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Research Article

Comparative study of phytic acid estimation by enzymatic and indirect assays in maize germplasm (*Zea Mays*.L)

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Abstract
Phytic acid is an abhorrent anti-nutritional factor ascertaining several micronutrient deficiencies to monogastric animals by their chelating ability. Hence, screening of germplasm accessions by a rapid assay is necessary to identify the potential donors for low phytic acid in maize. This compound involves in a complex pathway inhabiting the role of several genes, establishes the difficulties in the molecular screening and elaborates the importance of an initial rapid assay in facilitating the screening of germplasm resources. Although, several methods have been followed for phytic acid content estimation, the direct estimation of phytic acid by Megazyme kit is found to be more precise than any other methods adopted. This Megazyme kit utilizes the natural phytase enzyme to liberate the free phosphorous from the samples and measures the phytate phosphorous content. But, estimating by this kit increases the cost of estimation while going for screening of a large number of samples. Thus an alternate rapid method that estimates the phytic acid content with a similar efficiency to this Megazyme assay has to be followed to facilitate the screening in a larger population. Among all the known methods, the Indirect assay described by Davies and Reid (1979) is found to be rapid and easy to be carried out in the initial screening of germplasm resources. Hence, a comparative study of phytic acid content estimated by these two protocols in a set of fifty-eight lines were subjected to a chi-square and paired t test. The phytic acid estimated by direct assay ranged from 2.04 to 15.59 mg/g and by indirect assay the range was observed from 2.77 to 16.70 mg/g. Although there were minor variations, there was not much difference observed between the two protocols. The chi- square test revealed a perfect goodness of fit between the protocols (χ^2Calculated<χ^2 Table). Simultaneously, the paired t test between the means of phytic acid estimated from two protocols also exhibited a null difference (t calculated < t table) among them.

Thus, we can conclude that the rapid indirect assay described by Davies and Reid (1979) could be effectively followed for initial screening of large number of germplasm accessions to identify the spontaneous donors of low phytic acid content in maize.

Key words
Phytic acid, Megazyme kit, Indirect assay, paired t test and chi-square test

Introduction

Phytic acid in maize is an anti-nutrient compound. Due to its high polyanionic nature, it chelates the positively charged mineral cations like iron, zinc, calcium and phosphorous (Zhou *et al*.,1995). This reduces the absorbability of these minerals in monogastic animals due to the lack of phytase in their guts. Phytic acid in maize is accumulated in embryo unlike the other cereals (O'Dell *et al*.,1972), leading to its direct consumption in diet. Prominently, as 60 percent of the maize seeds is being fed by the poultry sector, it is an essential concern to breed for low phytic acid maize lines to combat the nutritional requirement in the world. Thus several breeding programs have been developed to reduce the phytic acid in maize (Raboy *et al*.,2000). Phytic acid has been a part of the branching myoinositol pathway that produces several polysaacharides like raffinose and galactose (Shi *et al*.,2005). This reveals the constraints in identifying the target genes to accomplish molecular screening programs. Hence, it is necessary to identify an easy and rapid protocol to facilitate the screening of germplasm resources for identifying low phytate maize lines. This standardized protocol should act as a supplementary method to ensure the composition of phytic acid in maize lines to screen the low phytic acid lines from a larger population. These lines can be further promoted to identify the key genes beneath this trait. Several methods have been described by the scientists for the estimation of the phytic acid content in maize and among them, the direct estimation by Megazyme enzymatic kit and the indirect estimation of phytate by Davies and Reid (1979) are the eminent protocols for estimating the amount of phytic acid content in maize. The first Megazyme enzymatic assay is a direct estimation of phytic acid by the use of crude

phytase enzyme and the latter method is an easy rapid in direct estimation of the ferric phytate complex given by Davies and Reid (1979). Considering both these protocols, it is necessary for the breeders to analyze the efficiency of the rapid indirect estimation as it is less laborious and reduces the cost of estimation of phytic acid per sample. Hence, this study was conducted to analyze the efficiency of the indirect estimation of phytic acid against the direct Megazyme analysis kit. For this study, a set of fifty-eight inbred lines (Table.1) was subjected to phytic acid estimation by both the protocols. Their results were compared and analysed by a chi-square and paired t test.

Materials and Methods

For analyzing the efficiency of the protocols, the phytic acid content was estimated in both of the Megazyme and Davies and Reid (1979) method in a set of fifty-eight lines (Table 1). The seeds of the fifty-eight entries were subjected to both of these protocols and their results were compared by means of a chi-square and paired t test.

Methodologies in Phytic acid estimation:

Reagents required for phytic acid estimation:

Ferric Ammonium Sulphate (FAS) - The stock solution was prepared freshly by dissolving 21.60 g of FAS in 100ml distilled water (2.16mg/ml). The working solution was prepared by mixing one part of stock solution and twenty-four parts of distilled water.

Chen's reagent for HIP assay – The calorimetric reagent for inorganic phosphorous was prepared by mixing 6N H_2SO_4: 2.5% Ammonium molybdate: 10% Ascorbic acid: H_2O in a proportion of 1:1:1:2 by volume (Chen *et al*. 1956).

Buffer solution for enzyme extraction – There are four steps involved in the preparation of buffer solution required for the phytase extraction from maize seeds.

Step 1. 11.50 g of acetic acid or 15ml of glacial acetic acid was added to 200ml distilled water and stirred until the acetic acid was completely dissolved. The volume was made to 1000ml with distilled water. The concentration of the prepared solution is 200mM.

Step 2. 16.40 g of sodium acetate or 27.20 g of Sodium acetate trihydrate was added to 200ml distilled water and stirred until the salt was completely dissolved. The volume was made to 1000ml with distilled water. The concentration of the prepared solution is 200mM.

Step 3. 10.50 ml of acetic acid solution or 14.80 ml of glacial acetic acid solution prepared in step 1 was mixed with 39.50ml of sodium acetate or sodium acetate trihydrate solution and the final volume was made to 100 ml with distilled water. The resultant stock buffer solution was having a pH of 5.2 with a concentration of 200mM.

Step 4. For 50mM acetate buffer solution, 100ml of stock buffer was added to 300ml distilled water. The resultant buffer was 50mM and its pH was 5.2.

Trichloroacetic acid (50% w/v) - 50 g of trichloroacetic acid was added to 60 mL of distilled water and dissolved by continuous stirring. The volume was made to 100 mL with distilled water.

Sodium Hydroxide (0.75M) – 3 g of sodium hydroxide pellets were dissolved in 40 ml distilled water. After the pellets were completely dissolved, the volume was made to 100 ml with distilled water.

Indirect method for phytic acid estimation

Davies and Hilary Reid (1979)

Ten milliliters of 0.5M HNO_3 was added to 0.5g of finely ground seed samples and kept on magnetic stirrer for about 3 hours. Two biological replicates were taken for each sample. It was then filtered through Whatman No.1 filter paper to obtain the extract. From each biological replicate (extract), two technical replicates of 0.2 ml were taken separately. To this 0.2 ml of the extract, 0.2 ml of FAS working solution was added in a 2ml centrifuge tube. Then the centrifuge tubes were kept in boiling water bath for 20 minutes. After the tubes got cooled, 1ml Iso Amyl Alcohol was added. To that, 0.02ml of Ammonium Thiocyanate (5g/50ml) was added for the color reaction to occur. The centrifuge tubes are then kept in centrifuge at 3000 rpm for 10 minutes. Finally, 0.2 ml of the supernatant was transferred to a well-plate and the color was read at 465 nm using a multi-mode reader.

Standards series. A series of standards were run along with each analysis. The standard stock solution was prepared by dissolving 50mg Sodium phytate (from rice) in 20ml distilled water and making the final the volume to 100ml with distilled water. The working solution was of 0.5mg/ml concentration.The series of standards for phytic acid from the working solution is given in Table 2.

Free inorganic phosphorous assay

The seeds were grounded to a fine powder and 0.5g of the flour obtained was extracted for 3 hours by adding 10 ml 0.5 M HNO_3at room temperature on a magnetic stirrer. It was then filtered through Whatman No. 1 filter paper to obtain the extract. One hundred microliter of extract was taken in 2ml centrifuge tubes. To this, freshly prepared 900 µl of Chen's reagent (6N H_2SO_4:2.5% ammonium molybdate: 10% ascorbic acid: H_2O [1:1:1:2, v/v/v/v]) was added (Chen *et al.,* 1956). After incubation of these tubes for 30 minutes in a boiling water bath, the blue coloured phosphomolybdate complex whose color intensity

is proportional to the free phosphate content is measured by using a spectrophotometer (λ = 660 nm).

Standard series. The stock standard solution (1M concentration) for phosphorous was prepared by dissolving 6.8045g KH_2PO_4 in 20ml distilled water. The final volume was made to 50ml with distilled water. To prepare the working standard solution (1mM), 50µl of stock solution was made to 50ml volume with distilled water. The series of standards for HIP assay from the working solution is given in Table 3.

Enzymatic estimation of phytic acid

Phytase extraction:Homogeneous seed of a wheat variety (HW2507) grown at IARI Regional Station, Wellington was used for the isolation and purification of phytase. Phytase was extracted from 500 g of whole meal flour using 5000 ml of extraction buffer (50 mM acetate buffer, pH 5.3). The samples were soaked in extraction buffer over night at 4°C and centrifuged at 4000 rpm for 30 minutes. The supernatant was collected and stored in 2ml centrifuge tubes at 4°C for convenience.

Phytate Estimation:The phytate content was estimated by a modified method of AOAC Method 986.11.

The seeds were ground to a fine powder and 0.5g of flour obtained was extracted for 3 hours using 15 ml 0.5 M HNO_3at room temperature on a magnetic stirrer. It was then filtered through Whatman No. 1 filter paper to obtain the extract. From this, 0.5 ml of extract was taken and appropriate volumes of 0.75M NaOH and water were added such that the pH becomes 6.0-7.0 (neutralized). One hundred micro liter of neutralized extract were used for the phytate assay To this neutralized extract, 0.6ml distilled water and 0.1ml crude phytase enzyme extracted from wheat seeds were added. The tubes were then kept in hot water bath of 50°C for two hours. After that, 0.4ml trichloroacetic acid was added to the tubes to stop the enzymatic reaction. The tubes were then centrifuged at 13000rpm for 10 minutes. 1ml of the supernatant from each centrifuge were taken and to that, 0.5ml of Chen's reagent (6N H_2SO_4:2.5% ammonium molybdate: 10% ascorbic acid: H_2O [1:1:1:2, v/v/v/v]) (Chen *et al.,* 1956) was added. After incubation for 30 minutes in a boiling water bath, the blue coloured phosphomolybdate complex proportional to the phosphate content was measured using aspectrophotometer (λ = 660 nm). To find the phytate phosphorous content a conversion factor was used which is given below.

Calculation of phytate phosphorous content. The total phosphorous content obtained through enzymatic estimation was in terms of phytate phosphorous. This was labelled as **A**. The free inorganic phosphorus content measured through HIP assay was labelled as **B**. The total phosphate content (**A**) is multiplied by 0.282 (the conversion factor) to get it in terms of inorganic phosphorus. This was labelled as **C**. By subtracting **B** from **C,** we found out the phytate content in terms of inorganic phosphorous. This was labelled as **D.** Now, the component **D** was divided by 0.282 to get the actual phytate content. The series of standards for Phytic acid by enzymatic assay from the working solution is given in Table 4.

Statistical Analysis:

Test for goodness of fit (Karl Pearson, 1900)

This test was used to know whether the given objects are segregating in a theoretical ratio or whether the two attributes are independent in a contingency table.

The expression for χ^2 –test for goodness of fit is calculated by the formula given below:

$$\chi^2 = \frac{(O-E)^2}{E}$$

where Oi = observed frequencies

Ei = expected frequencies

n = number of cells (or classes)

The table value of chi-square is viewed in n-1 degrees of freedom. If the table value is greater than the calculated value, then there is a perfect goodness of fit between the observed and the expected frequencies or samples.

Paired T- Test (William Sealy Gosset, 1908):

The paired t test is a statistical conformation of null difference between two sample means. This statistically conforms the similarity present among two samples and was analysed using Microsoft-office Excel.

Results and Discussion

A set of fifty-eight lines were analysed for their phytic acid content by two protocols *i.e* The direct enzymatic assay (Megazyme)and indirect assay (Davies and Reid,1979). The results of their estimations are given in Table 5. There was not much difference absorbed between the results of Enzymatic and the indirect estimation protocol. The phytic acid estimation by the direct protocol exhibited a range of 2.04 to 15.59 mg/g of phytic acid. The phytate phosphorous ranged from 0.57 to 4.83 mg/ g. The highest value of phytic acid was found in the line UMI 1005-1. The indirect protocol by Davies and Reid method gave similar results to that of the direct assay. The phytate phosphorous ranged from 0.80 to 4.63 mg/g. The phytic acid content by indirect assay ranged from 2.77 to 16.70 mg/g. The highest phytic acid accumulated entry by indirect assay was UMI-265. There were very few minor deviations from the

direct assay which could be overcome by replicating the samples under estimation (Table 5). Some false positive results were also observed from the enzymatic assays in replications and this indicates a non-uniform activity of the phytase enzyme which has to be kept in mind while estimating the phytate phosphorous by enzymatic assay.

In the indirect protocol given by Davies and Reid (1979) we assumed that all the ferric ions that were reduced from the definite amount of added ions were chelated with the phytic acid in the sample. The short comings or minor variations against the direct assay is due to the presence of other chemical compounds that chelates with the ferric ions. Hence, replicating the samples will help us to calculate the phytic acid content of the samples by indirect assay. The direct estimation of phytic acid by Megazyme Total phosphorous assay kit was found to be more precise due to the use of pure phytase enzyme (Lopez *et al*.,2017). The content estimated by this Megazyme kit uses crude phytase enzyme extracted from wheat seeds to liberate the phosphorous in maize samples. The wheat seeds were used for phytase extraction as only in the mature grains of rice and wheat, a prominent activity of phytase is observed.

Whereas, by the use of this kit only 50 samples can be analysed and this includes the replicates of the samples for estimation. Also the purchase of this kit for the estimation may increase the cost of phytate estimation per sample. Therefore, in order to reduce the time and cost, we need to go for an alternate protocol with similar efficiency to screen the larger number of samples in a population. Hence the indirect and direct methods were subjected to a comparative analysis. To conform the linearity of the results obtained by both the protocols a chi- square analysis was carried out. The detailed estimation of chi-square values estimated are given in Table 6. From these results, it showed that the calculated value was lesser than the table value. This indicates that the results obtained from both the estimations were nearly same. For further conformation, a paired t- test was also conducted between the phytic acid values obtained from both the protocols. This further showed a non-significant difference among them as the calculated t value was lesser than the table t value (Table 7). Thus, this study confirms that the phytic acid estimated by direct enzymatic and indirect assay was linearly correlated.
Considering all these results the phytic acid content estimated by the Indirect Davies and Reid method (1979) is more precise, less laborious, rapid with reduced cost and higher efficiency. Therefore, we can conclude that for analysing the phytic acid content in a large number of germplasm accessions, this indirect estimation by Davies and Reid method could be effectively adopted to identify the potential donors of low phytate in maize.

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Table 1. Genotypes used in the estimation of phytic acid in maize

No.	Genotype	No.	Genotype
1	Box No.1137-6	31.	UMI 614 A
2	IN-3	32.	UMI 679
3	IN-6	33.	UMI 687-A
4	IN-12	34.	UMI 779
5	UMI 113	35.	UMI 823
6	UMI 135	36.	UMI-857-1
7	UMI 158	37.	UMI 919-1
8	UMI 161	38.	UMI 955-2
9	UMI 163-3	39.	UMI 960-1
10	UMI 170-4	40.	UMI 1004
11	UMI 260	41.	UMI 1005-1
12	UMI 262	42.	UMI 1009-2
13	UMI 265	43.	UMI 1013-1
14	UMI 300-1	44.	UMI 1017
15	UMI 304	45.	UMI 1027
16	UMI 334-1	46.	UMI 1030
17	UMI 346-2 RS	47.	UMI 1031
18	UMI 351	48.	UMI 1036
19	UMI 363	49.	UMI 1054
20	UMI 375	50.	UMI 1100
21	UMI 447	51.	UMI 1101
22	UMI 467	52.	UMI 1105
23	UMI 473-1	53.	UMI 1110-1
24	UMI 504	54.	UMI 1112
25	UMI 507	55.	UMI 1113
26	UMI 510-1-2	56.	UMI 1124
27	UMI 51WS	57.	UMI 1126-1
28	UMI 550	58.	UMI 1156
29	UMI 607		
30	UMI 612		

Table 2. Standard series for the estimation of phytic acid by Davies & Reid method

Concentration (mg/ml)	Working Standard (ml)	0.5M HNO3 (ml)	Total volume (ml)
0.5	0.2	0	0.2
0.25	0.1	0.1	0.2
0.125	0.05	0.15	0.2
0.1	0.04	0.16	0.2
0.05	0.02	0.18	0.2
0.025	0.01	0.19	0.2
0	0	0.2	0.2

Table 3. Standard series for the estimation of Free inorganic phosphorous assay

1mM KH2PO4 (µl)	0.5M HNO3 (µl)	Total volume (µl)	P content (µg)
90	10	100	2.781
60	40	100	1.854
45	55	100	1.3905
30	70	100	0.927
10	90	100	0.309
5	95	100	0.1545
0	100	100	0.00

Table 4. Standard series for the estimation of Phytic acid by enzymatic assay

Concentration (mg/ml)	Working Standard (µl)	0.5M HNO3 (µl)	Total volume (µl)
0.5	100	0	100
0.25	50	50	100
0.125	25	75	100
0.1	20	80	100
0.05	10	90	100
0	0	100	100

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Table 5. Results of the phytic acid contents obtained from both of the protocols

Genotype	Enzymatic Assay (mg/g)			Davies & Reid Method (mg/g)			HIP assay (mg/g)
	Total P	Phyt P	PA	Total P	Phyt P	PA	Free P
1137-6	3.42	3.00	10.64	3.05	2.63	9.33	0.42
Inbred 3	1.30	1.01	3.58	2.41	2.12	7.51	0.29
Inbred 6	0.73	0.57	2.04	1.73	1.58	5.59	0.15
Inbred 12	2.20	1.92	6.80	2.05	1.77	6.26	0.29
UMI 51 WS	3.87	3.49	12.38	3.64	3.26	11.55	0.38
UMI 113	2.45	2.20	7.82	1.05	0.80	2.77	0.26
UMI 135	2.85	2.51	8.91	3.13	2.80	9.91	0.34
UMI 158	2.40	2.18	7.74	2.12	1.91	6.78	0.21
UMI 161	5.09	4.83	17.12	4.52	4.25	15.07	0.23
UMI 163-3	2.21	1.90	6.74	3.00	2.69	9.53	0.31
UMI 170-4	3.23	2.96	10.49	2.54	2.27	8.04	0.27
UMI 260	3.08	2.77	9.83	3.57	3.26	11.57	0.31
UMI 262	3.74	3.46	12.26	3.05	2.77	9.82	0.28
UMI 265	1.77	1.36	4.84	5.12	4.71	16.70	0.41
UMI 300-1	2.57	2.06	7.31	1.40	0.89	3.17	0.51
UMI 304	3.91	3.53	12.51	4.29	3.92	13.89	0.38
UMI 334-1	3.38	3.07	10.87	2.99	2.68	9.51	0.31
UMI 346-2 RS	3.59	3.06	10.86	3.18	2.64	9.37	0.53
UMI 351	3.12	2.46	8.73	3.17	2.51	8.89	0.66
UMI 363	2.99	2.57	9.12	3.37	2.95	10.48	0.42
UMI 375	3.04	2.60	9.21	3.78	3.33	11.81	0.45
UMI 447	2.24	1.97	6.99	2.46	2.19	7.78	0.27
UMI 467	2.53	1.84	6.51	2.23	1.15	5.50	0.69
UMI 473-1	3.50	3.09	10.96	4.75	4.33	15.37	0.41
UMI 504	3.56	3.11	11.04	2.96	2.51	8.90	0.45
UMI 507	4.48	4.12	14.60	3.53	3.18	11.26	0.36
UMI 510-2-2	0.29	0.12	0.44	2.55	2.39	8.48	0.17
UMI 550	2.73	2.49	8.83	3.00	2.76	9.80	0.24
UMI 607	3.29	2.98	10.56	3.86	3.55	12.60	0.32
UMI 612	3.14	2.90	10.30	3.45	3.22	11.41	0.24
UMI 614 A	3.68	3.20	11.36	3.26	2.79	9.88	0.48
UMI 679	3.48	3.20	11.35	3.09	2.81	9.95	0.28
UMI 687-1	3.59	3.07	10.88	4.04	3.52	12.48	0.52
UMI 779	4.46	3.87	13.73	5.23	4.63	16.41	0.59
UMI 823	3.41	3.18	11.27	3.03	2.79	9.90	0.24
UMI 857-1	3.86	3.49	12.37	4.62	4.25	15.07	0.37
UMI 919-1	2.99	2.71	9.62	2.86	2.59	9.17	0.28
UMI 955-2	3.92	3.56	12.61	3.90	3.53	12.51	0.37
UMI 960-1	3.18	2.64	9.36	3.15	2.62	9.28	0.54
UMI 1004	2.45	2.21	7.83	2.70	2.45	8.69	0.24
UMI 1005-1	4.69	4.40	15.59	3.40	3.11	11.02	0.29
UMI 1009-2	4.03	3.80	13.48	3.57	3.34	11.86	0.23
UMI 1013-1	2.90	2.52	8.94	2.88	2.50	8.87	0.38
UMI 1017	2.41	1.98	7.03	2.79	2.36	8.38	0.43
UMI 1027	3.55	3.08	10.92	3.28	2.80	9.94	0.47
UMI 1030	3.16	2.82	10.02	2.80	2.47	8.74	0.34
UMI 1031	2.97	2.59	9.19	2.63	2.25	7.99	0.38
UMI 1036	3.39	3.20	11.34	3.01	2.81	9.97	0.20
UMI 1054	2.54	2.27	8.04	2.79	2.52	8.94	0.27
UMI 1100	2.58	2.37	8.39	2.26	2.05	7.25	0.22
UMI 1101	2.57	2.32	8.22	3.04	2.78	9.87	0.26
UMI 1105	2.42	2.03	7.19	2.93	2.54	8.99	0.39
UMI 1110-1	3.88	3.45	12.23	4.26	3.83	13.59	0.42
UMI 1112	2.96	2.64	9.38	3.25	2.94	10.42	0.31
UMI 1113	4.33	4.05	14.36	3.83	3.56	12.62	0.27
UMI 1124	2.41	2.24	7.95	2.48	2.31	8.20	0.17
UMI 1126-1	4.42	4.08	14.46	3.93	3.58	12.68	0.34
UMI 1156	3.46	3.17	11.23	3.07	2.77	9.83	0.30

Total P: Total phosphorous (mg/g), Phyt P: Phytate phosphorous (mg/g), PA: Phytic acid (mg/g), Free P: Free phosphorous (mg/g)

Table 6. Chi-square test for goodness of fit

S.No	Genotype	Observed (O)	Expected (E)	$\chi^2 = \frac{(O-E)^2}{E}$
1	1137-6	10.64	9.33	0.18
2	Inbred 3	3.58	7.51	2.06
3	Inbred 6	2.04	5.59	2.26
4	Inbred 12	6.80	6.26	0.05
5	UMI 51 WS	0.44	8.48	7.62
6	UMI 113	12.38	11.55	0.06
7	UMI 135	7.82	2.77	9.16
8	UMI 158	8.91	9.91	0.10
9	UMI 161	7.74	6.78	0.14
10	UMI 163-3	17.12	15.07	0.28
11	UMI 170-4	6.74	9.53	0.82
12	UMI 260	10.49	8.04	0.75
13	UMI 262	9.83	11.57	0.26
14	UMI 265	12.26	9.82	0.61
15	UMI 300-1	4.84	16.70	8.42
16	UMI 304	7.31	3.17	5.41
17	UMI 334-1	12.51	13.89	0.14
18	UMI 346- 2 RS	10.87	9.51	0.19
19	UMI 351	10.86	9.37	0.24
20	UMI 363	8.73	8.89	0.00
21	UMI 375	9.12	10.48	0.18
22	UMI 447	9.21	11.81	0.57
23	UMI 467	6.99	7.78	0.08
24	UMI 473-1	6.51	5.50	0.19
25	UMI 504	10.96	15.37	1.27
26	UMI 507	11.04	8.90	0.51
27	UMI 510-2-2	14.60	11.26	0.99
28	UMI 550	10.64	9.33	0.18
29	UMI 607	8.83	9.80	0.09
30	UMI 612	10.56	12.60	0.33
31	UMI 614 A	10.30	11.41	0.11
32	UMI 679	11.36	9.88	0.22
33	UMI 687-1	11.35	9.95	0.20
34	UMI 779	10.88	12.48	0.20
35	UMI 823	13.73	16.41	0.44
36	UMI 857-1	11.27	9.90	0.19
37	UMI 919-1	12.37	15.07	0.48
38	UMI 955-2	9.62	9.17	0.02
39	UMI 960-1	12.61	12.51	0.00
40	UMI 1004	9.36	9.28	0.00
41	UMI 1005-1	7.83	8.69	0.09
42	UMI 1009-2	15.59	11.02	1.90
43	UMI 1013-1	13.48	11.86	0.22
44	UMI 1017	8.94	8.87	0.00
45	UMI 1027	7.03	8.38	0.22
46	UMI 1030	10.92	9.94	0.10
47	UMI 1031	10.02	8.74	0.19
48	UMI 1036	9.19	7.99	0.18
49	UMI 1054	11.34	9.97	0.19
50	UMI 1100	8.04	8.94	0.09
51	UMI 1101	8.39	7.25	0.18
52	UMI 1105	8.22	9.87	0.28
53	UMI 1110-1	7.19	8.99	0.36
54	UMI 1112	12.23	13.59	0.14
55	UMI 1113	9.38	10.42	0.10
56	UMI 1124	14.36	12.62	0.24
57	UMI 1126-1	7.95	8.20	0.01
58	UMI 1156	14.46	12.68	0.25
		χ^2Calculated		**49.75**
		χ^2 table		**75.62**

Table 7. Paired t test between the phytic acid contents estimated by both protocols

	Direct assay	Indirect Assay
Mean	9.79	10.02
Variance	9.49	7.95
Observations	58	58
Pearson Correlation	0.56	
Hypothesized Mean Difference	0	
Df	57	
t Stat	-0.60	ns
P(T<=t) one-tail	0.27	
t Critical one-tail	1.67	
P(T<=t) two-tail	0.55	
t Critical two-tail	2.00	

***ns: non-significant**