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Insights of Colorimetric Estimation of Phytic Acid in Rice Grain

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Abstract

Phytic acid present in rice grain is an anti-nutrient that hinders the absorption of iron (Fe) and zinc (Zn) ions in the human digestive system. Estimation of phytic acid is a part of the biofortification studies targeted to enhance micronutrient content in the rice grain. Among the various methods of phytic acid estimation, colorimetric method is highly amenable to most of the laboratories due to its simple requirements. Wade reagent has been widely used in phytic acid estimation in various crops. However, details like the range of phytic acid concentrations required for standard graph preparation and maximum limits of detection were not clearly mentioned and the volume of Wade reagent differed. Hence, the objective of this study is to identify the range of phytic acid concentrations that is suitable to develop phytic acid standard graph followed by the determination of the maximum limit of Wade reagent volume. The results of this study will be useful in the future studies to understand the inherent details like the volume of Wade reagent, volume of biological extract, optimum volume as well as concentration optimum for standard graph preparation and the factor for calculating phytate-P from phytic acid.

Keywords: Phytic acid, Colorimetric assay, Spectrophotometric assay, Wade reagent.

Introduction

Rice is consumed as a staple diet by more than half of the world's population. Rice grain provides more energy than nutrients. Elements like iron, zinc, phosphorous, aluminium, cadmium, calcium, cobalt, copper, magnesium, manganese, molybdenum, etc. are also considered as micronutrients (Longvah, 2017). In general, elements are in minute quantities and are often lower than the prescribed recommended dietary allowance (RDA) which is 10-15 mg for iron and 12-15 mg for zinc (Sanjeeva Rao *et al*., 2014, 2020; Suman *et al*., 2021). Of these, iron and zinc are considered as priority elements due to the prevalence of anaemia and stunted growth in the global population.

Iron is required for the formation of heme (protoporphyrin) which is the prosthetic group of haemoglobin and redox components of various electron transfer chains of human beings, microbes and plants. Similarly, zinc is a prosthetic group of zinc fingers that control gene expression of some operons in eukaryotes and required for around 300 enzymatic reactions (McCall *et al*., 2000).

Phytic acid (PA), also referred to as the main phosphorus (P) storage compound found in seeds (Kumar *et al*., 2021), plays a crucial role in negatively binding positively charged minerals (**Figure 1**), rendering them inaccessible to the human digestive system (Raboy *et al*., 2001; Prasanna *et al*., 2021). Seeds accumulate an excess of phosphorus beyond what is necessary for normal cellular functions during germination. Rice grain contains around 7% phytic acid and binds to 70% of the phosphorus stored in the grain (Perera *et al*., 2018). This trait in rice germplasm was less studied or exploited with phytic acid content ranging from 22.64 to 41.31 µg/grain in brown rice,

2.67 to 6.42 µg/grain in embryo and 22.24 to 38.70 µg/grain in endosperm of wild and low phosphate mutant (Liu *et al*., 2004), 3.98 mg/g to 32.36 mg/g in the few rice germplasm sets and it noted negative association with grain zinc as well as iron contents (Gyani *et al*., 2021). It contains multiple negative charges due to the presence of phosphate groups that bind to divalent cations like iron and zinc and makes them unavailable for absorption in the human digestive tract (Graham *et al*., 2001).

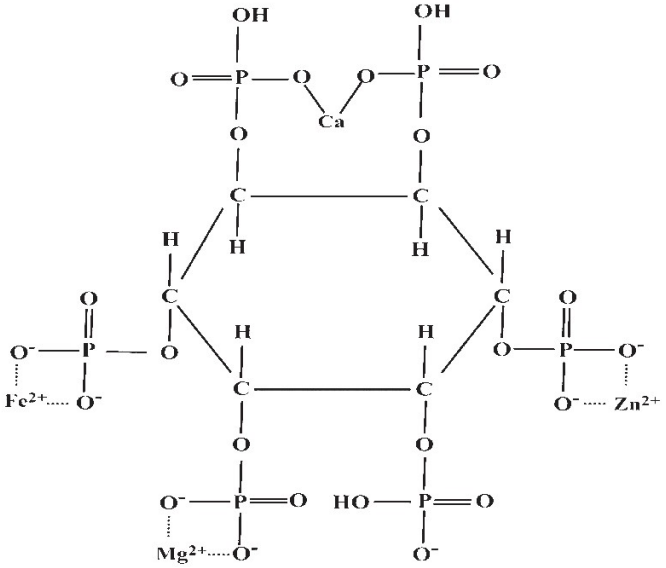

Figure 1: Phytic acid multi-cation complex

In addition to yield, enhancing nutrition in cereal crops is one of the targets of millennium development goals. As some of the other molecules in the grain can act as anti- or pronutrients and influence the absorption of the zinc or iron, assessing the bioavailability of the zinc or iron also became a pre-requisite. Since phytic acid is considered as an anti-nutrient, screening the germplasm or mapping population or advanced material for phytic acid is gaining focus in the past few years. Reducing PA content not only enhances the nutritional value of food but also addresses concerns related to malnutrition (Bohn *et al*., 2008; Ragi *et al*., 2022).

The two decades of consistent research on biofortification by various groups resulted in the release of few better zinc biofortified rice varieties through AICRPR biofortification (Sanjeeva Rao *et al*., 2014; Neeraja *et al*., 2018; Kapoor *et al*., 2019; Pradhan *et al*., 2020; Bollinedi *et al*., 2020; Sanjeeva Rao *et al*., 2020; Suman *et al*., 2021; Anusha *et al*., 2021; Uttam *et al*., 2022). Phytic acid is one of the phosphoric acid esters which was initially estimated by descending paper chromatography where phosphorus is released in the presence of acid and reacts with molybdate to form blue colour phosphor-molybdate complex (Hanes and Isherwood, 1949). However, in the presence of acid, paper is degraded that affects the further analysis. Hence, instead of acid molybdate solution, $FeCl_3$. $6H_2O$ in 80% alcohol followed by salicyl sulphonic acid in 80% alcohol were sprayed on the paper and these chemicals have been used as Wade reagent (Wade and Morgan, 1953).

A colorimetric method for the estimation of the phytic acid using Wade reagent (0.03% $FeCl_3$-$6H_20$ and 0.3% sulfosalicylic acid in distilled water) was developed (Latta and Eskin, 1980) without extracting phytate by precipitation as iron phytate or by ion-exchange chromatography (Vaintraub and Lapteva, 1988). This Wade reagent was modified (0.125% $FeCl_3$-$6H_20$ and 1.25% sulfosalicylic acid in distilled water) by adjusting the pH to 3.05 using NaOH (Lorenz *et al*., 2007).

Apart from these, phytic acid was also indirectly derived by estimating iron and multiplying it with a factor of 4.2 in Barley (Dai *et al*., 2007) or estimating inorganic phosphorus and dividing it with a factor of 3.55 in Soyabean (Raboy and Dickinson, 1984). Phytate-P was calculated by multiplying the estimated value of phytate with a conversion factor of 0.282 in Rice (Gyani *et al*., 2021). Eventually, colorimetric / spectrophotometric method is always simpler and can be carried out in many laboratories as spectrophotometer is a common equipment in comparison with sophisticated equipment like HPLC (Liu *et al*., 2007; Lee *et al*., 2014).

Although Wade reagent is popular, the quantity of the biological sample, volume of extract and volume of Wade reagent varied in the above reports. In the case

of standard graph, regression values were mentioned without the range of working standard solution. Moreover, the intensity of the colour decreases with the increase in phytic acid concentration in the extract (Latta and Eskin, 1980) and the faint colour formed above the maximum limit of the reagent also fades in few minutes. Our efforts indicate that the identification of maximum limit of Wade reagent (1 mL to 2 mL) plays an important role in the phytic acid estimation. As the range of standard graph is not mentioned in the earlier reports, every researcher has to independently make efforts to identify the concentrations required for the standard graph.

Hence, the objective of this study is to identify the range of phytic acid concentrations to make a standard graph, identify the minimum and maximum detection limits of the Wade reagent to get a stable final colour intensity, to identify a plausible quantity of rice powder and the volume of extract. The results presented here will be useful to the researchers planning to work on phytic acid estimation in rice grain.

Materials and Methods

Preparation of reagents

Stock solution of phytic acid standard (5 mg/mL) was prepared using phytic acid dodecasodium salt from corn (Sigma P-8810) in 0.65 *M* HCl. The working standard solution was prepared by adding 25 mL of stock solution to 25 mL of 0.65 *M* HCl. Wade reagent was prepared by taking 0.125% $FeCl_3$. $6H_20$ and 1.25% 5- Sulfosalicylic acid hydrate 95% (Sigma-390275) in distilled water and pH was adjusted to 3.05 using NaOH. This is also called as modified Wade reagent which could be stored in a refrigerator for one month (Lorenz *et al*., 2007).

Preparation of standard graph

Phytic acid ranging from 50 µg to 1000 µg was prepared by taking series of 10 µL to 200 µL of working standard solution into pre-labelled 2 mL Eppendorf tubes (**Table 1**). The final volume in each tube was made upto 300 µL with 0.65 *M* HCl. 1.5

Table 1: Development of phytic acid standard graph with Wade reagent.

S. No.	Volume of WSS (µL)	Phytate (µg)	Volume of 0.65 M HCl (µL)	Volume of Wade reagent (mL)	Average OD at 490 nm
1	10	**50**	290	1.5	**1.48**
2	20	**100**	280	1.5	**1.34**
3	30	**150**	270	1.5	**1.23**
4	40	**200**	260	1.5	**1.13**
5	50	**250**	250	1.5	**1.01**
6	60	**300**	240	1.5	**0.92**
7	70	**350**	230	1.5	**0.80**
8	80	**400**	220	1.5	**0.76**
9	90	**450**	210	1.5	**0.68**
10	100	**500**	200	1.5	**0.64**
11	110	**550**	190	1.5	**0.53**
12	120	600	180	1.5	0.50
13	130	650	170	1.5	0.46
14	140	700	160	1.5	0.42
15	150	750	150	1.5	0.38
16	160	800	140	1.5	0.38
17	170	850	130	1.5	0.35
18	180	900	120	1.5	0.33
19	190	950	110	1.5	0.29
20	200	1000	100	1.5	0.29

*Bold values indicate the range of phytic acid that can be determined by 1.5 mL of Wade reagent.

mL of Wade reagent was added, contents were made uniform by mixing and all the tubes were incubated at room temperature (37 °C) for 20 minutes. The colour intensity was measured at 490 nm in a UV-Visible spectrophotometer (Biochrom Ultrospec™ 7000) using 0.65 *M* HCl as blank.

The phytic acid concentration was converted to phytate-P by dividing with 3.55 (Lorenz *et al*., 2007) or by multiplying with 0.282 (Gyani *et al*., 2021) following the range of phytic acid-P (1.2 to 1.8 mg/g) which is equal to 4.2 to 6.5 mg/g of phytic acid identified by applying low nutrient P for cultivation in soya bean (Raboy and Dickinson, 1984).

Results and Discussion

In the past few years screening for phytic acid levels in rice grain gained attention due to its natural capacity (possess negative charge) to bind with the cations and making them unavailable for absorption in the human gut or digestive tract. Hence, identification of low phytic acid lines coupled with higher iron and or zinc contents, yield and the ability to complete all the other aspects of rice life-cycle (seed to seed) like high yielding rice varieties became a priority. As mentioned in the introduction, direct (Wade reagent) colorimetric or spectrophotometric method is preferred. However, the available reports varied with respect to the weight of the biological sample, volume of extract and volume of Wade reagent. Moreover, the range of standard solutions in the preparation of standard graph plays an important role in the final estimated values. Hence, the standard graph of phytic acid with Wade reagent is presented followed by maximum detection limits of the Wade reagent.

In the phytic acid standard graph, regression value of 0.99 was obtained with the known concentrations of phytic acid ranging from 50 µg to 350 µg **(Figure 2)**. The regression value decreased slightly from 0.99

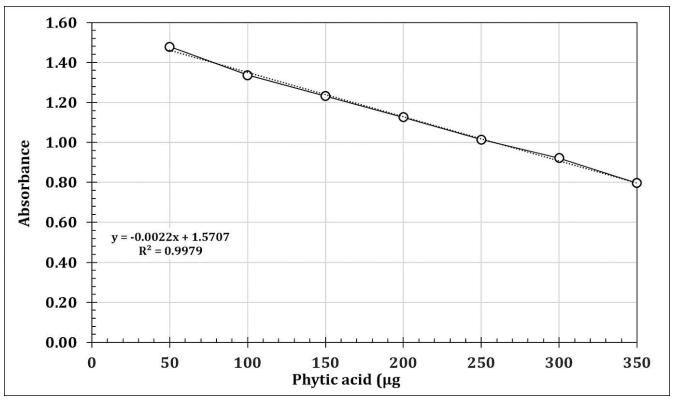

Figure 2: Standard graph indicating phytate concentration from 50 µg to 350 µg

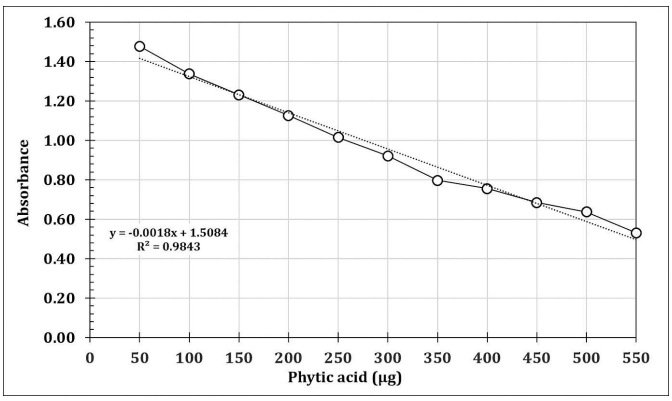

Figure 3: Standard graph indicating phytate concentration from 50 µg to 550 µg

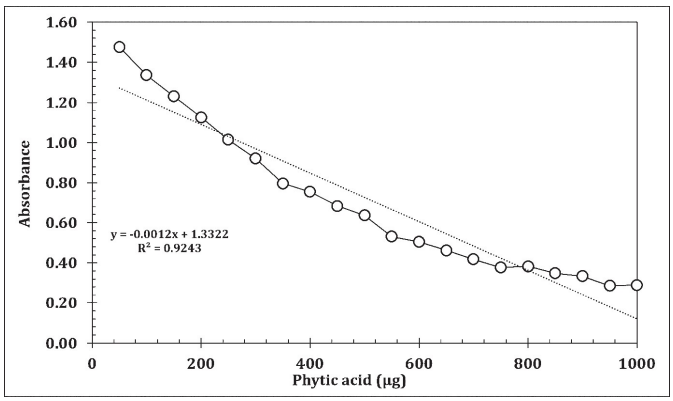

Figure 4: Standard graph indicating phytate concentration from 50 µg to 1000 µg

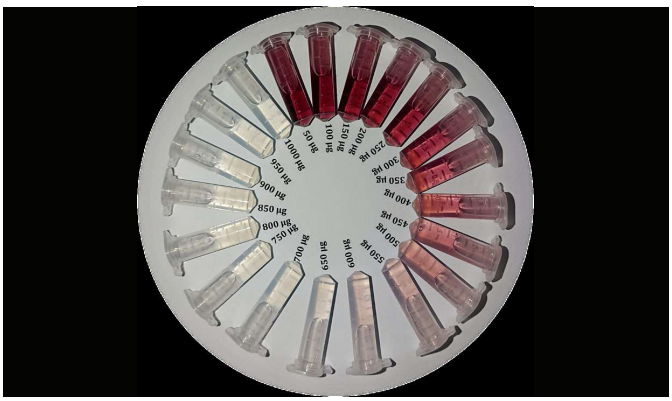

Figure 5: Change in colour intensity with increase in phytate concentration from 50 µg to 1000 µg

to 0.98 while including phytic acid concentration upto 550 µg in the standard graph (**Figure 3**). The regression value further decreased to 0.92 while phytic acid concentration upto 1000 µg in standard graph (**Figure 4**). Most importantly, the traces of pink colour also faded completely from 600 µg onwards. As the phytic acid reduces iron ion in Wade reagent the intensity of the pink colour decreased (**Figure 5**) with the increase in phytic acid concentration and eventually, the maximum detectable concentration of phytic acid was approximately 600 µg with 1.5 mL of Wade reagent. This maximum detectable phytic acid can change with the change in the usage of the volume of Wade reagent (Lorenz *et al*., 2007). In some studies, 0.2 mL of Wade reagent for 30 µL biological extract which is equivalent to 2.0 mL of Wade reagent and 300 µL of biological extract (Lorenz *et al.*, 2007), 1.25 mL (Gyani *et al*., 2021) of Wade reagent was used in both the preparation of standard graph as well as the determination of phytic acid in the biological samples. Moreover, it can also change with the change in the composition of ferric chloride concentration in the Wade reagent preparation.

In the case of biological samples, if faint pink colour appears, it indicates that the phytate concentration in the volume of the extract taken almost saturated or consumed or reduced most of the iron ions available in the Wade reagent. Hence, it is better to either decrease the volume of extract or dilute the extract to get dependable values or increase the volume of Wade reagent in both biological sample as well as the standard graph (the volume of the reagent, total volume of the reaction mixture must be same in all the gradations of the standard and biological samples). In our case, in some biological samples, even the faint pink colour that formed after adding the Wade reagent disappeared within 15 minutes of the incubation period.

The proportion of phytate-P in phytate was identified as 28.2% while cultivating soya bean at low nutrient P in pots (Raboy and Dickinson, 1984). This difference was converted into factor 3.55 to estimate phytic acid-P in maize (Lorenz *et al*., 2007; Nadeem *et al*., 2011; Abhijith *et al*., 2020), rice (Liu *et al*., 2004), Siberian oil seed (Russo and Reggiani, 2013; Colombini *et al*., 2014), in hemp seed (Russo and Reggiani, 2013), Marijuana (Galasso *et al*., 2016), Wheat (Safar-Noori *et al*., 2018) and Soya bean (Taliman *et al*., 2019). However, unlike maize where this factor 3.55 was determined, the factor is being applied in other crops too. Hence, although the global soils are deficient in phosphorus, it is also important to conduct studies on the relationship between phosphorous application and its uptake in other crops to know the applicability of this factor.

Conclusion

The standard graph range for phytic acid from 50 to 550 µg can be used along with 1.5 mL of Wade reagent with a total reaction volume of 1.8 mL. If the colour of reaction mixture is unstable then the biological extract can be diluted or the initial quantity of the sample (weight) can be decreased to get a stable colour and dependable values. Further, the response of rice crop with graded levels of phosphate nutrition can be studied to decide on applicability of the factor 3.55 identified for soya bean.

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