}{100\text{ mg protein}} = \frac{1.39}{9.0} \times 100 = 15.44$$

Reference

Mertz, E. T., R. Jambunathan and S. Misra. 1975. Simple chemical and biological methods used at Purdue University to evaluate cereals for protein quality. Stat. Bull. No. 70, Biochemistry Department, Purdue University, W. Lafayette, Indiana 47907.

LYSINE IN NON-PIGMENTED CEREALS

Introduction

An ideal simple method for direct determination of lysine in cereals has yet to be found. All methods have disadvantages, but the method developed by Tsai and co-workers as modified by Villegas 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 sorghums give unusually low lysine values.

Equipment

a) Centrifuge and centrifuge tubes

b) Oven at 65 C

c) Spectronic 20 spectrophotometer or equivalent and cuvettes

d) Mortar and pestle

e) Shaker

f) Vortex test tube mixer

g) Screw cap vials 20 x 65 mm (3 dram)

h) Test tubes 15 x 150 mm with rubber stoppers

i) Pipettes, 0.5, 1.0, and 5.0 ml

j) Vacuum source (water aspirator)

k) pH meter

l) Disposable or Pasteur pipettes

Reagents

a) Copper phosphate reagent

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

2) Solution b): 13.6 g $Na_3PO_4 \cdot 12H_2O$ in 200 ml distilled water.

3) Borate buffer (0.05 M, pH 9.0)

Combine solutions a) and b) with mixing, centrifuge and discard supernatant. Wash pellet 3 times by resuspension in 15 ml borate buffer and centrifugation. After the third wash, resuspend pellet in 80 ml borate buffer. This reagent must be prepared fresh every week.

b) Pyridine reagent

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

c) Amino acid mixture

Villegas has modified the Tsai procedure by using a simulated corn protein pattern for preparation of standard curve. To prepare mixture grind together in a mortar the following quantities of 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).

d) Papain solution

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

e) Ethyl acetate

Procedure

a) Place 100 mg finely ground, defatted endosperm or whole kernel sample into a 20 x 65 mm screw cap vial (3 dram) and add 5 ml of papain solution.

b) Cap vials and mix well. Incubate in a 65 C oven overnight. Include a blank (5 ml of papain solution) with every group of samples.

c) Remove vials from oven, shake and allow to cool to room temperature. Centrifuge and decant the clear supernatant solution digest.

d) 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.

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

f) 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 two hours at room temperature.

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

h) 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 pipette or equivalent connected to a water aspirator. This step should be repeated three times.

i) Determine the absorbance of the aqueous phase in a Spectronic 20 spectrophotometer at 390 mμ. Relate absorbance of cereal samples to the corresponding lysine value on the standard curve (see j) below) and report as lysine in grams per 100 grams protein (micro-Kjeldahl).

j) 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 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 six 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 e) to i).

Calculations

Lysine

Plot OD (absorbance reading) against µg of standard. Read off µg of samples from curve.

Sorghum Sample

OD 390	µg	% lysine	g lysine/100 g protein	% protein
0.082	39	0.195	1.84	10.61

$$\% \text{ lysine} = \frac{\mu g \text{ lysine}}{\mu g \text{ sample}} \times 100$$

$$= \frac{39}{200}$$

$$= 0.195$$

$$\frac{\text{gm lysine}}{100 \text{ g protein}} = \frac{0.195}{10.61} \times 100 = 1.84$$

References

Tsai, C. Y., L. W. Hansel, and O. E. Nelson. 1972. A colorimetric method of screening maize seeds for lysine content. Cereal Chem. 49:572-579.

Villegas, E. and E. T. Mertz, 1971. Chemical screening methods for maize protein quality at CIMMYT. Res. Bull. No. 20. CIMMYT, Londres 40, Mexico 6, D.F.

TRYPTOPHAN DETERMINATION

Principle

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 four parts of lysine to one 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. A breeding program for improved protein quality in maize has been carried on successfully at CIMMYT using the following tryptophan assay on maize endosperm. The most promising selections are usually analyzed for lysine to confirm the presence of acceptable levels of this amino acid.

Equipment

a) Three dram glass screw cap vials (20 x 65 mm)

b) Oven at 65 C

c) Centrifuge and centrifuge tubes

d) Pipettes, 1.0 and 5.0 ml

e) Vortex test tube mixer

f) Spectronic 20 spectrophotometer or equivalent

g) pH meter

Reagents

a) Color reagent

1) Dissolve 270 mg of $FeCl_3 \cdot 6H_2O$ in 0.5 ml distilled water and dilute to 1 liter with glacial acetic acid containing 2% acetic anhydride (do under hood).

2) 30 N sulfuric acid

3) Mix equal volumes of reagents 1) and 2) about 1 hr. before use to form the color reagent containing glyoxylic acid.

b) Papain solution

Dissolve papain-technical powder in 0.1 N sodium acetate buffer at pH 7.0 to a final concentration of 4 mg/ml. Prepare enzyme solution daily.

Sample Preparation

Endosperm is used for tryptophan analysis because the embryo 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:

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

b) Wash off pesticide if seeds have been treated.

c) 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.

Procedure

a) 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 overnight. Include a blank (5 ml of papain solution) with every group of samples.

b) Remove digest from oven, shake and allow to cool to room temperature. Centrifuge and decant the clear supernatant solution digest.

c) Pipette 1 ml of the clear digest into a test tube. Add 4 ml of color reagent. Mix using a Vortex mixer and allow the color to develop at 65 C for 15 minutes. Then cool the tubes to room temperature.

d) Place tubes in a Spectronic 20 and read the absorbance at 545 mμ.

e) 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 the color reagent (step c).

f) 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).

Calculations

Plot OD (absorbance) readings against mg of standard. Read off mg of samples from curve.

OD (545)	mg	100 mg/ml	% Trypt	gm/100 gm Prot.	% Protein
0.143	.122	12.2	0.061	0.71	8.62

% Trypt: 100 mg sample/5 ml papain

or: 20 mg/ml

or: 20,000 µg/ml

$$\text{\% Trypt:} = \frac{\text{Mg trypt}}{\text{Mg sample}} \times 100 = \frac{12.2}{20{,}000} \times 100 = 0.061$$

$$\frac{\text{gm Trypt}}{\text{100 gm Protein}} = \frac{\text{\% Trypt}}{\text{\% Protein}} \times 100 = \frac{0.061}{8.62} \times 100 = 0.71$$

Reference

Hernandez, H. and L. S. Bates. 1969. A modified method for rapid tryptophan analysis of maize. Res. Bul. No. 13, CIMMYT, Londres, 40, Mexico 6, D.F.

RAPID SECTIONING OF CEREAL GRAIN ENDOSPERM FOR EXAMINATION OF PROTEINS

Principle

A simple, rapid method for evaluating endosperm protein quality in cereal grains has been developed involving microscopic observation of subcellular protein. In this procedure untreated or glutaraldehyde infiltrated seed pieces are sectioned with a glass knife. Starch and alcohol soluble proteins are then removed from the sections leaving the protein bodies which are observable after staining.

Equipment

a) Coplin staining jar

b) Microtome specimen holder

c) Jeweler's saw or razor blade

d) File

e) Dowel with epoxy resin

f) Rotary microtome

g) Bunsen burner

h) Hot plate

i) Beakers (250 ml)

j) Slides

k) Steam bath

l) Glass wool

m) Test tube rack

n) Magnetic stirrer bar

o) N-funnel

p) Cheesecloth

Reagents

a) Glutaraldehyde

b) Sodium phosphate - mono and dibasic ($NaH_2PO_4 \cdot H_2O$) and (Na_2HPO_4). α-amylase (hog pancreas 2 x crystalline suspension in 0.5 saturated NaCl α-amylase.)

c) Autoradiographic gelatin adhesive

d) Iodine

e) Distilled water

f) Fast green, cosin crystal violet, buffalo black, and nile blue

g) Glycerol and dowel with epoxy resin

h) Silicone liquids (refractive index 1.40 x 1.53) or in cargille liquids ranging up to 1.70 in refractive index

i) 95% ethanol and chromium potassium sulfate

j) De-ashed gelatin (Eastman Kodak)

Preparation of Reagents

a) Phosphate buffer pH 7.4, 0.2N

1) Dissolve 6,898 g $NaH_2PO_4 \cdot H_2O$ in 250 ml of distilled water and 28.392 g Na_2HPO_4 in 1000 ml water.

2) Combine 190 ml $NaH_2PO_4 \cdot H_2O$ with 810 ml Na_2HPO_4. Adjust to pH 7.4 with a pH meter, (using NaH_2PO_4 if the solution is too basic or Na_2HPO_4 if the solution is too acidic).

b) Phosphate buffer pH 6.8, 0.1N

1) Dissolve 3.449 g $NaH_2PO_4 \cdot H_2O$ in 250 ml distilled water.

2) Dissolve 3.549 g Na_2HPO_4 in 250 ml distilled water. Combine 250 ml of 1) with 246 ml of 2) and adjust to pH 6.8 with pH meter.

c) Glutaraldehyde Solution

1) 5 cc glutaraldehyde

2) 7.5 cc H_2O

3) 12.5 cc, 0.2N phosphate buffer pH 7.4

d) Aqueous Glycerol Solution

1:1 or 1:2 water-glycerol, v/v

e) 80% Ethanol (v/v)

1) 80 ml absolute ethanol

2) 20 ml distilled water

f) Enzyme Solution

Dilute recrystallized hog pancreas α-amylase with pH 6.8, 0.1N phosphate buffer to about 2% of its original activity (10-20 units of α-amylase activity/ml).

Note: Source of α-amylase - Mann Research Laboratories, Division of Becton and Dickinson, New York, N.Y. 10006.

Preparation of Autoradiographic (ARG) Slides

a) Add 0.5 g (de-ashed) gelatin to 100 ml distilled water. Warm on a hot plate until gelatin is dissolved. Remove and add 50 mg chromium potassium sulfate. Stir until dissolved. Filter through glass wool into Coplin staining jar (gelatin solution).

b) Set up four 250 ml beakers. The first two containing distilled water, and the third and fourth containing 95% ethanol.

c) Dip a new slide into the first beaker of water, and wipe dry with cheesecloth. Then dip it into beakers 2, 3, and 4 draining after

each step on the edge of the beaker.

d) Pass the slide through the flame of a bunsen burner.

e) After cooling two or three seconds dip into the gelatin solution.

f) Place in a test tube rack to drain and dry.

Sectioning Procedure

Kernels with vitreous endosperm can be sectioned without any pretreatment. Trim the kernel with a jeweler's saw or razor blade and file smooth the surface to be sectioned. Large kernels like those of maize may be clamped in the jaws of the microtome specimen holder for sectioning. Glass knives used for sectioning are made exactly as if they were to be used in selectron microscopy. Special holders for the glass knive may be constructed or commercial holders may be readily adapted for holding it in a rotary microtome.

Because floury kernel varieties common to a number of cereal grains are too friable to be sectioned directly, such kernels should be cut into pieces of the desired size and steeped overnight at about 5 C in 10% glutaraldehyde in pH 7.4 phosphate buffer. The pieces are washed for 0.5 hr. in several changes of distilled water and then immersed in boiling water for three minutes.

With an ordinary microtome, sections are cut 3-4 μ thick. The sections are floated on a droplet of water and dried on slides coated with autoradiographic gelatin adhesive (ARG).

Preparation of Starch-Free Sections

Since starch makes up 80% or more of some endosperm tissues, it is necessary to destarch the sections to expose subcellular protein structure. Sections were destarched on the ARG slide by digesting them for 15-30

minutes in water bath at 35-40 C with a few drops of recrystallized hog pancreas α-amylase diluted to about 2% of its original activity with 0.1N phosphate buffer at pH 6.8. The sections are rinsed in several changes of distilled water.

Extraction of Alcohol-Soluble Protein

Alcohol-soluble protein is extracted from the destarched sections by heating the slides in a Coplin jar one hour at 65-75 C in 80% ETOH (v/v).

Staining

The endosperm proteins are stained over iodine vapor for visual examination or for photomicrography. Sections may be stained before or after extraction of zein in corn and kafirin in sorghum. Various dyes in aqueous solution that stain protein may be used in place of iodine. These include fast green, cosin crystal violet, buffalo black, and nile blue. Alcohol-soluble protein bodies stain more intensely in alcoholic solutions of the dyes than in aqueous solutions.

Mounting

Aqueous glycerol solutions (1:1 or 1:2 water glycerol, v/v) are convenient for visual observation in both bright field and phase contrast. For photomicrography in phase contrast, dry sections are mounted in silicone liquids (refractive index 1.40 - 1.53) or in Cargille liquids ranging up to 1.70 in refractive index.

References

Wolf, M. J. and U. Khoo. 1970. Mature cereal grain endosperm: Rapid glass knife sectioning for examination of proteins. Northern Regional Research Laboratory, USDA-ARS. Peoria, Illinois 61604.

PROTEIN FRACTIONATION PRINCIPLE

Principle

Endosperm proteins of grain sorghum can be separated into five fractions based on their solubility properties by the Landry-Moureaux procedure. The protein fractions which will be separated with the procedure are: Fraction I, albumins and globulins; Fraction II, kafirins; Fraction III, kafirin-like; Fraction IV, glutelin-like; Fraction V, glutelins and residues.

Equipment

a) Magnetic stirrers, stir bars

b) Cold room

c) Refrigerated centrifuge

d) Centrifuge tubes

e) pH meter

f) 50 ml volumetric flasks

Reagents

FRACTION I: NaCl (0.5 M)

Dissolve 29.23 g NaCl in 1 liter distilled water.

FRACTION II: Isopropanol (70%)

Bring 696 ml isopropanol alcohol up to 1 liter with distilled water.

FRACTION III: Isopropanol (70%) + (0.6%) 2-mercaptoethanol (2-ME)

Combine 696 ml isopropyl alcohol and 6 ml of 2-ME (0.6%) and bring up to 1 liter volume.

FRACTION IV: Borate buffer (pH 10) + 2-ME (0.6%)

Dissolve 6.18 g H_3BO_3, 29.22 g NaCl, and 6 ml of 2-ME (0.6%) in water. Bring pH to 10 with ~ 6.4 g NaOH. Make up to 1 liter with water. Note: Make sure 2-mercaptoethanol is added prior to adjustment of pH.

FRACTION V: Borate buffer (pH 10) + 2-ME (0.6%) + sodium dodecyl sulfate (SDS)

Dissolve 6.18 g H_3BO_3, 29.22 g NaCl, 6 ml of 0.6% 2-ME and 5 g SDS in water. Adjust pH to 10.0 with NaOH and bring to 1 liter with distilled water.

Note:

a) It is always advisable to check the pH for Fraction IV and V before use.

b) Reagents for Fraction I and II are stable, but Fractions III, IV and V are unstable, and should be prepared no more than one week before use.

c) Store the first fraction only in the refrigerator for total nitrogen determination.

Procedure

Fractionation

a) To obtain Fraction I, first weigh 1.0 g finely ground, defatted endosperm (or whole kernel) into a centrifuge tube. Add 10 ml of pre-chilled 0.5 M sodium chloride solution and a small magnetic stir bar and mix for 60 minutes at 4 C in a cold room.

b) Remove the magnetic stir bar from the centrifuge tube and centrifuge at 15,000 x g for 10 minutes at 4 C. Collect the supernatant solution in a 50 ml volumetric flask.

c) To the residue, add 10 ml of 0.5 M NaCl and stir for another 30 minutes in the cold. Centrifuge as above and composite supernatants in the 50 ml volumetric flask.

d) Extract the residue successively with another 10 ml NaCl for 30 minutes, 5 ml distilled water for 15 minutes, and 5 ml distilled water for another 15 minutes, each time collecting the supernatant solution. Bring composited supernatant solution up to 50 ml volume with NaCl. This is Fraction I which contains the albumins and globulins.

e) Proceed similarly to obtain Fractions II, III, IV, and V following the procedure in Table 1.

Micro-Kjeldahl determination of protein in fractions

a) Fractions I, IV, V

1) To about 5 ml of supernatant solution in a micro-Kjeldahl flask, add 2 ml concentrated H_2SO_4 and 2 g $KgO-K_2SO_4$ catalyst and a boiling stone.

2) Heat gently on a digestion heater to drive off water and prevent frothing.

3) Continue with normal micro-Kjeldahl digestion at high temperature.

b) Fractions II and III

1) Evaporate 5 ml of supernatant solution in a micro-Kjeldahl flask to dryness to remove isopropyl alcohol by heating in a sand or water bath under reduced pressure (optional).

2) Add 2 ml concentrated H_2SO_4, 2 g catalyst, 1 boiling stone and digest normally.

Calculations

Example: P-721 opaque endosperm (sorghum grain).

a) Total protein of endosperm by micro-Kjeldahl = 12.91%.

b) Assume sample weight = 1.0383 g (protein fractionation)

c) Therefore 12.91 : 100 = X : 1.0383

X = 0.1341 g or 134.1 mg

Table 1

Fraction	Solvent	Time of Agitation	Temperature	Protein Fraction
I	NaCl 0.5 M	10 ml for 60 min.	4 C	Albumins
	"	10 ml for 30 min.		Globulins
	"	10 ml for 30 min.		
	Distilled H_2O	5 ml for 15 min.		
	"	5 ml for 15 min.		
	Note: Keep NaCl extractant refrigerated. Don't exceed 50 ml for any extraction.			
II	Isopropanol 70%	15 ml for 30 min.	20 C	Kafirins
	"	15 ml for 30 min.		
	"	10 ml for 30 min.		
III	Isopropanol 70%	20 ml for 30 min.	20 C	Kafirin-like
	+ 2 ME 0.6%	20 ml for 30 min.		
IV	Borate buffer	15 ml for 60 min.	20 C	Glutelin-like
	pH 10 + 2 ME 0.6%	15 ml for 30 min.		
		10 ml for 15 min.		
V	Borate buffer	15 ml for 60 min.	20 C	Glutelin residue
	pH 10 (2 ME 0.6%	15 ml for 30 min.		
	+ SDS 0.5%)	10 ml for 15 min.		
	→ pH 10 by NaOH			

Note: 1) You can stop within all fractions, or between fractions I and II for no more than one day. You can stop between fractions III, IV and V over the weekend.

2) At the end of each fraction store the residue in the next fraction reagent, if you are stopping over night.

d) Blank HCl = 0.04

e) ml of sample Fraction I = 5 ml

f) ml of HCl : 0.24 ml

g) Corrected HCl : 0.94 - 0.04 = 0.90

h) mg N : 14 x 0.02 = 0.2828

0.90 x 0.2828 = 0.255 mg N

i) mg Protein : 0.255 x 6.25 = 1.59 in 5 ml

j) mg Protein in 50 ml: $1.59 \times \frac{50}{5} = 15.90$

k) % of total protein recovery for Fraction I: $\frac{15.90}{134.1} = \frac{x}{100}$

$$X = \frac{15.90 \times 100}{134.1} = 11.86$$

<u>% Total Protein</u>: Fraction I = 11.86
II = 14.09
III = 19.76
IV = 5.41
V = 44.42
95.54 Total recovery for all the fractions.

Tabulated Results:

Fractions	ml HCl	corr. HCl	mg N	mg P/5 ml	mg P/50 ml	% recovery
I	0.94 blank = 0.04	0.90	0.255	1.59	15.90	11.86
II	1.12 blank = 0.05	1.07	0.303	1.89	18.90	14.09
III	1.50 blank = 0.0	-	0.424	2.65	26.50	19.76
IV	0.44 blank = 0.03	0.41	0.116	0.725	7.25	5.41
V	3.37 blank = 0.0	-	0.953	5.957	59.57	44.42
						95.54

Reference

Misra, P. S. E. T. Mertz, and D. V. Glover. 1975. Endosperm protein changes in single and double endosperm mutants of maize. Cereal Chem.52:161-166.

Landry, J. and T. Moureaux. 1970. Heterogeneite des glutelines du grain de mais. Extraction selective et composition and acids amines des trois fractions isolees. Bull. Soc. Chim. Biol. 52:1021.

GEL ELECTROPHORESIS USING DODECYL-SULFATE-POLYACRYLAMIDE

Principle

Electrophoresis, the migration of charged colloidal particles in an electric field, has been used extensively in separating proteins. Gel electrophoresis, which separates compounds on the basis of shape, size, and charge, is very useful in this application, especially when employing acrylamide gels due to the ability to control their pore size and cross linking. Although it is relatively difficult to quantify proteins by acrylamide gel electrophoresis, the technique does offer important advantages:

a) Resolution is excellent, even for components with similar net charges, but with small differences in molecular weights or shapes.

b) Small quantities of protein (10-20 μg) are easily resolved.

c) The apparatus is relatively easy to operate. The following procedure describes a modification of the acrylamide gel technique for determination of the molecular weight of various proteins and protein subunits (demonstrated here using 5 purified proteins). It can be used for determining molecular weight of the proteins in different fractions obtained from protein fractionation.

Equipment

a) Magnetic stirrer and stir bars

b) Balance and weighing paper and spatulas

c) Griffen beakers

d) Volumetric flasks, 10, 25, 1500 ml

e) pH meter

f) Parafilm

g) Ten ml syringe with needle

h) Water bath

i) Water aspirator

j) Needle

k) Gel electrophoresis apparatus

l) Eye dropper squeezer

m) Flat dish

n) Measuring test tube

o) Forceps

p) Whatman # 1 paper

q) Centrifuge tubes

r) Small test tubes

s) Vortex mixer

t) Pasteur pipettes

u) 6 mm x 10 cm gel tubes

v) Destaining tubes

Reagents

a) Egg albumin

b) Pepsin

c) Trypsin

d) β-Lactoglobulin

e) Lysozyme

f) Acrylamide

g) Sodium Dodecyl Sulfate (SDS)

h) Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$)

i) Sodium hydrogen phosphate (Na_2HPO_4)

j) N,N^1 methylene bisacrylamide (BIS)

k) 2-mercaptoethanol (2-ME)

l) Ammonium persulfage

m) N,N,N^1,N^1 tetramethylenediamine (TEMED)

n) Coomassie blue

o) Methanol

p) Glacial acetic acid

q) Distilled water

r) Bromophenol blue

s) Sucrose

Reagent Preparation

<u>Incubation Solution</u>: (0.01 M phosphate buffer pH 7.0)

a) Dissolve 0.138 g $NaH_2PO_4 \cdot H_2O$ in 100 ml H_2O

b) Dissolve 0.13680 g $Na_2HPO_4 \cdot H_2O$ in 100 ml H_2O

c) Mix 49 ml of a) with 50 ml b).

d) Add 1 ml 2-ME and 1 g SDS. Stir until dissolved, and adjust to pH 7.0.

Dialysis buffer: (0.01 M phosphate buffer pH 7.0)

a) Dissolve 0.690 g $NaH_2PO_4 \cdot H_2O$ in 100 ml H_2O.

b) Dissolve 1.340 g $Na_2HPO_4 \cdot 7H_2O$ in 100 ml H_2O.

c) Mix 35 ml of a) with 65 ml b).

d) Add 0.5 ml 2-ME and 0.5 g SDS.

e) Make up to 1400 ml with distilled water.

Tracking dye:

a) 0.001% bromphenol blue is added to an 80% sucrose solution, and made up to pH 7.1.

10% acrylamide solution:

a) Dissolve 22.2 g of acrylamide and 0.6 g BIS in 100 ml water. (This gives the proper cross-linkage for fraction I-IV of corn and sorghum, especially fractions II and III.)

b) Filter with Whatman # 1 filter paper and store at 4 C in dark.

8.5% acrylamide solution:

a) Dissolve 18.87 g of acrylamide + 0.51 g of BIS in 100 ml water. (This is good for fraction V.)

b) Filter with Whatman # 1 filter paper and store in the dark at 4 C in dark bottle.

7.5% acrylamide solution:

a) Dissolve 16.65 g acrylamide + 0.45 g BIS in 100 ml water.

b) Filter with Whatman # 1 filter paper and store in dark bottle at 4 C.

Ammonium persulfate:

a) Dissolve 150 mg ammonium persulfate in 10 ml distilled water. (Prepare freshly just before use).

Staining solution:

a) Dissolve 1.25 g coomassie blue in 454 ml of 50% methanol + 46 ml glacial acetic acid.

b) Filter through Whatman # 1 filter paper.

Destaining solution:

a) Combine 75 ml glacial acetic acid 50 ml methanol and 875 ml distilled water.

Storing solution:

a) Dilute 75 ml glacial acetic acid to 1 liter.

Procedure

Preparation of protein solutions:

a) Place 10 mg of 5 purified proteins used as standards: egg albumin, pepsin, trypsin, β-lactoglobulin, and lysozyme, in 25 ml volumetric flasks. In addition, prepare a composite sample consisting of 10 mg each of the above five proteins in a 25 ml volumetric flask (0.4 mg/ml for each protein).

b) Make flasks up to volume with 0.01 M phosphate buffer containing 1% SDS and 1% 2-ME.

c) After mixing until dissolved, incubate protein solutions in a water bath at 37 C for 2½ hours to insure complete solution. Then store in a refrigerator until use.

d) Make up lyophylized protein fractions in 0.01 M phosphate buffer at 0.4 mg/ml as above and dialyze overnight at room temperature against 500 ml 0.01 M sodium phosphate buffer pH 7.0 containing 0.1% SDS and 0.1% 2-ME.

e) Centrifuge dialyzed solutions and transfer supernatant solutions to 100 ml Erlenmeyer flasks.

f) Transfer 2.5 ml of protein standards (0.4 mg/ml) and dialyzed supernatant solutions to properly labeled test tubes.

g) Add 4-5 drops of tracking dye to each of the test tubes.

h) Mix test tubes on a vortex mixer and store in a refrigerator until use.

<u>Preparation of gels for running 12 tubes</u>:

a) Mix 15 ml of gel buffer and 13.5 ml 10% or 25% acrylamide solution, and deaerate solution 15 minutes using a vacuum source, such as a water aspirator. Omission of this step will result in bubbles in the gel.

b) Add 1.5 ml ammonium persulfate (freshly prepared) plus 0.045 ml (2 drops) of TEMED and mix thoroughly.

Note: This mixture solidifies in 15-20 minutes, hence it is necessary to fill the 12 glass tubes (6 mm x 10 cm) within this period of time.

c) Twelve gel electrophoresis tubes are placed on a piece of parafilm in the gel tube holder and pushed down to prevent leakage (gently so as not to break the parafilm). Level the gel holder and mark the tubes with a pen to demarcate gel volume.

d) Add acrylamide solution to each tube using a Pasteur pipette starting near the bottom of the tube and moving upward to the marked line to prevent inclusion of air bubbles.

e) Layer several drops of distilled water carefully on top of each gel before they harden by use of a Pasteur pipette, letting the water flow down the tube wall.

f) The gel will solidify in 15-20 minutes and the water-gel interface will be apparent. The water layer is then removed carefully with a Pasteur pipette.

g) Apply 3-6 drops of protein solutions to each gel with a Pasteur pipette (tubes should be numbered to avoid confusion and error). One drop ≈ 0.025 ml protein concentration = 0.4 mg/ml, therefore, 1 drop ≈ 0.01 mg protein.

h) Place tubes in the electrophoresis apparatus.

i) Dilute gel buffer 1:1 with distilled water and carefully layer about 2 ml with Pasteur pipette on each gel.

j) Fill two compartments of the electrophoresis apparatus each with 400 ml of diluted gel buffer.

Running the electrophoresis:

a) Perform electrophoresis at constant current of 8 milliamps per gel with the positive electrode in the lower chamber.

b) Stop current when the marker dye has moved through 3/4 of the gel (4 hours).

Gel removal, destaining, and reading:

a) To remove the gels from the tubes, insert a needle (bevel towards tube wall) to its full length between the tube wall and gel under constant water pressure from a syringe. Then rotate the gel tube to free gel from tube with continued water pressure. Finally withdraw the syringe and needle while maintaining water pressure.

b) Extrude from gel tube under air pressure by attaching a Pasteur pipette bulb to one end and applying pressure. Extrude gels into a flat dish containing water, and transfer them with the aid of forceps into a graduated test tube containing 7.5% acetic acid storage solution after marking the location of the tracking dye by inserting a bent-tipped needle 2/3 of the way through the gel at the mid-point of the tracking dye band.

c) Record distance of movement of marking dye (X) and total gel length (Y). (The entire gel length minus the height of the tracking dye equals the length of tracking dye migration (Y-X) when the top of the gel is also at the top in the graduated tube.)

d) Transfer the gels into destaining tubes and place them in a beaker filled with staining solution and incubate at room temperature for 30-40 minutes with stirring.

e) Remove gels from stain, rinse with distilled water and place in 150 ml destaining solution overnight. (If a destaining apparatus is not available, this may require several days with 3-4 changes of destaining solution.) Destaining solution is stirred on a magnetic stirrer.

f) After destaining, gels are again transferred to graduated test tubes and total gel length (Y^1) and location of the blue-stained protein bands (Z) are recorded. (Note: the violet-purplish stained end should be at the top of the measuring test tube, and the lightest region should be at the bottom.)

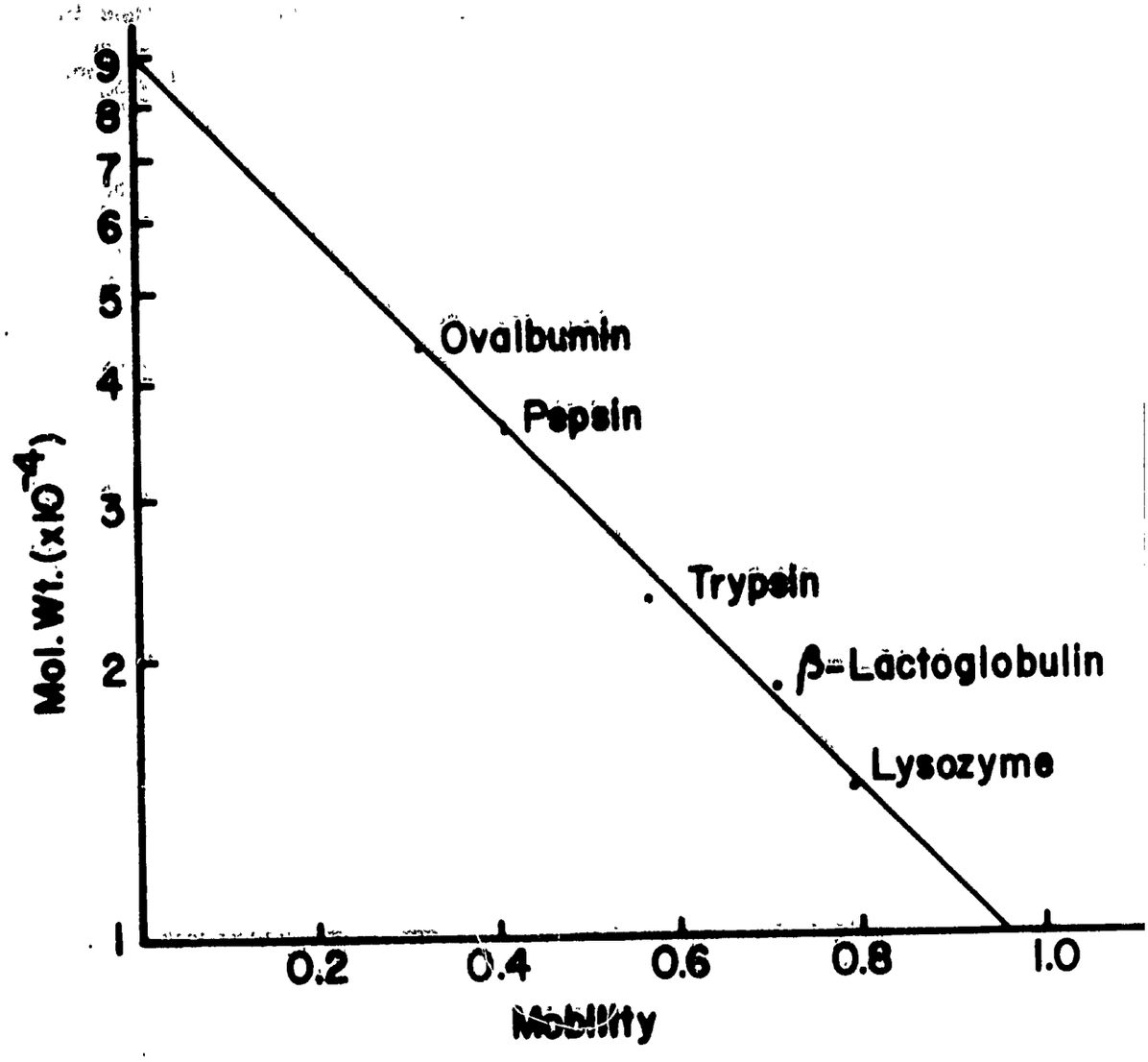


Figure 2. Standard curve for gel electrophoresis.

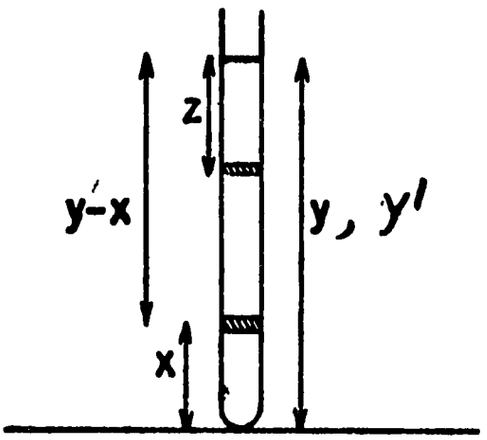
REFERENCE MOBILITY - 10% ACRYLAMIDE

Amount Used	Sample		(Y) Length Before Staining (cm)	(Y-X) Distance Of Dye Migration (cm)	Ave.	(Y^1) Length After Destaining (cm)	(Z) Distance Of Protein Migration (cm)	Ave.	Mobility	Average Mobility	M.W.
30 mg	Ovalbumin	1)	9.3	6.8 ;6.0	6.4	10.35	1.9 ;2.55	2.23	0.312	0.319	43,000
		2)	9.1	6.3 ;5.7	6.0	10.3	1.7 ;2.6	2.15	0.316		
		3)	9.3	6.5 ;7.0	6.75	10.2	2.2 ;2.7	2.45	0.331		
		4)	9.15	5.85;6.25	6.05	10.2	2.05;2.2	2.13	0.315		
60 mg	Pepsin	1)	9.3	6.6 ;5.8	6.2	10.2	2.4 ;2.9	2.65	0.390	0.406	35,000
		2)	9.1	6.2 ;7.0	6.55	10.05	2.75;3.15	2.95	0.407		
		3)	9.0	6.4 ;7.0	6.7	10.05	2.9 ;3.2	3.05	0.409		
		4)	9.12	5.62;6.32	5.97	10.05	2.65;2.85	2.75	0.418		
60 mg	Trypsin	1)	9.1	6.5 ;5.7	6.1	10.2	3.6 ;4.0	3.8	0.555	0.565	23,000
		2)	9.1	6.1 ;6.7	6.4	10.1	3.9 ;4.2	4.05	0.571		
		3)	9.25	5.55;6.25	5.90	10.15	3.65;3.75	3.7	0.570		
40 mg	β-lactoglobulin	1)	9.3	5.9 ;6.5	6.2	10.1	4.6	-	0.685	0.708	18,400
		2)	9.2	5.6 ;6.2	5.9	10.05	4.55;4.75	4.65	0.721		
		3)	9.28	5.58;6.08	5.83	10.20	4.5 ;4.7	4.6	0.718		
30 mg	Lysozyme	1)	9.2	6.0 ;6.8	6.40	9.9	5.1 ;5.6	5.35	0.776	0.792	14,300
		2)	9.1	5.7 ;6.2	5.95	10.0	5.0 ;5.5	5.25	0.803		
		3)	9.3	5.6 ;6.2	5.90	10.15	4.95;5.35	5.15	0.798		

STANDARD MOBILITY - 8.5% ACRYLAMIDE

Amount Used	Sample		(Y) Length Before Staining (cm)	(Y-X) Distance Of Dye Migration (cm)	Ave.	(Y^1) Length After Destaining (cm)	(Z) Distance Of Protein Migration (cm)	Ave.	Mobility	Average Mobility	M.W.
30 mg	Ovalbumin	1)	8.95	6.25;6.85	6.55	9.78	2.58;2.78	2.68	0.375	0.383	43,000
		2)	9.1	6.4 ;5.9	6.65	10.0	2.75;2.95	2.85	0.390		
60 mg	Pepsin	1)	9.1	5.8 ;6.6	6.2	9.98	3.18;3.38	3.28	0.482	0.482	35,000
		2)	9.0	5.8 ;6.5	6.15	9.9	3.15;3.35	3.25	0.482		
60 mg	Trypsin	1)	9.2	5.9 ;6.6	6.25	10.0	4.25;4.45	4.35	0.639	0.604	23,000
		2)	9.2	5.9 ;6.5	6.2	9.98	4.23;4.48	4.35	0.648		
40 mg	β-lactoglobulin	1)	9.0	5.9 ;6.5	6.2	9.8	5.25;5.5	5.38	0.796	0.793	18,400
		2)	9.12	5.85;6.65	6.25	9.92	5.27;5.47	5.37	0.789		
30 mg	Lysozyme	1)	9.05	6.05;6.65	6.35	9.88	5.98;6.38	6.18	0.890	0.892	14,300
		2)	9.1	6.1 ;6.7	6.4	9.9	6.0 ;6.4	6.2	0.893		

Calculation



Y - gel length before destaining

X = distance between marking dye and base of gel tube, measured before destaining

Y-X = distance of marking dye from top of gel (calulated by subfraction)

Z = distance of protein migration, measured after destaining

Y^1 = gel length after destaining

$$\text{mobility} = \frac{Z}{Y^1} \times \frac{Y}{Y-X}$$

e.g., $\frac{2.23}{10.35} \times \frac{9.3}{6.4} = 0.312$ (table 1 : 10% acrylamide)

Mobilities of standard proteins are plotted against their known molecular weights on a semi-logarithmic scale to obtain a standard curve.

Reference

Weber, Klaus and Mary Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

TANNIN ANALYSIS

Introduction

Tannins are generally described as water-soluble high-molecular weight polyphenols which precipitate gelatin from solutions and which combine with hide fibers to form leather. Seed of numerous sorghum varieties contain tannins, primarily of the condensed type, which are associated with a pigmented testa layer in the pericarp. In addition to imparting an astringent flavor, tannins in sorghum grain have been found to reduce its biological value when fed to monogastric animals and humans.

Vanillin-Hydrochloric Acid Method

Principle

The Vanillin-Hydrochloric Acid procedure (VHCl) of Burns is based upon the acid catalyzed formation of an adduct of vanillin with tannin which absorbs maximally at 500 mμ. The reaction is specific for leucoanthocyanins and catechins although anthocyanins and dihydrochalcones may interfere to some extent. Although originally developed to estimate tannin content in forage legumes such as lespediza, tick clover, trefoil, and crown vetch, the VHCl procedure has equal utility for analysis of tannins in sorghum forage and grain. The test is rapid, and equipment and chemicals are inexpensive. In addition, our laboratory

has found that tannin estimated in sorghum grain by the VHCl method is highly correlated with biological value.

Equipment

a) Spectronic 20 spectrophotometer or equivalent

b) Test tubes

c) Automatic pipettor

d) Balance

e) Graduated cylinder (500 ml)

f) Erlenmeyer flasks (50 and 2000 ml)

g) Rubber stoppers to fit Erlenmeyer flasks

h) 100 ml volumetric flasks

Reagents

a) Vanillin-HCl reagent.

1) Make up 8% concentrated hydrochloric acid (HCl) in methanol (v/v).

2) Dissolve 4% vanillin in methanol (w/v).

3) Mix equal volumes of solutions just before use. Prepare daily. Do not use after trace of red appears.

Example: For 40 samples 400 ml are required. To 184 ml methanol add 16 ml concentrated HCl. Dissolve 8 g vanillin in 200 ml methanol. Mix these solutions.

b) Standard solutions.

1) Prepare a stock solution of 200 mg of NBC (Nutritional

Biochemicals Company) catechin in 200 ml methanol.

2) Make the following concentrations: 0.05, 0.10, 0.25, and 0.50 mg/ml by adding respectively 5, 10, 25, and 50 ml of stock solution to 100 ml volumetric flasks and making up to volume with methanol.

3) The five standard solutions, consisting of the stock solution and the four dilutions, can be stored several months in stoppered flasks in the refrigerator.

Procedure

a) Weigh out 0.5 g ± 3 mg samples of ground grain for a set of 40 samples into 50 ml Erlenmeyer flasks.

b) Dispense 25 ml absolute methanol into each flask (with automatic pipette) and stopper. Shake by swirling the contents, and allow them to extract overnight at constant temperature (24 to 32 C).

c) Prepare a listing sheet of code numbers, with space for duplicate absorbance readings beside them.

d) Prepare 90 test tubes, duplicates for each of the 40 samples and the five standard solutions. After 24 to 28 hours proceed as follows:

e) Turn on the Spectronic 20 or other spectrophotometer one hour before using.

f) Using a 1 ml automatic syringe, pipette 1 ml of each standard solution into its set of duplicate test tubes. Between con-

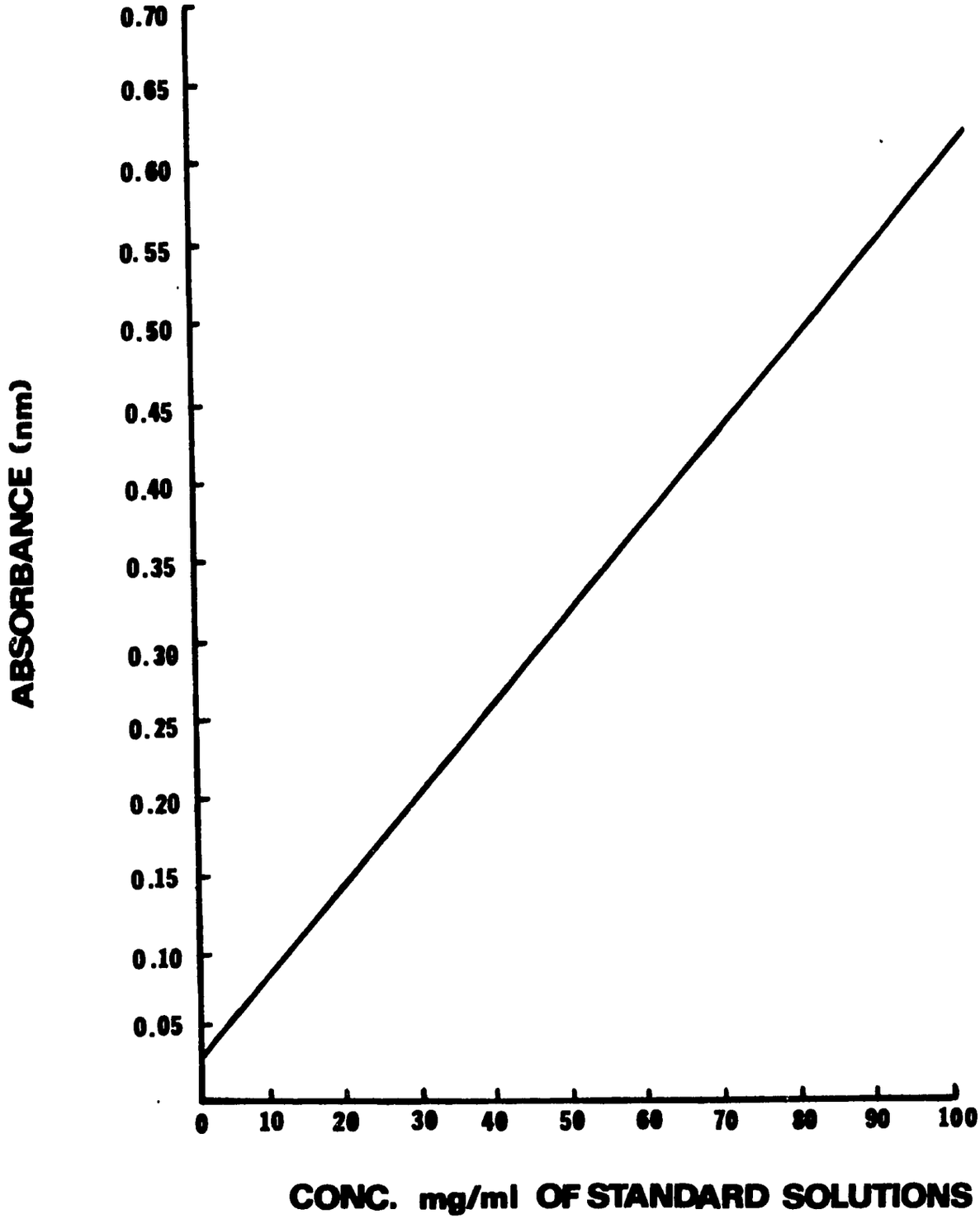


Figure 1. Standard curve for tannins.

centrations rinse the automatic pipettor with the standard solution to be used.

g) Pipette 5 ml of vanillin-HCl solution (with automatic pipettor) into the test tubes filling one test tube per minute. When test tube #21 is filled, the contents of the first test tube is poured into the cuvette and read in the colorimeter at 500 mμ. Discard the contents. Then fill test tube #22 using the same procedure, read the second test tube, and so on. After both replicates of one sample have been assayed, wash the cuvette with 5 ml of the vanillin-HCl solution.

Calculations

a) Prepare a standard curve (Figure 1) by plotting the averaged absorbance readings of the five standard solutions against NBC catechin concentrations in mg/ml.

b) Determine the slope of the standard curve. To do this, use only the line generated, and not any individual values.

c) Calculate catechin equivalent values (CE) for the unknowns according to the formula:

$$\frac{5}{\text{slope}} \times \text{average absorbance of unknown} = \text{catechin equivalent of unknown.}$$

The number 5 eliminates the steps to convert mg of catechin/ml to percent CE.

Note

Recent experimental results in our laboratory indicate that the VHCl procedure is rendered more reliable by use of blanks consisting of a 1 ml aliquot of sorghum extract incubated 20 minutes with 5 ml of a 4% HCl-methanol solution. Subtraction of a blank for each extract corrects for the absorbance at 500 mμ of non-tannin components in the extracts (pigments). Through the use of blanks we have found that many of the sorghum lines previously classified as low in tannin have, in actuality, no tannin at all.

Caution is advised in equating catechin equivalents from the VHCl test with any quantitative estimate of tannin content. Catechin is a uniform monomer while tannins are a heterogenous group of polymers of undefined length and unknown and probably differing extinction coefficients. Hence, while the VHCl test is useful in ranking sorghum lines for tannin content and in distinguishing between low and high tannin lines, its use as a quantitative estimation method is questionable at best.

Reference

Burns, R.E. 1963. Methods of tannin analysis for forage crop evaluation. Georgia Ag. Exp. Tech. Bull. No. 32:1-14.

RAPID SCREENING TEST FOR TANNINS

Principle

The test is a rapid, simple method for visual estimation of tannin content in sorghum grain. It is based on development of a blue complex of potassium ferricyanide with ferrous ions. Ferrous ions are produced during oxidation of tannin and other phenols by ferric ions. The test is easily performed with a minimum of equipment and technical skill required, lending itself readily to field or laboratory screening of large sorghum populations and to quick evaluation of tannin content of sorghum grain shipments at the elevator.

Equipment

a) 250 ml flask

b) 250 cc graduated cylinder

c) 2 ml measuring spoon

Reagents

a) Ferric chloride (0.1 M): Dissolve 16.2 g anhydrous ferric chloride or appropriate weight hydrated $FeCl_3$ in deionized water and bring to 1 liter with 0.1 N HCl (8.3 ml conc. HCl per liter). Filter by gravity to remove any fine, red, insoluble particles. The solution should be yellow. (Note: This solution is stable for months).

b) Potassium ferricyanide (0.1 M): Dissolve 32.9 g potassium ferricyanide ($K_3Fe(CN)_6$) in deionized water and bring up to 1 liter.

c) Ferric chloride (0.008 M in).008 N HCl): Dilute 20 ml of solution (a) 0.1 M $FeCl_3$, to 250 ml deionized water.

d) Potassium ferricyanide (0.004 M): Dilute 4 ml of solution (b) 0.1 M $K_3Fe(CN)_6$, to 1 L with deionized water.

(Note: solutions a, b and d above are stable for several months).

Procedure

a) Scoop up finely ground sorghum grain sample using a 2 ml (approx. 0.7 g) measuring spoon. Level off sample without packing and place into a 250 ml flask.

b) Add 200 ml 0.004 M $K_3Fe(CN)_6$ (solution d) and swirl.

c) Add 10 ml 0.008 M $FeCl_3$ (solution c), swirl and observe change in color of solution. The solution will quickly become light green if little or no tannin is present (group I) or only tannin of the group II type is present. The solution will turn dark green in the presence of intermediate amounts of tannin and deep blue in the presence of high tannin concentrations (group III).

Note: - Group I and Group II have high biological values while Group III has low biological value.

- The bleach test can be used to divide grains into two categories: those with testa (group II and III) and those with testa (group I).

- The rapid screening test can be used to divide the grain into a category consisting of varieties with no water soluble tannin (group I and II) and a category containing all water soluble tannin (group III). It can, furthermore, give information as to relative tannin content in the latter, depending on the shade and depth of color.

Cautions

a) Since the test measures relative amounts of tannin, color developed with unknown samples should always be compared with that of several standards or checks of known tannin content (e.g. BR-54, high tannin: NK-300, intermediate tannin; RS-610, low tannin). Absolute tannin content of the standards can be determined by the Vanillin-HCl test (see pages 96-101).

b) Always note the color within a few minutes since it gradually changes with time.

c) The amount of grain, purity of reagents, method and fineness of grinding, etc. can cause changes. Hence, you <u>must</u> experiment with a high and low tannin sorghum until finding just the right conditions to obtain optimum blue and yellow color (no tannin or other phenols) formations respectively. The following conditions may shift color development towards the blue end:

   1) Use of more grain.

   2) Finer grind of grain.

   3) Addition of less potassium ferricyanide.

   4) Increasing time before reading.

d) Any polyphenol will give a positive test. In sorghum the main interefering substances are the colored anthocyanins. As a result, all colored grain will give a small positive response even if there is no tannin present. But of 35 sorghum varieties examined, the amount of these interfering substances was found to be low in comparison to the amount of tannin present in bird resistant varieties. This method is useful for rapid screening. However, varieties which appear to be especially high in tannin should be checked in the laboratory by the Vanillin-HCl test to confirm that tannins are causing the color.

e) If flasks become stained, they may be cleaned after soaking overnight in an oxalic acid solution.

Reference

Price, M.L. and L.G. Butler. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907, in press. J. Agric. Food Chem.

USE OF A BLEACH TEST TO SCREEN FOR TESTA LAYER IN SORGHUM

Principle

A bleach test used in market grading to determine the percent brown seeds in a sample was investigated for its usefulness as a selection technique. This test is an unofficial means of identifying a pigment testa layer (which has been associated with high tannin content) in sorghum seed. The importance of this procedure as a screening tool can be readily seen by the differences in both tannin values and percent seeds with a testa. Selection of plants without a testa should lead to the development of populations low in tannin. The presence of a testa layer is controlled by two complementary dominant genes, B_1 and B_2. Thus, selection for or against this trait should be accomplished easily.

Equipment

a) Test tubes

b) Test tube rack (40 test tubes)

c) Paper towels or blotters

d) Small tea strainer

e) Water bath 60-70 C

f) Protective clothing: gloves, apron, goggles

Reagents

a) Household bleach (6% NaOCl by weight)

b) Potassium hydroxide pellets.

c) Prepare a 1:5 (w/r) concentration of KOH:bleach.

Procedure

a) Place approximately 30-50 seeds in each test tube.

b) Add 10 ml of solution (c) in each test tube and swirl for a few minutes

c) Place the whol rack with 40 test tubes in a water bath, 60-70 C, for 10-15 minutes and shake occasionally.

d) After 15 minutes of bleaching, pour into tea strainer and rinse thoroughly with running water.

e) Shake off excess moisture and dump sample onto two or more towels. When the bulk of the water is absorbed, transfer to fresh towels and examine.

f) Seeds with a testa layer turn black, while seeds without a testa layer remain light in color when tested by this method.

Precautions

a) The seeds tested should be glume-free because they contain phenols which may give biased results.

b) Both bleach and KOH are corrosive to the skin. Handle with caution.

c) Wear protective clothing; gloves, apron and goggles.

d) If spilled, immediately flush skin with large amounts of water and neutralize area with vinegar.

Reference

Kofoid, K.D., J.W. Maranville and W.M. Ross. 1976. A test for determining the presence of testa in sorghum. University of Nebraska, Lincoln. Published as paper no. 77-846, Abstract Service, Nebraska Agricultural Experiment Station.

CHEMICAL DEHULLING OF SORGHUM GRAIN

Principle

Alkali dehulling of sorghum grain may be of interest to those concerned with grain quality. Tannins, which are thought to complex with the grain protein upon ingestion, thereby reducing its biological availability, are localized in the pericarp and hence can be removed by the dehulling process. In addition, dehulling improves sorghum grain quality for industrial processing. Blessin et al. reports fiber content of dehulled products are approximately 50% of the whole grain level. Dehulled grain starch is whiter than that of whole grain. Also, larger quantities of cleaner germ with higher oil content are recovered from dehulled grain. Under the conditions of this procedure the aleurone layer is not removed, and ash, ether extract and protein levels are not significantly affected. Average yield of dehulled sorghum grain is reported at 92% of the whole grain.

Procedure

a) Temper 1000 g or smaller sample of sorghum grain with water at 160 F for three to four minutes.

b) Drain, and heat sample at 106 F in a 20% (w/w) aqueous solution of sodium hydroxide for six to eight minutes depending on the sorghum line used.

c) Drain the dehulled grain and wash six to eight times with water at 160 F to prevent further action of alkali.

d) Neutralize residual alkali by submerging in 5% glacial acetic acid (V/V) for five minutes at 160 F.

e) Rinse sample again in 160 F water and air dry.

f) The sorghum grain should be stirred continuously during the alkali-treating, neutralizing, and rinsing operations.

References

Blessin, C. W., R. A. Anderson, W. L. Deatherage, and G. E. Inglett. 1971. Effect of alkali dehulling on composition and wet milling characteristics of sorghum grain. Cereal Chem. 48:528.

Blessin, C. W., W. L. Deatherage, and G. E. Inglett. 1970. Chemical dehulling of dent corn. Cereal Chem. 47:303.

DIETHYL SULFATE INDUCED MUTATION PROCEDURE

Principle

Working with induced mutation one should keep in mind the following points:

a) Have a specific objective - e.g., to increase concentration of lysine in sorghum grain.

b) Use a large population - in higher plants it is recommended to start with 1,000,000 seeds.

c) Employ an efficient selection technique to handle the large population.

Among the chemical mutagens under alkylating agents (monofunctional), diethyl sulfate (DES) is one of the most effective mutagen treatments on sorghum grain. The reason for its mutagenic effect is due to its ability to donate an alkyl group to N-7 position of guanine in DNA which causes mispairing or deletions.

Equipment

a) 6000 ml Erlenmeyer flask

b) Sieve or cheesecloth

c) Pro-pipette bulb

d) Pipettes

e) Hair dryer

f) Cookie pan or equivalent

Reagent

a) Diethyl sulfate

Procedure

a) For best results, treat at the rate of 75 grams of seed per 1000 ml of a solution containing 0.75 - 1.25 ml DES.

b) For treating 300 g seed, add 4 ml DES to 4000 ml distilled H_2O in a 6000 ml Erlenmeyer flask. Shake well to completely disperse DES in solution.

c) Add seed to flask and soak 3 hr, agitating thoroughly every ½ hr.

d) Place treated seeds in a sieve or cheese cloth and rinse 5 min. (minimum) with running H_2O (alternatively soak seed in water 1 hr).

e) Spread washed seeds on a cookie pan and air dry seed surfaces with a hair dryer.

f) Treat with fungicide and plant as soon as possible.

Precautions

It is necessary to remove all DES quickly by washing because the mutagen is easily hydrolyzed to ethyl sulfonic acid (C_2H_5-O-S-OH^-) which is toxic to seeds and reduces germination.

BIOLOGICAL ASSAY IN RATS

Principle

Several methods are available for the determination of the protein quality of cereals with rats. Most of them 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 rats 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 ratio 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 and in India found opaque-2 maize to be 90% 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 for humans.

Procedure

A recommended procedure to compare the feeding value of different lines of sorghum follows:

Arrange sixty 20-day old male weanling rats (albino) (*Rattus rattus*) belonging to a single strain with nearly similar inital weight in groups of 20 to make their average weight close to their overall mean weight: House each rat in individual wire mesh cages and supply with ground feed and water *ad libitum* for 28 days. Weigh each rat at weekly intervals for 28 days and make an accurate record of food consumption. Rats should be given unrestricted access to the diet and water. Change water on alternate days. The use of special feed cups will minimize the feed spill to a negligible level. From rat weight gain and feed consumption data, feed efficiency ratio (FER, feed consumed per gram of body weight gain), and protein efficiency ratio (PER, weight gain per gram of protein consumed) are calculated.

Example

Details of a typical bioassay of different cereal grains as carried out in the laboratory of Dr. Mertz using the PER method are presented below:

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. 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 1

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 PM-132, triticale	14.6	6.4	1.6	2.6	1.9
ANRC casein	Animal Nutrition Research Council reference casein*	91.0	4.8	-	-	2.3

*Sheffield Chemical Co., Union, N.Y.

Table 2

Composition of Diets Containing 8.8% Protein

Diet	Protein Source	Cottonseed Oil	Mineral[a] Mix	Vitamin[b] Mix	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 Crop., 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.

Table 3

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

Items Compared	CM-4856 Opaque-2 Maize	CM-4858 Normal Maize	OH43xB14 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)*
Percent 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 parentheses. These three were studied in a separate feeding test with their own casein control.

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 percent of casein for sorghum, to a high of 87 percent of casein for opaque-2 maize in the 28-day feeding trial. At the end of the first seven days the calculated percent 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 percent as effective as casein in 14 days. Average amount of cereal grain consumed by the ten rats in seven 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 seven 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 six male weanling rats for 14 days instead of 10 animals for seven 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.

Reference

Mertz, E. T., R. Jambunathan, S. Misra. 1975. Simple chemical and biological methods used at Purdue University to evaluate cereals for protein quality. Station Bulletin No. 70, Biochemistry Department, Purdue University, West Lafayette, Indiana 47907.

SECTION II

CHEMICAL AND BIOLOGICAL METHODS FOR FORAGE SORGHUM

INTRODUCTION

Laboratory evaluation of forage sorghum is essentially aimed at obtaining analytical data that predict the extent of biological degradation under specified conditions, animals, organisms, and time. In general, an *in vitro* rumen fermentation reflects the known and unknown factors limiting availability of forage to the digesting organism. The analyst can assay only known constituents. The assays for things that are expedient to handle are known, or are considered important, and its evaluation is valid only if the principles concerned are dominant in the sample. For example, it makes little sense to expect lignin to be a good predictor of digestibility if silica or some other factor is a more important variable.

The Weende proximate analysis system for analyzing food and animal feed includes the determination of moisture (dry matter), ether extract, crude protein, ash, crude fiber, and nitrogen free extract (NFE).

In the past, these analyses have been the starting point for more detailed analyses of specific nutrients. If the feed and feces are analyzed for the proximate analyses, total digestible nutrient (TDN) may be calculated.

Expanded knowledge of nutrient requirements of animals and their nutrient utilization from feeds has revealed serious errors in the methods and assumptions underlying the crude fiber nitrogen-free extract and ether extract components. It is recommended that the TDN system be discarded for more modern methods of partitioning the nutrients of feeds.

For evaluating forages the procedures of Van Soest are being used in preference to the Weende system. For making up feed composition tables the important analyses are dry matter, protein, amino acids, energy values according to the caloric system, minerals, and vitamins.

WEIGHING SAMPLES BY DIFFERENCE FOR CHEMICAL ANALYSIS

Principle

The sample is kept covered so that moisture is not lost while weighing.

Equipment

a) Analytical balance to weigh to an accuracy of three decimal points (x.xxx)

b) 50 ml weighing bottles

c) Small spoon made from aluminum or plastic

d) 10 to 25 ml weighing bottles

e) Pipettes

Procedure

Solid samples

Fill a 50 ml weighing bottle about half full of the sample. Place a small spoon made out of a piece of aluminum or plastic in the weighing bottle. Place the lid on the weighing bottle. Weigh the correct amount of sample by difference for each determination.

Arrange to weigh out samples for several analyses at the same time. For example, samples for dry matter, nitrogen, ash, cell walls, etc., could be weighed from one weighing bottle. This would mean that only one weighing would be needed to make each sample for a single analysis or two weighings for duplicate analyses.

After the samples have been weighed for the various determinations,

empty out the weighing bottle, wipe it and the spoon with a clean cloth. The bottle is then ready for the next sample. If contamination is a problem between samples, such as when working with trace minerals or insecticides, use a separate weighing bottle and spoon for each sample.

Do not weigh exact amounts of sample. This takes more time to weigh and may result in errors in sampling.

Liquid samples

Stir sample well if sediment is on bottom. While stirring, pipette sample into a tared weighing bottle. Weigh the sample plus the weighing bottle. The difference in weight is the weight of the sample delivered by the pipette. Use the same pipette to pipette a separate sample into the container used for chemical analysis.

This procedure is helpful if a large amount of material such as urine needs to be weighed and sampled. If the above procedure is used, results are reported on a weight basis.

Reference

Harris, Lorin E. 1970. Nutrition research techniques for domestic and wild animals. Animal Science Department, Utah State University, Logan, Utah 84321

DRY MATTER AND PARTIAL DRY MATTER

Principle

The moisture (water) of the sample is removed by volatilization caused by heat or freeze drying. The percentage of sample left after removing this moisture is known as the percent dry matter or percent partial dry matter.

The aim is to:

a) obtain "as fed" dry matter or "as collected" dry matter,

b) prepare the samples for chemical analyses, and

c) report the chemical analyses on a dry basis.

Dry matter content of feed samples and other materials is expressed on three dry matter bases: 1) as fed, 2) partially dry, and 3) dry, as defined below:

1) "As fed" refers to the feed as it is consumed by the animal; the term "as collected" is used for materials which are not usually fed to the animal, i.e., urine, feces, etc. If the analyses on a sample are affected by partial drying, the analyses are made on the "as fed" or "as collected" sample. Similar terms: "air dry"; i.e., hay; "as received"; fresh, green, wet.

2) "Partially dry" refers to a sample of "as fed" or "as collected" material that has been dried in an oven (usually with forced air) at a temperature usually about 60 C or freeze dried and has been equilibrated with the air; the sample after

these processes would usually contain more than 88% dry matter (12% moisture); some materials are prepared in this way so they may be sampled, chemically analyzed and stored. This analysis is referred to as "partial dry matter % of "as fed" or "as collected" sample". The partially dry sample must be analyzed for dry matter (determined in an oven at 105C) to correct subsequent chemical analyses of the samples to a "dry" basis. This analysis is referred to as "dry matter % of partial dry sample". Similar term: Air dry (sometimes air dry is used for as fed).

3) "Dry" refers to a sample of material that has been dried at 105C until all the moisture has been removed. Similar terms: 100% dry matter; moisture free. If dry matter (in an oven at 105C) is determined on an "as fed" sample it is referred to as "dry matter of as fed sample". If dry matter is determined on a partial dry sample it is referred to as "dry matter of partial dry sample". It is recommended that analyses be reported on the "dry" basis (100% dry matter or moisture free), and in addition the "as fed dry matter" should be reported.

As explained in the definitions there are essentially two kinds of samples: 1) those dry enough to grind and analyze immediately (more than 88% dry matter) and 2) those which need to be partially dried or handled in a special way (less than 88% dry matter). All samples with less than

88% dry matter are partially dried before analyzing. However, the chemist should use his judgment and decide whether partial drying will affect the results of the analyses. If it does, then arrangements should be made to analyze the sample on the "as fed" or "as collected" basis.

Recently it has been shown that the fiber fractions of esophageal samples are affected by drying samples at too high of a temperature. (Lesperance and Bohman, 1963). Therefore, care should be exercised in analyzing these samples.

Recently freeze drying equipment has become available. If this equipment is available this method should be substituted for partial drying in a force draft oven at 60C.

Example Calculations

a) Dry matter % on as fed basis =

$$\frac{(\text{wt of dried sample and container} - \text{wt of drying container})}{\text{wt of sample in g on as fed basis}} \times 100$$

Example: If the as fed sample weighed 1.9444 g, the sample container weighed 12.3278 and the dried sample (after drying in 105C oven) and container weighed 14.1011 g.

Dry matter % on as fed basis =

$$\frac{(14.1011 - 12.3278)}{1.9444} \times 100 = 91.2\%$$

b) Partial dry matter % on as fed or as collected sample =

$$\frac{\text{wt of dried sample}}{\text{wt of sample in g on as fed or as collected basis}} \times 100$$

Example: This determination is made when as fed samples contain less than 88% dry matter such as silages and fresh forages, and a large sample is dried for further analysis. If the as fed or as collected sample weighed 468.2 g, the sample after drying at 60C in a forced draft oven and equilibrating with moisture in the air weighed 155.4 g.

Partial dry matter % on as fed or as collected sample =

$$\frac{155.4}{468.2} \times 100 = 33.2\%$$

c) Dry matter % on partial dry sample =

$$\frac{(\text{wt of dry sample and container} - \text{wt of container})}{\text{wt of sample on partial dry basis}} \times 100$$

Example: When the partial dry sample is equilibrated with the water content of the air, another dry matter determination is necessary to correct it to the dry matter basis. If the sample of partial dry material weighed 2.3146 g, the dried sample and container after drying at 105C weighed 14.3957 g and the drying container weighed 12.2140 g.

Dry matter % on partial dry sample =

$$\frac{(14.3957 - 12.2140)}{2.3146} \times 100 = 94.3\%$$

d) To calculate as fed or as collected dry matter when partial dry matter was obtained on sample such as on silages or fresh forages and dry matter was determined on the partial dry sample.

Dry matter % of as fed or as collected sample =

$$\frac{\text{partial dry matter (\%) of as fed or as collected sample}}{100} \times \frac{\text{dry matter (\%) on partial dry sample}}{100} \times 100$$

Example: If partial dry matter of as fed or as collected sample was 33.2%, and the dry matter on the partial dry sample was 94.3%.

Dry matter % of as fed or as collected sample =

$$\frac{33.2\%}{100} \times \frac{94.3\%}{100} \times 100 = 0.332 \times 0.943 \times 100 = 31.3\%$$

e) All other chemical analyses must also be converted to the dry basis. The following is an example of the calculation using cellulose analysis for the example.

Cellulose % on as fed basis =

$$\frac{(\text{wt of crucible and cellulose} - \text{wt of crucible and ash})}{\text{wt of sample in g on as fed basis}} \times 100$$

Example: If wt of crucible and cellulose after drying is 35.4375 g and wt of crucible and ash is 35.1131 g and the as fed sample used weighed 1.0341 g.

Cellulose % on as fed basis =

$$\frac{(35.4375 - 35.1131)}{1.0341} \times 100 = 31.4\%$$

To adjust as fed sample to dry basis:

$$\frac{\text{cellulose \% on as fed basis}}{\text{dry matter \% of as fed sample}} \times 100$$

Example:

$$\frac{\text{31.4\% cellulose on as fed basis}}{\text{91.2\% dry matter on as fed sample}} \times 100 = \text{34.4\% cellulose on dry basis}$$

or

Cellulose % on partial dry basis =

$$\frac{(\text{wt of crucible and cellulose} - \text{wt of crucible and ash})}{\text{wt of sample in g on partial dry basis}} \times 100$$

Example: If the sample weighed 1.0114 g, the crucible and dry cellulose 34.8393 g and the crucible and ash 34.5114 g.

Cellulose % on partial dry basis =

$$\frac{(34.8393 - 34.5114)}{1.0114} \times 100 = 32.4\%$$

To adjust partial dry sample to dry basis:

$$\frac{\text{cellulose \% on partial dry basis}}{\text{dry matter \% of partial dry sample}} \times 100$$

Example:

$$\frac{\text{32.4\% cellulose on as fed basis}}{\text{94.3\% dry matter on as fed sample}} \times 100 = \text{34.4\% cellulose on dry basis}$$

Reference

Harris, Lorin E. 1970. Nutrition research techniques for domestic and wild animals. Published by L. E. Harris, 1408 Highland Drive, Logan, Utah, USA, 84321.

(Example heading for recording chemical analyses in bound books)

CELLULOSE

Date_____

Analyst_____

Lab No.	Beaker and crucible nos.	Weights to obtain sample wt. g	Crucible and Cellulose g	Crucible and Ash g	Cellulose g	Cellulose of det. %	Ave. Cellulose of Sample %	Dry Matter of Sample* %	Cellulose on dry basis %
70	3	76.3223	34.8393			32.16	32.4	91.30	34.4
380	27	75.3028		34.5114					
260A		1.0195			.3279				
70	4		36.2591			32.68			
380	28	74.2923		35.9289					
260B		1.0105			.3302				

* This is dry matter of partial dry sample in this example.

DETERMINATION OF DRY MATTER

Principle

The moisture of the sample is lost by volatilization caused by heat. The amount of material left after the removal of the moisture is the dry matter.

Equipment

a) Oven, 105 C.

b) Covered aluminum dishes, 50 mm diameter with covers, or similar containers.

Determination

a) Brush out the aluminum dishes between samples. If ether extract is to be determined on the sample rinse the dishes with 95% ethyl alcohol and finally with ethyl ether. Let the ether evaporate under a hood. Dry the dishes in 105 C oven for at least one hour. Place in desiccator, cool and weigh. Handle dishes with metal tongs.

b) Weigh by difference 1.5 to 2.0 g of sample into a weighed dish. Put top of dish on bottom of dish and place in 105 C oven overnight. Remove dishes, put cover on top and place in desiccator and cool. Remove from desiccator and weigh as quickly as possible.

c) The samples may be stored in a desiccator for use in ether extract determination.

Calculation

$$\% \text{ dry matter} = \frac{(\text{wt of dish} + \text{wt of dried sample}) - \text{wt of dish} \times 100}{\text{wt sample before drying}}$$

$$= \frac{\text{wt of dry sample} \times 100}{\text{wt sample before drying}}$$

Note: If the sample was analyzed on an as fed basis the dry matter is labeled "dry matter of as fed sample".

If the sample was analyzed on a partial dry basis the dry matter is labeled "dry matter of partial dry sample".

Reference

Official methods of analyses of the Association of Official Agricultural Chemists. 1960. 9th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D.C.

DETERMINATION OF GROSS ENERGY

Principle

The amount of heat, measured in calories, that is released when a substance is completely oxidized in a bomb calorimeter, is called the gross energy (GE) of the substance. A sample of the material to be tested is weighed into a combustion capsule. The combustion capsule is placed in an oxygen bomb containing 25 to 30 atmospheres of oxygen. The oxygen bomb is covered with 2000 g of water in an adiabatic calorimeter. After the bomb and calorimeter have been adjusted to the same temperature, the sample is ignited with a fuse wire. The temperature rise is measured under adiabatic conditions. From the hydrothermal equivalent of the calorimeter times the temperature rise minus some small corrections for fuse wire oxidation and acid production, the caloric content of the sample is calculated.

Equipment

a) Parr oxygen bomb calorimeter and accessories or equivalent.

b) The calorimeter may be equipped with an automatic temperature controller (Fisher Scientific Co., Catalog No. 4-364V2).

c) Solution or trip balance with capacity to 3000 g and accurate to 0.1 g.

Reagents

a) Standard sodium carbonate solution, equivalent to 1 cal/ml (3.658 g Na_2Co_3 per liter).

b) Methyl orange indicator.

c) Benzoic acid combustion tablets or primary standard grade crystals.

Procedure

a) Weigh by difference approximately 1.0 g of sample and place in a clean, empty combustion capsule. Samples may be pelleted, but this is usually not necessary.

b) Attach a 10 cm length of fuse wire between the electrodes of the bomb (oxygen) and set the combustion capsule with sample in place in the loop electrode. Adjust the fuse wire so it is touching the sample.

c) Place about 1 ml of water in the bomb cylinder and swirl it around to wet the sides. This is not necessary if the bomb is still wet from a preceding determination.

d) Assemble the bomb, tighten the screw cap, close the pressure release valve and fill with oxygen to 25 atmospheres gauge pressure. Place the bucket (oval) in the calorimeter, set the bomb in the bucket, and attach the clip terminal.

e) Weigh 2000 g distilled water on the solution or trip balance (use a 2000 ml volumetric flask to hold the water) and carefully pour into the calorimeter bucket. The water temperature must be within the range of the calorimeter thermometers.

f) Close the cover (be sure thermometers are up), lower the thermometers and start the water circulating motor. Remove the cap from the calorimeter jacket cover and fill the cover with water until it runs out the drain hose (only necessary at the beginning of the day's run).

g) Adjust the temperature of the water in the outer jacket to approximately equal that of the calorimeter by adding hot or cold water, and allow one minute to attain equilibrium. Then carefully adjust the temperature to be exactly equal and check the calorimeter temperature at one-minute intervals for three minutes.

h) Read and record the initial temperature to the nearest 0.0002 C and ignite the sample. Turn in hot or cold water to keep the jacket temperature equivalent to the calorimeter temperatures during the period of rise.

i) Compare and adjust the temperature of the outer jacket to the inner jacket of the calorimeter temperature frequently and carefully to insure adiabatic condition or that the temperatures are equal. Read and record the final temperature after the same temperature is observed in three successive one-minute intervals.

j) Raise the thermometers. Open the calorimeter, take the bomb from the calorimeter bucket, release the residual pressure of the bomb and open. Carefully remove the remaining pieces

of fuse wire from the electrodes; straighten and measure the combined total length in centimeters. The calories of wire burned can be determined with the measuring scale that is supplied with the wire.

k) Rinse all inner bomb surfaces with a stream of neutral distilled water and collect all washings in a clean beaker. Titrate the washings with the standard sodium carbonate solution using methyl orange indicator to determine the amount of acid formed from the incidental oxidation of nitrogen and sulfur compounds. A correction is made to take care of the heat liberated in the formation of the acid.

l) Correct the initial and final temperatures from the calibration curve supplied with the thermometer.

Calculations

a) GE (cal/g) on as fed basis = $\dfrac{\left(\text{final temp.} - \text{initial temp.}\right) \text{ hydrothermal equiv. of bomb} - \left(\text{length of fuse wire burned} \times \text{cal per cm}\right) - \text{ml } Na_2Co_3}{\text{wt. of sample}}$

Example: A 1.0214 g sample of feed (as fed basis) had an initial temperature of 23.13 C and a final temperature of 25.25 C. The hydrothermal equivalent of the bomb is 2412 cal per degree C. There was 7.0 cm of fuse wire burned with a correction of 2.3 cal per cm of wire and 6.0 ml of Na_2Co_3 titrated (equivalent of 6.0 cal).

GE (cal/g) = $\dfrac{(25.25-23.13)\ 2412 - (7.0 \times 2.3) - 6.0}{1.0214 \text{ g sample on as fed basis}}$ = 3804 cal/g or 3804 kcal/kg

Note: Report the data on the source form as kcal/kg.

Adjusting to dry basis:

$$\frac{\text{GE(kcal/kg) on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{GE (kcal/kg) on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

DETERMINATION OF ASH

Principle

The sample is ignited at 600 C to burn off all organic material. The inorganic material which does not volatilize at that temperature is called ash.

Equipment

a) Muffle furnace

b) Porcelain crucibles (for trace element analyses)

c) Desiccator, with magnesium perchlorate desiccant.

Procedure

a) Place new or clean crucibles in a muffle furnace at 600 C for one hour. Transfer crucibles from furnace to a desiccator and cool to room temperature. Weigh them as quickly as possible to prevent moisture absorption. Use metal tongs to move the crucibles after they are ashed or dried.

b) Weigh by difference 1.5 to 2.0 g sample into a tared porcelain crucible. Place in a muffle furnace and hold the temperature at 600 C over night.

c) Transfer the crucible to a desiccator and cool to room temperature. When cool, weigh the crucible as quickly as possible to prevent moisture absorption.

d) Save the ash sample if mineral determinations are to be made.

Calculations

$$\text{Ash (\%) on partial dry or as fed basis} = \frac{\text{wt of ash} \times 100}{\text{wt of sample}}$$

Adjusting to dry basis:

$$\frac{\text{ash \% on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{ash \% on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

Reference

Official Methods of Analysis of the Association of Official Agricultural Chemists. 1965. 10th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D. C.

DETERMINATION OF CRUDE FIBER

Principle

A moisture-free and ether-extracted sample is digested first with a weak acid solution, then a weak base solution. The organic residue is collected in a filter crucible. The loss of weight on ignition is called crude fiber.

Equipment

a) Extraction apparatus consisting of individually controlled heaters and water-cooled condensers designed to maintain constant volume of solution throughout digestion.

b) Digestion containers. Use digestion containers of such size and shape that the solution is not less than 25 mm or more than 38 mm in depth. A 500 ml wide mouth Erlenmeyer flask or 600 ml tall form beaker is recommended, depending on the type of condenser used.

c) Fit condensers into the top of two 5 liter round bottom flasks and put into a heating element that fits the flasks. Arrange to have a siphon made from rubber tubing. The purpose of this apparatus is to be able to preheat the sulfuric acid and sodium hydroxide to boiling before adding to the digestion containers.

d) Linen cloth with about 20 threads per cm or No. 40 filtering cloth made by National Filter Media Corporation, New Haven, Connecticut.

Reagents

a) Sulfuric acid solution. 0.255 N, 1.25 g H_2SO_4/100 ml. Check normality by titration and adjust if necessary to 0.255 N.

b) Sodium hydroxide solution. 0.313 N, 1.25 g NaOH/100 ml, free from Na_2CO_3. Check normality by titration and adjust if necessary to 0.313 N.

c) Asbestos. Gooch grade, medium fiber, acid-washed, and ignited is usually satisfactory. If it needs testing, digest on steam bath for eight hours with about 5% NaOH; wash thoroughly with hot water. Digest in similar manner eight hours with HCl (1+3) and again wash with hot water. Dry and ignite at bright red heat.

d) Dow-Corning Antifoam A, spray or octyl alcohol.

e) Ethyl alcohol.

Procedure

a) The residue from the ether extract determination may be used (use original weight of sample before drying and extracting with ether). If this residue is not used, proceed as follows to obtain a dry ether extract free sample.

b) Weigh by difference 1.5 to 2.0 g of sample into a clean alundum thimble. Dry in an oven at 105 C overnight (weigh 6 samples as outlined under e).

c) Extract with ether (see ether extract determination page 141).

d) Transfer the extracted residue, together with approximately 0.5 g asbestos to the digestion containers. Spray a small amount of Dow-Corning Antifoam A into the container, or add a drop or two

of octyl alcohol. Siphon 200 ml of boiling sulfuric acid solution into the digestion container. Place the beaker on preheated extraction heater and cover with condenser. The solution should start boiling within one minute. Rotate the container frequently until the sample is thoroughly wetted. Take care to keep material from remaining on the sides of the beaker out of contact with the solution. (A blast of air conducted into the flask serves to reduce frothing of liquid.) Repeat procedure on other samples at 5-minute intervals.

e) When the first sample has boiled for exactly 30 minutes, remove it and filter onto a linen cloth in a fluted funnel, and wash with boiling water until washings are no longer acid. Wash the sample back into the container with the 200 ml of boiling sodium hydroxide solution. Place beaker back on hot plate. The solution should come to a boil within one minute. Allow a total of five minutes from time of removal of sulfuric acid treated sample from hot plate until the sodium hydroxide treated sample starts to boil. (With this timing sequence, six determinations can be run at a time by one operator.)

f) When the first sodium hydroxide solution has boiled exactly 30 minutes, remove it and filter onto linen cloth as before. Wash the sample with boiling water. Filter with suction into a Gooch crucible with thin layer of asbestos in bottom. This is prepared by first having asbestos in water solution and pouring a small quantity into Gooch crucible. Finally, wash sample in Gooch

crucible with about 15 ml of 95% ethyl alcohol.

g) Dry the sample in a 105 C oven overnight. Cool to room temperature in a desiccator and weigh.

h) Ignite the contents of crucible in a muffle furnace at 600 C for an hour or longer. Cool in a desiccator to room temperature and weigh. Report the loss in weight as crude fiber. If the crude fiber samples are difficult to filter through the Gooch crucibles, asbestos may be added to samples prior to addition of sulfuric acid solution. Also, washing the sample while on the linen just prior to filtering through Gooch crucible with hot 10% K_2SO_4 solution may be helpful. The K_2SO_4 solution may be added during filtering whenever filtration becomes difficult.

Calculations

Crude fiber (%) on partial dry or as fed basis =

$$\frac{\text{loss of wt on ignition} \times 100}{\text{wt of sample before drying and extracting with ether}}$$

Adjusted to dry basis:

$$\frac{\text{crude fiber \% on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{crude fiber \% on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

Reference

Official Methods of Analysis of the Association of Official Agricultural Chemists. 1965. 10th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D. C.

DETERMINATION OF ETHER EXTRACT

Principle

Ether is continuously volatilized, then condensed and allowed to pass through the sample, extracting ether soluble materials. The extract is collected in a beaker. When the process is completed, the ether is distilled and collected in another container and the remaining crude fat is dried and weighed.

Equipment

a) Goldfisch, fat extraction apparatus

b) Solvent beakers

c) Extraction thimbles, alundum

Reagents

a) Diethyl ether, anhydrous

If anhydrous ether is not available, proceed as follows: Wash commercial ether with 2 or 3 portions of water, add solid sodium hydroxide or potassium hydroxide, and let stand until most of the water is extracted from the ether. Decant into dry bottle, add small pieces of carefully cleaned metallic sodium and let stand until hydrogen evolution ceases. Keep ether, thus dehydrated, over metallic sodium in loosely stoppered bottles. Add zinc pellets to containers of both new and used ether to prevent formation of dangerous peroxides.

Procedure

a) Weigh by difference a 1.5 to 2.0 g sample into a clean previously extracted alundum thimble.

b) Dry the sample at 105 C overnight (or use sample from dry matter determination).

c) Clean and dry solvent beakers in 105 C oven for an hour. Cool them to room temperature in a desiccator, weigh and record the tare weight.

d) Place thimble and sample in sample container and fix under the condenser of the Goldfisch extraction apparatus. Add 30 to 40 ml of diethyl ether to the solvent beaker and place on condenser with screw ring, which is tightened as much as possible by hand. Turn on the water that cools the condenser. Raise the hot plates until they are in contact with the beakers and turn on heaters. Check for ether leaks after the ether starts to boil and condense. When level of ether goes down in beaker to a constant level (because a portion is volatilizing and condensing) the apparatus may be left with occasional checks. Extract 16 hours.

e) After the extraction is completed, lower the heater and allow the thimble to drain empty. Remove the samples and place glass ether reclaiming tubes under the condenser. Replace beaker, raise hot plate and distill ether into the reclaiming tubes. Just before ether in the beaker evaporates to dryness, lower the hot plate. Remove beakers. Pour ether from reclaiming tubes into a container for used ether. Complete drying of ether in beakers

in open air and allow ether beakers to remain on bench. Dry the ether extract in a 105 C explosion proof oven for 30 minutes. Cool in desiccator to room temperature and weigh.

Calculations

Ether extract (%) partial dry or as fed basis =

$$\frac{\text{wt ether extract} \times 100}{\text{wt of sample}}$$

Adjusting to dry basis:

$$\frac{\text{ether extract \% on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{ether extract \% on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

Reference

USA Official Methods of Analysis of the Association of Official Agricultural Chemists. 1960. 9th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D. C.

DETERMINATION OF NITROGEN FREE EXTRACT

Principle

Nitrogen free extract (NFE) of a feed is determined by difference after the analyses have been completed for ash, crude fiber, ether extract and crude protein. NFE is necessary for the calculation of TDN.

Calculation

NFE (%) on dry basis =

100%-[ash (%) on dry basis + crude fiber (%) on dry basis + ether extract (%) on dry basis + protein (%) on dry basis].

Example: If the ash on dry basis was 7.2%; crude fiber, 32.7%; ether extract, 3.3%; and the crude protein, 15.3%, then:

NFE (%) on dry basis = 100% - [7.2% + 32.7% + 3.3% + 15.3%] = 41.5%

Adjusting to as fed basis =

$$\frac{\text{constituent on dry basis (\%)}}{100} \times \frac{\text{as fed dry matter (\%)}}{100} \times 100$$

Example:

If as fed dry matter = 89.3%, then: NFE (%) on as fed basis =

$$\frac{41.5}{100} \times \frac{89.3}{100} \times 100 = 0.415 \times 0.893 \times 100 = 37.1\%$$

Reference

Crampton, E. W., and L. E. Harris. 1969. Applied Animal Nutrition, The use of feedstuffs in the formulation of livestock rations. Second Edition. W. H. Freeman and Co. San Francisco, California.

DETERMINATION OF NITROGEN AND CRUDE PROTEIN

Principle

The nitrogen of protein and other compounds are transformed into ammonium sulfate by acid digestion with boiling sulfuric acid. The acid digest is cooled, diluted with water, and made strongly basic with sodium hydroxide. The ammonia is released and distilled into a boric acid solution. The ammonia in the boric acid solution is titrated with standardized sulfuric acid.

An alternate procedure is also given that uses standardized acid to collect the ammonia and standardized base to back-titrate the excess acid. Either method will give good results but there is a small saving in reagent costs with the alternate method. However, the boric acid procedure saves considerable time since the boric acid does not have to be standardized or measured accurately and there is no problem of absorbing all of the ammonia distilled over with high nitrogen samples.

Equipment

a) Macro Kjeldahl nitrogen digestion and distillation apparatus

b) Kjeldahl flasks, 650 ml

c) Erlenmeyer flasks, 500 ml

d) Two burettes

Reagents

a) Indicator solution (0.1% methyl red and 0.2% bromocresol green in alcohol).

b) Standardized sulfuric acid solutions, 0.1 N. Standardize acid against tris (hydroxymethyl) aminomethane, also known as 2-amino-2-(hydroxy-methyl)-1,3-propanediol, which has an equivalent wt of 121.14 g. A 0.3029 g sample dissolved in water is equivalent to 25 ml of 0.10 N H_2SO_4. Use the indicator solution under a). Make at least three determinations and use the average normality of the three.

c) Concentrated sulfuric acid, 93-98%, reagent grade.

d) Catalyst mixture. Mix 7% reagent grade fine crystalline copper sulfate with reagent grade potassium sulfate (premeasured catalyst packages are also available).

e) Zinc, mossy.

f) Sodium hydroxide solution, nitrogen-free (dissolve 450 g sodium hydroxide flakes per liter of solution). Usually 18 to 25 liters are mixed at a time. Mix in a large metal container by alternately adding small amounts of water and sodium hydroxide. Stir with metal or glass rod until completely dissolved after each addition of sodium hydroxide. Let stand overnight to cool then transfer by using a porcelain pitcher to a polyethylene storage bottle.

g) Boric acid solution, 4%. Dissolve 40 g boric acid reagent per liter and add 5 ml indicator solution.

Alternate Procedure Reagents

a) All reagents as described in b) through f) above.

b) Sodium hydroxide solution, 0.1 N. Add 4 g of NaOH (reagent grade) per liter of distilled water (free from CO_2). Standardize the

sodium hydroxide solution against potassium biphthalate which has an equivalent wt of 204.228 g. A 0.5106 g sample dissolved in water is equivalent to 25 ml of 0.10 N NaOH.

c) Methyl red indicator (0.2% methyl red in 95% ethyl alcohol).

Procedure

a) Weigh by difference a sample to contain approximately 25 to 50 mg N. For feed samples this is 1.5 to 2.0 g. For fresh feces this is 4 to 6 g. For fresh urine use 5.0 ml, but weigh the material because urines vary considerably in specific gravity. Place a folded filter paper in a small funnel set in a small bottle. Place solid samples in the filter paper. Twist the top of the filter paper around the sample and introduce into a Kjeldahl flask.

b) Run two reagent blanks (with filter paper) through all the steps of the procedure and subtract the blank titration from the sample titrations. Usually the average of two blanks are sufficient for a day's run.

c) Add 10 g of the catalyst mixture with a small measuring scoop followed by 25 ml of concentrated sulfuric acid. Increase the amount of sulfuric acid used by 10 ml for each gram of organic matter in sample above 2 g (for samples very low in nitrogen).

d) Place the flask on the digestion heaters of the Kjeldahl apparatus and turn on heat and exhaust fan. Watch the digestion process until frothing ceases. If the froth of a sample starts up the neck of the flask remove it from the heat to allow froth to subside

and return to heater. Frothing is common with liquid samples. Continue digestion, turning flasks occasionally for 30 minutes after the solution clears and all carbon has been oxidized. Turn off the heat and allow the flasks to cool on the heaters until fuming ceases; then remove the flasks and continue cooling on a rack.

e) Before the digest solidifies, carefully add 250 ml cold tap water while cooling the flask under running cold water. If the digest cooled to a solid state dissolve the solids by swirling before continuing the procedure.

f) Add 50 ml of the boric acid solution to the 500 ml Erlenmeyer flasks and set them under the condensers with the tips beneath the surface of the solution (see alternate procedure below).

g) Turn on the water for the condenser. Turn on the heaters on the distillation rack so that they are hot when the distillation begins. This prevents the boric acid from being drawn up into the distillation flasks.

h) Carefully add 110 ml of NaOH with the flask tilted so that the reagent runs down the side to the bottom of the solution. (If additional H_2SO_4 was used add 35 ml of NaOH for each 10 ml of H_2SO_4.) Add a few pieces of mossy zinc (1-2g) and quickly attach the flask to the distillation condenser. With the stopper in place, gently mix the contents of the flask with a rotary motion. The copper ions will form a dark blue copper ammonium complex that

indicates that enough NaOH was added to neutralize the excess sulfuric acid and release the ammonia.

i) Distill about two-thirds of the liquid in the flask or until about 100 ml of liquid are contained in the receiving flask. Lower the receiving flasks and allow the condensers to drain for five minutes while the Kjeldahl flasks are cooling on the distillation heaters. Rinse the bottom of the condensers with neutral water (titrated with indicator and sulfuric acid).

j) Titrate the ammonia with standardized 0.1 N sulfuric acid to a faint purple or colorless endpoint.

k) Empty the Erlenmeyer flasks and let drain upside down on a rack. They are then ready for the next run without washing.

l) Drain Kjeldahl flasks through a screen to collect the zinc. (The undissolved pieces of zinc can be reused.) Rinse in tap water and drain upside down on a rack. They are then ready for the next run without washing.

Alternate Procedure

a) Weigh sample, digest and dilute digest as in a) through e) above

b) Add from a burette or volumetric pipet exactly 50 ml of standardized 0.1 N sulfuric acid to the 500 ml Erlenmeyer flasks (rather than the boric acid) and set them under the condensers as in f) above. Continue the procedure through the distillation as in g), h), and i) above.

c) Add 5 drops of the methyl red indicator solution. Back-titrate the sulfuric acid that has not been neutralized by ammonia with the standardized 0.1 N sodium hydroxide solution. (If the back-titration is zero then the sample contained too much ammonia for 50 ml of 0.1 N H_2SO_4 to absorb. Repeat the determination with a smaller sample or larger volume of standardized sulfuric acid.

d) Continue the procedure as in k) and l) above.

Calculations

a) Calculate the percent nitrogen of sample when using boric acid as follows: Nitrogen percent of sample =

$$\frac{\left[\left(\begin{matrix}\text{ml acid}\\ \text{titrated}\end{matrix} - \begin{matrix}\text{ml blank}\\ \text{titration}\end{matrix}\right)(\text{acid N})\right]}{\text{wt of sample in g}} \times 0.014 \times 100$$

b) Calculate the percent nitrogen of sample when using sulfuric acid to collect the ammonia as follows: Nitrogen percent of sample =

$$\frac{(\text{ml acid} \times \text{acid N}) - \left(\begin{matrix}\text{ml base}\\ \text{titrated}\end{matrix} \times \text{base N}\right)}{\text{wt of sample in g}} \times 0.014 \times 100$$

c) To convert percent nitrogen to percent crude protein:

crude protein = N % x 6.25

d) Adjust percent crude protein to dry basis:

$$\frac{\text{protein \% on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{protein \% on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

Reference

Scales, F. M., and A. P. Harrison. 1920. Boric acid modification of the Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem. 12:350-352.

MICRO KJELDAHL DETERMINATION OF TOTAL NITROGEN IN PLANT MATERIAL

Principle

Total nitrogen in plant material is generally estimated by a two-step Kjeldahl procedure. First, the sample is subjected to a high temperature digestion with concentrated H_2SO_4 and catalysts to convert organic and inorganic forms of N to ammonium ions. Secondly, acidimetric titration following alkaline distillation of ammonia, or colorimetric techniques. Modified digestion procedures have been proposed for quantitative recovery of nitrate based on the conversion of a proportion of nitrate to ammonia. The Dumas procedure has been suggested for estimation of total N in plant materials, but has not been widely adopted because of the time required per analysis and of the investment required for equipment.

Macro-Kjeldahl procedures have been widely used for total N analyses of plant material. However, semimicro modifications have been gaining favor in recent years because of the increased speed of analysis, the decreased cost per sample, and the reduced volumes of corrosive acid and alkali that are handled by the analyst. The maximum number of total N analyses that can be performed daily is generally limited by a lack of sample digestion facilities, a direct result of the relatively high cost of Kjeldahl digestion units and of the considerable laboratory space required for these units.

Equipment

a) Heating block capable of refluxing H_2SO_4

b) A steam distillation apparatus

c) A titration apparatus

Preparation of Reagents

a) Salicylic acid - H_2SO_4 mixture

5 g salicylic acid

200 ml conc. H_2SO_4

b) Salt - catalyst mixture

100 g K_2SO_4

10 g $CuSo_4 \cdot 5\ H_2O$: (1 g Se)

Note: Use of selenium as a catalyst is optional. It will reduce time of digestion considerably. However, extreme caution should be used since selenium is highly toxic.

c) 10 N NaOH

Add distilled water to 400 g NaOH to make 1 liter. Caution: Mix in cold water bath. Store in a plastic container.

d) Methyl red - bromocresol green indicator

Dissolve in 100 ml of ethyl alcohol:

0.330 g bromocresol green

0.165 g methyl red

e) Boric acid indicator

Dissolve 20 g Boric acid (H_3BO_3) in 700 ml hot water. Add

cooled solution to 200 ml ethanol. Add 20 ml of above indicator. Add approximately 0.05 N NaOH until an equal volume of water turns the boric acid indicator solution from pink to pale green. Dilute solution to volume (1 liter) with water.

Procedure for standardization of acid

A. THAM Primary Standard

a) Put about 1 g of Tris (Hydroxymethyl) Aminomethane (THAM) in a clean weighing bottle and dry in an oven at 100 C for 4 hours. Be sure Primary Standard grade of THAM is used.

b) Place in a desiccator until the sample has cooled.

c) Weigh out three portions of THAM with accuracy of 0.1 mg into 125 ml Erlenmeyer flasks. Determine size of portion according to the following formula:

$$g\ THAM = 8 \times 121.14 \times \frac{5}{1000} \times \text{desired Normality of Standard}$$

For example: if the desired normality is 0.01, the equation works out to .0484 g THAM. Therefore, weigh out three portions of THAM weighing between 0.045 and 0.050 g.

d) Add exactly 5 ml of Boric Acid Indicator (from total N procedure) to each flask.

e) Titrate with acid solution. About 40 ml of acid should be required.

f) Calculate the normality of the acid and average the three values. The average deviation should not be greater than 0.0002 N.

Calculation

$$\text{Normality of Acid} = \frac{\text{grams of THAM}}{(121.14)\ (\%\text{ of acid titrated})}$$

Procedure (samples containing high NO_3^- conc.)

a) Weigh out 100 mg plant samples in Folin-Wu N.P.N. tubes (<40 mesh).

b) Add 4 ml of salicylic acid - H_2SO_4 mixture and leave at room temperature for 2 hours.

c) Add 0.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) followed by cautious heating in the aluminum block at 3.5 until frothing stops, (about 1½ hours).

d) Add 1.1 g of salt - catalyst mixture and heat at 3.5 until foaming stops, wrap aluminum block in tin foil and heat on high until solution is green.

e) Cool tubes and dilute with distilled water.

f) Mix by stoppering tubes and inverting several times.

g) Take 10 ml aliquot, add 8-10 ml of 10 N NaOH, and steam distill into erlenmeyer beaker containing about 10 ml of boric acid indicator.

h) Titrate with dilute, standardized HCl.

Plant materials are dried at 60 C for 24 hours, ground in a Wiley mill to pass a 40 mesh screen, and stored in stoppered glass bottles. The proposed procedure described involves digestion of plant materials in graduated pyrex Folin-Wu N.P.N. tubes (Kimax 47125 or Corning 7940) heated

in an aluminum block. Put an asbestos board (1/8" thick) extending from the base of the block to the upper heat shield. This is necessary to prevent temperature gradients from developing when the block is heated in a well-ventilated fume hood.

Procedure A - For estimating total N in plant materials containing trace amounts of nitrate.

A 100 mg plant sample (<40 mesh) is placed in a clean, dry Folin-Wu digestion tube and 1.1 g of salt catalyst mixture (100 g K_2SO_4: 10 g $CuSO_4$: 1 g Se) and 4 ml of concentrated H_2SO_4 are added. The tube is swirled to mix the sample and digestion reagents and then placed in the aluminum heating block preheated to 300 C. A small glass funnel (25 mm diameter) is placed in the mouth of the tubes to insure efficient refluxing of the digestion mixture and prevent loss of H_2SO_4. The sample is digested at the boiling point of the mixture for 60 minutes past the time of clearing, removed from the heating block, and allowed to cool to room temperature. The digest is diluted with distilled water to the 50 ml mark inscribed on the digestion tube, stoppered, and mixed by inverting several times. Ammonium in the digest is then determined by making an aliquot (e.g., 10 ml) alkaline with 10 N NaOH and performing a steam distillation analysis.

Procedure B - For estimating total N in plant materials containing high nitrate concentrations.

A 100 mg plant sample is placed in a Folin-Wu digestion tube and 4 ml of a salicylic acid - H_2SO_4 mixture (5 g of salicylic acid per 200 ml of conc. H_2SO_4) is added. After allowing the mixture to stand at 25 C for 2 hrs,

0.5 g of $Na_2S_2O_3 \cdot 5\ H_2O$ are added followed by cautious heating in the aluminum block until frothing ceases. After removing the tube from the heating block and cooling to about 25 C, 1.1 g of salt - catalyst mixture is added, the sample is digested, and ammonium in the digest is determined as described above.

Calculations

$$\% N = \frac{\text{ml acid x normality x 14 x dilution factor x 100}}{\text{sample dry wt.}}$$

% crude protein - 6.25 x % N

Reference

Nelson, D. W., L. E. Sommers. 1973. Determination of total nitrogen in plant material. Purdue University, Lafayette, Indiana 47907.

DETERMINATION OF EXCHANGEABLE AMMONIUM BY STEAM-DISTILLATION METHOD

Principle

Determines the percent of exchangeable ammonium-nitrogen.

Reagents

a) 2N potassium chloride

Add distilled water to 155 g KCl until 1 liter of solution is obtained.

b) Magnesium oxide

Heat heavy MgO in muffle furnace at 600 C for 2 hrs. Cool in a desiccator containing KOH pellets and store in a tightly stoppered bottle.

c) Boric acid indicator

1) 800 g boric acid dissolved in 5 liters of boiling distilled water and diluted to 20 liters.

2) Indicator made by mixing 1 g bromcresol green and 0.4 g methyl red in 200 ml ethyl alcohol.

3) Add 80 ml of indicator to 20 liter of boric acid solution. Adjust pH so that equal volume of water turns color of boric acid indicator solution.

Procedure

a) Weigh out 0.5 g samples of ground samples into 50 ml centrifuge tubes.

b) Add 30 ml 2N KCl, stopper tubes, and shake for 1 hour.

c) Take 10 ml aliquot, add 0.2 g MgO and 10 ml distilled water, and steam distill into an Erlenmeyer flask containing 5 ml boric acid indicator. Regulate distillation so that about 30 ml is distilled in 2-4 min.

d) Titrate with dilute, standardized HCl.

Calculations

$$\% \ NH_4^+\text{-}N = \frac{\text{ml acid x normality x 14 x dilution factor x 100}}{\text{sample dry weight}}$$

Reference

Black, C. A., A. C. Dinaur, et al. 1965. Methods of soil analysis - Part 2. Chemical and Microbiological Properties. American Society of Agronomy, Inc., Publisher, Madison, Wisconsin.

RAPID FIELD SCREENING PROCEDURES FOR NITRATE ACCUMULATION

Principle

The following tests are based on the rapid development of color by a reagent powder or solution when placed in contact with plant sap allowing a rough quantitative estimate of nitrate concentrations. As such, they are widely used in plant tissue and soil fertility test kits. An additional utility of these tests for the plant breeder results from the rather high positive relationship found between nitrate accumulation of a line or cultivar and its grain protein content under uniform nitrogen fertility. This property enables rapid, non-destructive field screening for high protein lines by a nitrate test in situations where a direct chemical protein analysis is too slow or unavailable.

Diphenylamine Test

Reagents - Equipment

a) Dissolve 1 g of diphenylamine in 100 ml concentrated H_2SO_4. Store in a bottle equipped with an eye dropper. Discard if solution turns blue or black.

b) Knife or razor blade.

Procedure

a) Split the midrib of the first fully expanded leaf of a plant in boot stage (prior to flowering) with a knife or razor blade.

b) Apply several drops of diphenylamine solution to cut surface.

c) Observe color development. Formation of a deep blue color within 1/2 - 1 min. indicates high nitrate content. Absence of blue color indicates low nitrate.

Cautions

a) Diphenylamine solution is strongly acidic and carbonizes some tissue turning it deep brown or black. The discoloration should not be confused with blue color obtained when nitrates are present.

b) Avoid contact of reagent with clothes or skin.

Alpha-naphthylamine Test

Equipment

a) Knife or razor blade.

b) Powder bottle or atomizer (non-light transparent).

c) Whatman number 1 filter paper or equivalent (optional).

d) Needle nose pliers (optional).

Reagents

a) Bray nitrate powder

1) 110 g $BaSO_4$

2) 10 g $MnSO_4 \cdot H_2O$

3) 2 g finely powdered zinc

4) 75 g citric acid

5) 4 g sulfonilic acid

6) 2 g alpha-naphthylamine

- Grind any coarse materials to a fine powder mixing 1), 2), 3), 4), and 5) together. Then mix one part of alpha-naphthylamine to 100 parts of the mixture. Then thoroughly mix the whole. Use extreme care to have room, table tops and equipment free of nitrate and nitrite. Store in a thoroughly blackened bottle as light affects the alpha-naphthylamine.
- Glycerol may be added to the powder to permit easier application. Two parts of glycerol to one part of powder.

Procedure

a) Split the lower portion of the midrib of the third leaf from top of a plant in boot stage (prior to flowering) with knife or razor blade.

b) Sprinkle small amount of nitrate powder or powder glycerol mixture on the cut portion of the midrib.

c) The powder turns <u>red</u> within 1/2 - 1 min. in the presence of high nitrate concentrations. Nitrate concentrations are proportionately lower for light red, pink and white colored powder.

Cautions

a) For uniform comparisons, all color readings should be determined after a set time of 1/2 - 1 minute.

b) It is advisable to test all plants at approximately the same

time or day and under uniform weather conditions (mid-day in full sunshine is most desirable) in order to obtain the best estimate of variation between cultivars. Evaluate, when rate of photosynthesis is at a maximum. Rate of nitrate uptake is influenced by weather conditions.

c) Alpha-naphthylamine has been classified as a cancer producing chemical in animals by OSHA. Extreme caution should be exercised in preparation and use of Bray's nitrate powder to avoid breathing or contact with the skin.

d) Address for ordering alpha-naphthylamine:

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO. 63178, U.S.A.

References

Ohlrogge, A.J. 1962. Soil and plant tissue tests. Station Bulletin 635. Purdue University Agric. Station, West Lafayette, Indiana, 47907

Morgan, N.D., G.A. Wickstrom. 1956. Give your plants a blood test. American Potash Institute, Washington, D.C.

DETERMINATION OF NITRATES AND NITRITES IN FORAGE

Nitrates and nitrites are known to have injurious effects on animals when excess amounts are ingested. Nitrites are much more toxic but nitrates are reduced to nitrites by bacterial action in the digestive tract.

Nitrites (NO_2^-) are rarely found in plants in appreciable amounts except under very unusual conditions such as severe disease infections; therefore, the results are usually reported in terms of nitrate (NO_3^-). The usual method is to determine the total nitrate and nitrite in terms of nitrate. However, the method can also be used to determine the nitrite content of a feed separately if a nitrite problem is suspected.

Principle

Nitrate is reduced to nitrite by zinc and manganese (II) sulfate. The reaction then consists of diazotization of sulfanilic acid by the nitrite ion and subsequent coupling with 1-naphthylamine to form a red dye. The reaction is optimum in the pH range of 1.7 - 3.0. Interfering iron is complexed with citrate. Copper from 0.2 to 1.0 ppm aids the reaction but over 1.2 ppm interferes.

Equipment

a) Spectronic "20" spectrophotometer or equivalent.

b) Centrifuge.

Reagents

a) Standard nitrate solution. Dissolve 1.37 g of sodium nitrate in 1 liter of water for a stock solution of 0.1% nitrate (1000 micrograms per ml).

b) To 200 ml of acetic acid, add 5.0 ml of $CuSO_4$ solution (0.1572 g of $CuSO_4 \cdot 5H_2O$ per liter) and dilute to 1000 ml. This solution is then 20% acetic acid with 0.2 ppm Cu.

c) Powder mixture (Bray's indicator). One hundred g of barium sulfate (dried at 105 C), 10 g of manganese (II) sulfate monohydrate, 2 g of finely powdered zinc, 75 g of powdered citric acid, 4 g of sulfanilic acid, and 2 g of 1-naphthylamine. Grind any coarse material to a fine powder. Mix the manganese (II) sulfate, powdered zinc, sulfanilic acid, and 1-naphthylamine separately with portions of barium sulfate. Then mix thoroughly all ingredients including the barium sulfate and citric acid. Use extreme care to have room, table top, and equipment free of nitrate and nitrite. Store the powder in a blackened bottle, since light affects the 1-naphthylamine. The reagent is stable for many months in a bottle painted on the outside with black paint. If the determination of nitrite in the presence of nitrate is desired, make up a separate powder mixture that does not contain the powdered zinc and manganese (II) sulfate.

d) 0.1 N HCl. 8.18 ml of 37-38% HCl per 1.0 liter of water. Exact standardization is not necessary.

e) Activated charcoal. Merck number 18351 activated charcoal N.F. powder.

Sampling of Feeds

The nitrate content of plants or feeds from different parts of a field, stack or silo often varies extremely. For example, the nitrate content of plants in a field vary between: different plant species, different stages of plant growth, different parts of a plant, different areas in a field, different times of day, and different days as climatic conditions vary. Nitrate contents of feeds in stacks and silos vary because of the above factors and because of differences in: how the hay or silage is handled in the field (including the loss of nitrate by leaching from rain); and losses and movement of nitrates during fermentation in the silo.

Therefore, at any one sampling time it is desirable to obtain separate samples from different places in the field, stack or silo and analyze each sample separately; for plants growing in the field, samples should be collected and tested at different times.

Nitrates may be lost from moist plant or silage samples between the time the sample is collected and the time it is analyzed if the samples are not handled properly. Such losses may be due to enzymatic or microbiological reduction of nitrates in the moist sample or to volatilization of nitrogen gases or acids during drying. Therefore, <u>either the moist plant or feed samples should be frozen as soon as possible after they are collected and kept frozen until they are analyzed, or plant samples (but</u>

not silage samples) may be dried immediately in an oven with adequate ventilation at 60 C.

Procedure

a) Weigh by difference approximately 1.0 g of air dry plant material or 3.0 to 5.0 g of moist sample. The sample should contain 1 to 8 milligrams nitrate to be within the range of the standard curve. Place the sample in a 125 ml glass stoppered Erlenmeyer flask.

b) Add 100 ml of 0.1 N HCl. Stopper the flask and shake the contents until all parts of the sample are wetted. Let the sample stand with occasional shaking for one hour to extract nitrate. If the extract is strongly colored, decolorize it by adding about 1 g of activated charcoal to the extract in the Erlenmeyer flask and shake well. Filter the extract through filter paper.

c) Pipet 1 ml of the extract and 9 ml of the 20% acetic acid solution to a glass stoppered centrifuge tube. Add about 0.5 g of the Bray's indicator powder with a small measuring scoop. Stopper and shake each tube 1 minute. Shade the solution from strong light.

d) Place the tubes in the centrifuge tube holder and centrifuge for five minutes at 3,000 revolutions per minute or until the supernatant liquid is clear. Remove any film on top and pour the clear red solution into a spectrophotometer cuvette.

e) Observe the optical density at 520 millimicrons. Set the instrument at 100% transmittancy with a blank solution that has been treated exactly as the sample solutions.

f) Compare the observations to a standard nitrate curve ranging from 0 to 10 micrograms nitrate per ml of solution in the cuvette. (Make the standard curve by diluting 0, 2, 5, and 10 ml aliquots of the standard nitrate solution to 100 ml in volumetric flasks with water. Transfer 1 ml of the diluted solution to centrifuge tubes and continue the procedure from (c) on. The solution in the cuvettes will contain 0, 1, 2, 5, and 10 micrograms nitrate per ml. The remaining solution in the 100 ml volumetric flasks may be transferred to polyethylene bottles and stored for several months and reused as needed.)

g) When nitrites are to be determined in the presence of nitrates follow the same procedure except use the powder mixture that does not contain powdered zinc or manganese (II) sulfate.

Calculations

a) To determine the nitrate plus nitrite content, the powder mixture containing zinc and manganese sulfate is used and 11 nitrates are reduced to nitrites. The result is the total of nitrate and nitrite in terms of nitrate when the standard curve under (f) is used.

Total nitrate (%) on partial dry or as fed basis =

$$\frac{\text{concentration of nitrate in final solution in micrograms per ml} \times 1000 \times 100}{\text{wt of sample in g} \times 1{,}000{,}000} = \frac{NO_3 \text{ microgram/ml from curve}}{\text{g sample} \times 10}$$

b) To determine only nitrite in the presence of nitrate, the zinc and manganese sulfate are omitted from the powder mixture.

The nitrite % on partial dry or as fed basis =

$$\frac{\text{concentration of nitrogen in final solution in micrograms per ml} \times 1000 \times 100}{\text{wt of sample in g} \times 1{,}000{,}000} = \frac{NO_2 \text{ in microgram/ml from curve}}{\text{g sample} \times 10}$$

The nitrite determination is used only to add more information when nitrite poisoning is suspected in local areas. The amount will be included in the analysis of total nitrate and therefore need not be reported separately on the source form.

c) Adjusting to dry basis for total nitrate (nitrate plus nitrite):

$$\frac{\text{\% nitrate in as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

or

$$\frac{\text{\% nitrate in partial dry sample}}{\text{dry matter \% of partial dry sample}} \times 100$$

Note: report % of nitrates (nitrate plus nitrite) on the source form under other analyses.

Reference

Easley, J. F., J. T. McCall, G. K. Davis and R. L. Shirley. 1965. Analytical methods for feeds and tissues. Nutrition Laboratory, Department of Animal Science, University of Florida.

Hanway, J. J., J. B. Herrick, T. L. Willrich, P. C. Bennett and J. T. McCall 1963. The nitrate problem. Special report no. 34, Iowa State University, Ames, Iowa.

Nelson, J. L., L. T. Kurtz and R. H. Bray. 1954. Rapid determination of nitrates and nitrites. Analytical Chemistry 26:1081.

Rider, B. F. and M. G. Mellon. 1946. Colorimetric determination of nitrites. J. of Industrial and Engineering Chemistry, Analytical Edition 18:96.

Woolley, J. T., G. P. Hicks and R. H. Hageman. 1960. Rapid determination of nitrate and nitrite in plant material. J. of Agricultural and Food Chemistry 8:481.

DETERMINATION OF TOTAL NON-STRUCTURAL CARBOHYDRATES

Principle

Carbohydrates are the primary source of reserve energy stored in the vegetative organs of forage plants. Reserves of available carbohydrates are essential to survival and to the production of plant tissues during periods when carbohydrate utilization exceeds photosynthetic activity.

Total available carbohydrate levels and trends in storage organs are useful in indicating the periods of storage and usage and are useful in evaluating the potential of plants for regrowth and production following defoliation. A number of methods have been used to extract total available carbohydrates from forage plant tissue. However, the method of extraction used will influence greatly the values obtained because forages vary in type of carbohydrate stored and may contain substances which interfere with the extraction procedure. The latter is the case in forage sorghum where tannins contained in leaf materials have been shown to inhibit enzyme activity such as amyloglucosidase, the chief extraction agent of the second method below. Under these circumstances the acid extraction procedure is the method of choice.

Acid Extraction Procedure for Removing Total Non-Structural Carbohydrate

Equipment

a) Refluxing device.

To prepare simple refluxing devices, draw a fine capillary end

on 30 cm sections of 7 mm diameter glass tubing. Insert undrawn ends into rubber stoppers fitted to 100 ml centrifuge tubes. Construct a rack to support 50 such centrifuge tubes so that they can be suspended in a boiling bath. Vapor from the extraction mixtures condenses on the sides of the long thin glass vents and no measurable volume loss occurs.

b) Oven at 60 C or freeze-dried

c) Whatman #42

d) 100 ml beakers

e) 100 cc graduated cylinders

Reagent

a) H_2SO_4

Procedure

a) Reflux 0.25 g oven dried or freeze dried forage in 50 ml of 0.2 N H_2SO_4 for 1 hour.

b) After cooling, filter mixture through Whatman #42 filter paper supported on the rim of a 100 ml beaker.

c) Dilute 5 ml of filtrate up to 100 ml with distilled water.

d) Analyze carbohydrate level by the phenol-sulfuric acid colorimetric technique. (See page 177)

Amyloglucosidase Procedure for Removing Total Non-structural Carbohydrates

Procedure

a) Place approximately 250 mg of ground plant material into 50 ml centrifuge tubes.

b) Add 15 ml deionized water and boil 2-3 minutes.

c) Add 10 ml acetate buffer.

d) Add 10 ml 0.5% amyloglucosidase solution.

e) Stopper and incubate 44 hours at 38 C.

f) Filter through Whatman #42 paper and save hydrolyzate for carbohydrate determination.

Note: Enzyme blanks must be included in order to adjust carbohydrate determination for carbohydrate in the enzyme solution.

Reagents

a) Acetate buffer (pH 4.45). Mix three parts of 0.2 N acetic acid with two parts 0.2 N sodium acetate. Add a small amount of powdered thymol to prevent microorganism growth.

0.2 N acetic acid - dilute 11.45 ml of glacial acetic acid (99.5% pure) to 1 liter with distilled water.

0.2 N sodium acetate - dissolve 16.4 g anhydrous sodium acetate with distilled water to make 1 liter.

b) 0.5% Amyloglucosidase. Add 5 g amyloglucosidase to 1 liter

distilled water. Add a small amount of powdered thymol and store in a brown bottle.

Phenol-Sulfuric Acid Colorimetric Method for Total Carbohydrate

Reagents

a) 5% phenol - 50 g redistilled reagent grade phenol. Dissolve above in water and dilute to 1000 ml. Store in glass stoppered brown bottle.

b) Sulfuric acid - 96% reagent grade.

c) Standards: glucose - 1500 mg/ml, diluted to 15, 30, 45 mg/ml.

Procedure

a) 1 ml aqueous solution containing v/w 10 to 60 µg sugar is pipetted into test tube.

b) If less than 1 ml sample is used, bring the volume up to 1 ml total.

c) 1 ml of 5% phenol reagent is added with automatic pipette. Mix samples.

d) Add 5 ml of conc. H_2SO_4 (96%) to each tube rapidly <u>while mixing</u>. It is important that the H_2SO_4 addition be reproducible from tube to tube. Use the acid syringe for adding the acid.

e) Let stand for ten minutes, then stir again.

f) After 20 minutes, read the O.D. at 490 mu for hexoses (480 for pentoses or uronic acids). Use a water blank.

g) If necessary, these can be left overnight and the color does not deteriorate excessively.

Precautions

a) Keep samples covered. This procedure is easily contaminated with lint, dust, paper, etc.

b) H_2SO_4 is easily contaminated. Do not lay the acid syringe down when using unless it is thoroughly washed before re-use. Do not pour unused acid back into the bottle.

Reference

Smith, D., G. M. Paulsen, and C. A. Raguse. 1964. Extraction of total available carbohydrates from grass and legume tissue. Plant Physiol. 39:960-962.

Lechtenberg, V. L. 1971. Diurnal variation in various nonstructural carbohydrates of alfalfa (Medicago sativa), tall fescue (Festuca arundinacea), and sudangrass (Sorghum sudanense). Ph.D. Thesis. Purdue University.

Smith, D. 1969. Removing and analyzing total nonstructural carbohydrates from plant tissue. Wisc. Res. Rep. No. 41. Madison, Wisconsin.

Whistler, R. L., and M. L. Wolform. Methods in carbohydrate chemistry. Vol. 1:388-389. Academic Press, New York.

ORGANIC MATTER

Organic matter is the material which burns in the presence of oxygen. The ash or mineral matter remains.

If the percent ash is determined on the sample, the organic matter may be calculated by difference.

% organic matter on a dry basis = % dry matter - % ash on dry basis

DETERMINATION OF CELL CONTENTS

Principle

The more readily utilizable nutrients are enclosed by the cell wall and can be grouped as cell contents. These include the protein, soluble carbohydrate, soluble minerals and lipids. A high percentage of cell contents is a good index of high nutritive value of a feed. The cell content is determined as the difference between the % cell walls and 100%.

Calculation

Cell contents (%) on a dry basis = 100% - cell walls (%) on dry basis.

Example:

If the % cell walls on the dry basis is 59.3% then:

cell contents (%) on dry basis = 100% - 59.3% = 40.7%

Adjusting to as fed basis:

$$\frac{\text{constituent on dry basis (\%)}}{100} \times \frac{\text{as fed dry matter \%}}{100} \times 100$$

Example:

If as fed dry matter = 89.3%, then:

cell contents (%) on as fed basis = $\frac{40.7\%}{100} \times \frac{89.3\%}{100} \times 100 =$

$0.407 \times 0.893 \times 100 = 36.3\%$

Reference

Van Soest, P. J. 1967. Development of a comprehensive system of feed analysis and its application to forages. J. Animal Sci. 26:119.

DETERMINATION OF CELL WALLS (NEUTRAL DETERGENT FIBER) AND CELL CONTENTS

Principle

The neutral-detergent procedure for cell walls (CW) is a rapid method for the total fiber in fibrous plant feedstuffs. It appears to divide the dry matter of feeds very near the point which separates the nutritively available and soluble constituents from those which are completely available or dependent on a microbial fermentation. This method cannot be used with feeds that have a high protein and low fiber content.

Equipment

a) Refluxing apparatus. Use any conventional apparatus suitable for crude fiber determinations. Berzelius beakers (600 ml) and condensers made from 500 ml round flasks are preferred.

b) Scintered glass crucibles. Use tall form, coarse porosity with plate 40 mm in diameter, and large enough to contain 40-50 ml of liquid.

c) Cleaning of crucibles. After much use, crucibles tend to become clogged with residual matter, resistant to the commonly used chromic acid filtration. A reliable cleaning procedure is to ash at 500 C (if not already ashed) and force water in reverse flow through the filter plate. When crucibles become clogged with mineral particles after several uses a hot solution of 20%

KOH, 5% Na_3PO_4, and 0.5% disodium ethylenediaminetetra-acetate forced through the scintered glass plate in reverse flow will usually be effective. Overuse of the alkaline cleaning solution should be avoided, as it tends to erode the glass.

Reagents

a) Neutral detergent solution. Add 30 g sodium lauryl sulfate, USP grade, 18.61 g disodium dihydrogen ethylenediaminetetra-acetate dihydrate reagent, 4.56 g disodium hydrogen phosphate reagent, 6.81 g sodium borate decahydrate reagent, and 10 ml 2-ethoxyethanol (ethylene glycol, monoethyl ether), purified grade, per liter of distilled water and agitate to dissolve. Check pH of solution to be within range of 6.9 to 7.0 and adjust as necessary.

b) Decalin, reagent grade decahydronaphthalene.

c) Acetone, reagent grade.

d) Sodium sulfite, reagent grade.

Procedure

a) Weigh by difference approximately a 1.0 g sample that has been ground to pass through a 1 mm screen and place the sample in a Berzelius beaker for refluxing.

b) Add, in order, 100 ml cold (room temperature) neutral-detergent solution, 2 ml decahydronaphthalene, and 0.5 g sodium sulfite

with a calibrated scoop. Heat to boiling in 5-10 minutes. Reduce heat as boiling begins, to avoid foaming. Adjust boiling to an even level and reflux for 60 minutes, timed from onset of boiling.

c) Place previously tared crucibles on filtering apparatus. Swirl beaker to suspend solids and fill crucible. Do not admit vacuum until after crucible has been filled. Use low vacuum at first, increasing it only as more force is needed. Rinse sample into crucible with a minimum of hot (80 C) water. Remove vacuum, break up mat, and fill crucible with hot water. Filter liquid and repeat washing procedure.

d) Wash twice with acetone in same manner and suck dry. Dry crucibles at 105 C overnight and weigh.

e) Report yield of recovered neutral-detergent fiber as cell walls.

f) Estimate cell content (soluble material) by subtracting this value from 100.

Calculations

Cell walls % on as fed or partial dry basis =

$$\frac{\text{wt of crucible and cell walls} - \text{wt of crucible}}{\text{wt of sample}} \times 100$$

Adjusting to dry basis:

$$\frac{\text{cell walls \% on as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

or:

$$\frac{\text{cell walls \% on partial dry sample}}{\text{dry matter \% of partial dry sample}} \times 100$$

Note: Refer to page 174 for calculation of cell contents.

Reference

Van Soest, P. J., and R. H. Wine. 1967. Use of detergents in the analysis of fibrous feeds. IV. The determination of plant cell wall constituents. J. Assn. Offical Anal. Chem. 50:50.

DETERMINATION OF ACID DETERGENT FIBER

Principle

The acid-detergent fiber procedure provides a rapid method for determination of lignocellulose in feed stuffs. The residue, however, also includes silica. The difference between the cell walls and acid-detergent fiber is an estimate of hemicellulose, although this difference does include some protein attached to cell walls. The acid-detergent fiber is used as a preparatory step for lignin determination.

Equipment

a) Same as used with cell wall analysis, neutral detergent fiber procedure.

Reagents

a) Acid-detergent solution. Sulfuric acid, reagent grade standardized to 1 N. This is achieved by adding 49.04 g (correct for assay) H_2SO_4 within each liter of distilled water. For 18 liter of solution it requires 882.72 g of H_2SO_4. Add 20 g of cetyl trimethylammonium bromide (CTAB), technical grade, to one liter of 1 N H_2SO_4 solution, or 360 g per 18 liters. Stir to facilitate solution.

b) Decalin, technical grade dicahydronaphthalene

c) Acetone, reagent grade

d) Hexane, reagent grade

Procedure

a) Weigh by difference approximately 1 g of sample into a beaker or container suitable for refluxing.

b) Add 100 ml cold (room temperature) acid-detergent solution and 2 ml decahydronaphthalene. Heat to boiling in 5-10 minutes. Reduce heat to avoid foaming as boiling begins. Reflux 60 minutes from onset of boiling, adjusting boiling to a slow, even level.

c) Filter on a previously tared crucible, which is set on the fiber manifold, using light suction. Break up the filtered mat with a rod and wash twice with hot water (90-100 C). Rinse sides of the crucible in the same manner.

d) Repeat wash with acetone until it removes no more color, breaking up all lumps so that the solvent comes into contact with all particles of fiber.

e) Wash with hexane. Hexane should be added while crucible still contains some acetone. (Hexane can be omitted if lumping is not a problem.) Suck the acid-detergent fiber free of hexane and dry at 105 C eight hours or overnight, cool in a desiccator to room temperature and weigh.

Calculations

Acid detergent fiber % on partial dry or as fed basis =

$$\frac{(\text{wt of crucible + fiber - tare wt of crucible}) \times 100}{\text{wt of sample}}$$

Adjusted to dry basis =

$$\frac{\text{acid detergent fiber \% on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{acid detergent fiber \% on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

Reference

Van Soest, P. J. 1963. Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. J. Assoc. Official Agr. Chem. 46(5):829.

DETERMINATION OF ACID DETERGENT LIGNIN

Principle

The acid-detergent lignin procedure utilizes the acid-detergent fiber procedure as a preparatory step. The detergent removes the protein and other acid-soluble material which would interfere with the lignin determination. The principle of the procedure is that the acid-detergent fiber residue is primarily lignocellulose of which the cellulose is dissolved by the 72% H_2SO_4 solution. The remaining residue consists of lignin and acid-insoluble ash; however, with samples containing large amounts of cutin, this also is measured as part of the lignin.

Equipment

a) All equipment required for determination of cell walls. (See page 182)

b) Glass tray

c) Muffle furnace, temperature controlled at 500 C.

Reagents

a) Sulfuric acid, 72%. Calculation of g acid and water needed in 1 liter of solution:

$$\frac{100 \times 98.08 \text{ molecular weight} \times 12 \text{ moles}}{H_2SO_4 \text{ assay } (\%)} = \text{g } H_2SO_4 \text{ needed.}$$

$$1000 \times 1.634^{+} - \text{g } H_2SO_4 = \text{g } H_2O \text{ needed.}$$

$^{+}$specific gravity of 72% H_2SO_4.

Weigh the water needed for 1 liter in a 2000 ml beaker. Weigh

the acid needed in a separate container. Gradually add the acid to the water with occasional stirring while the solution is being cooled in a cold water bath (sink). Check the specific gravity by measuring a 5 ml aliquot at 20 C with a volumetric pipette, placing it in a covered weighing bottle and weighing it on an analytical balance. Adjust the specific gravity to 1.634 at 20 C by adding small measured amounts of water or acid and recheck the specific gravity as before.

Procedure

a) The first step is to prepare acid-detergent fiber as described on page 187.

b) Place the crucibles in the glass tray. Have one end of the tray 2 cm higher so acid will drain away from the crucibles.

c) Cover the contents of the crucible with cooled (5 C) 72% H_2SO_4 and stir with a glass rod to a smooth paste, breaking all lumps. Fill crucible about halfway with acid and stir. Let glass rod remain in crucible. Refill with 72% H_2SO_4 and stir at hourly intervals as acid drains away. Crucibles do not need to be kept full at all times. Three additions suffice.

d) Keep crucible 20-23 C.

e) After three hours, filter off as much acid as possible with vacuum. Rinse the residue once with 72% acid and filter it off.

f) Wash contents with hot water (85-95 C) until free from acid. Rinse and remove stirring rod.

g) Dry crucible overnight at 100 C and weigh.

h) Ignite crucible in a muffle furnace at 500 C for three hours. Cool to 250 C and transfer to a desiccator. Cool to room temperature and weigh.

Calculations

Lignin (%) on as fed or partial dry basis =

$$\frac{(\text{wt of crucible and lignin} - \text{wt of crucible and ash})}{\text{wt of sample}} \times 100$$

Adjusting to dry basis:

$$\frac{\text{lignin \% on as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

or:

$$\frac{\text{lignin \% on partial dry sample}}{\text{dry matter \% of partial dry sample}} \times 100$$

Reference

Van Soest, P. J. 1963. Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. J. Assoc.Official Agr. Chem.46:829.

DETERMINATION OF PERMANGANATE LIGNIN, CELLULOSE AND SILICA (INSOLUBLE ASH)

Principle

An indirect method for lignin utilizing permanganate allows the determination of cellulose and insoluble ash in the same sample. The insoluble ash is an estimate of silica content, which in many grasses is a primary factor in reducing digestibility. The permanganate lignin method is an alternative procedure to the 72% sulfuric acid method; each has its own advantages. Choice of methods will depend on materials analyzed and on the purpose for which the values are to be used. Advantages of the permanganate method over the 72% acid method include: 1) a shorter procedure, 2) the permanganate reagents are much less corrosive and require no standardization, and 3) values are less affected by heat damage artifacts and are closer to a true lignin figure.

However, cutin, which is important in many seed hulls, is not measured. A variation for the analysis of seed hulls is to prepare the permanganate cellulose and treat with 72% sulfuric acid and asbestos for three hours. This will result in the partitioning of crude lignin into two fractions as described below.

One disadvantage of the permanganate method is that large particles

are poorly penetrated by the reagents and yield low values. Consequently materials high in water content must be partially dried and ground through a 1 mm screen, and the method is not applicable to fresh feces and forages which have been ground in a meat grinder. Because of higher sensitivity to heat damage, 72% acid is preferred for assaying artifact lignin.

Theory of the method: Interfering matter is removed by preparing acid-detergent fiber, which is chiefly composed of lignin, cellulose, and insoluble minerals. Lignin is oxidized with an excess of acetic acid-buffered potassium permanganate solution, containing trivalent iron and monovalent silver as catalysts. Deposited manganese and iron oxides are dissolved with an alcoholic solution of oxalic and hydrochloric acids leaving cellulose and insoluble minerals. Lignin is measured as the weight lost by these treatments, while cellulose is determined as the weight loss upon ashing. The ash residue is mainly silica and much of the non-silica matter can be removed by leaching with concentrated hydrobromic acid.

Equipment

a) Same as for acid detergent lignin. (See page 189)

Reagents

a) Saturated potassium permanganate. Dissolve 50 g reagent-grade $KMnO_4$ per liter of distilled water, 900 g $KMnO_4$ per 18 liters of distilled water. Keep out of direct sunlight.

b) Lignin buffer solution. For one liter dissolve 6.0 g ferric nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$) and 0.15 g silver nitrate in 100 ml distilled water. Combine with 500 ml glacial acetic acid and 5.0 g potassium acetate (reagent grades). Add 400 ml tertiary butyl alcohol and mix (reagent grade). For 12 liters use the following amounts of chemicals:

ferric nitrate nonahydrate	72 g
silver nitrate	1.8 g
acetic acid	6.0 liter
potassium acetate	60 g
t-butyl alcohol	4.8 liter
distilled water	1.2 liter

c) Combined permanganate solution. Combine and mix saturated potassium permanganate and the lignin buffer solution in the ratio of 2:1 by volume before use. Unused mixed solution may be kept about a week in a refrigerator in the absence of light. Solution is useable if it is purple in color and contains no precipitate.

d) Demineralizing solution. For one liter, dissolve 50 g oxalic acid dihydrate in 700 ml 95% ethyl alcohol. Add 50 ml concentrated (approximately 12 N) hydrochloric acid and 250 ml distilled water and mix. For 18 liters use the following amounts of chemicals:

oxalic acid	900 g
95% ethanol	12.6 liter
concentrated hydrochloric acid	900 ml
distilled water	4.5 liter

e) Ethyl alcohol 80%. For one liter mix 155 ml distilled water and 845 ml 95% ethyl alcohol. For 18 liters, mix 2.8 liters

distilled water and 15.2 liters 95% ethyl alcohol.

f) Hydrobromic acid, reagent grade.

Procedure

a) The residue from the acid-detergent fiber determination may be used (use original weight of sample). If this residue is not used, proceed as outlined under b), c), and d).

b) Weigh approximately a 1.0 g sample that has been ground through a 1 mm screen. On sample containing over 15% lignin, use 0.5 g.

c) Place crucible in a muffle furnace at 500 C for an hour or longer. Cool in a desiccator to room temperature and weigh.

d) Prepare acid-detergent fiber according to the procedure outlined on page 187.

e) Place crucibles containing the acid detergent fiber in a shallow glass tray containing approximately 1 cm cold water. Fiber in crucibles should not be wet.

f) Combine and mix saturated potassium permanganate and buffer solution 2:1 by volume, and add approximately 25 ml to the crucibles. Do not overfill. Adjust level of water in pan to reduce flow of solution out of crucibles. Place a short glass rod in each crucible to stir contents, to break lumps, and to draw permanganate solution up on sides of crucibles to wet all particles.

g) Allow crucibles to stand 90 ± 10 minutes at a temperature of

20 to 25 C, adding more mixed permanganate solution if necessary. Purple color must be present at all times.

h) Remove crucibles to filtering apparatus and filter off visible liquid. Do not wash. Place in a clean enamel or glass pan, and fill crucibles no more than half full with demineralizing solution. After about five minutes, filter off visible liquid and refill halfway with demineralizing solution. Care must be taken to avoid spillage by foaming. Repeat the demineralizing solution treatment a third time if the filtrate from the second treatment is very brown. Rinse down the sides of the crucibles with a fine stream of demineralizing solution from a squeeze bottle. Treat until fiber is white. Total time required is 20 to 30 minutes.

i) Fill and thoroughly wash crucible and contents with 80% ethyl alcohol, filter off liquid and repeat wash two times. Wash and filter twice in similar manner using acetone.

j) Lignin content. Dry at 105 C overnight, cool in a desiccator and weigh. Calculate lignin content as loss in weight from acid-detergent fiber.

k) Cellulose content. Ash at 500 C for three hours, cool in a desiccator and weigh. Loss in weight equals cellulose.

l) Silica content. A presumptive analysis for silica may be obtained by leaching contents of crucibles under k) with concentrated hydrobromic acid (48%) until no further color is removed. Wash

sample with acetone (use no water), and filter. Ash at 500 C for three hours, cool in a desiccator and weigh (this step is not necessary if residual ash is less than 2% of the original sample).

Precautions: Crucibles containing fiber of a high lignin content will require more permanganate solution; however, avoid additions of more solution than is necessary. Appearance of a yellow or brown color indicates exhaustion of permanganate. If crucible is full, filter solution off on a vacuum and add more reagent. A yellow color persisting after treatment of fiber with demineralizing solution indicates incomplete removal of lignin. This will occur only in materials of a very high lignin content. Cutin material present in seed coats and other plant parts is not oxidized by permanganate and thus is neither determined as lignin nor bleached with the treatments. Seed coats will appear as colored flecks among white cellulose particles and should not be confused with incomplete oxidation.

Excessive flow of permanganate solution through the crucibles should be avoided with samples of low lignin content, particularly in the case of immature grasses. Here a single addition of permanganate solution suffices. Fiber from immature grasses is very rapidly delignified and there is danger in these materials of loss of cellulosic carbohydrates if there is too much flow. Reduction in flow is accomplished by adjusting pan water level to near that in the crucibles. These precautions are not needed with the demineralizing solution.

Calculations

Lignin % on partial dry or as fed basis =

$$\frac{(\text{wt acid detergent fiber} - \text{wt permanganate fiber residue})}{\text{wt of sample before determination of acid detergent fiber}} \times 100$$

Cellulose % on partial dry or as fed basis =

$$\frac{(\text{wt crucible and permanganate fiber residue} - \text{wt crucible and ash})}{\text{wt of sample before determination of acid detergent fiber}} \times 100$$

Silica % on partial dry or as fed basis =

$$\frac{(\text{wt of ash after hydrogen bromide washing})}{\text{wt of sample before determination of acid detergent fiber}} \times 100$$

Adjusting to dry basis:

$$\frac{\text{analysis \% on as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

or:

$$\frac{\text{analysis \% on partial dry sample}}{\text{dry matter \% of partial dry sample}} \times 100$$

Reference

Van Soest, P. J., and R. H. Wine. 1968. Determination of lignin and cellulose in acid-detergent fiber with permanganate. J. Assoc. Official Anal. Chem. 51:780.

PREPARATION OF ASH SOLUTION FOR THE DETERMINATION OF CALCIUM, MAGNESIUM, AND PHOSPHORUS

Principle

The ash which s soluble is put into solution in an acid medium. The insoluble part is filtered off.

Equipment

a) Hot plate

Reagent

a) Concentrated HCl

Procedure

a) Add 5 ml of concentrated HCl acid to the porcelain crucibles containing the ashed sample which was saved from the determination of ash procedure.

b) Add about 20 ml of distilled water and place on 100 C hot plate. Evaporate the samples to about 10 ml.

c) Add 10 ml distilled water and heat to around 90 C. Cool and filter the samples through Sharkskin S & S filter paper (filter paper of low ash content) in long stem funnels into 100 ml volumetric flasks. Rinse crucible and filter paper with distilled water.

d) Make the solutions to volume with distilled H_2O.

e) Save for determinations of calcium, magnesium, and phosphorus.

References

Easley, J. F., J. T. McCall, G. K. Davis, and R. L. Shirley. 1965. Analytical methods for feeds and tissues. Nutrition Laboratory Department of Animal Science, University of Florida, Gainesville, Florida.

Snell, Foster Dee and Cornelia T. Snell. 1949. Colorimetric methods of analysis. 3rd Edition. Vol II. D. Van Nostrand Company, Inc. Princeton, New Jersey.

DETERMINATION OF CALCIUM

Principle

Ethylenediaminetetraacetic acid (EDTA) forms, in basic solution, a soluble and slightly ionized complex with calcium and magnesium as well as many other metals. When the magnesium ion is present, a good end point is observed with Eriochrome Black T indicator. Calcium is complexed by EDTA before magnesium. Therefore, an unknown calcium solution containing a known amount of magnesium may be titrated with EDTA to determine the calcium present. Interference by other ions must be eliminated.

Equipment

a) Micro burettes

Reagents

a) Buffer solution. Mix 67.5 g of ammonium chloride with 570 ml of concentrated ammonium hydroxide and dilute to 1 liter.

b) Indicator. Dissolve 0.25 g of Eriochrome Black T in 50 ml of diethanolamine.

c) Magnesium sulfate solution. Dissolve about 2.0 g of $MgSO_4.7H_2O$ in a liter of water. An exact EDTA titer must be determined by titrating the magnesium sulfate solution with the EDTA solution. (1 ml of magnesium sulfate solution needs to be evaluated in regard to how many ml of the EDTA solution it is equivalent to).

d) EDTA solution. Dissolve 6.65 g of reagent grade disodium ethylene-diaminetetraacetic (EDTA) in sufficient water to make 1 liter of solution. (1.0 ml of EDTA needs to be evaluated in regard to how many mg of calcium it is equivalent to.)

e) Saturated ammonium oxalate.

f) Concentrated hydrochloric acid.

g) Methyl red indicator. 0.1% alcoholic solution. Dissolve 0.1 g in 95% ethyl alcohol and dilute to 100 ml.

h) Calcium standard (one mg Ca per ml). Dissolve 2.4973 g pure calcium carbonate in about 100 ml of dilute HCl. Dilute to exactly 1 liter with water.

i) Ammonium hydroxide, 1%. Dilute 10 ml of reagent NH_4OH to 1 liter with water.

Procedure*

a) Pipette an aliquot of the ash solution containing from 0.05 mg to 1.5 mg of calcium into a 15 ml conical-tipped centrifuge tube containing three ml of saturated ammonium oxalate.

b) Add 1 drop of methyl red indicator and adjust the pH to 5.0-5.5 (faint pink color of indicator) using a dilute solution of hydrochloric acid or ammonium hydroxide. Mix the contents thoroughly, let stand for one hour and centrifuge for five minutes at 3,000 rpm.

c) Carefully pour off the supernatant liquid, resuspend the precipitate and wash the sides of the tube with about 3 ml of 1%

* See page 193 for preparation of ash solution.

ammonium hydroxide, centrifuge, again pour off the supernatant liquid.

d) Dissolve the precipitate in 0.5 ml of concentrated hydrochloric acid. Wash the sample with distilled water into a 100 ml beaker. Dilute to about 30 ml, add 5.0 ml of the buffer and several drops of the Eriochrome Black T indicator.

e) Add 0.5 ml of the magnesium sulfate solution from a burette. The color should be wine red.

f) Add EDTA solution from a burette until a true blue color is obtained, then add 0.1-0.5 ml extra. Back titrate with the magnesium sulfate solution until the very first wine red tinge appears in the blue solution.

g) If a clear end point is not obtained, add about 0.25 g of sodium cyanide to the sample after the buffer has been added.

Calculations

Calcium (%) on either partial dry or as fed basis =

$$\frac{\text{calcium titer of EDTA [ml EDTA} - (\text{Mg titer of EDTA} \times \text{ml } MgSO_4)] \times 100}{\text{ml aliquot of ash solution} \times \text{wt of sample before ashing in g}} \times \frac{1000}{100}$$

or:

$$\frac{\text{calcium titer of EDTA [ml EDTA} - (\text{Mg titer of EDTA} \times \text{ml } MgSO_4)]}{\text{ml aliquot of ash solution} \times \text{wt of sample before ashing in g}} \times 10$$

Adjusting to dry basis:

$$\frac{\text{calcium \% on as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

or:

$$\frac{\text{calcium \% on partial dry sample}}{\text{dry matter \% of partial dry sample}} \times 100$$

Reference

Easley, J. F., J. T. McCall, G. K. Davis, and R. L. Shirley. 1965. Analytical methods for feeds and tissues. Nutrition Laboratory. Department of Animal Science. University of Florida, Gainesville, Florida.

Welcher, Frank J. 1957. The analytical uses of ethylenediaminetetraacetic acid. D. Van Nostrand Company, Inc., Princeton, New Jersey.

DETERMINATION OF MAGNESIUM

Principle

Ethylenediaminetetraacetic acid (EDTA) forms, in basic solution, a soluble and slightly ionized complex with calcium and magnesium as well as many other metals. All calcium ions are complexed before the magnesium ions. When using Eriochrome Black T indicator, a good end point is observed in the presence of magnesium ions. The calcium is determined as in the calcium procedure, and the equivalent amount of EDTA is subtracted leaving the amount due to magnesium ions. Using the magnesium equivalent of the EDTA solution titer, the milligrams of magnesium in sample may be calculated.

Equipment

a) Micro burettes

Reagents

a) Buffer solution. Mix 67.5 g of ammonium chloride with 570 ml of concentrated ammonium hydroxide and dilute to 1 liter.

b) Indicator. Dissolve 0.25 g of Eriochrome Black T in 50 ml of diethanolamine.

c) Magnesium sulfate solution. Dissolve about 2 g of $MgSO_4 \cdot 7H_2O$ in a liter of water. The EDTA equivalent of this solution must be determined by titration with the EDTA solution. (1 ml of magnesium sulfate solution needs to be evaluated in regard to how many milliliters of EDTA solution it is equivalent to.)

d) Magnesium standard. One milligram per ml. Dissolve 8.3606 g of reagent grade magnesium chloride 6-hydrate in a small volume of water and dilute to 1 liter with water.

Procedure+

a) Pipette an aliquot of the ash solution containing 0.02-1.5 mg of magnesium into a 100 ml beaker and dilute to about 30 ml with distilled water.

b) Add 5 ml of the buffer solution and a few drops of the indicator solution.

c) Add 0.2-0.5 ml of magnesium sulfate solution and titrate to a true blue color with the EDTA solution.

d) Add 0.2-0.5 ml extra EDTA solution to the first faint wine-colored tinge in the blue solution.

e) If a clear end point is not obtained, add about 0.25 g of sodium cyanide to the sample after the buffer has been added. Clean equipment with care so that it does not contaminate other equipment or yourself. If interference persists, see reference below.

+See page 193 for preparation of ash solution.

Calculations

Magnesium (%) partial dry or as fed basis =

$$\frac{\text{magnesium equivalent of EDTA solution} \left\{ \text{ml EDTA titrated} - \text{ml EDTA equivalent of calcium in aliquot} - \text{ml EDTA equivalent of } MgSO_4 \text{ added} \right\} \times 10}{\text{ml aliquot ash solution} \times \text{wt ashed sample in g}}$$

Adjusting to dry basis:

a) $\frac{\text{magnesium \% on as fed basis} \times 100}{\text{dry matter \% of as fed sample}}$

or

b) $\frac{\text{magnesium \% on partial dry basis} \times 100}{\text{dry matter \% of partial dry sample}}$

Reference

Welcher, Frank J. 1957. The analytical uses of ethylenediaminetetraacetic acid. D. Van Nostrand Company, Inc., Princeton, New Jersey.

DETERMINATION OF PHOSPHORUS

Principle

The orthophosphate ion reacts with ammonium molybdate to form a phosphomolybdate compound. The phosphomolybdate compound is reduced to molybdenum blue with 1-amino-2-naphthol-4-sulfonic acid. The blue color formed is in direct proportion to the orthophosphate present.

Equipment

a) Spectronic "20" spectrophotometer or equivalent.

Reagents

a) Standard phosphorus solution, 0.1 ml phosphorus per ml. Dissolve 0.4394 g of pure, dry KH_2PO_4 in 300 ml of distilled water and 200 ml of 1 N sulfuric acid. Add a few drops of 2% potassium permanganate as a preservative and dilute with water to 1 liter. Dilute as needed for standards.

b) Sulfuric acid solution, 10 N. Carefully pour 280 ml of concentrated sulfuric acid into 720 ml water.

c) Ammonium molybdate reagent. Dissolve 12.5 g of ammonium molybdate in about 100 ml of water. Add the molybdate solution to 150 ml of 10 N sulfuric acid in a 500 ml volumetric flask and dilute to volume with water. Store in refrigerator.

d) Sodium bisulfite solution, 15%. Dissolve 15 g of sodium bisulfite in water and dilute to 100 ml.

e) Sodium sulfite, 20%. Dissolve 10 g of anhydrous sodium sulfite in water and dilute to 50 ml.

f) Amino-naphthol sulfonic acid reagent (ANSA). Weigh 250 mg of reagent grade 1-amino-2-naphthol-4-sulfonic acid. Make a paste with a drop or two of the 15% sodium bisulfite solution. Then add 97.5 ml of the 15% sodium bisulfite solution and 2.5 ml of the 20% sodium sulfite solution. If all the aminonaphthol sulfonic acid does not readily dissolve, add more of the 20% sodium sulfite solution, 0.5 ml at a time, until solution is complete. Do not add an excess. Let stand overnight, then filter through No. 41 Whatman filter paper or equivalent. Store in a brown glass bottle.

Procedure+

a) Pipette an aliquot of the ash solution containing 0.01 mg to 0.2 mg of phosphorus into a 50 ml volumetric flask. Add about 25 ml of distilled water. Add 5 ml of the ammonium molybdate reagent. Swirl the flask to mix the solution. Add 2 ml of ANSA reagent. Fill to volume with water. Stopper and mix well.

+See page 199 for preparation of ash solution.

b) Record the absorbancy of the solution at exactly 20 minutes after addition of the ANSA reagent. Compare the absorbancy to a reagent blank using distilled water in place of sample aliquot. Read the phosphorus concentration by comparing the absorbancy to a previously prepared standard phosphorus curve ranging from 0.01 to 0.25 mg phosphorus per 50 ml.

Calculations

Phosphorus % partial dry or as fed basis =

$$\frac{\text{mg phosphorus in aliquot} \times 10}{\text{ml aliquot ash solution} \times \text{wt ashed sample}}$$

Adjusting to dry basis =

a) $\frac{\text{phosphorus \% on as fed sample} \times 100}{\text{dry matter \% on as fed basis}}$

or

b) $\frac{\text{phosphorus \% on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$

References

Bolts, D. F., and M. G. Mellon. 1948. Spectrophotometric determination of phosphorus as molybdiphosphoric acid. Anal. Chem. 20:749.

Fiske, C. A., and I. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375.

TRACE ELEMENT ANALYSIS

In the determination of trace elements (usually considered less than 0.01%) certain precautions should be taken. Glassware as free as possible from traces of the element desired should be used. In the case of ashing crucibles, this means using Vycor brand, which is 96% silica glass, the remainder being boric oxide, aluminum, sodium, iron, and arsenic. This is comparable to fused quartz in thermal and chemical resistance. It is very stable with acids and mild alkalies. All other glassware used should be of Pyrex brand quality. This is a low-alkali-content, boro-silicate glass. It is free from magnesia-lime-zinc group elements, heavy metals, arsenic, and antimony. For boron determinations, use alkali-resistant glass which is free from boron.

All glassware used must be washed well with a detergent, rinsed with tap water, rinsed with 10% hydrochloric acid solution and then rinsed with triple distilled water.

Special care should be taken in preparation of samples if the trace metals are to be determined. Samples prepared for proximate analyses are frequently ground in Wiley mills, generally equipped with bronze sieves. The bronze sieves have been shown to contribute significant quantities of trace metals to the ground material. Stainless steel sieves are available for the Wiley mill which should reduce contamination and should be used when

trace metals are to be determined on the ground sample. If stainless steel sieves for the grinding mill are not available, stainless steel scissors can be used with certain types of forages for cutting or chopping the sample in lieu of grinding. In this case, sub-samples of greater weight than those usually taken can be used to aid in obtaining representative material if the sample is cut rather than ground. If at all possible, it should be established that the preparation procedure being utilized does not contaminate the samples with metals to be determined.

Care must also be taken to prevent gross contamination of the sample through accidental introduction of metal from common articles of the laboratory -- orin from ring stands, nickel from crucible tongs, copper and zinc from burners, zinc from rubber, etc.

Water used in trace mineral analysis should be glass distilled.

A blank should be carried through all the steps of the determination proper. This takes account of the amounts of constituent contributed by the vessels and by the reagents. A zero blank is not necessarily an indication that all is well. It may mean that a foreign substance in the reagent is combining with the desired constituent and preventing its reaction with the color reagent in colorimetric analysis.

Dilute solutions of metal ions may undergo a serious loss in strength on standing as a result of absorption on, or cation exchange with, the glass container. The loss is greatest in neutral or slightly basic medium, but may occur as well in slightly acidic solution. The weaker the solution,

the greater will be the relative decrease in concentration. Therefore, all standards and solutions should be stored in polyethylene containers. Solutions in organic solvents should never be stored in polyethylene bottles.

Fungus growth may remove metals from solution.

Standard solutions may be prepared from pure metals or from pure salts of definite composition. If a good grade of "reagent-quality" salt is available, it may, as a rule, be used without further purification for the purposes of colorimetry. Whenever possible, anhydrous salts are used. These should be powdered and dried at 100 C or higher to remove most of the vacuole water. Hydrated salts may, in many instances, be used with satisfaction if they are not deliquescent or markedly efflorescent. The crystals should be large enough to permit the picking out of clear uneffloresced individuals. Such air-dry crystals may contain a few parts per thousand of foreign water but this will rarely matter in trace analysis.

In practically all cases the organic material must be destroyed before trace analysis. This may be done by dry ashing in a muffle furnace at 500-600 C or by wet digestion with acids.

References

Easley, J. R., J. T. McCall, G. K. Davis and R. L. Shirley. 1965. Analytical methods for feeds and tissues. Nutrition Laboratory, Department of Animal Science, University of Florida, Gainesville, Florida.

Sandell, E. B. 1959. Colorimetric determination of traces of metals. 3rd Edition. Interscience Publishers, Inc., New York, New York.

DETERMINATION OF COBALT

Principle

Cobalt is extracted from ash solution with dithizone. Cobalt forms a red complex with sodium 1-nitroso-2-naphthol-3, 6-disulfonate, nitroso-R salt, which is stable in boiling nitric acid. This last property makes possible a determination that is nearly specific for cobalt. Three moles of reagent combine with one of cobalt. This reaction furnishes a most sensitive method for the determination of traces of cobalt such as those that occur in soils, plants, and animal organs.

Equipment

a) Spectronic "20" or equivalent spectrophotometer.

b) 5 liter separatory funnel.

Reagents

a) Nitroso-R salt (sodium 1-nitroso-2-hydroxynaphthalene-3, 6-disulfonate), 0.2% aqueous solution. This solution undergoes no change for months if kept in the dark.

b) Standard cobalt stock solution, 0.0100% cobalt. Dissolve 0.040 grams of $CoCl_2 \cdot 6H_2O$ in water, add 1 ml of concentrated HCl and dilute to 100 ml. From this solution prepare a more dilute one (e.g., 10 micrograms of cobalt per milliliter).

c) Citric acid, 0.20 M. Dissolve 4.2 g of the monohydrate in water and dilute to 100 ml.

d) Buffer solution. Use 6.2 g of boric acid, 35.6 g of disodium phosphate dihydrate, and 500 ml of 1.00 N sodium hydroxide in a total volume of 1 liter.

e) Dithizone. Dissolve 0.5 g dithizone in 600-700 ml CCl_4. Filter into 5 liter separatory funnel containing 2.5-3.0 liters of 0.02 N NH_4OH, shake well, and discard CCl_4 layer. Shake with 50 ml portions of high grade CCl_4 until CCl_4 phase, as it separates, has pure green color. Add 1 liter of CCl_4 and acidify slightly with HCl (1 + 1). Shake the dithizone into CCl_4 layer and discard aqueous layer. Store in cool, dark place, preferably in refrigerator.

f) Ammonium citrate solution, 40%. Dissolve 800 g of citric acid in 600 ml of water and, while stirring, add slowly 900 ml NH_4OH. Adjust pH to 8.5, if necessary. Dilute to 2 liters and extract with 25 ml portions of dithizone solution until aqueous phase stays orange and CCl_4 remains predominantly green. Then extract solution with CCl_4 until all orange color is removed.

g) Hydrogen Peroxide, 30%.

h) Concentrated sulfuric acid.

i) Concentrated nitric acid.

j) Concentrated hydrochloric acid.

k) Phenolphthalein, 1% alcoholic solution. Dissolve 1 gram in 95% ethyl alcohol and dilute to 100 ml.

l) Ammonium hydroxide (1 + 1). Distill concentrated NH_4OH into equal volume of water.

m) HNO_3 + H_2SO_4 solution. Combine 45 ml concentrated nitric acid and 30 ml concentrated sulfuric acid.

Procedure

See instructions on how to prepare samples (page 211). Weigh about 6 g sample (or larger amount) into a Vycor crucible. Ash at 500 C overnight. Cool. Dissolve ash with 3 ml concentrated HCl and add 25 ml water. Evaporate two-thirds of solution. Add 15 ml water. Evaporate two-thirds of solution. Add 15 ml water. Evaporate one-half of solution. Filter into 25 ml volumetric flask and make to volume. Transfer all or part of this solution to a 150 ml separatory funnel. Add 5 ml of the ammonium citrate solution and one drop phenolphthalein solution; adjust to pH 8.5 with NH_4OH (1 + 1). If precipitate forms, add additional ammonium citrate. Add 10 ml of the dithizone in CCl_4 and shake the solution five minutes. Drain CCl_4 phase into 100 ml beaker. Repeat as many times as necessary, using 5 ml quantities of dithizone solution and shaking five minutes each time. Extraction is complete when aqueous phase remains orange and CCl_4 phase remains predominantly green. Then add 10 ml CCl_4, shake five minutes, and combine with CCl_4 extract. Final

10 ml CCl_4 should be pure green. If not, extraction was incomplete and must be repeated. Add 2 ml of HNO_3-H_2SO_4 solution and evaporate just to dryness. Cool. Add 20 ml water. Add 2 ml 30% H_2O_2 until solution is clear. Evaporate solution to 5 ml volume. Add 1 ml of citric acid solution and 1.2 ml of phosphate-boric acid buffer. The pH should be 8.0; if not, add more buffer until pH 8.0 is reached. Add exactly 0.5 ml of nitroso-R salt solution while stirring. Boil for one minute, add 1 ml of concentrated nitric acid, and again boil for exactly one minute. Cool immediately in the dark and make up to 10 ml after transferring to volumetric flask. Observe the optical density of the solution at 420 millimicrons. Compare the observed optical density to a standard cobalt curve ranging from 0 - 10 microgram cobalt per 10 ml final volume.

Calculations

Cobalt (milligrams per kg) on partial dry or as fed basis =

$$\frac{\text{micrograms cobalt in sample}}{\text{wt of sample}}$$

Adjusting to dry basis:

a) $$\frac{\text{cobalt (milligrams per kg) on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or

b) $$\frac{\text{cobalt (milligrams per kg) on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

References

Easley, J. F., J. T. McCall, G. K. Davis and R. L. Shirley. 1965. Analytical methods for feeds and tissues. Nutrition Laboratory. Department of Animal Science, University of Florida, Gainesville, Florida.

Official Methods of Analysis of the Association of Official Agricultural Chemists. 1960. 9th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D. C.

Marston, H. R. and D. W. Dewey. 1940. Australian J. Expt. Biology and Medical Sci. 18:343.

Sandell, E. B. 1959. Colorimetric determination of traces of metals. 3rd Edition. Interscience Publishers, Inc., New York, N.Y.

Snell, Foster Dee and Cornelia T. Snell. 1949. Colorimetric methods of analysis. 3rd Edition, Volume II. D. Van Nostrand Company, Inc. Princeton, New Jersey.

DETERMINATION OF MANGANESE

Principle

The colorimetric determination of manganese by oxidation to permanganate in acid solution is both sensitive and specific. Periodate oxidizes manganous salts smoothly to permanganate. The reaction is as follows:

$$2Mn^{++}+5IO_4^{-}+3H_2O \rightarrow 2MnO_4^{-}+5IO_3^{-}+6H$$

Equipment

a) Spectronic "20" or other spectrophotometer

b) Vycor ashing crucibles or equivalent

Reagents

a) Concentrated H_2SO_4

b) Concentrated HNO_3

c) Phosphoric acid (85%)

d) Sodium or potassium periodate (meta)

e) Hydrofluoric acid (48%)

f) Standard manganese solution. This is prepared by dissolving pure electrolytic manganese metal in dilute nitric acid, boiling out oxides of nitrogen, and diluting to volume. A standard solution may also be obtained by weighing reagent grade, crystalline potassium permanganate, acidifying with sulfuric acid, and reducing with a little sulfite.

Procedure

a) See instructions on how to prepare samples (page 211).

b) Ash sample of plant tissue or biological material of suitable size in a muffle furnace at 500 C, cool.

c) Add 10 ml of 48% hydrofluoric acid. This will prevent loss of manganese as insoluble silicate. Warm to dissolve, cool, add 10 ml concentrated sulfuric acid and evaporate under a fume hood until fumes of sulfur trioxide come off. Transfer quantitatively to a 100 ml volumetric flask and dilute to volume. The ash may also be taken up directly in 10 ml concentrated nitric acid and diluted to volume.

d) The solution, ready for analysis, (0.1 to 1.0 mg manganese), should contain 10 ml concentrated sulfuric or 15 to 20 ml nitric acid; and, in addition, 5 to 10 ml of 85% phosphoric acid in a volume of 100 ml. However, if the amount of manganese present is of the order of a few micrograms, make the solution 2 N in sulfuric acid and add 20 mg of silver nitrate, in addition to phosphoric acid.

e) Add 0.3 grams of potassium meta-periodate, or equivalent amount of sodium meta-periodate for each 100 ml of solution. Heat to boiling with stirring, and keep at or slightly below the boiling point for 10 minutes (or at least one hour for any small amounts of manganese); cool.

f) Dilute to volume and observe the optical density at 525 millimicrons in a spectrophotometer. Compare the observed optical density to a standard manganese curve ranging from 0.1 to 1.0 mg of manganese per 100 ml.

Calculations

Manganese (milligrams per kg) partial dry or as fed basis =

$$\frac{\text{Milligrams manganese in sample} \times 1000}{\text{wt of sample}}$$

Adjusting to dry basis:

a) $$\frac{\text{manganese (milligrams per kg) on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or

b) $$\frac{\text{manganese (milligrams per kg) on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

References

High, J. H. 1943. Amount of periodate required to oxidize manganese. Analyst 68:368.

Official Methods of Analysis of the Association of Official Agriculture Chemists. 1960. 9th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D. C.

Read, F. E. 1939. Determination of manganese in biological material. Analyst 64:586.

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Snell, Foster Dee and Cornelia T. Snell. 1949. Colorimetric methods of analysis. 3rd Edition, Volume II. D. Van Nostrand Company, Inc. Princeton, New Jersey.

DETERMINATION OF CAROTENE IN FEEDS AND FORAGES

Principle

Carotene and other pigments are extracted from plant material by an organic solvent. The carotene is eluted on a chromatographic column which retains other pigments. The carotene concentration is determined by comparing to a standard curve on a spectrophotometer.

Equipment

a) Solvent extraction apparatus. Goldfisch or equivalent.

b) Spectronic "20" spectrophotometer or equivalent.

c) Chromatographic tubes. 20 x 400 mm, with fritted glass filter.

Reagents

a) Acetone. Dry, alcohol-free.

b) Hexane. B. P. 60-70 C.

c) Activated Magnesia. SeaSorb 43, Fisher Scientific Company, or equivalent.

d) Diatomaceous earth. Hyflo Super-cel, Johns-Manville or Fisher Scientific Company.

Procedure

a) For hays and dried plants, grind sample through a 1 mm screen. Weigh by difference 1.000 to 4.000 g depending on amount of

carotene in sample in relation to the standard curves and place in flask of extractor (Goldfisch). Add 30 ml acetone-hexane mixture (3 + 7) to flask and reflux one hour on high heat setting. Cool to room temperature.

b) Filter extract into 100 ml volumetric flask, wash residue, and dilute to volume with hexane (this solution now contains 9% acetone).

c) For fresh plant materials and silages, cut material finely with scissors or knife. If analysis cannot be performed immediately, blanch in boiling water for five to ten minutes and store in frozen condition. Place weighed sample, 2 to 5 g, in high speed blender; add 40 ml acetone, 60 ml hexane, and 0.1 g $MgCO_3$, and blend for five minutes. Let residue settle and decant into separatory funnel, wash residue with two 25 ml portions of acetone, then with 25 ml of hexane, and combine extracts. Wash acetone from extract with five 100 ml portions of water, transfer upper layer to 100 ml volumetric flask containing 9 ml acetone, and dilute to volume with hexane.

d) Prepare chromatographic column with 1 + 1 mixture of activated magnesia and diatomaceous earth. To prepare column, add loose adsorbent to 15 cm depth, attach tube to suction flask, and apply full vacuum of water. Use flat instrument (such as inverted cork mounted on a rod) to gently press adsorbent and flatten

surface (packed column should be about 10 cm deep). Place 1 cm layer of a hydrous Na_2SO_4 above adsorbent. All materials must be completely dry. If necessary, dry them in 105 C oven.

e) With vacuum continuously applied to flask, pour extract into column. Use 50 ml acetone-hexane (1 + 9) or slightly more if necessary, to develop chromatogram and wash visible carotenes through adsorbent. Keep top of column covered with layer of solvent during entire operation. Collect entire eluate. (Carotenes pass rapidly through column; bands of xanthophylls, carotene oxidation products, and chlorophylls should be present in column when operation is complete).

f) Transfer eluate, which has been reduced in volume by loss of vapor through water vacuum, to 100 ml volumetric flask, and dilute to volume with acetone-hexane (1 + 9). Determine carotene content of this solution at 436 millimicrons on the spectrophotometer.

g) A standard curve may be run with a range of 0.0001 to 0.005 mg beta-carotene per ml of acetone-hexane mixture (1 + 9); or 0.01 to 0.5 mg beta-carotene per 100 ml of solution.

Calculations

Carotene (milligrams per kg) on partial dry or as fed basis =

$$\frac{\text{mg carotene in sample} \times 1000}{\text{wt of sample in g}}$$

Adjusting to dry basis:

a) $\frac{\text{carotene (milligrams per kg) on as fed basis} \times 100}{\text{dry matter \% of as fed sample}}$

or

b) $\frac{\text{carotene (milligrams per kg) on partial dry basis} \times 100}{\text{dry matter \% of partial dry sample}}$

Reference

Official Methods of Analysis of the Association of Official Agricultural Chemists. 1960. 9th Edition. Published by the Association of Official Agricultural Chemists, P.O. Box 540, Benjamin Franklin Station, Washington, D. C.

DETERMINATION OF CYANIDE IN PLANTS

Principle

Cyanide is distilled from a chloroform and water solution into KOH solution, forming KCN. KCN is titrated with silver nitrate; $2KCN+AgNO_3 = KCN \cdot AgCN+KNO_3$. An excess of $AgNO_3$ produces insoluble AgCN, which is the end point of the titration: $Ag(CN)_2K+AgNO_3 = 2AgCN+KNO_3$.

Equipment

a) Kjeldahl distillation apparatus (macro)

b) 5 ml burette (micro)

Reagents

a) 2% KOH solution. Dissolve 2 g KOH in 100 ml H_2O.

b) Chloroform. Spectranalyzed, Fisher Certified, A.C.S. or equivalent.

c) Potassium iodide.

d) Potassium chloride. Recrystallized three times with H_2O, dry at 105 C, and then heat at about 500 C to constant weight.

e) Potassium chromate solution. 5% solution of K_2CrO_4 in H_2O.

f) Silver nitrate solution - 0.1 N. Dissolve slightly more than theoretical quantity of $AgNO_3$ (equivalent weight - 169.89) in halogen-free H_2O and dilute to volume. Thoroughly clean glassware,

avoid contact with dust, and keep prepared solution in amber glass-stoppered bottles away from light. Standardize as follows: weigh accurately sufficient quantity of KCl to yield titration of about 40 ml (about 0.3 g for 0.1 N solution) and transfer to 250 ml glass-stoppered Erlenmeyer flasks with 40 ml H_2O. Add 1 ml 5% K_2CrO_4 solution and titrate with the $AgNO_3$ solution until appearance of first perceptible pale red-brown color. Subtract from titration milliliters of the $AgNO_3$ solution required to produce end point color in 75 ml H_2O containing 1 ml of the K_2CrO_4 solution. Calculate normality of $AgNO_3$ solution.

Procedure

Cut sample of forage into one-fourth inch lengths. Mix well. Place an 8 g sample in an 800 ml Kjeldahl flask. Add about 200 ml distilled water and 5 ml chloroform. Steam distill into a 100 ml test tube containing 5 ml 2% KOH, with the adaptor end of the condenser below the surface of the KOH solution. Distill over 60-70 ml and transfer to 600 ml beaker. Add enough H_2O to make 300 ml. Add a few crystals of KI and titrate to faint opalescence with the $AgNO_3$ solution. A drop of the silver salt in excess will produce a permanent turbidity, owing to the following reaction:

$Ag(CN)_2K + AgNO_3 = 2AgCN + KNO_3$, the insoluble AgCN being formed. (In making this titration, it is advantageous to have beaker over black surface.) From milliliters 0.1 N $AgNO_3$ titrated, percent CN is calculated. The reaction is represented by the equation:

$2NaCN + AgNO_3 = NaCN \cdot AgCN + NaNO_3$; hence 1 ml 0.1 N $AgNO_3$ = 0.005204 g CN.

Calculations

Cyanide (milligrams per kg) on partial dry or as fed basis =

$$\frac{52 \text{ ml } AgNO_3 \times N\ AgNO_3 \times 1000}{\text{wt sample in g}}$$

Adjusted to dry basis:

$$\frac{\text{cyanide (mg per kg) on as fed sample} \times 100}{\text{dry matter \% of as fed sample}}$$

or:

$$\frac{\text{cyanide (mg per kg) on partial dry sample} \times 100}{\text{dry matter \% of partial dry sample}}$$

References

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EVALUATION OF FEEDS

The simplest method of measuring the value of any feed is to determine the amounts of digestible nutrients that it supplies. Another method, theoretically more accurate, is to determine the amount of net energy supplied by the feed; but this method is more complicated and more expensive. The best way to determine the true value of any feed for a particular class of livestock is to conduct actual feeding experiments under practical conditions with this class of livestock. Such experiments must be carefully planned and conducted under controlled conditions for a sufficient length of time.

Procedure

Feed records are a necessary feature of all feeding trials. In many feeding experiments the animals can be fed as a group. This is the simplest procedure from the standpoint of equipment needed and labor cost, but in many experiments it introduces complications in the interpretation of results. Such complications arise when there is a wide variability in the individual behavior within the lot, as to both production and feed consumption. The performance of the individual can be removed from consideration, but the food which it ate cannot. Individual feeding eliminates these disadvantages. It makes possible the correlation of the individual performance record with the feed intake.

Individual records are much more useful from the standpoint of statistical treatment. For example, to compare the feeding value of three different lines of sorghum, animals (the number can range from 20 to 60) are separated into three groups. Each group receives a ration composed of all the requirements for maximum performance (protein, energy, vitamins, minerals) which are kept constant in the three rations. The only variable will be the sorghum line to be studied.

In the case of sheep, each animal would be kept in a "crate". Water would be given ad libitum and the feeding of the ration closely controlled.

A digestion trial is a type of experiment in which a record of the nutrients consumed and the amount of nutrients eliminated in the feces is kept. In such a trial, the same amount of feed is given to the animal each day. The feces are carefully collected (separately from the urine) and weighed, and samples are analyzed. It is necessary to collect the feces uncontaminated by the urine because the substances in the urine are not undigested feed, but waste products from the organs and tissues of the body. Various methods are available for feed collection. In the case of omnivora and carnivora some "marker" may be used. The marker is fed just before the beginning of the ingestion of the ration to be tested and again at the end.

In the case of herbivorous animals with their much larger and more complicated digestive tracts, the use of a marker is a less suitable method. For these species, the ration to be tested is fed in constant daily amounts for an extended period. After allowing a certain number of

days to elapse as a "preliminary" period to clear the digestive tract from previous residues, the collection of the feces is then begun and continued through the collection period.

For ruminants, these periods are from 8 to 10 days; for pigs 3 to 5 days.

The difference between the amount of each nutrient fed daily and the amount found in the feces is the amount of the nutrient that is digested. From the information, the percentage of each nutrient which is digested from the feed is then computed.

To show the manner in which the digestibility of a feed is determined, let us suppose that during a 10-day digestion experiment a cow was fed 20 lbs. of clover hay each day containing 13.26% of protein. The amount of the hay would therefore contain 2.65 lbs. of protein. During the experiment the cow excreted, on the average, 47.3 lbs. of feces daily, which contained 2.32% protein on a total of 1.10 lbs. of protein. Then subtracting 1.10 lbs. (amount of protein in feces) from 2.65 (amount of feed) we find that 1.55 lbs. of protein were digested. Next we divide 1.55 lbs. by 2.65 lbs. and multiply the quotient by 100 to obtain the digestion coefficient for protein in the hay. This is 58.5%.

The digestion coefficients for all other components of the feed can be found in a similar manner.

Calculations

% digestion coefficient of a compound (x)

$$= \frac{(\text{amount of (x) in feed}) - (\text{amount of (x) in feces})}{\text{amount of (x) in feed}} \times 100$$

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