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Quantification of Phytic Acid in Grains

Inorganic Geochemistry, Centre for Environmental Geochemistry

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Quantification of Phytic Acid in grains

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Foreword

This report is the published product of a study by the British Geological Survey (BGS) for the validation of a laboratory procedure to quantify phytic acid in grain samples.

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Contents

1. Summary

This report describes the validation of a cost effective method for quantifying phytic acid in grains, namely, rice and wheat, using UV/Vis spectroscopy. Background information describing phytic acid and its impact on human biological systems and hence the importance of its analysis is included in this report.

The validation method involved a range of tests to determine accuracy, precision and reproducibility of the method. Multiple sample matrices were used including standards and spiked samples as described in the validation plan and criteria in Appendix 2.

The method employed a commercially available assay kit from Megazyme® and was found to give accurate reliable data according to the performance characteristics attained. This method also has the potential for transfer to laboratories with limited resources, in particular developing countries. It is applicable to survey scale and small batch analysis owing to its relatively low start up and running costs, fast analysis time and ease of instrument set up for each analytical batch compared to established methods using ion chromatography.

2. Introduction

Mineral micronutrient deficiencies (MNDs) are an important global health problem, affecting up to two billion people worldwide (WHO, 2009, 2015). The common mineral MNDs include; iodine (Andersson *et al.* 2012*;* Watts *et al*. 2015; Zia *et al.* 2015), iron (Siyame *et al*. 2014; Gibson *et al*. 2015), selenium (Hurst *et al*. 2013) and zinc (Ahmad *et al*. 2012; Joy *et al*. 2015; Kumssa *et al*., 2015b). Estimates of deficiency for some minerals (Fe, I, Se, Zn), are often based on direct measurement of mineral concentrations or indicators in blood, urine or other tissues (Ku *et al*. 2015; Fairweather-Tait *et al*. 2011). Alternatively, for elements including Mg, food consumption or food supply data can be used to calculate dietary mineral intakes to estimate the risk of deficiency (Kumssa *et al*. 2015a; Ecker and Qaim, 2011) and national Food Balance Sheets (FBSs) available from the United Nations Food and Agriculture Organisation (FAO, 2014; Broadley *et al*. 2012; Joy *et al*. 2012; 2014). Local food composition data has improved estimates of mineral deficiencies, and for some MNs demonstrated a strong influence of soil type on dietary composition (Chilimba *et al*. 2011; Hurst *et al*. 2013; Joy *et al*. 2015), resulting in significant spatial variation.

Mineral MNDs in developing countries, particularly in Sub-Saharan Africa are exacerbated by a lack of dietary diversity, with reliance on a limited range of staple foods for calorific intake (e.g. maize, rice). Developing countries most effected by MNDs often have a high reliance on a plant based diet, with the consumption of meat and dairy products limited in availability (Joy *et al*. 2012; Joy *et al*. 2014). This lack of dietary diversity can often lead to an insufficient intake of Fe and Zn, (Hunt *et al*. 2003), whilst also increasing the intake of phytic acid (or phytate). Foods possessing large concentrations of phytic acid result in significant reductions to the bioavailability of Zn (Cakmak *et al*. 1998). Phytic acid is often present in seeds, serving as a storage for *myo*-inositol and phosphorus, which is utilised during seed germination and seedling growth (Bentley *et al.* 2015). Phytic acid is a strong chelator of Fe^{2+} and Zn^{2+} *in-vivo* and poses a major risk of anti-nutrient deficiency throughout Africa and worldwide (Hunt *et al*. 2003; Kumssa *et al.*, 2015b), limiting the bioavailability of these essential minerals from an already deficient dietary intake. Measurement of phytic acid in foodstuffs is an important consideration to improve population estimates for mineral deficiency in combination with direct human biomonitoring, FBS, food composition data and better understanding of the spatial controls on their soil-to-crop transfer.

This report describes the analytical method used to quantify phytic acid in grain samples using simple and relatively low-cost UV/Vis spectroscopy, which could easily be applied in a developing world situation. Whilst measurement by Ion Chromatography provides very high sensitivity and specificity for phytate (Harlanda *et al.* 2004), it requires expensive equipment and consumables, a high degree of technical competency and takes approximately 20-30 minutes per sample for analyses following a complex extraction process to measure phytic acid in solution. A commercially available kit (K-PHYT 12/12 Megazyme, Ireland) was reported by Xue et al. (2015) to determine the distribution of stable Fe^{57} and Zn^{68} isotopes in tissues of wheat lines with respect to phytic acid content, with sufficient sensitivity suitable for phytic acid in the majority of common grains (e.g. maize, rice). There is also the potential for high throughput with analyses taking only 6 minutes per sample, whilst using relatively low cost equipment that requires little maintenance and effort for calibration for each analytical batch. This methodology employed a commercially available assay kit from Megazyme® for measuring phytic acid by enzymatic and redox chemistry.

This report describes the validation and implementation of this method recently completed at BGS, with the aim of undertaking cost effective measurements of phytic acid in a range of food grains to improve estimations for dietary mineral intake.

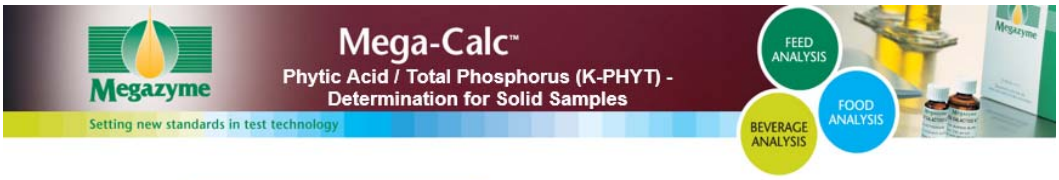
3. Methodology

This section provides describes the experimental procedure used in the quantification of phytic acid in foodstuffs. Included is an overview of the extraction of both phytic acid and inorganic phosphorus from the grain samples, as well as the equipment required for analysis. A screenshot of the calculation software is also included below (Figure 1) that was used to determine phytic acid concentrations from absorbance measurements.

Phytic acid was extracted from grain samples using a 0.66 M solution of HCl. Following extraction, a number of enzymatic reactions were used to release inorganic phosphorus (P_i) from the extracted phytic acid as described by Megazyme®. To produce a solution quantifiable by UV/Vis spectroscopy, P_i was reacted with ammonium molybdate before redox chemistry formed a final solution containing molybdenum blue.

To quantify phytic acid in each sample, a Lambda 35 spectrometer from Perkin Elmer was used to measure absorbance of molybdenum blue at 655 nm. Proportionality between P_i and molybdenum blue allowed the software to calculate the concentration of P_i and hence phytic acid in the original sample.

Using this UV/Vis method it was possible to analyse up to 48 samples per day. With software calculating phytic acid concentrations directly from absorbance results (Figure 1). No additional staff time was required for data interpretation. This not only decreased costs but also increased time efficiency and greatly simplified the analytical process.



Sample details

Instructions
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Standard absorbance values

Phosphorus Standard	Phosphorus (µg)	Abs 1	Abs 2
STD0	0.00		
STD1	0.50		
STD2	2.50		
STD3	5.00		
STD4	7.50		

Results

ΔAbs (standard)	M (µg/ΔAbs)

Mean M

Sample absorbance values

	Sample identifier	Free Phosphorus	Total Phosphorus	Sample weight (g)	Extraction volume (mL)
1				1.0000	20.000
2				1.0000	20.000
3				1.0000	20.000
4				1.0000	20.000
5				1.0000	20.000

Results

ΔAbs (Phosphorus)	Phosphorus (g/100 g)	Phytic acid (g/100 g)

Figure 1. Megazyme phytic acid calculation software.

A. EQUIPMENT

UV/Vis spectrometer	Vortex mixer
Water bath (stable at 40°C)	Microcentrifuge and 1.5 mL tubes
Glassware	Micro-cuvettes (1.5 mL)
Analytical balance	Timer
Pipettes (20 µL to 5 mL)	Megazyme calculation software

B. REAGENTS

Megazyme® phytic acid assay kit	Powdered ascorbic acid
Concentrated Sulfuric acid	Sodium hydroxide pellets
Hydrochloric acid	Powdered ammonium molybdate
Powdered trichloroacetic acid	

C. STABILITY OF REAGENTS

The Megazyme phytic acid assay kit provided solutions stable for over two years at 4°C:

Two buffer solutions (pH 5.5 and 10.4)	Phytase suspension
Alkaline phosphatase	Phosphorus standard solution
Oat flour reference material.	

Other reagent solutions not supplied included the following,

Ascorbic acid (10% w/v)/Sulfuric acid (1M)	Stable for one week at 4°C
Ammonium molybdate (5% w/v)	Stable for one month at 4°C
Trichloroacetic acid (50% w/v)	Stable for 6 months at 4°C
Hydrochloric acid (0.66 M)	Stable at room temperature
Sodium hydroxide (0.75 M)	Stable at room temperature

The supplied phosphorus standard was used to prepare a five phosphorus concentrations from 0 to 7.5 µg of phosphorus for calibration, including DI water, which were stable for one week at 4°C. The method employs a colour change in sample solutions as a result of the reaction of ascorbic acid and ammonium molybdate solutions in a 5:1 ratio. Due to its instability the complex/sample was prepared on the day of analysis (Appendix 1).

4. Validation

The procedure for the quantification of phytic acid using UV/Vis spectroscopy is described in Appendix 1. This also contains instructions on the preparation of standards and specific solutions required for the analysis. Appendix 2 outlines the validation plan devised to test the rigidity and reproducibility of the method, respectively. The stability of each reagent used was described previously in the Methods section, with storage duration/conditions described for each reagent summarised in Appendix 1.

The validation process began with the analysis of four samples covering a range of typical phytic acid concentrations from rice and grain samples (1534-10,964 mg kg^{-1}). Each sample was analysed in triplicate for n=5. Percentage relative standard deviation (%RSD), was used to determine both within and between run variations. Standards at 7 and 80% of the top calibration standard (7.5 µg/mL phosphorus equating to 29598 mg kg^{-1} phytic acid) at (n=5) were analysed to confirm accuracy and precision. Since standards were prepared using a phosphorus solution, calculations were required to convert these values to phytic acid concentrations prior to analysis. Phytic acid concentrations for the 7 and 80% of the highest calibration standard were 1790 and 23,679 mg kg^{-1}, respectively. This corresponded to phosphorus concentrations of 517 and 6000 mg kg^{-1}. A rice sample spiked with a phytic acid standard was also used to measure extraction recovery performance. A further two analytical runs were carried out by a second operator to establish whether analyst variation had a significant influence on performance criteria.

**Note: 5% of the top calibration standard resulted in a solution below the limit of quantification; hence a higher standard (7%) was used to overcome sensitivity issues.*

Detection limits (DL), were quoted as 400 mg kg^{-1} in the assay procedure provided by Megazyme® using a smallest absorbance difference of 0.05. Using techniques described by Gonzalez and Herrador (2007), our own detection limits (DL) were obtained using standard deviations (SD), of 10 blank solutions. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using a value of three and ten times SD, resulting in values of 413 mg kg^{-1} and LOQ = 1408 mg kg^{-1}, respectively (Gonzalez *et al*. 2007).

5. Results and Discussion

A. OAT FLOUR REFERENCE MATERIAL

Although no certified reference material was commercially available, a sample of oat flour with established phytic acid values was provided with the phytic acid assay kit (Megazyme®) was initially used to measure method accuracy. The average measured value of 17,862 ± 838 mg kg^{-1} showed good agreement with the established concentration of 17,700 mg kg^{-1}, representing a bias of 1% (Table 1).

Table 1: Accuracy and precision of measured values versus target values for an oat flour reference material (n=10).

	Phytic acid
Target Value (mg kg^{-1})	17,700
Measured mean (mg kg^{-1})	17,862
Standard Deviation (mg kg^{-1})	838
Precision (%RSD)	5
Bias (%)	**+1**

B. PRECISION AND ACCURACY FOR HIGH AND LOW STANDARDS

Standards at both 1790 and 23,679 mg kg^{-1} phytic acid (7 and 80% of the top calibration standard) were analysed (n=5). Since a phosphorus calibration standard (not phytic acid), was used, phosphorus concentrations were also included in the analysis. The 7 and 80% standards corresponded to 517 and 6000 mg kg^{-1} phosphorus, respectively. Tables 2 and 3 display the accuracy and precision for each standard. The accuracy of measurements for a solution equivalent to 7% of the top standard concentration was 92% for phosphorus and 94% for phytic acid. Table 3 shows the accuracy for phosphorus and phytic acid in a solution equivalent to 80% of the top standard concentration was 99 and 90% respectively.

Note: A 7% standard was used in place of a 5% standard due to the limits of quantification stated previously (a higher concentration standard was required to obtain reliable data).

Table 2: Accuracy and precision of a phytic acid measurement at 7% of the top calibration concentration (n=5).

	Phosphorus	**Phytic Acid**
Target value (mg kg^{-1})	517	1790
Mean measurement (mg kg^{-1})	474	1679
Bias (%)	**-8**	**-6**
Measured SD (mg kg^{-1})	34	122
Precision (%RSD)	**7.2**	**7.2**

Table 3: Accuracy and precision of a phytic acid measurement at 80% of the top calibration concentration (n=5).

	Phosphorus	**Phytic Acid**
Target value (mg kg^{-1})	6000	23679
Mean measurement (mg kg^{-1})	5970	21233
Bias (%)	**-1**	**-10**
Measured SD (mg kg^{-1})	298	902
Precision (%RSD)	**5.0**	**4.2**

C. WITHIN AND BETWEEN RUN VARIATION

To calculate both within and between run variation, four samples (2 rice and 2 wheat grain samples), were measured in triplicate (n=5*). Anova analysis was then used to calculate each source of variation (within and between run). All within run variations were below 10% and hence passed the validation criteria set in Appendix 2. Between run variation was often higher (with rice 1 as the exception). Rice 2 had extremely low levels of phytic acid verging on the LOQ for the analysis. To reduce between run variation in these low-phytate samples there are two possible solutions. More sample could be used in each assay (e.g. 2 gram of sample for the same extracting volume). This would raise the measured phytic acid concentrations (reducing the variation), which would be accounted for in the calculations. Alternatively all samples with measured phytic acid concentrations below 2000 mg kg^{-1} will be run in duplicate to ensure more accurate results. Large between run variations seen in the grain samples was due to a lack of homogeneity within the sample. As phytic acid is predominantly stored in grain husks, homogeneity has a significant influence on between run variations. Again this variation could be reduced in two ways. A larger sample and extracting acid volume (e.g. 3g sample, 60 mL acid), would allow for a more representative sample being analysed with no need for further sample preparation. Alternatively, additional sample preparation techniques could be investigated such as the use of a mortar and pestle to produce a finer more homogenised sample material. All results for within and between run variation can be found below (Table 4).

**Note n=4 used for rice 1.*

Table 4: Within and between run variation of an analysed rice and grain sample using %RSD.

	Rice 1	**Rice 2**	**Grain 1**	**Grain 2**
Between run (%RSD)	1	33	15	10
Within Run (%RSD)	5	9	7	8

D. OVERALL PRECISION

Overall precision was calculated based on all of the data obtained from two rice and two grain samples (Table 5). Rice 2 had very low phytic acid concentrations that approached the limit of quantification (1408 mg kg^{-1}). Hence it is seen to have an associated high %RSD of 19%. Thus, any sample with a measured concentration below 2000 mg kg^{-1} should be run in duplicate to ensure accurate results. It was also noted that the wheat grain samples were less homogenous than the rice. Although the %RSD is still acceptable (≤10%), further sample preparation may result in more accurate results in future.

Table 5. Overall Precision of phytic acid for two rice and two grain samples (n=15).

	Rice 1	**Rice 2**	**Grain 1**	**Grain 2**
Overall Average (mg kg^{-1})	6442	1534	10964	9809
Overall SD (mg kg^{-1})	271	292	1132	899
Overall Precision (%RSD)	**4**	**19**	**10**	**9**

E. SPIKE RECOVERY

A solution spiked with a calibration standard was used to determine the percentage recovery in a rice sample with a known concentration of phytic acid. 2 mL of a 100,000 mg kg^{-1} (assuming 1 kg L^{-1}) solution was added to the extracted sample containing 20 mL HCl. This provided a total spike of 9090 mg kg^{-1}, resulting in a total target concentration of 15,532 mg kg^{-1}. Table 6 displays an observed recovery of 94%. This value was calculated after the exclusion of an outlier using the Dixons test (Table 7). The likely reason for the outlier is the presence of a large air bubble during a pipetting step.

Table 6: Spike recovery data (n=4).

	Phytic Acid
Target spike value (mg kg^{-1})	9090
Mean spike measurement (mg kg^{-1})	8550
Average recovery	**94%**
Measured SD (mg kg^{-1})	65
Precision (%RSD)	0.8

Table 7: Dixons test to exclude a result as an outlier.

Spike concentration (mg kg^{-1})	**Dixons parameter**	***Value***
7084	**Gap**	1385
8470	**Range**	1531
8525	**Q-Score**	0.905
8589	**Q-score for exclusion (99%)**	0.821
8615	**Result**	**Excluded at 99%**

F. ANALYST VARIATION

A Students T-test was used to evaluate the null hypothesis; no significant variation occurred between multiple analysts. Grocery store bought red split lentils were analysed to confirm this hypothesis. First, an F-test confirmed equal variance of the two datasets with a value of 1.35, (lower than the critical value 8.85). The students T-test produced a T-value of 0.11 which was much lower than the 2.20 critical value. The null hypothesis was confirming at the 95% confidence level (Appendix 2) with a P value of 0.91 (Table 8).

Table 8: F-Test and T-Test results for analyst variation.

	F-Test	T-Test
Calculated Value	1.35	0.11
Critical Value	8.85	2.20
P Value	0.44	0.91

6. Conclusion

A reliable method for the measurement of phytic acid in typical grain samples using UV/Vis spectroscopy and a commercially available assay kit from Megazyme® was verified. Phytic acid concentrations for a control sample of oat flour with a known concentration of 17,700 mg kg^{-1} produced a measured average of 17,862 mg kg^{-1}, (within 1% of the known phytic acid concentration). Samples with concentrations approaching the LOQ of 1408 mg kg^{-1} demonstrated a lower precision than at higher concentrations. It is therefore recommended that all samples with a measured concentration below 2000 mg kg^{-1} phytic acid will be run in duplicate.

Standards at 7 and 80% of the top calibration concentration were found to show accuracies of 94 and 90%, respectively. Due to no commercially available certified reference material (CRM) being identified during validation, the oat powder sample provided by Megazyme® can be used as a quality control sample until a CRM can be sourced.

Reproducibility for wheat grain samples was demonstrated with a precision of 10% between separate runs, meeting the validation criteria outlined in Appendix 2. Rice samples were likely more homogenous and hence produced measurements within these limits, excluding the sample with concentrations below 2000 mg kg^{-1} phytic acid.

Spike recovery data for phytic acid were well within the limits set by the validation plan. Recovery was 94%, with an extremely high precision of 0.8%. The total spike concentration fell within the calibration concentration range of 45 to 55% with measured concentrations between 13,500 and 15,100 mg kg^{-1} of phytic acid. Using the Students T-Test, the null hypothesis for analyst variation was confirmed by a P-value of 0.91.

In summary, all validation tests passed the initial requirements of the validation plan with an exception of samples containing a phytic acid concentration below 2000 mg kg^{-1}, close to the LOQ. The method described is fit-for-purpose for typical concentrations of phytic acid in common grain samples. In addition, the method through its simplicity is easily reproducible between operators, and could be transferred easily to labs with minimal infrastructure. The fast throughput and low cost per sample will allow for large scale or routine analysis to better inform the impact of phytic acid mineral dietary intakes and enable improved mitigation approaches. The simplicity of the method will also allow for responsive analysis and is appropriate for small and large sample batches, due to the fast set-up of instrumentation compared to ion-chromatography.

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Appendix 1.

1.1 SUPPLIED REAGENTS

Solution 1. Buffer (25 mL, pH 5.5) and sodium azide (0.02% w/v) as a preservative,

Solution 2. Phytase suspension (1.2 mL),

Solution 3. Buffer (25 mL, pH 10.4) plus $MgCl_2$, $ZnSO_4$ and sodium azide (0.02% w/v) as a preservative,

Solution 4. Alkaline phosphatase suspension (1.2 mL),

Solution 5. Phosphorus standard solution (24 mL, 50 µg/mL) and sodium azide (0.02% w/v) as a preservative,

Bottle 6. Oat flour control powder (5 g; Phosphorus content displayed on bottle).

1.2 PREPARATION OF REAGENTS

Solution A – Stable for 1 week at 4°C
In a fume cupboard prepare the 10% ascorbic acid by adding 10 g ascorbic acid to 90 mL of de-ionised water (100 mL flask).

To this, add 5.35 mL of concentrated (95%) sulphuric acid to dissolve the ascorbic acid powder and make up to the 100 mL volume by adding de-ionised (DI) water.

Solution B – Stable for 1 month at 4°C
Dissolve 1.25 g ammonium molybdate to 20 mL of de-ionised water in a 25 mL flask.

Make up to volume using DI water

Colour Reagent – Prepare on use
Mix together solutions A and B in the ratio 5:1, A:B allowing for 0.6 mL per sample. i.e. (0.5 mL solution A and 0.1 mL solution B per sample).

Trichloroacetic acid – Stable for 6 months at 4°C
In a fume cupboard, add 50 g trichloroacetic acid to 60 mL of de-ionised water and dissolve. Make up to volume in a 100 mL volumetric flask

Hydrochloric acid
Add 54.5 mL of 37% HCl to 945.5 mL of de-ionised water in a 1 L volumetric flask (under fume hood).

Sodium hydroxide
In a fume cupboard, add 6 g of sodium hydroxide pellets to 180 mL of de-ionised water and dissolve (200 mL flask).

1.3 EQUIPMENT NEEDED

UV/Vis spectrometer	Vortex mixer
Water bath (stable at 40°C)	Microcentrifuge and 1.5 mL tubes
Glassware	Micro-cuvettes (1.5 mL)
Analytical balance	Timer
Pipettes (20 µL to 5 mL)	Megazyme calculation software

1.4 ASSAY PROCEDURE

1.4.1 Phytate extraction

Weigh 1 g (±0.001g) of sample material into a 75 mL glass beaker.

Add 20 mL of 0.66 M HCl, cover with foil and stir vigorously for a minimum of 3 hrs (or overnight for convenience),

Transfer 1 mL of the extract to a 1.5 mL microfuge tube and centrifuge for 10 minutes,

Immediately after, transfer 0.5 mL of the supernatant to a fresh 1.5 mL microfuge tube add 0.5 mL of 0.75 M sodium hydroxide.

1.4.2 Enzymatic dephosphorylation reaction

Add the following solutions to two separate Eppendorf tubes labelled free and total phosphorus,

Table 1. First enzymatic dephosphorylation reaction

Reagents	Free phosphorus*	Total phosphorus*
DI	0.62 mL	0.60 mL
Solution 1	0.20 mL	0.20 mL
Sample extract	0.05 mL	0.05 mL
Solution 2	-	0.02 mL

*Note both of these are required

Mix using the vortex and place in a water bath at 40°C for 10 minutes

After 10 minutes add,

Table 2. Second enzymatic dephosphorylation reaction

Reagents	Free phosphorus*	Total phosphorus*
DI	0.02 mL	-
Solution 3	0.20 mL	0.20 mL
Solution 4	-	0.02 mL

*Note both of these are required

Vortex and place in a water bath at 40°C for 15 minutes,

After 15 minutes, add 0.30 mL of trichloroacetic acid to stop the reaction.

Centrifuge the final solution for 10 minutes. **DO NOT MIX AFTER CENTRIFUGATION**

1.4.3 Preparation of the calibration curve

Table 3. Calibration preparation

	Standard 0 (0 µg)	Standard 1 (0.5 µg)	Standard 2 (2.5 µg)	Standard 3 (5 µg)	Standard 4 (7.5 µg)
DI water	5.00 mL	4.95 mL	4.75 mL	4.50 mL	4.25 mL
Solution 5	-	0.05 mL	0.25 mL	0.50 mL	0.75 mL

1.4.4 Colourimetric determination of phosphorus

Pipette into a 1.5 mL centrifuge tube, 1.0 mL of sample/standard and 0.5 mL of the colour reagent prepared earlier (Appendix 1.2)

Mix by vortex and place in a 40°C water bath for 1 hour.

After 1 hour, mix by vortex and transfer 1 mL into a micro-cuvette for UV/Vis analysis at 655 nm within three hours.

1.4.5 UV/Vis parameters

Wavelength: 655 nm
Cuvette: 1 cm light path
Temperature: room temperature
Final volume: 1.5 mL
Sample concentration: 0.5-7.5 µg/mL of phosphorus
Read against water

1.5 CALCULATIONS

Determine the absorbance (A_{655}) for each standard. Subtract the absorbance of STD 0 from all other standards hence obtaining ΔA (phosphorus)

Calculate **M** as follows for standards 1 to 4

$$\boldsymbol{M} = \frac{P\ (\mu g)}{\Delta A\ (phosphorus)}$$

Calculate the **mean M** as follows

$$\frac{\Sigma M(STD\ 1-4)}{4}$$

Use the **mean M** to calculate the phosphorus content of tested samples.

Phosphorus and phytic acid content

Determine the absorbance (A_{655}) for both the "free phosphorus" and "total phosphorus" samples. Calculate ΔA (phosphorus) using the following,

$$\Delta A\ (phosphorus) = A\ (total\ phosphorus) - A\ (free\ phosphorus)$$

The concentration of phosphorus can be calculated using,

$$c = \frac{mean\ M\ \times 20\ \times F}{10000\ \times 1.0\ \times v} \times \Delta A\ (phosphorus)$$

Mean M = mean value of phosphorus standards
20 = original sample extract volume (mL)
F = dilution factor
Δ A (phosphorus) = absorbance change of sample
10,000 = conversion from µg/g to g/100g
1.0 = weight of original material
v = sample volume (used in colourimetric determination step)

Hence the equation simplifies to:

$$\boldsymbol{c = mean\ M\ \times 0.1112\ \times \Delta A\ (phosphorus)}$$

For Phytic Acid (g/100g)

$$\boldsymbol{c = \frac{[phosphorus](\frac{g}{100g})}{0.282}}$$

Appendix 2

1. Background

The purpose of the UV/Vis validation method is to describe the process used to validate the procedure and provide confidence in the robustness, accuracy and reproducibility of the method.

2. Scope

By using the UV/Vis method outlined in this report, concentrations of phytic acid will be measured in a range of staple African food sources. The method will predominantly focus on the quantification of foods containing high levels of phytate (> 600 mg kg^{-1}).

3. Test samples

The validation method will use wheat, rice and lentil samples. Pakistan wheat and Malawi rice samples were obtained during field work by BGS, whereas lentil data was obtained using a store bought product (UK). These contrasted samples showed phytate concentrations between 900-12000 mg kg^{-1}. Resultantly, a wide range of phytate concentrations were tested within the validation method.

4. Calibration standards

As part of the Megazyme® phytic acid assay kit, a stock solution (50 µg/mL phosphorus), was provided. This was then diluted to create a range of calibration standards containing 0, 0.5, 2.5, 5.0 and 7.5 µg/mL of phosphorus.

5. Validation tests

To validate the method, both grain and rice samples were analysed, in quintuplicate, over 3 separate analytical runs. This allowed the determination of both within and between run variations. A number of runs were also carried out by a second analyst to allow the evaluation of analyst variation.

A stock solution of phytic acid was used to evaluate method accuracy at 7 and 80% of the top calibration standard during a separate run.

To validate the limit of detection (LOD) stated by Megazyme®, a blanks were run at n=10. The standard deviation (SD) was then calculated allowing the LOD and LOQ to be determined using the 3 and 10x SD respectively.

To determine spike recovery, 2 mL of a 100,000 mg kg^{-1} standard was added to a known sample (6442 mg kg^{-1} phytic acid), during extraction. This created a spiked addition of 9090 mg kg^{-1}. Analysis was then carried out for n=5 spiked samples to determine an average spiked concentration and hence percentage recovery.

6. Acceptance criteria

To confirm the validation of the method, the following criteria must be attained,

- Oat flour percentage difference ≤10%
- Accuracy of 7 and 80% calibration standards ≥90%;
- Within and between run variation ≤10%;
- Spike recovery percentage ≥90%;
- Confirmation of no analyst variation at the 95% confidence level.

Appendix 3.

3.1 BLANK DATA FOR DETECTION LIMIT AND LIMIT OF QUANTIFICATION

Table 5: Blank raw data

	[phytic acid] mg kg^{-1}
Blank 1	1034
Blank 2	772
Blank 3	912
Blank 4	981
Blank 5	1160
Blank 6	832
Blank 7	772
Blank 8	912
Blank 9	981
Blank 10	1160

3.2 OAT FLOUR REFERENCE MATERIAL RAW DATA

Table 6: Oat flour reference material raw data

Oat Powder (g/100g)	Oat Powder (mg kg^{-1})
1.8466	18466
1.843	18430
1.7794	17794
1.6441	16441
1.8179	18179

3.3 STANDARDS AT 7 AND 80% OF THE TOP CALIBRATION STANDARD RAW DATA

Table 7: Standards at 7 and 80% of the top calibration standard raw data

	5% standard	
	Phosphorus	Phytic acid
Target Value (mg kg^{-1})	517	1790
Mean measurement (mg kg^{-1})	474	1679
Accuracy	92%	94%
Measured SD (mg kg^{-1})	34	122
Precision (%RSD)	7.2	7.2

	80% standard	
	Phosphorus	Phytic acid
Target Value (mg kg^{-1})	6000	23679
Mean measurement (mg kg^{-1})	5970	21233
Accuracy	99%	90%
Measured SD (mg kg^{-1})	298	902
Precision (%RSD)	5.0	4.2

3.4 ALL RICE AND GRAIN SAMPLE RAW DATA

Table 8: All rice and grain sample raw data

	Run 1	Run 2	Run 3	Run 4	Run 5
	Phytate (mg kg^{-1})				
Rice 1	6383	6619	6587	**NA**	6311
Rice 1	6430	6371	5849	**NA**	6215
Rice 1	6616	6343	6932	**NA**	6650
Rice 2	1463	1856	1121	1301	1796
Rice 2	1826	1963	1285	1274	1632
Rice 2	1585	1743	1364	1033	1775
Grain 1	9688	12274	11998	9628	11069
Grain 1	9784	12772	11533	10831	10709
Grain 1	9953	12373	9159	11097	11587
Grain 2	9272	10337	8748	10921	9433
Grain 2	9629	10235	9209	11569	9878
Grain 2	8984	9799	9161	8675	11285

3.5 SPIKE RECOVERY RAW DATA

Table 9: Spike recovery raw data

	Phytic acid
Target Value (mg kg^{-1})	9090
Mean Measurement (mg kg^{-1})	8550
Average Recovery (%)	94
Measured SD	65
Precision (%RSD)	0.8

3.6 ANALYST VARIATION RAW DATA

Table 10: Analyst variation raw data

ADZ lentils [Phytic acid mg kg^{-1}]	DR Lentils [Phytic acid mg kg^{-1}]
6762	6129
7791	7791
6310	6310
8173	6762
	7486
	8968
	6335
	7633
	8496