Maize nutrition quality and plant tissue analysis laboratory

Laboratory Protocols 2008

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(Compilers and editors)

CIMMYT

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CIMMYT MR
International Maize and Wheat Improvement Center

The International Maize and Wheat Improvement Center, known by its Spanish acronym, CIMMYT® (www.cimmyt.org), is an international, not-for-profit research and training organization. With partners in over 100 countries, the center applies science to increase food security, improve the productivity and profitability of maize and wheat farming systems, and sustain natural resources in the developing world. The center's outputs and services include improved maize and wheat varieties and cropping systems, the conservation of maize and wheat genetic resources, and capacity building. CIMMYT belongs to and is funded by the Consultative Group on International Agricultural Research (CGIAR) (www.cgiar.org) and also receives support from national governments, foundations, development banks, and other public and private agencies.

Correct citation: L. Galicia, E. Nurit, A. Rosales, N. Palacios-Rojas. Laboratory protocols 2009: Maize nutrition quality and plant tissue analysis laboratory. Mexioc, D.F.: CIMMYT.

ISBN: 978-970-648-169-6
AGROVOC descriptors: *Zea mays*; Maize; Wheats; Triticum; Transgenic plants; Genetic engineering; Gene transfer; Genetic transformation; DNA hybridization; Phenotypes; Selection; Tissue culture; In vitro culture; Biolistics; Research projects; Methods; Molecular cloning; PCR

AGRIS category codes: F02 Plant Propagation
F30 Plant Genetics and Breeding

Dewey decimal classification: 631.523

Printed in Mexico.

Contents

Abbreviations / Acronyms

°C	Celsius degree
AACC	American Association of Cereal Chemists
AM	Amylose
AOAC	Association of Official Analytical Chemists
BHT	Butylhydroxytoluene
cm	centimeters
Conc.	Concentration
ddH2O	Deionized water
d_f or FD	Dilution factor
d_w	Original weigh of flours
F-C	Folin Ciocalteu reagent
g	Gram(s)
g (Centrifuge)	Times acceleration or gravity
Gall	Gallic acid
h	Hour(s)
h_f	Starch hydrolysis factor
HPLC	High performance liquid chromatography
I.D.	Identification
ICP-OES	Inductively coupled plasma optical emission spectrometry
Inj vol	Injection volume
kg	kilograms
l	liter
Lys	Lysine
M	Molar
mg	Milligram(s)
min	Minute(s)
mL	Milliliters
mm	Millimeters
mM	Millimolar
N	Normality (concentration)
ng	Nanogram(s) = 10^{-9} gram
NIR	Near infrared reflectance
nm	Nanometers
OD	Optical Density
Pel	Pelargonidine chloride
pH	Potential of hydrogen
ppm	Parts Per Million
QPM	Quality protein maize
rpm	Revolution per minute (Centrifuge rotor speed)
s	Second(s)
Stock	Stock concentration
TEA	Triethylamine
TFA	Trifluroacetic acid
Trp	Tryptophan
U	enzymatic activity unit
μg	Microgram(s) = 10^{-6} gram
μL	Microliter(s) = 10^{-6} liter

Introduction

Biochemical and chemical analysis of seeds and green plant tissues are essential in several breeding programs including nutritional and industrial enhancement, plant physiology, and plant pathology.

The objective of CIMMYT's maize nutritional quality and plant analysis laboratory is to develop and/or adopt methodologies to support the breeding programs. Therefore, wherever possible, we seek robust, cheap, and fast methodologies that allow us to provide accurate data to breeders for further field decisions. Ninety-six well microplate readers and near infrared reflectance (NIR) are two of the options we are currently using in the establishment of the biochemical and chemical platform for analysis.

All methodologies presented here have been validated either by comparison with other methods or by inter-laboratory assessments.

This manual compiles all current methodologies used in the laboratory and aims to serve as a guide for other laboratories or institutions that are interested in the methodologies.

- For quality protein maize assurance we present the tryptophan, lysine, and protein determination protocols.
- For provitamin A enhancement, the high performance liquid chromatography (HPLC) carotenoid protocol is included.
- For micronutrient analysis we present current iron and zinc determination by inductively coupled plasma-optical emission spectroscopy (ICP-OES).
- Other analysis performed in our lab are soluble sugars determination, anthocyanin content, total and free phenols, ash content, oil content, starch determination and amylase/amylopectin determination.

For further information please contact us the maize nutritional quality and plant analysis laboratory, CIMMYT (Natalia Palacios: **n.palacios@cgiar.org**).

Safety Recommendations

Some of the presented methodologies involve chemicals that must be handled with caution. As a general rule, the use of a lab coat, gloves, goggles, and a mask is recommended. The use of a fume hood is also very important in the preparation of some of the reagents. Please check safety recommendation sheets given by suppliers to ensure proper handling, storage of the chemicals, and appropriate procedures in case of an accident.

Plant Sample Preparation: General Considerations

Sampling

Samples must be representative of the study area, type of material (i.e. open pollinated maize varieties vs. inbred lines), and purpose of the study. Chemical composition varies according to growth, environmental conditions, physiological age, and the part of the plant sampled. It is recommended to carefully sample the material according to the experimental design and objectives of the analysis. When analyzing maize kernels, we recommend avoiding kernels from the edges of the cob.

For micro-element analysis like iron and zinc, special care must be taken in the field (Stangoulis, J. and Sision, C., 2008):

1. Ensure field team is aware of contamination risks.
2. Harvest after physiological maturity without removing the husk.
3. Place the ears (in husk) in a clean bag and avoid soil contact until in a clean area.
4. Dehusk manually (remember to remove all jewelry as it can be a source of contamination).
5. Put the cobs into a clean basket (e.g. clean plastic woven bag that is kept solely for this purpose).
6. Put in clean drying trays (e.g. clean plastic) and dry at 40 ^{o}C for five days in a non-contaminating oven. It is also possible to dry the cobs with the husk in the sun (but over a clean area) and then dehusk and shell them.
7. Shell with clean, bare hands onto a clean plastic tray or paper envelops and thoroughly mix kernels.
8. To gain a representative sample for analysis, pile the grains evenly on a clean surface, flatten the pile and spread it into a circle (Figure 1). Make a cross by dividing the circle into four roughly equal parts. Discard two diametrically opposite quarters and re-mix the remaining two parts. Repeat the quartering procedure until the amount is reduced to approximately 250 g.
9. Mill fine (grains to pass through a 30-mesh sieve) using a non-contaminant analytical mill (e.g. Retsch mill with teflon chambers and zirconium balls) and then sub-sample, taking sample for analysis, being careful to place each sample in new, clean, labeled paper bags or tubes.

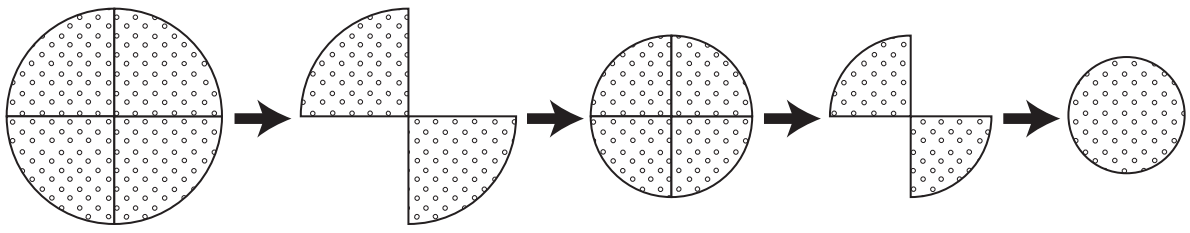


Figure 1. Illustration of quartering grains to get a representative sample.

Washing to remove contaminates

Decontamination by washing must be done prior to drying plant tissue. This can be done in the field as plants are collected, or by keeping collected plant tissue in a fully turgid state in a cool and moist atmosphere until washed in the laboratory.

It is known that metabolic activity can alter the composition of plant tissue material. To keep metabolic activity to a minimum, keep the samples cold, frozen, or as dry matter.

Oven drying

Drying fresh plant tissue should be done in a dust-free, forced draft oven at a temperature of 80 °C, to remove moisture without appreciable thermal decomposition. Drying temperatures less than 80 °C may not be sufficient to remove all the moisture, and temperatures above 80 °C can result in thermal decomposition.

Grinding

In order to reduce the dried plant tissue to a particle size suitable for laboratory analysis, and to ensure a greater degree of uniformity in sample composition, the tissue is mechanically ground using an intermediate Wiley Mill with stainless steel contact points or the Tecator Cyclotec Mill, to pass through a 0.5 mm mesh sieve of stainless steel. Large samples are first ground through a standard Wiley Mill using a 2 mm sieve of stainless steel.

Sample homogeneity is a key issue for reliable results in both chemical and non-destructive analysis. Particle size is of great importance when samples are analyzed by near infrared reflectance (NIR). Ground samples are transferred to glass bottles, paper bags, or sealed polyethylene bags, labeled clearly, and stored for further analysis.

Washing of treated seeds

When maize or wheat seeds for lab analysis are treated with a preservative or chemical product, you must:

- Wash seeds with tap water, then with distilled water, and lastly with deionized water. Personnel protection must be worn in case the products being washing are toxic.
- Place washed seeds in plastic trays with a laboratory identification number and dry them at room temperature for 24 hours.
- Weight dry samples and pack them into yellow paper bags, with correct laboratory number.

Endosperm separation

- For endosperm analysis, soak seeds in distilled water for 20-30 minutes. Peel off the pericarp and remove the germ with tweezers and a scalpel.
- Air dry the remaining endosperm material overnight at room temperature.

Laboratory Methods

Ash Quantification

Near infrared reflectance curve is in place for ash quantification in maize leaves. The chemical method of reference is Total Ash, AACC Method 08-01, 1995. This method is also used for ash determination in maize kernels.

Reagent	Specifications	Special recommendations
Calcium Chloride	$CaCl_2$ (in pieces)	Use as desiccator agent
Apparatus	**Specifications**	**Special recommendations**
Electric muffle furnace	With pyrometer for indicating temperature	
Muffle's Gripper		
Ashing dishes	Preferably of platinum or silica	
Desiccator		

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Be sure all seed samples have similar moisture content.
3. Grind each sample to a very fine powder.

Incineration

4. Dry the samples for 24 hours at 80 ºC.
5. Label all ashing dishes according to laboratory number.
6. Place the ashing dishes in a muffle furnace for 1 hour at 600 ºC.
7. Cool the samples and ashing dishes in a desiccator.
8. Weigh the ashing dishes and record their weight.
9. Weigh 2 g of the sample.
10. Place in a muffle furnace for 6 to 8 hours at 600 ºC.
11. Cool in a desiccator and weigh soon after room temperature is reached.

Calculation:

$$\%Ash = \frac{Weight\ of\ residue}{Sample\ weight} * 100$$

Ether Extract (fat crude)

Near infrared reflectance curve is in place for oil determination in maize flour. The chemical method of reference is the AOAC Method 7.044, 1975.

Procedure:

Sampling and Grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Be sure all seed samples have similar moisture content.
3. Grind each sample to a very fine powder.

Extraction

4. Weigh 2 g of the sample into the extraction thimble (sample weight).
5. Turn on the refrigeration system.
6. Pre-heat the heaters for 8 to 10 minutes.
7. Select heat rate required (normally level 5).
8. Place thimble containing sample into sample tube.
9. Place sample into condensator.
10. Fix the condensator into the system.
11. Place between 25 to 35 ml of petroleum ether (solvent) in each beaker.
12. Fix the beakers in to the system ensuring they are properly attached.
13. Ensure all beakers are properly fixed without any leaking solvent.
14. Unlock the heater and push it up until it touchs the beakers.
15. Keep refluxing for 6 hours.
16. Once the extraction is completed, put heater covers on heaters.
17. Recover the solvent.
18. Dry the extract with swing beaker holder. After most of the solvent is evaporated, transfer the beaker to a drying oven for 1 hour at 130 ^{o}C.
19. Weight beakers with sample (weight of ether extract).

Calculation:

$$\%Fat\ crude = \frac{Weight\ of\ ether\ extract}{Sample\ weight} * 100$$

Nitrogen Determination

Near infrared reflectance curve is in place for nitrogen determination in ground wheat tissue and maize flour. We have used the two methodologies described below as reference methods.

The determination of nitrogen is based on a colorimetric method in which an emerald-green color is formed by the reaction of salicylate and hypochloride with ammonia.

Determination of nitrogen with the Technicon AutoAnalyzer II method.
Technicon AutoAnalyzer II. Industrial method #334-74, 1977

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
100 µg/ml Ammonium sulphate		Weigh 1.179 g and dissolve it in 250 ml of distilled water.	Store at 4 °C for one month maximum. Keep in a light-protected container.
Sulfuric acid (analytical grade, 98%)			Store at room temperature in a light-protected container.
Catalyst mixture	Potassium sulphate Selenium	Mix very well 1 kg of K_2SO_4 with 5 g of selenium.	Handle very carefully; Selenium is an extremely dangerous reagent. Store at room temperature.
Reagent mixture 1	Sodium chloride Sulfuric acid Brij 35 purified	Dissolve 200 g of sodium chloride, 15 ml of sulfuric acid and 2 ml of Brij 35. Complete volume to 2 l with distilled water.	Store at room temperature.
Reagent mixture 2	Sodium phosphate dibasic anhydrous Sodium hydroxide	Dissolve 71 g of sodium phosphate dibasic anhydrous and 20 g sodium hydroxide and complete volume to 1 l with distilled water.	Store at room temperature.
880 mM Potassium tartrate	Potassium L- tartrate tetra-hydrated	Dissolve 200 g of potassium tartrate in 1 l of distilled water.	Store at room temperature.
5 M sodium hydroxide	Sodium hydroxide	Dissolve 200 g of sodium hydroxide in 1 l of distilled water.	Store at room temperature.
Reagent mixture 3	Reagent mix 2 880 mM Potassium L-tartrate tetra-hydrated 5 M sodium hydroxide Brij 35	Mix 400 ml of reagent mixture 2, 500 ml of 880 mM potassium tartrate, 500 ml of 5 M sodium hydroxide, and 1ml of Brij 35. Complete volume to 2 l with distilled water.	Store at room temperature in darkness for a maximum of 15 days.
Reagent mixture 4	Sodium salycilate (99.5%) Sodium nitro-prusiate Brij 35	Dissolve 300 g of sodium salycilate (99.5%) and 600 mg sodium nitro-prusiate. Add 2 ml of Brij 35 and complete volume to 2 l with distilled water.	Store at room temperature in darkness.
Sodium hypochloride	Sodium hypochloride	Use 6 ml of sodium hypochloride and complete volume to 100 ml with distilled water.	Store at room temperature in darkness.
Ammonium sulphate (100 µg/ml)	Ammonium sulphate	Dissolve 10 mg of ammonium sulphate in 100 ml of distilled water.	Store at 4 °C. Keep stable for one month.

Procedure:

Sample digestion

1. Weigh between 40 mg of ground sample. Include two check samples.
2. Transfer the sample to the bottom of a 75 ml digestion tube.
3. Include one or two tubes as blanks for digestion (without any samples).
4. Add 2.0 g of catalyst mixture to each tube and 2.5 ml concentrated H_2SO_4. Let stand until the reaction ceases.
5. Digest, under the fume hood, in pre-heated digester block at 380 ^oC for 90 minutes.

Sample analysis

6. Remove the rack of tubes from the digester, let them cool to room temperature and add 75 ml of distilled water to avoid crystal formation. Ensure the digest solution is totally clear.
7. Close tubes tightly with a rubber cap and mix by inverting the tubes several times.
8. Transfer 2 ml of the solution to Technicon vials and place the samples into the Technicon AutoAnalyzer.
9. Establish the baseline by pumping each of the four reagents: reagent mixture 1, reagent mixture 3, reagent mixture 4, and sodium hypochloride.
10. Set at 0% on the chart using the blank digestion solution.
11. Run four vials of blank digestion solution and recheck the 0% baseline.
12. Run four vials of 20 μg N/ml standard and set the peak at 70% on the chart.
13. Run check samples and unknown samples.

Preparation of N standard:

1. Prepare 100 μg/ml of ammonium sulphate solution in distilled water.
2. Every time you analyze samples, make a dilution of 20 μg/ml of ammonium sulphate in blank digestion solution.

Calculation of nitrogen percentage:

20 μg N/ml is set at 70% on the chart.
Where:
1% on the chart = 0.2857 μg N/ml in digest.
μg N/ml in digest = %chart reading x 0.2857 μg N/ml
or
μg N in 75 ml digest = %chart reading x 0.2857 μ x 75

$$\text{Calculation factor} = \frac{20\ \mu g\ N/ml}{Set\ of\ chart\ divisions\ x\ 1{,}000}\ x\ Digestion\ volume\ x\ 100\%$$

$$\%N = \frac{2.1427 \text{ x chart reading}}{\text{Weight of sample (mg)}}$$

Special recommendations:

a) Soap can be use to clean the digestion tubes, but you must remove all residues with deionized water.
b) If necessary, the digested samples can be stored at room temperature (if protected from air) for a maximum of 7 days before sample analysis. However, the sooner you analyze the digested samples, the better.
c) Clean the Technicon vials by washing 3-4 times with deionized water only. Do not use any soap.

d) Always include at least two standards with every set of 34 samples analyzed.
e) Calibrate the Technicon every time you start any measurement.

Troubleshooting table

Problem	Solution
Baseline is too high or variable	Check that all reagents are being pumped into the system. If you have prepared a new reagent, ensure it was done properly. Prepare new reagents.
Changes in values of your check samples	Weigh samples accurately. Ensure that sample digestion was complete. Be sure that reagent mixture 3 is not oxidized. If so, prepare a new one. Ensure quality of reagents. Prepare new ones.
Digestion solution is not clear	Ensure that sample is placed on the bottom of the tube. Ensure that catalyst mixture is placed on the bottom of the tube. When you add the sulfuric acid, do it carefully. Try to wash the wall of the tube as you are introducing the sulfuric acid.
Black/ yellow spots in the digestion solution	Check temperature of digester. Check that digester wells are clean. Extend digestion time for 20 minutes.

Protein determination

Thus the protein can be estimated from nitrogen value and in the case of maize the calculation is:

% Protein = % of nitrogen x 6.25 (conversion factor for maize)

Tryptophan Determination in Maize Grain Using Glyooxilic Acid

Nurit et al., 2009

Reagent / mixture	Specific reagents	Preparation	Special recommendations
Acetate solution: 0.165 M NaH3CCOOH	Sodium acetate	• Weigh 13.6 g of sodium acetate for 1 l of distilled water. • Adjust to pH 7.0 with NaOH (or with acetic acid if pH is basic after the preparation of the solution).	Keep as stock at 4 °C and stable for several weeks
Papain solution 1 mg/ml	Papain (crude extract: 2.5 units/mg)	• Weigh 40 mg of papain for 40 ml of solution (prepare it always fresh and in excess; you need 3ml per sample). • Dissolve the papain in the sodium acetate solution at room temperature.	Prepare it every time you will use it. Ensure that the sodium acetate buffer is at room temperature. Ensure that papain powder is well dissolved.
30 N sulfuric acid (Reagent C)	Sulfuric acid (analytical)	• Place a bottle on ice. • Mix at the same time 833.3 ml of sulfuric acid (96%) and 166.7 ml of distilled water to prepare a 30 N H_2SO_4 solution. • Complete final volume with distilled water.	
7 N sulfuric acid	Sulfuric acid (analytical)	• Place a bottle on ice. • Mix at the same time 35 ml of 30 N sulfuric acid and 115 ml of distilled water to prepare a 150 mL of 7N H_2SO_4 solution. • Complete final volume with distilled water.	
Reagent A: 0.1 M Glyoxilic Acid		• Weigh 0.9205 g of glyoxilic acid and place it in a 100 ml flask. • Add 50 ml of 7 N H_2SO_4. • Shake very slowly the flask until the glyoxilic acid is completely dissolved. • Adjust volume to 100 ml with 7 N H_2SO_4.	Prepare it daily.
Reagent B: 1.8 mM Ferric chloride		• Dissolve 0.050 g of $FeCl_36H_2O$ in100mL of reagent A.	Prepare it daily. Be sure everything is dissolved. Be aware that $FeCl_3$ highly hygroscopic.
Reagent C: 30 N sulfuric acid			
Reagent D: Colorimetric reagent		• One hour before use, mix 20 mL of reagent and 20 ml of reagent C.	Prepare it daily. Protect it from light and oxygen.
Tryptophan stock solution (100 μg/ml)	DL-Tryptophan	• Dissolve 10 mg of DL-Tryptophan in 100 ml of 0.1 N sodium acetate buffer pH 7.	Prepare it weekly and store it a 4 ºC. Vortexes thoroughly before you prepare dilutions for standard curve.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Grind each sample to a very fine powder.

Defatting

3. Transfer each sample in a commercial filter paper envelope (for example, 10x11 cm).
4. Defat samples for 6 hours with approximately 300 ml of hexane per balloon in a Soxhlet-type continuous extractor.
5. Air dry samples and ensure all hexane is evaporated.

Digestion

(These amounts are for reaction in 15 ml tubes. See modifications in table below).

6. For each sample, weigh 80 mg of defatted powder in a 15 ml falcon tube. A technical replicate per sample is recommended to be done.
7. Add 3 ml of papain solution.
8. Always include at least 2 blank controls, 4 checks (of known tryptophan concentration: 2 QPM, 2 normal), and the standard curve (see details below).
9. Close the tubes, ensuring no evaporation will take place during incubation
10. Vortex thoroughly the samples and place them in an oven at 64 ºC for 16 hours (overnight). If possible, vortex them twice more—one hour after being placed in the oven, and one hour before they complete the 16 hour incubation time.
11. Take the tubes out of the oven and let them cool down at room temperature.
12. Vortex the tubes immediately before centrifuging them at 3600 g for 5 minutes. Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.

Colorimetric reaction

13. Take 1 ml of hydrolysate (supernatant) and carefully transfer it to a glass tube.
14. Add 3 ml of reagent D (colorimetric reagent).
15. Vortex thoroughly each sample for 3 to 5 seconds.
16. Incubate tubes at 64 ºC for 30 minutes for color development.
17. Take the samples out of the oven and let them cool down at room temperature.
18. Read absorbance at 560 nm in a spectrophotometer.

Special recommendations

a) Defatting of maize flour is important to improve accuracy and repeatability of results. When samples are not defatted, an average of 0.8% less tryptophan is detected using this protocol.
b) Make sure that there are no particles of samples stuck to the wall of the tube or floating in the supernatant after centrifuging your samples in step 17. If there are some particles, vortex the sample again and centrifuge it for 15 minutes.
c) The reaction, as any analytical method, is very sensitive to pipetting precision. Ensure that your pipettes and/or dispensers are properly calibrated.
d) Always include one standard curve for every set of samples analyzed in a day.
e) Always measure the papain blank from the same batch. Papain is a protein that contains large amounts of tryptophan itself (every papain molecule contains 7 tryptophan units). This has to be subtracted for the calculations of each sample.

Scale down of digestion and colorimetric reaction

Reagent	Step	15 ml tubes	Eppendorf / Microplate
Ground maize kernel	Digestion	80 mg	30 mg
Papain	Digestion	3 ml	1.125 ml
Hydrolysate	Colorimetric reaction	1 ml	50 μL
Colorimetric reagent	Colorimetric reaction	3 ml	150 μL

Standard curve

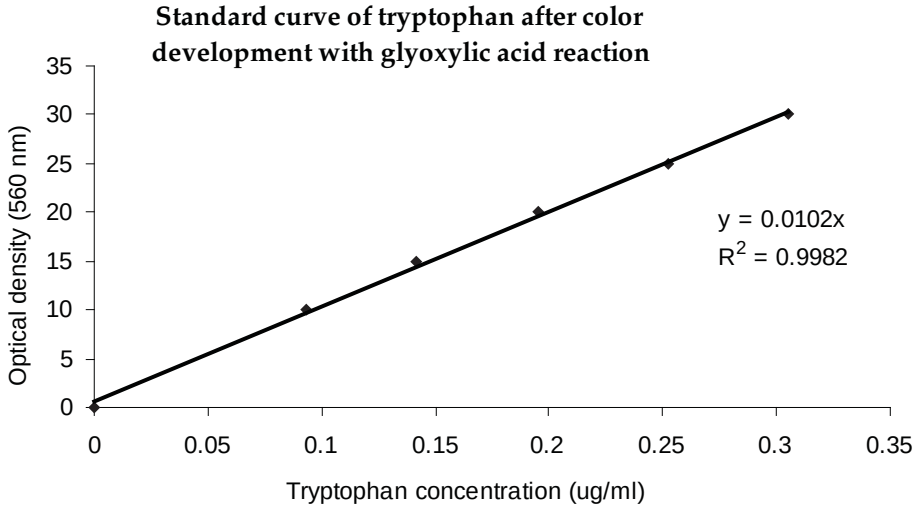
1. Prepare a stock solution of 100 μg / ml tryptophan in 0.1 M sodium acetate solution pH 7 (Prepare it weekly and store it a 4 ºC).
2. In 15 ml falcon tubes, prepare daily 0, 10, 15, 20, 25, and 30 μg / ml dilutions (in 0.1 M sodium acetate solution pH 7). Vortex properly before further use.
3. Do a colorimetric reaction (steps 16 to 20) using 1 ml of those dilutions.

Tube number	Stock trp 100 μg / ml (ml)	Sodium acetate 0.1 N, pH 7.0 (ml)	Total volume (ml)	Concentration μg trp / ml
1	0.0	10.0	10.0	0.0
2	1.0	9.0	10.0	10.0
3	1.5	8.5	10.0	15.0
4	2.0	8.0	10.0	20.0
5	2.5	7.5	10.0	25.0
6	3.0	7.0	10.0	30.0

Calculations:

Standard curve for tryptophan (calibration curve)

Develop a calibration curve using known amounts of tryptophan, ranging from 0 to 30 μg / ml. Plot the absorbance readings at 560 nm as a function of concentration and calculate the slope (m) of that standard curve. Note that the slope has the unit of OD*ml / μg.



Calculation of percentage of tryptophan

The amount of tryptophan (trp) for each sample is then estimated using the following equation:

$$\%\text{trp} = \frac{\text{OD}_{560\text{nm}}}{\text{slope}} \times \frac{\text{hydrolysis volume}}{\text{sample weight}} \times 100\%$$

Example:

$$\%\text{trp } (\mu g / \mu g) = \frac{0.5}{0.0095 \frac{\text{OD}}{\mu g/\text{ml}}} \text{ x } \frac{3\text{ml}}{80{,}000 \mu g} \text{ x } 100\%$$

However, this amount includes the tryptophan of the sample plus the tryptophan from the papain. To calculate the trp content of the biological material (defatted grain powder) you need to subtract the papain value.

Therefore, % trp should be calculated from the corrected absorption value:

$$\% \textbf{ trp} = \textbf{OD}_{\textbf{560nm corrected}} \textbf{ x Factor}$$

Where:

$$\text{OD}_{\text{560nm corrected}} = \text{OD}_{\text{560nm sample}} - \text{OD}_{\text{560nm average of papain blanks.}}$$

$$Factor = \frac{0.00375}{\text{slope}}$$

Note that: $\frac{3 \text{ ml}}{80{,}000 \ \mu g}$ *100 = 0.00375

In general, a sample with more than 0.070% of tryptophan in whole grain is considered as QPM. However, this also depends on the protein content and therefore the quality index value (%trp/protein).

Troubleshooting table

Problem	Solution
No color development in the reaction	1. Test another batch of colorimetric reagent.
Changes in factor curve values / OD measurements of tryptophan standard curve	2. Ensure quality of tryptophan standard curve. 3. Ensure that sulfuric acid is 30 N. 4. Ensure quality of all reagents. Prepare new ones. 5. Ensure that all quantities of reagents are properly measured.
OD for the Papain blank is too high	6. Ensure that the amount of papain is correct. 7. Use another batch of papain.
OD for the Papain blank is too low	8. Ensure that the amount of papain is correct. 9. Use another batch of papain.
Low values of control samples	10. Ensure that samples have been properly defatted (we recommend 6 hours defatting using hexane). 11. Ensure digestion of samples is done properly: a. Be sure that after sample digestion there are no particles on tube wall. If so, vortex the sample and centrifuge them again for 15 minutes. b. Ensure incubation was done at 64 °C for 16 hours. 12. Ensure quality and quantity of the reagents used. 13. Ensure quality of your tryptophan standard curve: a. Be sure that stock solution of tryptophan is properly dissolved before you do the dilutions. b. Mix well the stock solution of tryptophan before you do the dilutions. c. Prepare new stock solution of tryptophan.
OD measurements between replicates vary too much	14. Ensure that samples have been grinded properly to a fine powder. 15. Ensure accuracy of the sample weights. 16. Ensure replicates are analyzed equally, using the same batch of reagents. 17. Ensure samples have cooled down to room temperature before reading. 18. Set "zero" again in the spectrophotometer and be sure it is stable before you read your samples.
Papain does not dissolve	19. Ensure that the acetate solution is at room temperature.

Lysine Determination in Maize Grain

The colorimetric procedure for lysine quantification is based on two steps. The first step is the protection of the amino group in ∝ of lysine chain by reaction with copper which also blocked the amino group of low molecular weight peptides presents in the hydrolysate. The second step is the reaction of the 2-chloro-3-, 5-dinitropyridine with the amino group in ξ of protected lysine chain to give a colored ξ-dinitropyridil lysine which is determined spectroscopically at 390 nm.

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
0.03 M Phosphates buffer solution, pH 7.4	Sodium phosphate dibasic Na_2PO_4 Potassium phosphate monobasic KH_2PO_4	• Dissolve 3.19 g of sodium phosphate for 400 ml of deionized water. • Dissolve 1.04 g of potassium phosphate for 300 ml of deionized water. • Mix both solution and complete the final volume at 1 l. • Adjust the pH at 9 with HCl.	Keep as stock at 4°C and stable for several weeks.
Papain solution 4 mg/ml	Papain (crude extract: 2.5 units/mg)	• Weigh 1.6 g of papain for 400 ml of solution. (Prepare it always fresh and in excess. You need 5 ml per sample) • Dissolve the papain in the 0.03 M phosphates buffer solution with pH 7.4 at room temperature	Prepare it every time you will use it. Ensure that the phosphates buffer solution is at room temperature. Ensure that papain powder is well dissolved.
Papain solution 5 mg/ml	Papain (crude extract: 2.5 units/mg)	• Weigh 125 mg of papain for 25 ml of solution. (Prepare it always fresh. You need 20 ml for made the standard curve.) • Dissolve the papain in the 0.03 M phosphates buffer solution with pH 7.4 at room temperature.	Prepare it every time you will use it. Ensure that the phosphates buffer solution is at room temperature. Ensure that papain powder is well dissolved.
0.05 M Carbonates buffer solution, pH 9	Sodium carbonate Na_2CO_3 Sodium bicarbonate $NaHCO_3$	• Dissolve 6.36 g of sodium carbonate for 100 ml of deionized water. • Dissolve 25.2 g of bicarbonate sodium for 500 ml of deionized water. • Mix both solution adjust the pH at 9 with HCl.	Keep as stock at 4 °C and stable for several weeks
0.05 M Borate buffer solution, pH 9	Sodium borate solution decahydrate $Na_2B_4O_7 \cdot 10H_2O$	• Dissolve 19.07 g of sodium carbonate for 1 l of deionized water.	Keep as stock at 4 °C and stable for several weeks
Cupper phosphate suspension	Cupric chloride dihydrate, crystal $CuCl_2 \cdot 2H_2O$ Sodium phosphate tribasic dodecahydrate $Na_3PO_4 \cdot 12H_20$	• Dissolve 2.8 g of cupric chloride for 100 ml of deionized water. • Dissolve 13.6 g of sodium phosphate tribasic for 200 ml of deionized water. • Mix both solutions by shaking; divide the volume for 8 centrifuge tubes (approx. 37.5 ml per tube) centrifuge at 2000 rpm for 5 minutes, remove the supernatant and re-suspend the pellet for three times with 15 ml of borate buffer solution, removing the supernatant in the three times. • Finally, you have to re-suspend all pellets in 80 ml of borates buffer solution. You can use 10 ml of solution for each centrifuge tube and collect the 8 solutions.	Keep as stock at 4 °C and stable for one week.

Reagent / mixture	Specific reagents	Preparation	Special recommendations
1.2 N Hydrochloric acid	36.5 – 38% analytical hydrochloric acid	• Mix 100 ml of HCl and 300 ml of deionized water. • Complete the final volume at 1 l.	Prepare this solution under fume hood.
Aminoacids mixture	L-aminoacids	• Weight 20 mg of each: Cystine, Methionine, Proline • Weight 30 mg of each: Histidine, Alanine, Isoleucine, Threonine, Tyrosine • Weight 40 mg of each: Glycine, Phenylalanine, Valine • Weight 50 mg of each: Arginine, Serine • Weight 60 mg of Aspartic Acid • Weight 80 mg of Leucine • Weight 300 mg of Glutamic Acid • Mix all aminoacids powders and weight 100 mg for 10 ml of carbonates buffer solution.	Keep as stock at 4°C and stable for several months.
2-Chloro-3,5-dinitropyridine reagent (3% in methanol)	Sigma-Aldrich, Cat: 224049	• Dissolve 0.240 g of 2-Chloro-3,5-dinitropyridine for 8 ml of methanol.	Prepare it daily. Protect it from light and oxygen. Be sure that dinitropyridine is completely dissolved.
Lysine stock solution (2500 μg/ml)	L-Lysine monohydrochloride or L-Lysine	• For L-Lysine monohydrochloride, dissolve 78.125 mg in 25 ml of 0.05 M carbonates buffer solution pH 9.0 • For L-Lysine, dissolve 62.5 mg in 25 ml of 0.05 M carbonates buffer solution pH 9.0	Prepare it weekly and store it a 4 ºC. Vortex thoroughly before you prepare dilutions for standard curve.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Be sure all seed samples have similar moisture content.
3. Grind each sample to a very fine powder.

Defatting

4. Transfer each sample in a commercial filter paper envelope (for example, 10x11 cm).
5. Defat samples for 6 hours with approximately 300 ml of hexane per balloon in a Soxhlet-type continuous extractor.
6. Air dry samples and ensure all hexane is evaporated.

Digestion

7. For each sample, weigh 100 mg of defatted powder in a falcon tube.
8. Add 5 ml of papain solution.
9. Always include at least 2 blank controls, 4 checks (of known lysine concentration: 2 QPM, 2 normal).
10. Close the tubes, ensuring no evaporation will take place during incubation.
11. Vortex thoroughly the samples and place them in an oven at 64 ºC for 16 hours (overnight). If possible, vortex them twice more—one hour after being placed in the oven, and one hour before they complete the 16 hour incubation time.
12. Take the tubes out of the oven and let them cool down at room temperature.
13. Vortex the tubes immediately before centrifuging them at 2500 rpm for 5 minutes Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.

Colorimetric reaction

14. Transfer 1 ml to centrifuge tube and add 0.5 ml of carbonates buffer solution and 0.5 ml cupper phosphate suspension.
15. Shake for 5 minutes and centrifuge at 2000 rpm for 5 minutes.
16. Transfer 1 ml of supernatant in a new tube.
17. Add 0.1 ml of 2-Chloro-3,5-dinitropyridine reagent and vortex thoroughly.
18. Keep the tubes at room temperature for 2 hours and shake each 30 minutes.
19. Add 5 ml 1.2 N HCl to each tube and vortex.
20. Add 5 ml of ethyl acetate.
21. Cover the tubes; mix the solution inverting the tubes 10 times.
22. Remove the up phase using a syringe with a polyethylene tube connected. Repeat two times.
23. Read absorbance at 390 nm in a spectrophotometer.

Standard curve

1. Prepare a stock solution of 2500 μg/ml lysine in carbonates buffer solution.
2. In 15 ml falcon tubes, prepare 0, 250, 500, 750, and 1000 μg/ml dilutions (in carbonates buffer solution 0.05 M at pH 9). Vortex properly before further use.

Tube number	Stock Lys 2500 μg / ml (ml)	Carbonates buffer solution 0.05m, pH 9.0 (ml)	Total volume (ml)	Concentration μg Lys / ml
1	0	10.0	10.0	0
2	1	9.0	10.0	250
3	2	8.0	10.0	500
4	3	7.0	10.0	750
5	4	6.0	10.0	1000

3. In new15 ml falcon tubes, prepare new concentrations of lysine from the first stock solution, using 1 ml each tube and 4 ml of 5 mg/ml papain solution. Vortex properly before further use.

Tube number	Concentrations Lys 0-1000 μg / ml (ml)	Papain solution 5 mg/ml (ml)	Total volume (ml)	Concentration with Papain μg Lys / ml
1	1	4	5	0
2	1	4	5	50
3	1	4	5	100
4	1	4	5	150
5	1	4	5	200

4. Do a colorimetric reaction (steps 15 to 23) using 1 ml of those dilutions, for make the standard curve, using the 0.05 M carbonates buffer solution pH 9.0 with the aminoacids mix.

Tube number	Concentration with Papain μg Lys / ml (ml)	Amino acid mix in 0.05 M carbonates buffer solution, pH 9.0 (ml)	Cupper phosphate suspension (ml)
1	1	0.5	0.5
2	1	0.5	0.5
3	1	0.5	0.5
4	1	0.5	0.5
5	1	0.5	0.5

Calculation of percentage of lysine

The amount of lysine for each sample is then estimated using the following equation:

$$\%\text{Lys} = \frac{\text{OD}_{390\text{nm}}}{\text{slope}} \text{ x } \frac{\text{hydrolysis volume}}{\text{sample weight}} \text{ x } 100\%$$

Example:

$$\%\text{Lys } (\mu g / \mu g) = \frac{0.25}{0.00165 \frac{\text{OD}}{\mu g/mL}} \text{ x } \frac{5 \text{ ml}}{100{,}000 \mu g} \text{ x } 100\%$$

$$\textit{Factor} = \frac{0.005}{\text{slope}}$$

Note that: $\frac{5 \text{ ml}}{10{,}000\ \mu g}$ *100 = 0.005

Estimation of Free and Total Phenolics Content in Maize Using Folin-Ciocalteu Reagent

The F–C assay is based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 765 nm. This assay is performed in microcentrifuge tubes and assessed in a 96-well plate reader.

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
50% Methanol	Methanol absolute	• Mix 500 ml of deionized water and 500 ml methanol	Prepare weekly and store at 4 ºC to avoid evaporation.
1.2 M Hydrochloric acid in methanol	Hydrochloric acid fuming (analytical with 37% purity)	• Mix 10 ml of HCl with 90 ml of methanol. • Complete final volume (100 ml) with methanol.	Prepare weekly and store at 4 ºC to avoid evaporation.
25 % Folin-Ciocalteu reagent	2 N Folin – Ciocalteu's phenol reagent	• Use an amber bottle with cap. • Mix 2.5 ml of F-C 2N with 7.5 ml of deionized water and vortex thoroughly.	Prepare weekly and store at room temperature. Be sure that the bottle is tightly closed. Vortex thoroughly before you uses it.
400 mM Sodium Carbonate (Na_2Co_3)	Sodium carbonate (analytical)	• Dissolve 4.25 g of Na_2CO_3 (99.9%) in 100 ml of deionized water.	Be sure everything is dissolved. Prepare weekly and store at room temperature.
Gallic acid stock solution (100 μg/ml)		• Dissolve 20 mg of gallic acid in 200 ml of methanol.	Protect it from light, covering the flask with aluminum, paper, or using an amber bottle. Prepare it weekly and store it a 4 ºC. Vortex it thoroughly before you prepare dilutions for standard curve.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material
2. Be sure all seed samples have similar moisture content.
3. Grind each sample to a very fine powder.

Drying

4. Dry the powder at 64-65 ºC for 16 hours.

Extraction of free or soluble phenolics

5. For each sample, weigh 20 mg of powder in an eppendorf tube.
6. Add 1.3 ml of methanol.
7. Close the tubes, ensuring no evaporation will take place during extraction.
8. Vortex thoroughly the samples and place them in a thermomixer for microtubes at 65 ºC and 900 rpm for 30 minutes.
9. Take the tubes out of the thermomixer and let them cool at room temperature.
10. Centrifuge the tubes at 14,000 rpm for 5 minutes. Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.
11. Make the colorimetric reaction.

Extraction of total phenolics

12. For each sample, weigh 20 mg of powder in an eppendorf.
13. Add 1.3 ml of 1.2 M hydrochloric acid in methanol.
14. Close the tubes, ensuring no evaporation will take place during extraction.
15. Vortex thoroughly the samples and place them in a thermomixer for microtubes at 42 ºC and 1100 rpm for 30 minutes.
16. Take the tubes out of the thermomixer and let them cool at room temperature.
17. Centrifuge the tubes at 14,000 rpm for 5 minutes. Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.
18. Take 500 μL of supernatant, put it in new eppendorf.
19. Reduce to dryness and resuspend the precipitate resulting in 1.3 ml of methanol.
20. Vortex thoroughly and make the colorimetric reaction.

Colorimetric reaction

21. Take 50 μL of supernatant and carefully transfer it to a hole of microplate.
22. Add 40 μL of 25 % Folin-Ciocalteu reagent. The F-C reagent should be added before the alkali to avoid the air-oxidation of phenolics.
23. Add 110 μL of 400 mM Na_2CO_3.
24. Cover the microplates with adhesive aluminum tape to avoid dropping of samples.
25. Vortex the microplate at 800 rpm for 10 seconds.
26. Incubate microplate at 42 ºC for 9 minutes for color development.
27. Take the microplates out of the oven and let them cool at room temperature, protect them from direct light.
28. Read absorbance at 765 nm in a spectrophotometer.

Standard curve:

1. Prepare a stock solution of 100 μg/ml gallic acid in 50 % methanol (Prepare it weekly and store it a 4 ºC)
2. In 15 ml falcon tubes, prepare daily 0, 10, 15, 20, 25, and 30 μg/ml dilutions (in 50 % methanol). Vortex properly before further use.
3. Make a colorimetric reaction (steps 14 to 20) using 1 ml of those dilutions.

Tube number	Stock gallic acid 100 μg / ml (ml)	50 % Methanol (ml)	Total volume (ml)	Concentration μg galic acid / ml
1	0.0	10.0	10.0	0.0
2	1.0	9.0	10.0	10.0
3	1.5	8.5	10.0	15.0
4	2.0	8.0	10.0	20.0
5	2.5	7.5	10.0	25.0
6	3.0	7.0	10.0	30.0

Calculations:

Standard curve for gallic acid (calibration curve)

Develop a calibration curve using known amounts of gallic acid, ranging from 0 to 30 μg/ml. Plot the absorbance readings at 765 nm as a function of concentration and calculate the slope of that standard curve. Note that the slope has the unit of OD*ml/μg.

Calculation of percentage of gallic acid for free phenolics
The amount of gallic acid for each sample is then estimated using the following equation:

$$\%\ \text{Gall} = \frac{D_{765nm}}{\text{slope}} x \frac{\text{hydrolysis volume}}{\text{sample weight}} \times 100\%$$

Example:

$$\%\ \text{Gall}\ (\mu g / \mu g) = \frac{0.345}{0.0155\ \frac{D}{\mu g/mL}} x \frac{1.3ml}{20{,}000\mu g} \times 100\%$$

However, this amount includes the absorbance of plate and methanol. To calculate the gallic acid content of the biological material (grain powder) you need to subtract the absorbance value of plate and methanol.

$$\%\ \textbf{Gall} = \textbf{OD}_{\textbf{765nm corrected}} \textbf{ x Factor}$$

Where:

$$OD_{765nm\ corrected} = OD_{765nm\ sample} - OD_{765nm\ average\ of\ methanol\ blanks}$$

$$Factor = \frac{0.0065}{\text{slope}}$$

Note that: $\frac{1.3}{20{,}000} * 100 = 0.0065$

Calculation of percentage of gallic acid for total phenolics
In the case of total phenollics you need to consider the dilution factor and volume dried. The amount of gallic acid for each sample is then estimated using the following equation:

$$\%\ \text{Gall} = \frac{OD_{765nm}}{\text{slope}} x \frac{\frac{\text{hydrolysis volume}}{\text{volume dried}}}{\text{sample weight}} \times 100\% \times FD$$

Example:

$$\%\ \text{Gall}\ (\mu g / \mu g) = \frac{0.225}{0.0155\ \frac{OD}{\mu g/mL}} x \frac{\frac{1.3\ ml}{0.5\ ml}}{20{,}000\mu g} \times 100\% \times 2.6$$

However, this amount includes the absorbance of the plate and methanol. To calculate the gallic acid content of the biological material (grain powder) you need to subtract the absorbance value of plate and methanol.

$$\%\ \textbf{Gall} = \textbf{OD}_{\textbf{765nm corrected}} \textbf{ x Factor}$$

Where: $OD_{765nm\ corrected} = OD_{765nm\ sample} - OD_{765nm\ average\ of\ methanol\ blanks}$

$$Factor = \frac{0.0338}{\text{slope}}$$

Note that:

$$\frac{1.3ml / 0.5}{20{,}000} \times 100 \times 2.6 = 0.0338$$

Troubleshooting table

Problem	Solution
No color development in the reaction	1. Test another batch of colorimetric reagents.
Changes in factor curve values / OD measurements of gallic acid standard curve	2. Ensure quality of gallic acid standard curve. 3. Ensure that sodium carbonate is 400 mM. 4. Ensure quality of all reagents. Prepare new ones. 5. Ensure that all quantities of reagents are properly measured.
OD for the 50% methanol or blank is too high	6. Ensure that the concentration of sodium carbonate is correct.
Low values of control samples	7. Ensure extraction of samples is made properly: a) Be sure that after sample extraction there are not particles on the tube wall. If so, vortex the sample and centrifuge them again for 5 minutes. b) Ensure extraction was made at 65 °C and 900 rpm for free phenollics or at 42 °C and 1100 rpm for total phenollics. 8. Ensure quality and quantity of the reagents used. 9. Ensure quality of your gallic acid standard curve: a) Be sure that stock solution of gallic acid is properly dissolved before you make the dilutions. b) Prepare new stock solution of gallic acid.
OD measurements between replicates vary too much	10. Ensure accuracy of the sample weights. 11. Ensure samples have cooled down to room temperature before reading.

Soluble Sugars Determination Using Anthrone Reagent

Near infrared reflectance curve is in place for soluble sugars determination in grinded wheat tissue. We have used the anthrone methodology described below as reference methods.

The anthrone procedure is based on the reaction of the anthrone (9,10-dihydro-9-oxoantraceno) with furfural conformation of carbohydates (treatment of carbohydrate in strong sulfuric acid) to give a colored hemiacetal which is determined spectroscopically at 630 nm.

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
Deionized water			
Sulfuric acid	Sulfuric acid (analytical)		
Anthrone reagent		• One hour before use, weigh 100 mg of anthrone reagent and dissolve in 50 ml of analytical concentrated sulfuric acid.	Prepare it daily. Protect it from light; keep on ice or in the fridge.
Sucrose stock solution (100 μg/ml) Sucrose stock solution (30 μg/ml)		• Dry sucrose for 1 hour at 105 ºC. • Dissolve 25 mg of sucrose in 250 ml of deionized water. • Dilute 30 ml of 100 μg/ml solution in a 100 ml volumetric flask with deionized water.	Prepare it weekly and store at 4 ºC. Vortexes thoroughly before you prepare dilutions for standard curve.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material
2. Be sure all seed samples have similar moisture content.
3. Grind each sample to a very fine powder.

Drying

4. Dry samples for 4 hours at 60 ºC.

Extraction

(These amounts are for reaction in 15 ml tubes)

5. For each sample, weigh duplicate 20 mg of dried powder in a 15 ml falcon tube.
6. Add 4 ml of deionized water, immediately covering and vortex thoroughly.
7. Put the sample rack in a water bath for 45 minutes at 70 ºC.
8. Vortex thoroughly every 15 minutes.
9. After incubation, place the samples on ice.
10. Centrifuge at 3,000 rpm for 10 minutes.
11. Use the supernatant to make the necessary dilutions depending on the material:

    Wheat: 1:50 (100 μl of supernatant in 4.9 ml deionized water)
    Maize: 1:20 (250 μl of supernatant in 4.75 ml deionized water)

Colorimetric reaction

12. Take 2.5 ml of dilution and carefully transfer it to a new falcon tube in cold water.
13. Using a burette, slowly add 5 ml of anthrone solution to each tube; keep shaking the tube in water with ice.
14. Vortex thoroughly each tube.
15. Close the tubes and put them in a water bath at ebullition for 7.5 minutes.
16. Take the tubes out of the water bath and let them cool at room temperature.
17. Read absorbance at 630 nm in a spectrophotometer.

Scale down the colorimetric reaction and special recommendations for microplate use

Reagent	Step	15 ml tubes	Microplate
Hydrolysate	Colorimetric reaction	2.5 ml	50 μL
Colorimetric reagent	Colorimetric reaction	5 ml	100 μL

a) Each sample has to be analyzed in triplicate in microplate.
b) The 96 well plates have to be maintained on ice.
c) Add the anthrone solution using a digital multichannel pipette. Do it carefully to ensure all 100 μl goes to the well.
d) Cover the plate with aluminum tape and vortex it very carefully until homogenous solution in each well is observed.
e) Incubate the plates at 100 °C for 20 minutes with maize samples, and for 10 minutes with wheat samples.
f) Cool the plates in the fridge for 10 minutes before reading them at 630 nm in a microplate reader.

Standard curve

1. Prepare a stock solution of 100 μg/ml dried sucrose in deionized water.
2. In 15 ml falcon tubes, prepare 0, 10, 15, 20, 25, and 30 μg/ml dilutions (in deionized water). Do the dilutions daily and vortex properly before further use.
3. Make a colorimetric reaction (steps 14 to 18) using 2.5 ml of those dilutions.
4. Always include one standard curve in duplicate for every set of samples analyzed in a day.

Tube number	Stock sucrose 100 μg / ml (ml)	Deionized (ml)	Total volume (ml)	Concentration μg sucrose / ml
1	0.0	10.0	10.0	0.0
2	1.0	9.0	10.0	10.0
3	1.5	8.5	10.0	15.0
4	2.0	8.0	10.0	20.0
5	2.5	7.5	10.0	25.0
6	3.0	7.0	10.0	30.0

Standard curve for microplate

1. Prepare two stock solutions of dried sucrose 100 μg/ml and 30 μg/ml.
2. In 15 ml falcon tubes, prepare 0, 6, 12, 18, 24, 30, 40, and 60 ug/ml dilutions. Prepare dilutions daily and vortex properly before further use.
3. Make a colorimetric reaction (steps 14 to 18) using 50 μl of those dilutions.
4. Always include one standard curve in duplicate for every set of samples analyzed in a day.

Tube number	Stock sucrose 30 µg / ml (ml)	Deionized (ml)	Total Volume (ml)	Concentration µg sucrose / ml
1	0.0	2.5	2.5	0.0
2	0.5	2.0	2.5	6
3	1	1.5	2.5	12
4	1.5	1.0	2.5	18
5	2	0.5	2.5	24
6	2.5	0	2.5	30

Tube number	Stock sucrose 100 ug / ml (ml)	Deionized (ml)	Total Volume (ml)	Concentration µg sucrose / ml
7	1.0	1.5	2.5	40
8	1.5	1.0	2.5	60

Calculations:

Calibration curve

Develop a calibration curve using known amounts of sucrose, ranging from 0 to 75µg (0 to 3 µg in microplate). Plot the absorbance readings at 630 nm as a function of the amount in µg and calculate the slope of that standard curve. Note that the slope has the unit of OD/µg.

Calculation of percentage of sucrose

The amount of sucrose for each sample is then estimated using the following equation:

$$\%\ \text{SUC} = \frac{\text{OD630}_{\text{nm}}}{\text{slope}} \text{x} \frac{\text{dilution}}{\text{sample weight}} \text{ x } 100\%$$

Example: Maize in microplate

$$\%\ \text{SUC}\ (\mu g / \mu g) = \frac{0.127}{0.110\ {}^{D}_{\mu g}} \text{x} \frac{(4/0.05)}{20{,}000 \mu g} \text{ x } 100\%$$

To calculate the sucrose content of the biological material (gain powder) you need to subtract the absorbance value of anthrone in sodium acetate.

% suc = $OD_{630nm\ corrected}$ x Factor x dilution (maize or wheat)

Where:

$$OD_{630nm\ corrected} = OD_{630nm\ sample} - OD_{630nm\ average\ of\ anthrone\ blanks.}$$

$$Factor = \frac{0.4}{\text{slope}}$$

Troubleshooting table

Problem	Solution
No color development in the reaction	1. Test another batch of colorimetric reagents.
Changes in factor curve values / OD measurements of sucrose standard curve	2. Ensure quality of sucrose curve. 3. Ensure quality of sulfuric acid. 4. Ensure quality of all reagents. Prepare new ones. 5. Ensure that all quantities of reagents are properly measured.
OD for anthrone blank is too high (the value should be in this range 0.045-0.06)	6. Ensure anthrone reagent has been properly prepared using non-oxidize sulfuric acid. 7. Prepare another color reagent. 8. Make the anthrone reagent using another batch of sulfuric acid.
Low values of control samples	9. Ensure extraction of samples is made properly: a) Be sure that after the extraction of the samples, there are not particles on the tube wall. If so, vortex the sample and centrifuge them again for 10 minutes. b) Ensure extraction was made at 60-70 °C and 3000 rpm. 10. Ensure quality and quantity of the reagents used. 11. Ensure quality of your sucrose curve: be sure that stock solution of sucrose is properly dissolved before you make the dilutions. 12. Ensure the quality and accuracy of the dilutions.
OD measurements between replicates vary too much	13. Ensure accuracy of the sample weights. 14. Ensure samples have been cooled before reading. 15. Ensure good pipeting.

Maize Carotenoids Determination

Kurilich et al., 1999; Howe et al., 2006

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
0.1% Butylhydroxytoluene (BHT) in ethanol		Dissolve 250 mg of BHT in 250 ml of ethanol absolute.	Prepared on the day of extraction.
80% Potassium hiyoxide		Weight 80 g of potassium hydroxide and place it in a 100 ml flask. Add 50 ml of deionized water. Shake the flask until the potassium hydroxide is completely dissolved. Adjust volume to 100 ml with deionized water.	Prepared on the day of extraction.
Hexane			
Acetonitrile:methanol: mehylene chloride (45:20:35)		To make 100 ml of solution: Mix 45 ml of acetonitrile with 20 ml of methanol and 35 ml of methilene chloride.	Prepared on the day of extraction.
Washing solution: Methanol:water (1:1)	Both of them HPLC grade		
Reagent A: Water	HPLC grade		
Reagent B: 0.1 BHT and 0.05 % triethylamine (TEA) in methylene chloride	Methylene chloride (HPLC grade)	Put 125 ml of methylene chloride in a bottle. Add 250 mg of BHT and 125 μL of TEA. Shake the flask until BHT and TEA are completely dissolved. Adjust volume to 250 ml with methylene chloride.	Prepared on the day of extraction.
Reagent C: 0.1% BHT and 0.05% TEA in acetonitrile	HPLC grade	Put 1 L of acetonitrile in a bottle. Add 2 g of BHT and 1 ml of TEA. Shake the flask until BHT and TEA are completely dissolved. Adjust volume to 2 l with acetonitrile.	Prepared on the day of extraction.
Reagent D: 0.1% BHT and 0.05% TEA in methanol	HPLC grade	Put 250 ml of methanol in a bottle. Add 500 mg of BHT and 250 μL of TEA. Shake the flask until BHT and TEA are completely dissolved. Adjust volume to 500 ml with methanol.	Prepared on the day of extraction.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Be sure all seed samples have similar moisture content.
3. If the seeds have been treated, wash thoroughly with tap water and then rinse with distilled water. Let the seeds dry out.
4. Grind each sample to a very fine powder. Pack the powder into yellow paper bags.
5. Seal immediately with tape and refrigerate at -20 °C. Don't store the milled samples for over 48 hours.

Extraction

The following steps must be done with yellow light, never with direct light.
The extract of the samples must be analyzed by HPLC just after extraction.

6. In 15 ml the falcon tubes, previously wrapped with aluminum paper, weigh 600 mg by sample.
7. Add 6 ml 0.1% BHT in ethanol.
8. Vortex thoroughly.
9. Incubate at 85 °C for 5 minutes.
10. Add 120 μL 80% KOH.
11. Vortex thoroughly.
12. Incubate for 10 minutes at 85 °C
13. Transfer the falcon tube to ice.
14. Add 3 ml of cold deionized water to each tube.
15. Add 3 ml of hexane, under the fume hood.
16. Vortex thoroughly.
17. Centrifuge the samples at 3,000 rpm for 10 minutes.
18. Transfer the upper phase to a new falcon tube, also wrapped with aluminum paper. Keep these tubes on ice. Immediately cover the tubes, avoid contact of the upper phase with the room air.
19. Extract two more times with hexane (repeat steeps 15 to 18).
    Be sure that when you add hexane, the sample is fully resuspended. Use plastic mixers to release the pellet.
20. Add 3 ml of deionized water to the three combined hexane fractions.
21. Vortex thoroughly.
22. Centrifuge at 3,000 rpm for 10 minutes.
23. Transfer the upper phase to a new falcon tube, also wrapped with aluminum paper. Beware of transferring only the upper phase; don't take the water.
24. Dry the hexane by putting the tubes under nitrogen, do it under the fume hood (normally delayed 1.5 to 2 hours).
25. Cover the tubes immediately.
26. Resuspend the samples with 200 μL of acetonitrile:methanol:methylene chloride (45:20:35).
    Resuspend the samples just before injected to HPLC. If they can not be injected immediately, save the samples without resuspending them. Store them at -20 °C for no longer than 24 hours.

Separation and quantification by HPLC
General conditions:
Column: YMC Carotenoid S-5 4,6x 150 mm column
Pre-column: YMC Carotenoid 5u, 4x2 Gd Cat.

Mobile phase: acetonitrile: methanol: methylene chloride (75:20:5) containing 0.05% triethylamine (TEA) and 0.1% BHT

Flow: 1.8 ml/min

Absorbance: 450 nm

Standards: Lutein, beta-cryptoxanthin, beta-carotene, alpha-carotene all resuspended in acetonitrile: metanol: metilencloride (75:20:5) containing 0.05% triethylamine (TEA) and 0.1% BHT.

1. Spectroscopically determine the concentration of the standard based on its extinction coefficient (E1%) at the maximum wavelength (450nm) of the carotenoid. In a standard 1 cm quartz cuvette, the E1% are:

   β-carotene: 2592
   Zeaxanthin: 2348
   Lutein: 2550

   Concentration of the standard (ng/ul) = Absrobance x 10,000 + E1%

2. Verify the purity of the standard by running it on the HPLC, it should be more than 98% pure.
3. Perform standard curve that includes the peak area of the maize carotenoid. Use linear regression to establish an equation to convert peak area to the amount of carotenoid.

   Example standard curve. Purified β-carotene has an absorbance of 0.7425. The concentration of β-carotene in this solution is 0.7425 × 10 000 ÷ 2592 = 2.86 ng/μl. If you inject 10, 50, 100, and 150 μL, you might observe areas of 420,000; 2,000,000; 4,250,000; 6,250,000. You can then plot ng injected vs area (or vice-versa) and using linear regression you can obtain a standard curve that can be used to calculate the amount of β-carotene in an injection from the area of the β-carotene peak. If you plot area (x-axis) versus ng β-carotene injected (y-axis), the resulting standard curve is y = 6.8015 x 10-5x + 1.9609 with an r2 = 0.9994 (make sure you use enough significant figures). If your sample has a β-carotene peak of 5,000,000 then you injected 342 ng β-carotene. You must next calculate the ng β-carotene/g maize analyzed. If 50 μL were injected into the HPLC and the total volume of the sample at the end of the analysis was 500 μL, then 10% of the sample was injected into the HPLC. So, 342 ng/50 μL × 500 μL = 3420 ng/μL in the sample. But, the 500 μL sample is equivalent to the 0.6 g maize used in the analysis procedure, so 3420 ng/μL × 0.6g = 2,052 ng β-carotene/g maize. If you used an internal standard, you would then divide 2,052 by the extraction efficiency in decimal form. If your extraction efficiency was 91%, then 2,052 ÷ 0.91 = 2,255 ng β-carotene/g maize.

Injection volume (μL)	**Area**	**Beta-carotene (ng) [inj vol x 2.86 ng/μL]**
10	420,000	28.6
50	2,000,000	143
100	4,250,000	286
150	6,250,000	429

Sample	Calculated ng injected
5,000,000	342 = (6.8015×10-5 × 5,000,000) +1.9609

Determination of Aluminum, Iron, and Zinc in Plants by Nitric and Perchloric Acid Digestion and Analysis by ICP-OES

This procedure is for the determination of Al, Fe, and Zn in maize and wheat seeds by nitric and perchloric acid digestion and then analysis by Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES).

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
Neutral detergent 2%	Neutrad, Decon Labs, CAT 3001	Dilute 20 ml of detergent and complete with one liter of deionized water.	
Nitric acid 10%	Nitric acid, 70%, redistilled 99.999+%, SIGMA-ALDRICH 225711 or equivalent.	10 m of nitric acid and complete with deionized water to one liter.	Use plastic flask. Store at room temperature for 2 months maximum.
Acid digestion mixture (nitric: perchloric = 10 : 1)	Nitric acid, 70%, redistilled 99.999+%, SIGMA-ALDRICH 225711 or equivalent. Perchloric acid, 70%, Mallinckrott 2766, CAS 7601-90-3.	100 ml of Perchloric acid and complete to 1 l with nitric acid.	Use plastic flask. Store at room temperature for 2 months maximum.
Diluting acid solution for digests (nitric 1%)	Nitric acid, 70%, redistilled 99.999+%, SIGMA-ALDRICH 225711 or equivalent.	1 ml of nitric acid and complete with deionized water to one liter.	Use plastic flask. Store at room temperature for 2 months maximum.
Standard curve points.	Perkin Elmer Pure Atomic Spectroscopy Standards of Al, Fe and Zn, 1000 μg/ml 2% HNO_3	Make a standard curve diluting the pure standards with nitric acid. Normally the range of the standard curve goes from 0 to 5 ppm	Use plastic flask. Store at 4 ºC for 2 months maximum.

Procedure:

1. Mill the samples in the mixer using zirconium containers and balls to avoid contamination. Always include control samples.
2. Transfer powder to non-contaminant paper envelopes.
3. Dry the samples at 60 ºC for 24 hours.
4. Weigh the appropriate amount of sucrose (500 mg) for each acid blank.
5. Place the samples into an appropriate desiccator and allow all samples to cool to room temperature.
6. Weigh 600 mg of each control and sample into its marked digestion tube.
7. Cover the samples with saran wrap.
8. Place the rack of weighed samples into a fume hood and add 10 ml of the mixture nitric / perchloric acid. It is recommended to use special acid dispensers.
9. Leave the tubes in the fume hood overnight (12–14 hours) for a cold pre-digestion.
10. Vortex tubes after cold pre-digestion to ensure all sample is mixed with the acid.
11. Place the tubes on the aluminum blocks at room temperature and carry out the nitric / perchloric acid digestion using the digestion program.
12. A range of temperature regimes can be used depending on sample types and weights; usually, we use the next regime temperature.

Table 2. Normal nitric / perchloric digestion.

	Grain (corn and wheat)		Vegetal tissue (corn and wheat)	
Step No.	**Temperature (°C)**	**Time (min.)**	**Temperature (°C)**	**Time (min.)**
1	Step to 50	Approx. 15 - 20	Step to 90	Approx. 20 - 30
2	Hold @ 50	30	Hold @ 90	50
3	Step to 100	Approx. 10	Step to 96	Approx. 5
4	Hold @ 100	30	Hold @ 96	30
5	Step to 120	Approx. 10	Step to 100	Approx. 7
6	Hold @ 120	45	Hold @ 100	25
7	Step to 124	Approx. 7	Step to 106	Approx. 7
8	Hold @ 124	20	Hold @ 106	40
9	Step to 130	Approx. 7	Step to 116	Approx. 7
10	Hold @ 130	20	Hold @ 116	20
11	Step to 140	Approx. 7	Step to 120	Approx. 7
12	Hold @ 140	10	Hold @ 120	60
13	Step to 150	Approx. 7	Step to 126	Approx. 7
14	Hold @ 150	10	Hold @ 126	10
15	Step to 160	Approx. 7	Step to 145	Approx. 7
16	Hold @ 160	10	Hold @ 145	30
17	Step to 225	Approx. 15	Step to 160	Approx. 10
18	Hold @ 225	5	Hold @ 160	30
19	Step to 230	Approx. 7	END	5 – 10
20	Hold @ 230	5	END	
21	END	5 - 10	END	

13. When the program has finished, remove the baffle and the rack to let the tubes stand by themselves in the block. Allow the tubes to cool down for 5-10 minutes and then remove the tubes from the block.
14. Cool the digests in a plastic racks for 10-15 minutes under the fume hood.
15. Add 20 ml of nitric acid 10%.
16. Cover tubes with saran wrap and place them overnight in constant temperature room (20 – 22 ºC)
17. The next day make volume to correct calibration point with fine tipped squeeze bottle.
18. Mix diluted samples thoroughly on the vortex mixer (ensure liquid vortex comes down to the bottom of the tube).
19. Transfer the clear solutions to 50 ml tubes.
20. Prepare tubes in racks:
    i. Mark tubes with I.D.
    ii. Transfer clear sample solutions to tubes, then transfer into 10 ml tubes, close them and keep them at room temperature before analysis by ICP-OES.
21. Deliver capped digest solutions to the ICP-OES ready for analysis.
22. Solutions are analyzed and reported as indicated below.

Calculation:
All raw results (ppm in solution) are generated by an ICP-OES.

The raw results are transferred to Excel where they are processed. The general formula used is:

$$\text{Final Conc. in Sample (mg/kg)} = \frac{(\textit{SampleConc.(ppm)} - \textit{BlankConc.(ppm)})}{1} \times \frac{\textit{Total dilution}}{\textit{Sample mass}}$$

Troubleshooting table

Problem	Solution
Color of the digest is yellow	The digestion is not complete and needs to continue until a clear colorless solution has been obtained.
After digestion the tube is dry or the solution very low in volume	Allow the tube to cool down and add up 1 ml of concentrated perchloric acid.
Presence of perchlorate crystals in the dilution after the digestion	Place tubes on the block digest which is < ~140 ºC for 2-5 minutes to dissolve the perchlorate crystals. The perchlorate crystals will dissolve when the solution is warm. Remove the tubes before the solutions boil as the temperature can reach above point.
Problems with the ICP	Check the equipment manual.

Anthocyanins Determination in Pigmented Maize Grain

Reagent / mixture	Specific reagents	Preparation	Special recommendations
80% Methanol	Methanol absolute	Mix 80 ml of methanol and 20 ml deionized water.	Prepare it weekly and store it at 4 ºC in a closed bottle.
1% TFA (Trifluoroacetic acid) in 80% methanol	Spectro grade	Mix 1 ml of TFA with 90 ml of 80% methanol. Complete final volume (100 ml) with 80% methanol.	Prepare it weekly and store it at 4 ºC in a closed bottle.
Pelargonidin chloride stock solution (100 µg/ml)	Analytical	Dissolve 2.5 mg of pelargonidin chloride in 25 mL of 1% TFA, and adjust pH to 1.4, with HCl.	Protect it from light, covering the flask with aluminum foil and store in a closed bottle. Store up to 1 month at 4 ºC.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material
2. Be sure all seed samples have similar moisture content.
3. Grind each sample to a very fine powder.

Drying

4. Dry the powder at 64-65 ºC for 16 hours.

Extraction

5. For each sample, weigh 20 mg of powder in an eppendorf tube.
6. Add 1.3 ml of 1% TFA.
7. Close the tubes properly.
8. Thoroughly vortex the samples and place them horizontally under ice (4 ºC).
9. Shake the samples for 90 minutes at 150 rpm.
10. After ice incubation, centrifuge the tubes at 14,000 rpm for 5 minutes. Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.
11. Read absorbances at 520 nm (first extraction).
12. Re-extract the pellet with 1.3 ml of 1% TFA.
13. Repeat steps 7 to 10. The incubation on ice is done for 1 hour only.
14. Read absorbances at 520 nm (second extraction).

Reading samples in the spectrophotometer

15. For each extraction, take 200 µL of supernatant and carefully transfer it to the microplate. Do a technical replicate.
16. Always include blank samples which correspond to 1% TFA and control samples with known values.
17. Cover the microplate with aluminum tape.
18. Vortex the microplate at 800 rpm for 5 seconds.
19. Read absorbance at 520 nm in a spectrophotometer.
20. For estimation of total anthocyanins content it is necessary to add the quantity of both extractions.

Standard curve

1. Prepare a stock solution of 100 μg/ml pelargonidin chloride in 1% TFA.
2. Prepare daily 0, 1, 3, 5, 10, and 15 μg/ml dilutions (in 1% TFA) in 5 ml glass tubes
3. Vortex properly before use.

Tube number	Volume of stock pelargonidin chloride 100 μg / ml (μL)	1% TFA	Total volume (ml)	Concentration μg pelargonidin chloride / ml
1	0.0	2,500	2.5	0.0
2	25	2,475	2.5	1.0
3	75	2,425	2.5	3.0
4	125	2,375	2.5	5.0
5	250	2,250	2.5	10.0
6	375	2,125	2.5	15.0

Calculations:

Calibration curve

Develop a calibration curve using known amounts of pelargonidin, ranging from 0 to 15 μg. Plot the absorbance readings at 520 nm as a function of the amount in μg and calculate the slope of that standard curve. Note that the slope has the unit of OD/μg.

Percentage of pelargonidin

The amount of pelargonidin for each sample is then estimated using the following equation:

$$\%\ \text{Pel} = \frac{\text{OD520nm}}{\text{slope}} \times \frac{\text{hydrolysis volume}}{\text{sample weight}} \times 100\%$$

Example:

$$\%\ \text{Pel}\ (\mu g / \mu g) = \frac{0.225}{0.032\ {}^{D}_{\mu g/ml}} \times \frac{1.3ml}{20{,}000\mu g} \times 100\%$$

However, this amount includes the absorbance of the plate and methanol. To calculate the pelargonidin content of the biological material (grain powder) you need to subtract the absorbance value of the plate and methanol.

$$\%\ \textbf{Pel} = \textbf{OD}_{\textbf{520nm corrected}} \times \textbf{Factor}$$

Where:

$$\text{OD}_{\text{520nm corrected}} = \text{OD}_{\text{520nm sample}} - \text{OD}_{\text{520nm average of methanol blanks.}}$$

$$Factor = \frac{0.0065}{\text{slope}}$$

Note that: $\frac{1.3ml}{20{,}000} \times 100 = 0.0065$

Total Starch Determination in Maize Grains Using a Modified Assay from Megazyme

Megazyme starch determination use enzymatic digestion to extract the polymer. In the essay format described, starch hydrolysis proceeds in two phases. In phase I, starch is partially hydrolyzed and totally solubilised. In phase II, the starch dextrins are quantitatively hydrolyzed to glucose by amyloglucosidase. Glucose is then quantified colorimetrically using anthrone reagent.

Reagents

Reagent / mixture	Specific reagents	Preparation	Special recommendations
MOPS BUFFER (50mM, pH 7.0)		• Add 11.55 g of MOPS (Sigma cat. No M1254) to 900 ml of deionized water. Adjust the pH to pH 7.0 by the addition of 1 M (4 g/100 ml) sodium hydroxide solution. Approximately 28 ml is required. • Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 liter and store at 4 °C.	
Sodium acetate buffer (200 mM, pH 4.5)		• Add 11.8 ml of glacial acetic acid (1.05 g/ml) to 900 ml of deionized water. Adjust the pH to 4.5 by the addition of 2 M (8 g/100 ml) sodium hydroxide solution. Approximately 50 ml is required. Adjust the volume to 1 liter. Store at 4 °C.	
α-Amylase (30 ml, 100U/ml)		• Dissolve 61 mg of α-Amylase (Sigma cat. No 10070, from Bacillus subtilis, 51.5 Units/mg) in 30 ml of MOPS buffer (50 mM, pH 7.0)	Prepare it daily and store it at 4 ºC. Vortexes thoroughly before you use it.
Amyloglucosidase (2ml, 200 U/ml)		• Dissolve 5 mg of Amyloglucosidase (sigma 10115, from Aspergillus niger) in 2 ml Sodium acetate buffer (200mM, pH 4.5)	Prepare it daily and store it a 4 ºC. Vortexes thoroughly before you use it.
Sulfuric acid	Sulfuric acid (analytical)		
Anthrone reagent		• One hour before use, weigh 100 mg of anthrone reagent and dissolve in 50 ml of concentrated sulfuric acid.	Prepare it daily. Protect it from light, keep it on ice.
Glucose stock solution (0.5 mg/ml)		• Dry glucose for 1 hour at 105 ºC. Dissolve 50 mg of glucose in 100 ml of deionized water	Prepare it weekly and store it a 4 ºC. Vortexes thoroughly before you prepare dilutions for standard curve.

Recommendations for anthrone reagent:

a) The sulfuric acid has to be an analytical reagent and stored in a dark room.
b) The anthrone reagent must be transfered in a falcon tube which has been covered by aluminum paper and kept in the fridge at 4 °C.
c) Safety considerations for the anthrone preparation include protective clothing, gloves, and a ventilation hood.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Be sure all seed samples have similar moisture content.
3. If the seeds have been treated, wash thoroughly with tap water and then rinse with distilled water. Let the seeds dry out.
4. Grind each sample to a very fine powder. If possible use a 0.5 mm setting of a cyclone mill.

Defatting

5. Transfer each sample in a commercial filter paper envelope (for example, 10x11 cm).
6. Defat samples for 6 hours with approximately 300 ml of hexane per balloon in a Soxhlet-type continuous extractor.
7. Air dry samples and ensure all hexane is evaporated.

Extraction

8. For each sample, weigh 20 mg of flour sample in a glass tube (20*150 mm). Tap the tube to ensure all of the sample drops to the bottom of the tube.
9. Always include two tubes with the standard of starch.
10. Wet with 40 μL of aqueous ethanol (80% v/v) to aid dispersion, wait 5 minutes and stir the tube on a vortex mixer.
11. Immediately add 600 μL of α-Amylase in MOPS buffer (50 mM, pH 7.0) and vigorously stir the tube on a vortex mixer. Incubate the tube in a boiling water bath for 6 minutes (stir the tube vigorously after 2 minutes and 4 minutes).

    Note: In this step it is essential that the tube is stirred vigorously to ensure complete homogeneity of the slurry (removal of lumps). Also stirring after 2 minutes prevents the possibility of some of the sample expelling from the top of the tube when the alcohol is evaporating.

12. Place the tube in a bath at 50 °C (wait 5 minutes to cool down the tube at 50°C); add sodium acetate buffer (800 μL, 200 mM, pH 4.5), followed by amyloglucosidase (20 μL, with a digital pipette.). Stir the tube on a vortex mixer and incubate it at 50 °C for 30 minutes.
13. Transfer the entire contents of the test tube to a 50 ml corning plastic tube. Pipette 540 μl of deionized water and use it to rinse the glass tube content. Mix the tube and transfer it to the 50 ml corning plastic tube. Using a bottle dispenser, rinse three times with 6 ml of deionized water the glass tube, mix thoroughly each time and transfer it to the 50 ml corning plastic tube.
14. Centrifuge the 50 ml corning plastic tube at 3,000 rpm for 10 minutes. Ensure that the supernatant does not have sample particles floating in it; if it does, centrifuge again.
15. Take 1 ml of hydrolyzate (supernatant) and carefully transfer it to a glass tube (20*150 mm).
16. Add 9 ml of deionized water, immediately covering and vortex thoroughly.

Colorimetric reaction

17. With a digital pipette take 50 μL of dilution and carefully transfer it in a 96 wells plate, maintain in an ice bath.
18. Each sample has to be analyzed in triplicate.
19. Add 100 μL of the anthrone solution using a digital multi-channel pipette. (Ensure the 100 μl has been well distributed as the solution is very viscous).
20. The plate has to be covered with an aluminum paper and vortexed very carefully until you see a homogenous solution in each well.
21. Plates have to be incubating at 100 °C for 10 minutes.
22. Plates have to be cooled in the fridge for 10 minutes and then have to be vortexed before reading them at 630 nm in a spectrophotometer.

Standard curve

1. Prepare a stock solution of 0.5 mg/ml dried glucose in deionized water (prepare it weekly and store it a 4 ºC).
2. In 50 ml glass tubes, prepare daily 0, 12.5, 25, 37.5, 75, 100 μg/mL dilutions (in deionized water). Vortex properly before further use.
3. Make a colorimetric reaction (steps 17 to 22) using 50 μl of those dilutions.
4. Always include one standard curve in duplicate for every set of samples analyzed in a day.

Tube number	Stock glucose 0.5 mg / ml (ml)	Deionized water (ml)	Sodium acetate buffer (ml)	Total volume (ml)	Concentration mg glucose / ml
1	0	19.5	0.5	20.0	0.0
2	0.5	19	0.5	20.0	0.0125
3	1	18.5	0.5	20.0	0.025
4	1.5	18	0.5	20.0	0.0375
5	3	16.5	0.5	20.0	0.075
6	4	15.5	0.5	20.0	0.100

Calculations:

Standard curve for glucose (calibration curve)

Develop a calibration curve using known amounts of glucose, ranging from 0 to 0.1 mg/ml. Plot the absorbance readings at 630 nm as a function of concentration and calculate the slope of that standard curve. Note that the slope has the unit of OD*ml/mg.

Calculation of percentage of glucose

The amount of glucose for each sample is then estimated using the following equation:

1. Determine regression equation relating glucose concentrations in standard solutions to absorbance reading on the spectrophotometer. The regression formula appears as:

   $$\mathbf{Yg = b(x)}$$

   Where **Yg** is the absorbance units at 630 nm, **b** is the slope, and **x** is the glucose concentrations.

2. Calculate glucose concentrations in the samples by removing blank value to sample absorbance reading and dividing the absorbance reading by the slope. The general equation for calculating the milligrams of starch in the sample is:

   $$\mathbf{Starch\ (mg/100mg)\ of\ flours = x d_f v h_f\ ^*100\ /d_w}$$

Where **x** is the glucose concentrations (mg/ml), $\mathbf{d_f}$ is the dilution factor (e.g., 10 for 1:9 dilution), **v** is the original volume of starch extract (20 ml), $\mathbf{d_w}$ is the original weight of flours (20 mg), and $\mathbf{h_f}$ is the starch hydrolysis factor 0.9.

To express the results as a percent of dry weight, moisture content has to be evaluated and the following formula is used:

$$\mathbf{=Starch\ \%\ (as\ is)\ x\ (100)/\ (100-\ moisture\ content\ (\%\ w/w)}$$

Amylose Determination in Maize Grains

Starch, the major storage component in mature maize kernels, is composed of two macromolecules with different structures: amylose, essentially a linear polymer of glucoses residues, linked by α(1→4)-glucosidic bond plus occasional α(1→6)- glucosidic linkages, and amylopectin, a highly branched polymer.

In the presence of tri-iodide, a complex is formed giving a blue color with λ_{max} at 620 nm. The iodine reaction has been proposed to quantify amylose content in maize starches using measurement made at one wavelength (620nm).

Reagent / mixture	Specific reagents	Preparation	Special recommendations
Ethanol, 95% (V/V).			
Sodium hydroxide 1 M		Dissolve 4 g sodium hydroxide 100 ml deionized water.	
Sodium hydroxide 0.09 M		Dilute 9 ml NaOH 1M in 100 ml deionized water	
Acetic acid 1 M	Acetic acid glacial	5.72 ml / 100 ml deionized water.	
Lugol's solution		Weigh 2.000g of KI and transfer it in a baker. Add a little bit of ddH20 in a way to obtain a saturate solution, and then add 0.2 g of I2. Assume all the iodine is dissolve before transfer of the entire baker content in a volumetric flask of 100 ml, complete with ddH_20 and homogenize the solution.	Prepare it daily. Protect it from light.
Amylose standard (1 mg/ml)	Amylose from potatoes type III, Sigma cat. No A0512	Weigh 100 mg of amylose in a 100 ml volumetric flask. Add 1 ml of ethanol 95% and try to rinse the wall of the flask (make sure that there are no particles of samples stuck to the wall of the flask). Add 9 ml of sodium hydroxide 1 M and let it rest at room temperature for 20 to 24 hours. The next day adjust volume to 100 ml with deionized water and vigorously shake the flask.	Prepare it weekly and store it at 4 °C.

Procedure:

Sampling and grinding

1. Take a random sample of 20-30 seeds as a representative of your material.
2. Be sure all seed samples have similar moisture content.
3. If the seeds have been treated, wash thoroughly with tap water and then rinse with distilled water. Let the seeds dry out.
4. Grind each sample to a very fine powder. If possible use a 0.5 mm setting of a cyclone mill.

Defatting

5. Transfer each sample in a commercial filter paper envelope (for example, 10x11 cm).
6. Defat samples for 6 hours with approximately 300 ml of hexane per balloon in a Soxhlet-type continuous extractor.
7. Air dry samples and ensure all hexane is evaporated.

Extraction

8. For each sample weigh 20 mg of defatted powder in a 50 ml corning tube.
9. Include always two tubes with the standard of amylose.
10. Add 0.2 ml of ethanol 95% and try to rinse the wall of the tube (make sure that there are no particles of samples stuck to the wall of the tube).
11. Add 1.8 ml of sodium hydroxide 1 M and let it sit at room temperature for 20 to 24 hours (don't shake the tube).
12. The next day adjust volume to 20 ml with deionized water (18 ml) and vigorously shake the tube.

Colorimetric reaction

13. Take 1 ml of solution and carefully transfer it to a 50 ml corning tube.
14. Add 0.2 ml of acetic acid 1 M and shake vigorously.
15. Then add 0.4 ml of Lugol's solution and adjust volume to 20 ml (18.4 ml deionized water). The mixture is mixed and the color allowed to develop for 20 minutes (protect the tubes from light).
16. Pipette 200 μL of standard curve solution and of each sample and transfer it to a 96 well-plate. Read them at 620 nm in a spectrophotometer.

Standard curve

1. Prepare a stock solution of 1 mg/ml amylose in deionized water (prepare it weekly and store it at 4 °C).
2. In 50 ml corning tubes, prepare daily 0, 0.2, 0.4, 0.6, 0.8, and 1 g of amylose in a final volume of 20 ml. Vortex properly before further use.
3. Make a colorimetric reaction (steps 13 to 16) using 200 μL of those dilutions.
4. Always include one standard curve in duplicate for every set of samples analyzed in a day.

Tube number	Stock amylose 1 mg / ml (ml)	Acetic acid (1 Mol/ml) (ml)	Iodine solution (ml)	NaOH (0.09 M) (ml)	Deionized water
1	0	0.2	0.4	1	18.4
2	0.2	0.04	0.08	0	19.68
3	0.4	0.08	0.16	0	19.36
4	0.6	0.12	0.24	0	19.04
5	0.8	0.16	0.32	0	18.72
6	1	0.2	0.4	0	18.4

Calculations:

Standard curve for amylose (calibration curve)

Develop a calibration curve using known amounts of amylose, ranging from 0 to 1 mg. Plot the absorbance readings at 620 nm as a function of amount and calculate the slope of that standard curve.

Calculation of percentage of amylose

The amount of amylose for each sample is then estimated using the following equations:

1. Determine regression equation relating amylose amount in standard solutions to absorbance reading on the spectrophotometer. The regression formula appears as:

$$\mathbf{Yg= a(x)+b}$$

Where **Yg** is the absorbance units at 620 nm, **a** is the slope, **x** is the amylose amount, and **b** is the intercept (this correspond to the AP "Absorption Approximation").

2. Calculate amylose amount in the sample by removing first blank value to sample absorbance reading, secondly by removing the intercept, and then by dividing the absorbance corrected by the slope. The general equation for calculating the amylose percent in starch sample:

$$\% \ \mathbf{AM = (x^{*}d)^{*}(100/f)}$$

Where **x** is the amylose amount (mg), **d** is the dilution factor (e.g., 20 for 1:19 dilution), and **f** is the original weight of flours (20 mg).

Annex 1

Expected Range of Readings for Standard Concentrations

1. Tryptophan determination with glyoxilic acid (readings in Spectronic 20)

Standard concentration (μg/ml)	Range of readings
0	0
10	0.07-0.090
15	0.130-0.150
20	0.175-0.205
25	0.230-0.260
30	0.295-0.325

Using this, the value for the factor curve should be in the range 0.3454-0.3804.

2. Tryptophan determination with glyoxilic acid (readings in microplate)

Standard concentration (μg/ml)	Range of readings
0	0.04-0.05
10	0.075-0.085
15	0.095-0.120
20	0.125-0.145
25	0.155-0.175
30	0.195-0.215

Using this, the value for the factor curve should be in the range 0.6818-0.7332.

3. Free and total phenolics content in maize using F-C reagent (extraction in eppendorf tube and readings in microplate)

Standard concentration (μg gallic/ml)	Range of readings
0	0.045-0.055
10	0.195-0.215
15	0.265-0.285
20	0.345-0.370
25	0.400-0.420
30	0.495-0.515

4. Soluble sugars determination in **maize** using anthrone reagent (extraction in big tube and readings in a Spectronic 20)

Standard concentration (μg sucrose / ml)	Range of readings
0	0
10	0.060-0.080
15	0.150-0.200
20	0.300-0.350
25	0.400-0.450
30	0.600-0.650

5. Soluble sugars determination in **maize** using anthrone reagent (extraction in big tube and readings in microplate)

Standard concentration (µg sucrose / ml)	Standard concentration (µg sucrose in 2.5 ml)	Range of readings
0	0	0.05-0.07
6	0.3	0.090-0.110
12	0.6	0.125-0.140
18	0.9	0.160-0.180
24	1.2	0.195-0.215
30	1.5	0.240-0.270
40	2.0	0.280-0.310
60	3.0	0.360-0.395

6. Soluble sugars determination in **wheat** using anthrone reagent (extraction in big tube and readings in microplate)

Standard concentration (µg sucrose / ml)	Standard concentration (µg sucrose in 2.5 ml)	Range of readings
0	0	0.05-0.07
6	0.3	0.090-0.115
12	0.6	0.145-0.165
18	0.9	0.190-0.210
24	1.2	0.250-0.270
30	1.5	0.290-0.310
40	2.0	0.360-0.390
60	3.0	0.510-0.540

7. Anthocyanins with 1% TFA at pH 1.4 (extraction in eppendorf tube and readings in microplate)

Standard concentration (µg Pel/ml)	Range of readings
0	0.040-0.045
1	0.085-0.095
3	0.165-0.175
5	0.255-0.275
10	0.485-0.525
15	0.710-0.780

8. Starch determination (readings in microplate)

Standard concentration (mg glucose /ml)	Range of readings OD 630 nm
0.0	0.05
0.0125	0.136
0.025	0.218
0.0375	0.300
0.075	0.540
0.100	0.700

9. Amylose determination (readings in microplate)

Amount of standard (mg of amylose)	Range of readings OD 620 nm
0	0.04
0.2	0.166
0.4	0.316
0.6	0.449
0.8	0.569
1	0.738

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ISBN: 978-970-648-169-6

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