Laboratory Standard Operating Procedure

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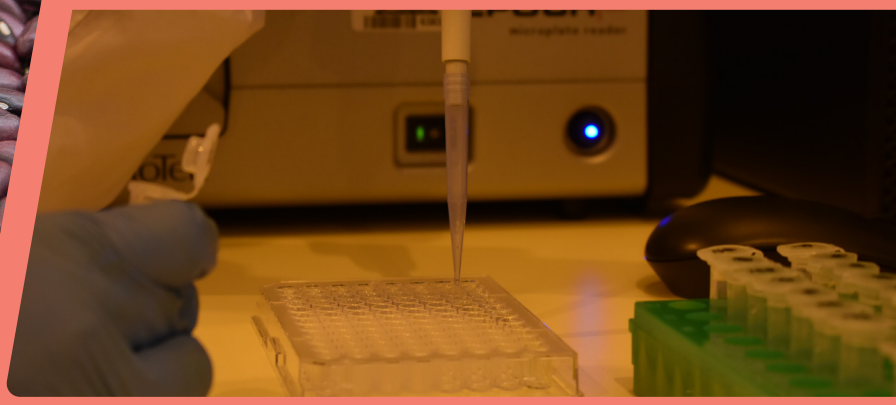

SOP for quantification of total phytates in beans, rice, and maize flour

Nutrition Quality Laboratory (NQL)

This report has been written in the framework of CGIAR Initiative on Market Intelligence, Work Package 2: Target product profile design.

The CGIAR Initiative on Market Intelligence aims to maximize CGIAR and partners' returns on investment in breeding, seed systems, and other Initiatives by creating a collaboration hub to develop institutional standards for inclusive and impact-driven market segmentation and product profiling and developing a global platform for sharing market intelligence and investment prioritization.

October 2024

Acknowledgements: This work was produced as part of the CGIAR initiative on Market Intelligence, which is supported by contributors to the CGIAR Trust Fund: cgiar.org/funders

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SOP for quantification of total phytates in beans, rice, and maize flour

Nutrition Quality Laboratory (NQL)

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To be cited as:

Orozco JC; Taleon V; Patiño G; Gallego S. (2024). SOP for quantification of total phytates in beans, rice, and maize flour. Nutrition Quality Laboratory Standard Operating Procedure. International Center for Tropical Agriculture (CIAT), Cali, Colombia. 19 p.

Contents

1 Scope and application

This SOP describes the stages involved in the treatment process of the grain samples to be analyzed (beans, maize, and rice) to subsequently quantify the total phytate content by spectrophotometry.

The step of selection and washing of bean and maize grains is carried out with the purpose of remove crop remains that have been left on the surface of these (clumps, remains of plant material, atypical grains). The step of drying process is carried out with the objective of eliminating a part of the moisture of the grain so that these are more permissible to obtain a homogeneous flour in the transformation process to flour. In addition, this drying process also helps avoid proliferation of insects and microorganisms in the grain (Arcos & Rojas, 2018). For rice grains, the steps of washing and sorting, in addition to drying, are unnecessary, due to the grain polishing process.

The protocol for the quantification of total phytates is also described in Taleon et al. (2020): Retention of Zn, Fe and phytic acid in parboiled biofortified and non-biofortified rice. *Food Chemistry: X* 8:100105. https://bit.ly/3XAqyea

2 Principles and definitions

Phytic acid is a natural component of plants, and its structure is of great interest in nutritional studies due to its ability to complex certain minerals of importance in the human diet. This method is a modification of the Latta and Eskin (1980) colorimetric method, which uses an anion exchange column to remove inorganic phosphorus and most inositol mono-, bis-, tri-, and tetrakisphosphates (IP1, IP2, IP3 and IP4) before the measurement of the change in color at 500 nm caused by the decolorization of a purple/pink complex of Fe+3/5-sulfosalicylic acid (Wade's reagent) due to the presence of phytic acid.

3 Apparatus

Chromatography columns: Poly-Prep prefilled columns, AG 1-X8 resin, 200–400 mesh, formate form, 0.8 x 4 cm

Pipettes: 1 mL, 5 mL, 10 mL

Centrifuge tubes: 2 mL, 15 mL, and 50 mL

Flasks: 25 mL, 250 mL, 500 mL

Beakers: 100 mL, 250 mL

Vortex: Mixer/Homogenizer, multivortex

Balance: Analytical, readability 0.1 mg

Centrifuge: >3,000 rpm (revolutions per minute)

Rack: For coupling ion exchange cartridges

Spectrophotometer: UV-ViS spectrophotometer

Fume hood or extraction cabin: At least 100 fpm

4 Reagents

Phytic acid: Standard or analytical grade

Ferric Chloride Hexahydrate ($FeCl_3 \cdot 6H_2O$): Analytical grade

5-Sulfosalicylic Acid ($C_7H_6O_6S \cdot 2H_2O$): Analytical grade

Hydrochloric Acid (HCl): Analytical grade

Sodium Chloride (NaCl): Analytical grade

Water: Type I (0.056 µS/cm to 25 °C max conductibility)

5 Preparation of diluted solutions and standard solution

Wade's reagent

A solution with ferric chloride (0.030 % $FeCl_3 \cdot 6H_2O$) and 5-sulfasalicylic acid dihydrate (0.30 %). Weigh 7.5 mg of ferric chloride ($FeCl_3 \cdot 6H_2O$) and 75 mg of 5-sulfosalicylic acid dihydrate. Add to a 25 mL amber flask and bring to volume with water type I.

Hydrochloric acid, 0.65 M

To prepare 500 mL of 0.65 M solution from 37% HCl. Take 26.91 mL of 37 % HCl (density=1.19 g/mL) and bring to volume in a 500 mL flask with water type I. From this solution, HCl solutions of lower concentration are prepared. Calculation:

$$500mL\ solution \times \frac{0.65\ mol}{1000\ mL} \times \frac{36.5\ g\ HCl}{1\ mol} \times \frac{100\ g}{37\ g\ HCl} \times \frac{1\ mL}{1.19\ g} = 26.9\ mL$$

Hydrochloric acid, 0.2 M

To prepare 250 mL of 0.2 M solution from 0.65 M HCl. Take 76.92 mL of 0.65 M HCl and bring to volume in a 250 mL volumetric flask with water type I. Calculation:

$$\frac{0.2\ M\ \times 250\ mL}{0.65\ M} = 76.9\ mL$$

Sodium Chloride, 1 M

To prepare 500 mL of 1 M NaCl solution. Add 29.4 g of NaCl to a 500 mL volumetric flask and bring to volume with water type I. Calculation:

$$500mL\ solution \times \frac{1\ mol}{1000\ mL} \times \frac{58.5\ g\ NaCl}{1\ mol} \times \frac{100\ g}{99.5\ g\ NaCl} = 29.4\ \text{g}$$

Sodium Chloride, 0.7 M

To prepare 500 mL of 0.7 M NaCl solution. Add 20.6 g of NaCl to a 500 mL volumetric flask and bring it to volume with water type I. From this solution, NaCl solutions of lower concentration are prepared. Calculation:

$$500mL\ solution \times \frac{0.7\ mol}{1000\ mL} \times \frac{58.5\ g\ NaCl}{1\ mol\ NaCl} \times \frac{100\ g}{99.5\ g\ NaCl} = 20.6\ g$$

Sodium Chloride, 0.07M

To prepare 500 mL of 0.07 M NaCl solution from 0.7 M sodium chloride solution, 50 mL of 0.7 M NaCl are taken and brought to volume in a 500 mL volumetric flask with water type I. Calculation:

$$\frac{0.07\ M\ \times 500\ mL}{0.7\ M} = 50\ mL$$

Phytic acid standard solution (200 mg/L)

Option 1: from the phytic acid dipotassium salt ($C_6H_{18}O_{24}P_6 \cdot 2K$, MW=736.22): Weigh 6.03 mg of phytic acid dipotassium salt, transfer to a 25 mL flask and dilute with 0.7 M NaCl to the gage line. Calculation:

{25 mL × (200 mg phytic acid) / 1000 mL} × (100 g phytic acid dipotassium salt, commercial product) / (82.91 g phytic acid) = 6.03 mg phytic acid dipotassium salt.

Note: Calculations were based on a purity of 92.5 % and phytic acid MW=660.

6 Phytic acid analytical curve

PASS1. Prepare the standard dilutions in 2 mL tubes using PASS1 and NaCl 0.7 M. Prepare at least 6 dilutions as shown in Table 1. The recommended dilutions along with the quantities of sample and aliquots of extract to be taken for analysis (recommended in the SOP) will be enough to cover the range 1–30 mg/g.

Table 1. Preparation of the phytic acid calibration curve from the 200 mg/L stock (PASS1)

Dilution ID	Target phytic acid concentration (mg/L)	PASS1 (mL)	NaCl 0,7 M (mL)	Final volume (mL)
PA-00	0	0.0	2.00	2.0
PA-05	5	0.05	1.95	2.0
PA-10	10	0.10	1.90	2.0
PA-20	20	0.20	1.80	2.0
PA-40	40	0.40	1.60	2.0
PA-60	60	0.60	1.40	2.0

7 Procedure

7.1. Sample preparation

7.1.1. Selection and clean

Select at least 12 g of the sample in good condition and free of particles (stones, plant remains, insects, etc.) (Figure 1).

Figure 1. Unselected grain, selected grain, and waste material (stones, plant remains, insects, etc.)

Put the selected grains (at least 12 g) in a plastic sieve and clean with cotton gauze or wash with type I water using a bash bottle (Figure 2). Washing can be omitted if grain looks very clean and free of dust, or if the washing step of a process is being evaluated. White (polished) rice samples should not be washed.

Figure 2. Grain washing

Regardless of whether the grain was rinsed with water or not, gently clear (or remove water from) the surface of sample using a piece of cotton gauze (non-sterile), taking care not to damage the grain's seed coat (Figure 3).

Figure 3. Manual drying of bean grains

Transfer the sample to a small paper bag (2½ x 4¼ inch, or similar) and dry in an oven at 60 °C for 3 days if the test material is beans/wheat/rice/millet, or 40 °C for 4 days if the sample is maize (Figure 4). If sample was not rinsed with water, dry only if sample moisture is higher than 12 % for cereals and >9 % for legumes until the samples reaches a moisture lower than these levels, this will ensure a proper grinding.

Figure 4. Material drying in oven

7.1.2. Milling

Weigh 5–6 g of grain (bean, or cereal grain) and transfer them to the grinding capsule and close with the lid equipped with a stainless-steel blade (for an IKA Tube Mill or handheld analytical mill) (Figure 5). The mill is set at a speed of 20,000 rpm for 1 minute. The sample must be finely ground (<0.5 mm particle size) before proceeding with analysis.

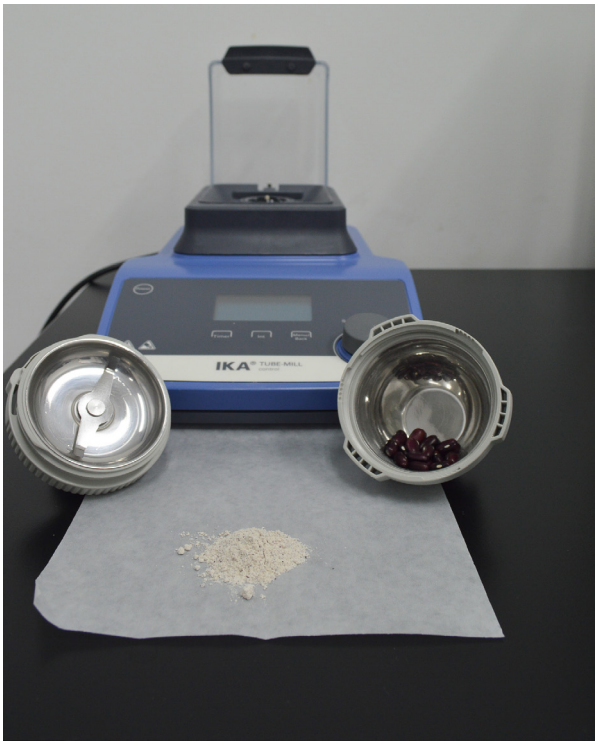

Figure 5. Milling grain using a IKA Tube Mill

The flour obtained is collected and transferred to a polyethylene flask with its respective lid (Figure 6).

Figure 6. Storage of the ground sample in polyethylene bottles

7.2. Extraction

Add 0.5 g of the ground sample, or 1.0 g if the sample it is expected to have a low content of phytic acid such as refined cereals (<3 mg/g), to a 50 mL tube (Figure 7). Examples: beans or whole grain cereal flours (0.5 g), refined cereal flours (1.0 g), LPA mutants (1.0 g).

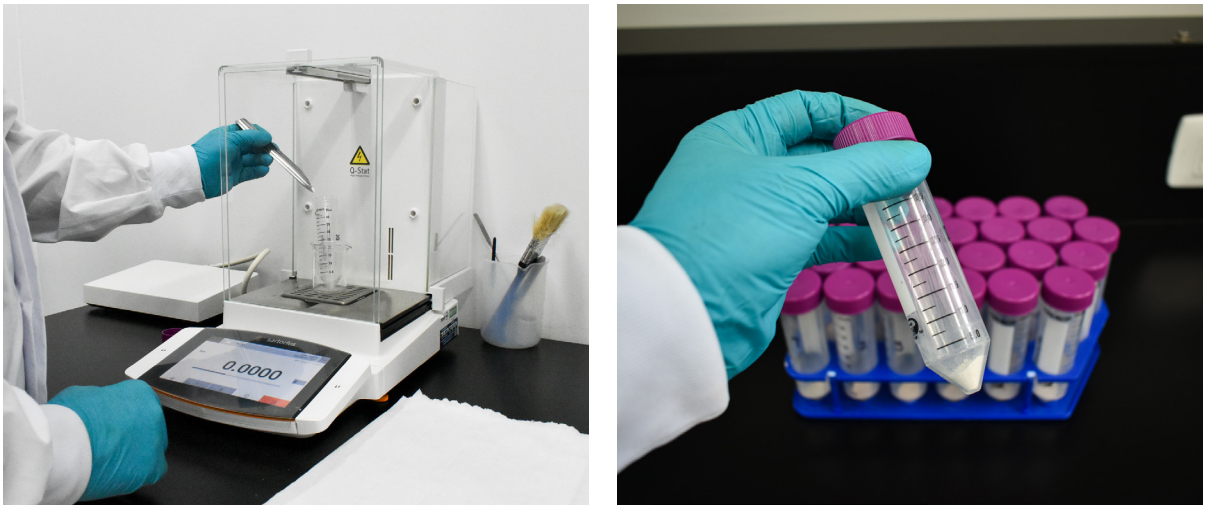

Figure 7. Sample weighing

For samples with fat content >2 % (or ~>10 mg of total fat expected in the sample to be extracted) an additional process must be performed to extract fat. Add 2.0 mL of hexane to the sample. Shake in a multivortex for 2 hours and later, centrifuge at 3,500 rpm for 10 minutes. Discard the extract (that will contain the fat) and take the tube with the sample (without lid) to an oven at 35 °C for a period of 12 hours. Add 20 mL of 0.65 M HCl and vortex for 20 seconds or until a homogeneous sample is obtained. Then put in the multivortex for 2 hours (Figure 8a) and centrifuge at 3,500 rpm for 15 minutes at room temperature (Figure 8b).

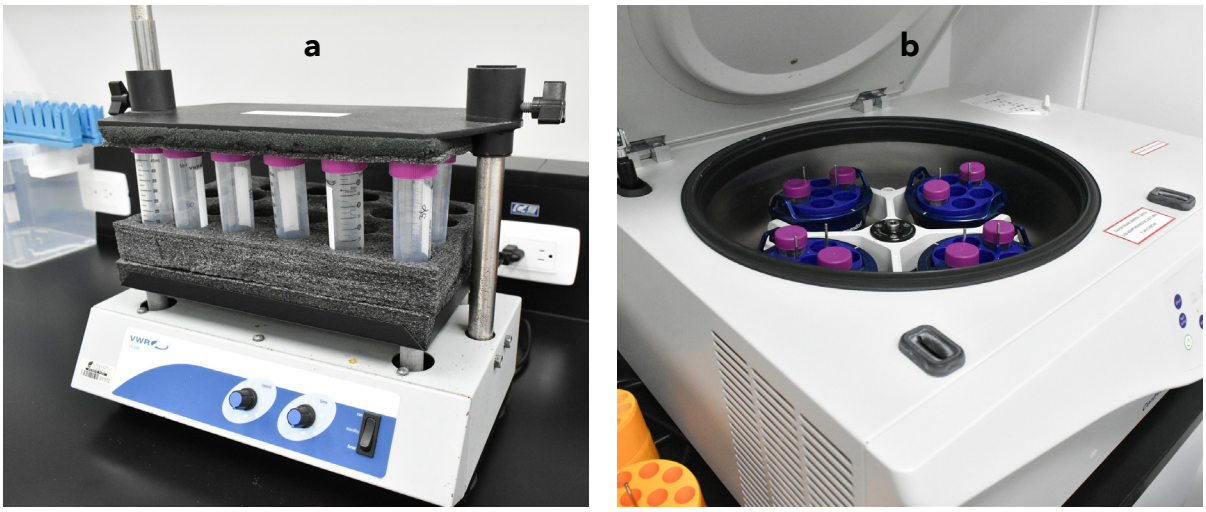

Figure 8. Vortex mixer with samples (a). Centrifuging samples (b)

Transfer the supernatant liquid (minimum 12 mL) to 15 mL tubes. Depending on the nature of the supernatant, it is possible to transfer it manually or with the help of a pipette (Figure 9).

Figure 9. Transferring the supernatant

Store the filtered extracts at -20°C if the purification process is the next day.

7.3. Separation and elution of total phytates

The separation and elution procedure is done by a gravimetric (without vacuum pump) procedure using a Poly-Prep Columns AG 1X8 BIO-RAD ion (anion, Chloride will be substituted by phosphates) exchange cartridge.

If the cartridges are new, break the snap-off seal and by gravity remove/drain the storage liquid solution (H_2O type I/or another solution used by Bio-Rad) (Figure 10). After draining liquid solution, place a cap at the bottom of the cartridge (Figure 11).

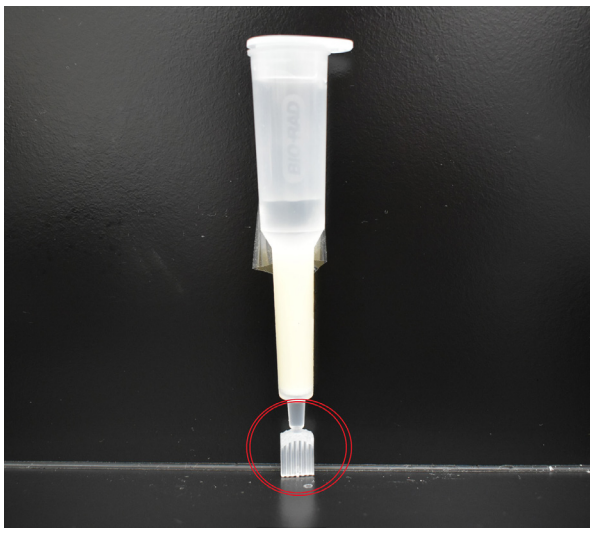

Figure 10. Break the snap (twist and remove the bottom part)

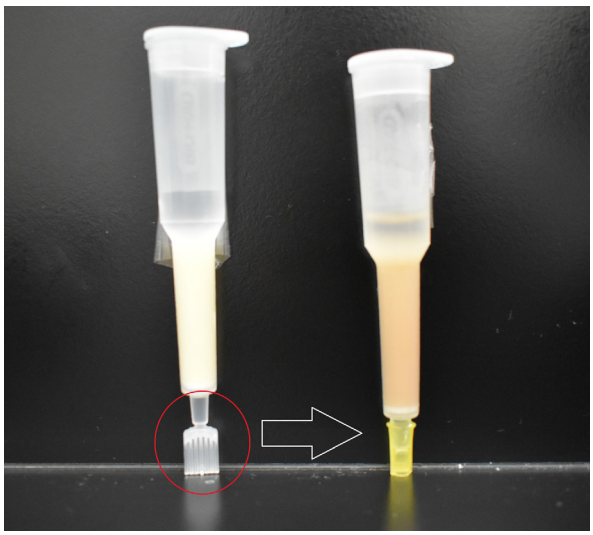

Figure 11. Cartridge with a cap

Insert/attach the Poly-Prep Columns AG 1X8 BIO-RAD ion exchange cartridges to the rack (Figure 12).

Figure 12. Ion exchange cartridges position in a rack (with the bottom closed with a cap)

Add 5 ml of type I water with a syringe with needle to rearrange the resin bed and elute (Figure 13). The rearrangement of the resin must avoid empty spaces (Figure 14). Empty spaces may have formed during transportation.

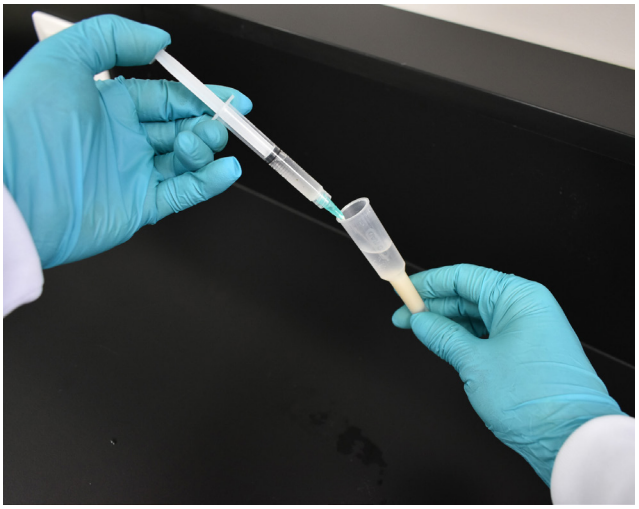

Figure 13. Resin rearrangement process

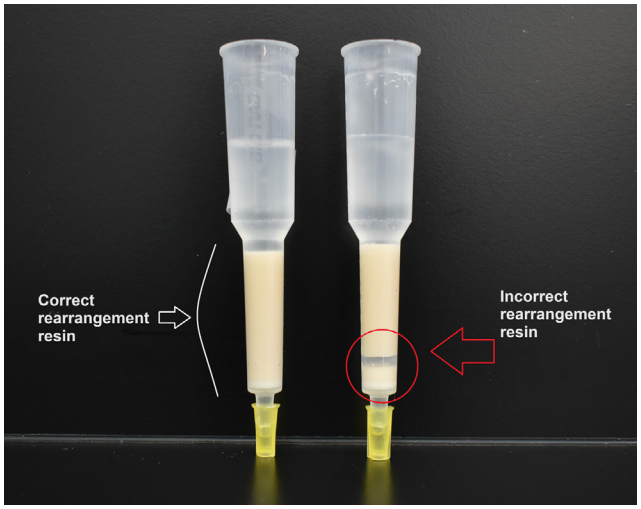

Figure 14. Resin rearrangement process (correct and incorrect)

Condition the cartridges with 5 mL of 0.2 M HCl solution and elute (remove the bottom cap to allow the solution to drain by gravity) (Figure 15).

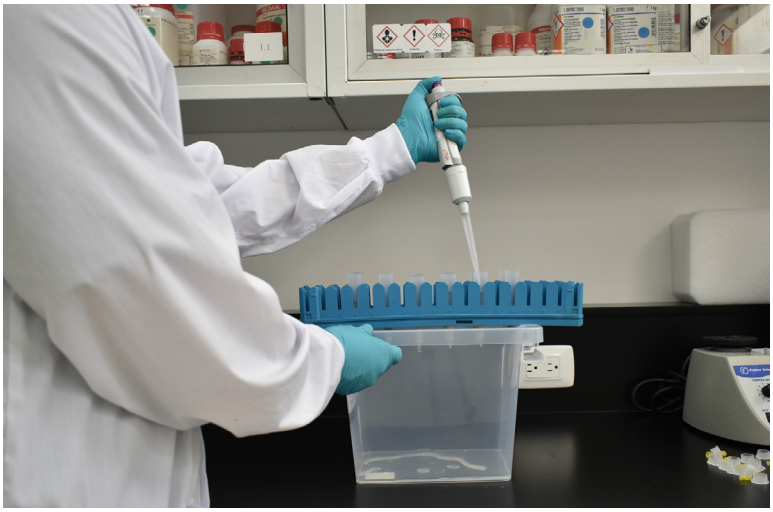

Figure 15. Cartridges conditioning

If cartridges were previously used and stored with water type I:

- Remove the storage liquid solution (open the bottom cap and allow solution to drain and close the cap).
- Add 5 ml of water type I with a syringe with needle to jet to rearrange the resin bed and elute (Figure 13 and Figure 14).
- Add 10 mL of 1 M NaCl solution and elute to remove residues of P (additional step).
- Add 5 mL of water type I and elute to flush the residues of 1M NaCl (additional step).
- Finally, condition the cartridges with 5 mL of 0.2 M HCl solution and elute.

With the bottom of the cartridge closed, load 8 mL of 0.65 M HCl and immediately add 2 mL of sample extract. If it is a sample with low phytic acid content (LPA bean, refined flours), load 2 mL of 0.65 M HCl and immediately add 8 mL of the extract. If it is maize, load 5 mL of type I, 0.65 M HCl and immediately add 5 mL of extract. If it is rice, only 10 mL of the extract will be loaded. Then proceed to elute.

Add 10 mL of 0.07M NaCl and elute (to elute other phosphorous types). Elute the purified phytates with 30 mL of 0.7 M NaCl solution (three 10-mL elutions), which are collected in 50 mL tubes with a whole to insert the tip of the cartridge (Figure 16).

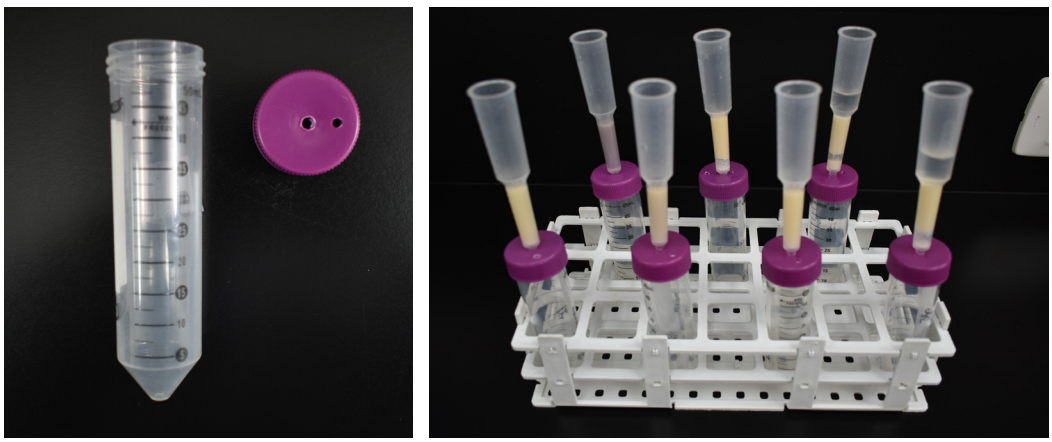

Figure 16. Phytate solution collection after purification

7.4. Quantification of phytates

The quantification of phytates in the samples is carried out as follows:

- Transfer a 0.9 mL aliquot of the sample and curve standards to 2 mL tubes.
- Add 0.3 mL of Wade's reagent to each tube.
- Vortex the tubes for 5 seconds or until a homogeneous mixture is obtained.
- Take 3 aliquots of 300 µL from each of the samples and standards, seed them in a multiwell plate (96 wells) and read the absorbance at 500 nm in a spectrophotometer (Figure 17).

Figure 17. Spectrophotometer reading

8 Calculations

$$\text{Phytic acid}\left(\frac{mg}{g}\right) = \left(\frac{Abs_{sample} - Intercept\ (mg)}{Slope\ (L)}\right) \times \frac{Vol.elution\ (mL)\ 30\ mL\ \ NaCl0{,}7M}{Vol.aliquot\ (mL)\ loaded} \times \frac{Vol.\,total\ extraction(L)\ 0{,}02\ L\ HCl}{Weigth_{sample}(g)}$$

Example:

$$\text{Phytic acid}\left(\frac{mg}{g}\right) = \left(\frac{Abs_{sample} - Intercept\ (mg)}{Slope\ (L)}\right) \times \frac{Vol.elution\ (mL)\ 30\ mL\ \ NaCl0{,}7M}{Vol.aliquot\ (mL)\ loaded} \times \frac{Vol.\,total\ extraction(L)\ 0{,}02\ L\ HCl}{Weigth_{sample}(g)}$$

9 Quality control

For samples with a known and validated matrix, for every sample received, do extraction in duplicate.

Variability of sample replicates: Repeat extractions if variability between the first two replicates (extraction replicates) is more than 10 %.

For samples of non-validated matrix: Do the extraction in triplicate.

If historical deviation is more than 7 % then, conduct an extensive revision of the extraction procedures to identify areas of improvement.

Backup sample: After analyzing a sample, the backup sample should be properly packaged, identified, and stored in case reanalysis/extraction of sample is needed.

Internal control sample: Analyze an internal control sample (in duplicate) every day of analysis. To prevent contamination (and moisture migration) of internal control sample, take several aliquots of the grain and put each in high barrier bags or amber glass bottles; finally place all aliquots in the same primary bag for storage (-20 °C).

10 Disposal of waste and cleaning of material

The waste obtained after the extraction, separation, and elution process of the total phytates can be discharged through the pipeline normally. The solid part is collected on a paper napkin and deposited in the garbage can.

All waste obtained from the colorimetric procedure (samples and standards mixed with Wade's reagent), must be collected in glass containers, labeled with the format of the department of health and safety at work and list the substances that are part of the mixture). Once the bottle is full, it must be taken to the CIAT hazardous waste warehouse at the times established by the department of health and safety at work, who manage hazardous waste.

Finally, the material used in the analysis must be placed in the ultrasonic bath for 15 minutes and then washed with citranox or alconox liquid soap and rinsed with distilled water from the CIAT network.

11 Annexes

There are no annexes documents related to this SOP.

12 References

Arcos J; Rojas DC. (2018). Recomendaciones para la producción de grano de fríjol biofortificado en Colombia. International Center for Tropical Agriculture (CIAT), Cali, Colombia. 34 p. https://hdl.handle.net/10568/100818

Latta M; Eskin M. (1980). A Simple and Rapid Colorimetric Method for Phytate Determination. *Journal of Agricultural and Food Chemistry* 28(6):1313–1315. https://doi.org/10.1021/jf60232a049

Taleon V; Gallego S; Orozco JC; Grenier C. (2020). Retention of Zn, Fe and phytic acid in parboiled biofortified and non-biofortified rice. *Food Chemistry: X* 8:100105. https://doi.org/10.1016/j.fochx.2020.100105

13 History of document modifications

Effective date of the SOP	Version number	Description	Reviewed by
September 2024	001	Original SOP	Sonia Gallego Victor Taleon

14 Security elements

- Reagent Safety Sheet
- Lab coat
- Long pants
- Closed toe shoes
- Chemical resistant gloves (Nitrile gloves)
- Safety glasses
- Extraction cabin

15 Notes and suggestions

- Chromatography columns: apart from the Poly-Prep prefilled columns, AG 1-X8 resin, 200–400 mesh, formate form, 0.8, several columns can be used. AG 1-X4 are less reliable. The particle size could also be 100–200. The chlorate form can also be used although it will need to be validated for the proportions of reagents described in this method.
- To carry out the analyzes on the samples, they must be taken in triplicate.
- Solutions must be shaken to obtain homogeneity.
- Take into account the procedure to follow when the cartridges are new or when they are going to be reused.
- Take three aliquots from each sample and/or standard to measure in the spectrophotometer.

Laboratory Standart Operating Procedure **Quantification of total phytates in beans, rice, and maize flour**	   
General information	
Process tittle: Quantification of total phytates in beans, rice, and maize flour	
Author: Juan Camilo Orozco Agredo	**SOP ID:** NQL-2023-001
E-mail: j.orozco@cgiar.org	**Date:** 12/15/2023
Company/Institution: Alliance Bioversity & CIAT	**Version:** 001
This document has been reviewed by:	
Victor Taleon Gelver Patiño Lenis Sonia Gallego Castillo	12/15/2023 01/22/2024 02/21/2024
Final validation by:	
Sonia Gallego Castillo	02/21/2024

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