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VALIDATION STUDY OF A RAPID COLORIMETRIC METHOD FOR THE DETERMINATION OF PHYTIC ACID AND INORGANIC PHOSPHORUS FROM SEEDS

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Phytate, as an important mineral storage compound in seeds, is vital for seed/grain development; it is often considered to be an antinutritional substance. The objective of this study was to develop a rapid and inexpensive colorimetric method of measuring phytate and inorganic P (P_i) concentrations from maize, soybean and sunflower seed/grain extracts, by combining adequate precision and simplicity, ideal for breeders interested in improving simultaneously P_i and phytate levels. The investigated extraction mediums: double distilled (DD) H_2O, 2.4 % HCl and 5 % trichloracetic acid (TCA) were proved to be suitable for the analysis of phytic acid and inorganic phosphorus in seed extracts. The advantages of 5 % TCA over to DD H_2O and 2.4 % HCl were reflected through the low limit of detection for both phytic acid and P_i and good recovery with low bias. A low detection limit for P_i is important for samples with naturally low P_i concentrations, such as soybean seeds.

KEY WORDS: phytic acid, inorganic phosphorus, seed, validation

INTRODUCTION

A very important mineral storage compound in seeds is phytate, a mixed cation salt of phytic acid (*myo*-inositol hexakis phosphoric acid). This compound is important for several reasons: it is vital for seed/grain development and successful seedling growth; it is often considered to be an antinutritional substance in diets, but it may have a nutritional role as an anti-oxidant agent; it represents a very significant amount of phosphorus being extracted from soils, and plays a role in eutrophication of waterways (1). Phosphorus bound in phytate is nutritionally unavailable to monogastric animals. Also, phytate can chelate certain minerals and exacerbate human mineral deficiencies.

In plant seed physiology, a novel role was assigned to phytic acid, *i.e.*, as protection against oxidative stress during the seed's life span. As in maize kernels, the greater part of phytic acid (and thus of metal ions) is concentrated in the embryo; thus its antioxidant action may be of particular relevance in this crop (2). Phytic acid and its derivatives are

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also implicated in RNA export, DNA repair, signalling, endocytosis, and cell vesicular trafficking (3). Phytate has an important antioxidant function in seeds during dormancy and it may be a substitute for the presently employed preservatives, many of which pose potential health hazards (4).

Our primary objective was to develop a rapid and inexpensive method for measuring phytate and inorganic P (P_i) concentrations in maize, soybean and sunflower grain. The combination of adequate precision and simplicity make the proposed method ideal for breeders interested in simultaneously improving P_i and phytate levels. Gas chromatography determinations show better sensitivity (5), but the analytical procedure lasts long. Also, suppressed conductivity, ion chromatography (6) had linearity over a wider range, with low detection limits for phytic acid and *Pi*. Veličković *et al.* (7) underlined the advantage of methods requiring reduced amounts of sample and reagents, with shorter extraction times, with no significant influence on accuracy.

Presently, most assays for phytic acid employ ferric chloride to precipitate ferric phytate. The precipitate may be digested and analyzed for either phosphorus or iron. Alternatively, sodium hydroxide may be used to convert the precipitate to sodium phytate and ferric hydroxide. It is convenient to analyze for iron after taking up ferric hydroxide in acid. Determination of residual iron or phosphorus in the supernatant after precipitation is also a rapid method (8). The addition of orthophosphate (up to 20 μg/ml of phosphorus) and chlorogenic acid (up to 50 μg/ml) does not impair the colorimetric assay of phytate based on the decolouration of the Fe^{3+}-sulphosalicylate complex (9).

The intention of this study was to readjust the colorimetric analytical procedure for phytic acid and inorganic phosphorus determination from the same seed extract, and to simplify and accelerate it for the analysis of a large number of samples.

EXPERIMENTAL

Preparation of samples

The seeds of four maize hybrids (ZPSC 704; ZPSC 836; ZPSC 341; ZPSC 666), five soybean varieties (Lidija; Olga; ZP 015; Lana; Nena) and five sunflower lines (KOZ/11; L/4/RU/4; PP/34; ALV/12; AZ/20) were ground in a Tecator Knifetec 1095 sample mill. The obstruction in the analytical procedure, due to the relatively high lipid contents in soybean and sunflower seeds, was surpassed by their removal by extraction with petroleum ether (40–60°C) during 14 hours, similar to the gas-chromatography procedure (5). Thus, the lipids were removed from soybean and sunflower flour.

Reagents and standards

The following reagents were used for the extraction of phytic acid and inorganic phosphorus: double distilled (DD) H_2O, 2.4 % HCl and 5 % trichloroacetic acid (TCA). The following reagents for determination were used for phytic acid: Wade reagent 0.3 g $FeCl_3$ $6H_2O$ + 3 g 5'-sulphosalicylic acid in one litre (this reagent can be stored for a long time in a dark bottle), and the reagent for inorganic phosphorus determination: 30 g of

ammonium heptamolybdate was dissolved in 500 mL of DD H_2O at 60 °C; 0.75 g of ammonium metavanadate was dissolved in 300 mL DD H_2O, after cooling 150 mL of HNO_3 was added and then the resulting solution was made up 500 mL with *DD* H_20. The ammonium heptamolybdate solution is then added with slow stirring into the ammonium metavanadate solution. Stock standard solutions: phytic acid (dodecasodium salt, from maize, Sigma P-8810) was made up to 200 mL DD H_2O (the concentration of phytic acid in the solution was 0.5 mg mL^{-1}) and KH_2PO_4 (Sigma P-5379) was made up to 50 mL *DD* H_2O (the concentration of phosphorus in the solution was 0.25 mg P_2O_5 mL^{-1}) and several drops of chloroform were added.

A series of standard phytic acid solutions was made from the stock standard solution by appropriate dilutions, with addition of extraction solutions, to simulate conditions similar to those in the samples: 17 mL of 5 % TCA or 6 mL of 2.4 % HCl. The concentrations of phytic acid in this series were as follows: 1; 5, 10; 15; 20; 25; 30; 35; 40 μmol 100 mL^{-1}. A series of standard inorganic phosphorus (P_i) solutions was made from the stock standard solution by appropriate dilutions to give the concentrations: 7.7; 15.5; 23.2; 31.0; 38.7; 45.5; 54.2; 62.0; 69.7; 77.4 nmol P_2O_5 100 mL^{-1}, with appropriate addition of extraction solutions: 50 mL of 5 % TCA or 2.4 % HCl (for maize and soybean), or 3 mL of extraction solution (for sunflower).

Extraction of phytic acid

A meal sample (0.25 g) was placed in an extraction flask and 10 mL of extraction solution was added. The flask was stoppered and shaken for 1 h on a mechanical shaker. Kwanyuen and Burton (10) obtained the best results with 1 h extraction of phytate from soybean meal. 5 mL of the extract were placed into a centrifuge tube and then centrifuged on an Eppendorf Centrifuge 5417R at 12,000 *g* for 15 minutes at 4°C. The method precision was tested by the spike procedure, with the addition of 0.75 ml of phytic acid stock or 50 μL of KH_2PO_4 stock to 5 mL of sample, before centrifuging. Six replicates were performed in all cases.

Colorimetric determination of the PA and P_i contents

The supernatant was diluted to an appropriate concentration suitable for the analysis; for the phytic acid determinations the dilution was 1:5 for maize, 1:16 for soybean and 1:30 for sunflower; for the P_i determinations, the dilutions were 1:1 for maize and soybean and 1:30 for sunflower. Then, to 1.5 mL aliquots of Wade reagent was added 0.5 mL for PA determination, or to 2 mL aliquots 0.5 mL of P_i reagent (ammonium heptamolybdate + ammonium metavanadate). Subsequently, the samples were centrifuged at 12,000 *g* for 10 minutes. The absorbance was read on a Shimadzu Spectrophotometer UV-1601, at λ = 500 nm for phytic acid and λ = 400 nm for P_i.

Minitab 14 (Minitab, 2004) was used for all statistical and graphical analyses.

RESULTS AND DISCUSSION

The lowest detection limit for *PA* (Table 1), with a low variation between the batches, was obtained with *DD* H_2O as the extraction medium. The lowest detection limit for inorganic phosphorus was obtained with 5 % TCA, but smaller variations were found for the *DD* H_2O batches. The obtained detection limits for *PA* and P_i are lower than those found using suppressed conductivity ion chromatography (6), where the detection limits for *PA* and P_i were 3.5 and 1.5 mg L^{-1}. Kikunaga *et al.* (12) denoted that detection limit for *PA* from small grain cereals, by using of HCl as extraction medium was 0.025 μmol mL^{-1}, while Gao *et al.* (13) found that the detection range for *PA* from soybean meal depends on applied method: for colorimetric method it was 1.91-6.20 mg g-1, for anion exchange column 1.35-7.28 mg g^{-1}, for HPLC 1.02-5.92 mg g^{-1}. It could be assumed that high acidity of the media provided for protein precipitation (12; 14), which could obstruct the later analytical procedure and could be crucial for soybean and sunflower seeds, which are rich in proteins. The use of an adequate amount of a suitable extraction medium reduced the extraction process to only 1 hour, which accelerated the analytical process compared to the method of Lorenz *et al.* (14), in which the extraction procedure lasted about 12 hours. The results obtained by Jočić (11) showed that multiple extraction of *PA* with 5 % TCA from maize meal is unnecessary and that 1 hour is sufficient to extract most of the *PA* from the maize meal. *PA* insoluble in water makes only 2.30-4.70% of total *PA* amount, while it is fully extracted with TCA as extraction medium.

Table 1. Limits of detection of phytic acid (PA) and inorganic phosphorus (P_i) in different extraction mediums

	PA / μmol ml^{-1} ± SD	**P_i / nmol ml^{-1} ± SD**
DD H_2O	0.00307 ± 0.00019	0.00069 ± 0.00014
5 % TCA	0.00416 ± 0.00025	0.00024 ± 0.000048
2.4 % HCl	0.00926 ± 0.00056	0.00026 ± 0.000053

The results of the linearity test for *PA* indicated that the standard curve was linear in the range of 0.05 – 0.30 μmol phytic acid ml^{-1} for DD H_2O and 5 % TCA as the extraction media (Figure 1), with a higher slope of 1.030 obtained using the TCA medium. A similar situation was present for the linearity test for P_i, for which the standard curve was linear in the range of 0.077 – 0.387 nmol P_2O_5 ml^{-1} for DD H_2O and 5 % TCA, with no remarkable difference in the slope value. Only the standard curves obtained when 2.4 % HCl was used as the extraction medium were linear in the ranges 0.05 – 0.20 μmol *PA* ml^{-1} and 0.155 – 0.387 nmol P_2O_5 ml^{-1}, which made this medium inapplicable for the P_i determination in samples with a low P_i content, such as in maize seeds.

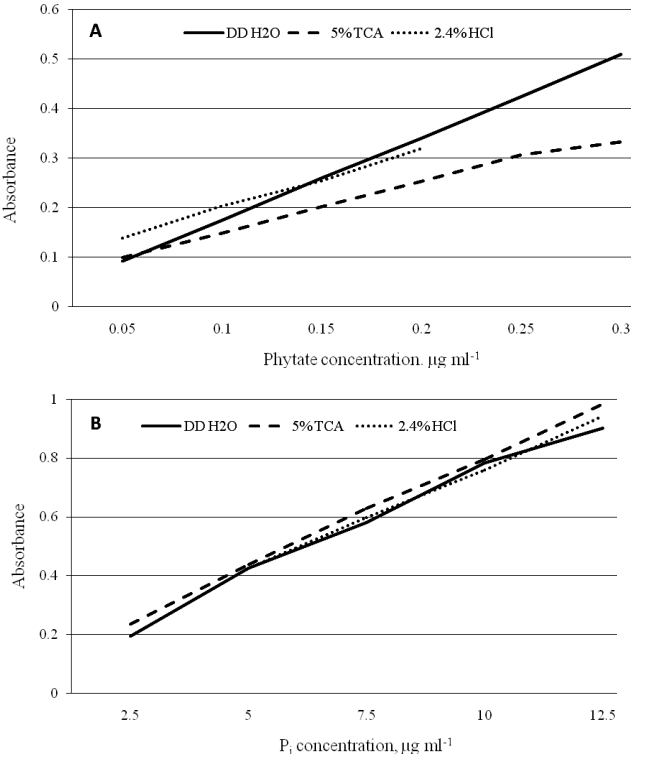

Figure 1. The linearity test of standard curve for phytic acid (A) and P_i (B) determination in DD H_2O, 5 % TCA and 2.4 % HCl, as extraction mediums

Table 2. Method accuracy for phytic acid determination in maize seed's extract, using DD H_2O and 5 % TCA as extraction mediums

	Sample	Nominal value* (nmol ml^{-1})	Recovery range (%)	Bias	CV[a] (%)
DD H_2O	*ZPSC 704*	0.1246	98 - 104	2.73	1.92
	ZPSC 836	0.1237	99 - 102	0.66	3.48
	ZPSC 341	0.3457	97 - 103	-0.12	0.87
	ZPSC 666	0.2445	98 - 103	1.18	0.65
5% TCA	*ZPSC 704*	0.1251	98 - 100	-1.63	2.28
	ZPSC 836	0.1014	96 - 100	-1.94	1.70
	ZPSC 341	0.3427	99 - 101	-0.52	1.04
	ZPSC 666	0.2386	99 - 102	1.18	1.67

* Least significant difference, Student's *T*-test, $P = 0.05$ (LSD = 0.0946)

[a] Coefficient of variation

The accuracy achieved by an analytical method is often evaluated by calculating the value for recovery, bias, and coefficient of variation. The results presented in Tables 2 and 3 indicate that there were no significant differences between the values of the content of *PA* and P_i in the maize sample extracts obtained using f DD H_2O and 5 % TCA. The *PA* amount in the DD H_2O extracts was 0.1237 – 0.3457 μmol ml^{-1}, while in the TCA extracts it was 0.1014 – 0.3427 μmol ml^{-1} (Table 2). Jočić (11) ascertained that there was no significant difference in the amount of *PA* soluble in DD H_2O and 5 % TCA extracted from maize seeds. The same author obtained 1.42-2.87 mg of total *PA* per maize seed by TCA extraction, while it was 1.25-2.84 mg of total PA per maize seed by extraction with deionised water. The P_i amount in the DD H_2O extracts was 9.385 – 17.458 nmol ml^{-1}, while in the TCA extracts it was 10.120 – 17.594 nmol ml^{-1} (Table 3).

Table 3. Method accuracy for P_i determination in maize seed's extract, using DD H2O and 5 % TCA as extraction mediums

	Sample	Nominal value* (nmol ml^{-1})	Recovery range (%)	Bias	CV[a] (%)
DD H_2O	*ZPSC 704*	14.832	98 – 101	-0.39	1.04
	ZPSC 836	17.458	96 – 102	-1.31	2.00
	ZPSC 341	9.385	97 – 100	-1.32	3.52
	ZPSC 666	12.672	98 - 104	2.77	2.91
5% TCA	*ZPSC 704*	15.397	100 – 101	0.39	1.55
	ZPSC 836	17.594	99 - 101	-0.17	2.00
	ZPSC 341	10.120	99 - 101	0.35	2.72
	ZPSC 666	12.300	100 - 101	1.01	1.60

* Least significant difference, Student's *T*-test, *P* = 0.05 (LSD = 2.397)

[a] Coefficient of variation

The differences between the samples, *i.e.*, maize hybrids, in terms of *PA* and P_i contents indicated that early maturity groups, *i.e*., smaller size seeds (such as ZPSC 341) had a higher *PA* content and *vice versa*, a lower P_i content, which is in agreement with experimental data of other authors (14). The values of the recovery range, closest to 100 %, as well as the lowest variations between sample replications for *PA* and P_i, indicate that 5 % TCA was a better extraction medium for maize seeds, irrespective of the fact that bias values were negligibly higher for *PA* when 5 % TCA was used as the extraction medium. It is important to emphasise that 2.4 % HCl was not an appropriate extraction medium for *PA* extraction from maize seeds, without neutralisation, which could prolong the analytic process.

The amount of *PA* in the DD H_2O extracts of soybean was 0.0135-0.0173 μmol ml^{-1}, and in the TCA extracts, it was 0.0139 – 0.0172 μmol ml^{-1}, while in the HCl extracts the values were higher, 0.069-0.0210 μmol ml^{-1} (Table 4).

Table 4. Method accuracy for phytic acid determination in soybean seed's extract, using DD H_2O, 5 % TCA and 2.4 % HCl as extraction mediums

	Sample	Nominal value* (nmol ml^{-1})	Recovery range (%)	Bias	CV[a] (%)
DD H_2O	Lidija	0.0135	99 – 102	0.85	4.90
	Olga	0.0152	99 – 101	0.06	4.46
	ZP 015	0.0160	100 – 102	1.17	2.48
	Lana	0.0140	100 – 101	0.84	5.13
	Nena	0.0173	99 - 102	0.85	5.32
5% TCA	Lidija	0.0139	99 – 102	0.48	3.62
	Olga	0.0154	97 – 102	0.68	3.72
	ZP 015	0.0169	97 - 102	0.73	2.69
	Lana	0.0145	99 – 101	0.23	2.27
	Nena	0.0172	98 - 101	-0.06	3.40
2.4% HCl	Lidija	0.0210	68 – 115	-9.81	4.57
	Olga	0.0165	84 – 125	-0.62	6.17
	ZP 015	0.0169	75 – 115	-4.57	5.12
	Lana	0.0149	97 – 105	-0.56	5.27
	Nena	0.0168	74 - 106	-6.55	5.45

* Least significant difference, Student's *T*-test, $P = 0.05$ (LSD = 0.0158)

[a] Coefficient of variation

Using all three extraction media for phytic acid extraction from soybean seeds indicated a statistically significant higher *PA* content in the samples of the Lidija variety when 2.4 % HCl was employed as extraction medium. It is also noticeable that the soybean HCl extracts had the highest range of recovery of 68 – 115%, with a high bias and variation between the batches. Moreover, the P_i determination from soybean by using of DD H_2O as the extraction medium could not be performed because of the precipitation caused by the high acidity of the reagent used for P_2O_5 analysis, which was hard to remove by centrifugation.

The P_i amount in the soybean TCA extracts was 0.0064 – 0.0090 nmol ml^{-1}, while it in the HCl extracts was 0.0047 – 0.0066 nmol ml^{-1} (Table 5). There was no statistical difference between the P_i values found in the extracts obtained using of 5 % TCA and 2.4 % HCl. Considering recovery, the best values were obtained using DD H_2O as the extraction medium for *PA* (99 – 102 %, Table 4) and using 5 % TCA for P_i extraction (98 – 103 %, Table 5). It was also noticeable that the bias values for both *PA* and P_i were the lowest in the TCA extracts and they were in the range of 0.06 – 0.73 for *PA* and 0.004 – 3.96 for P_i. Furthermore, the coefficient of variation for the TCA extracts was below 5 %, with the exception of that with the soybean variety Olga, where it was 5.05 %.

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Table 5. Method accuracy for P_i determination in soybean seed's extract, using 5 % TCA and 2.4 % HCl as extraction mediums

	Sample	Nominal value* (nmol ml^{-1})	Recovery range (%)	Bias	CV[a] (%)
5% TCA	Lidija	0.0090	99 – 103	3.96	3.94
	Olga	0.0069	98 – 101	0.004	5.05
	ZP 015	0.0064	100 – 102	3.56	4.83
	Lana	0.0089	98 – 102	2.24	2.04
	Nena	0.0067	98 - 101	0.27	4.27
2.4% HCl	Lidija	0.0066	85 – 89	-2.03	6.32
	Olga	0.0047	84 – 95	-11.24	6.58
	ZP 015	0.0047	80 – 96	-8.93	10.68
	Lana	0.0053*	95 - 102	-1.03	9.40
	Nena	0.0040	91 - 95	-6.07	6.77

* Least significant difference, Student's *T*-test, $P = 0.05$ (LSD = 0.0025)

[a] Coefficient of variation

Owing to the high dilution of the sunflower seed extracts, relatively low *PA* values were obtained, 0.0264 – 0.0341 μmol ml^{-1}, in the TCA extracts and 0.0287 – 0.0348 μmol ml^{-1} in the HCl extracts (Table 6), with no significant difference between the extraction media. A better recovery, of 99 – 100 %, and bias values below 1 (with the exception of the extracts of the variety AZ/20, for which the value was 1.44) showed that 5 % TCA was a better extraction medium. The coefficients of variation for the measurements obtained using 5 % TCA as the extraction medium were somewhat higher compared to those obtained using 2.4 % HCl. However, they also indicate good precision for both extraction media, since all of the values obtained were below 5 %. A high dilution of the sunflower extracts was also important for good precision of the P_i analysis. Diluting and pH buffering of the extracts may improve the results, since the recovery of phytate was increased when the extract pH was closer to 6.0 (9).

Table 6. Method accuracy for phytic acid determination in sunflower seed's extract, using 5 % TCA and 2.4 % HCl as extraction mediums

	Sample	Nominal value* (nmol ml^{-1})	Recovery range (%)	Bias	CV[a] (%)
5% TCA	KOZ/11	0.0341	100 – 102	0.37	2.58
	L/4/RU/4	0.0264	99 – 100	0.03	2.96
	PP/34	0.0316	99 – 102	0.61	3.76
	ALV/12	0.0327	99 – 102	0.74	1.82
	AZ/20	0.0328	99 - 102	1.44	2.24
2.4% HCl	KOZ/11	0.0348	96 – 109	2.26	1.80
	L/4/RU/4	0.0289	98 – 106	0.90	4.05
	PP/34	0.0299	97 – 100	-0.02	0.65
	ALV/12	0.0310	88 – 106	-1.69	1.18
	AZ/20	0.0287	89 - 107	-1.97	1.85

* Least significant difference, Student's *T*-test, $P = 0.05$ (LSD = 0.1168)

[a] Coefficient of variation

Table 7. Method accuracy for P_i determination in sunflower seed's extract, using 5 % TCA and 2.4 % HCl as extraction mediums

	Sample	Nominal value* (nmol ml^{-1})	Recovery range (%)	Bias	CV[a] (%)
5% TCA	KOZ/11	0.0028	99 – 101	0.22	2.31
	L/4/RU/4	0.0026	98 – 101	0.93	2.74
	PP/34	0.0029	99 – 102	0.96	1.71
	ALV/12	0.0030	99 – 101	-0.35	2.04
	AZ/20	0.0031	99 - 101	0.51	4.27
2.4% HCl	KOZ/11	0.0032	99 – 102	0.41	1.08
	L/4/RU/4	0.0027	99 – 101	0.03	2.05
	PP/34	0.0031	99 – 108	2.17	2.83
	ALV/12	0.0034	97 – 104	1.02	1.29
	AZ/20	0.0035	98 - 103	0.88	2.12

* Least significant difference, Student's *T*-test, $P = 0.05$ (LSD = 0.0225)

[a] Coefficient of variation

Besides, there was no statistical difference between the values of the P_i content in the TCA and HCl extracts; they were 0.0026 – 0.0031 nmol ml^{-1} in the TCA extracts and 0.0027 – 0.0035 nmol ml^{-1} in the HCl extracts (Table 7). Recovery values in the range of 99 – 102 %, as well as the bias values under 1, showed that 5 % TCA was a better extraction medium, irrespective of the lower coefficients of variation obtained in the samples obtained with 2.4 % HCl.

CONCLUSION

The investigated extraction media proved to be suitable for the determination of the *PA* and P_i contents in maize, soybean and sunflower seeds. The advantages of 5 % TCA for the seeds of all three examined species over DD H_2O and 2.4 % HCl, were reflected through the low limit of detection for *PA* and P_i, and good recovery with low bias values. A low limit of detection for P_i is important for the samples which naturally have low P_i levels, such as soybean seeds. The negligibly lower coefficients of variation obtained for the HCl extracts of sunflower seeds, in comparison to the TCA extracts could be overcome by increasing the number of replicates, which could be alternatively considered because the coefficients of variations for both extraction media were below 5 %.

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СТУДИЈА ВАЛИДАЦИЈЕ БРЗЕ КОЛОРИМЕТРИЈСКЕ МЕТОДЕ ЗА ОДРЕЂИВАЊЕ ФИТИНСКЕ КИСЕЛИНЕ И НЕОРГАНСКОГ ФОСФОРА У СЕМЕНУ

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Фитат је важан за развој семена и служи за складиштење минерала. Он се углавном сматра за антинутритивну супстанцу. Циљ истраживања је био да се развије брза и јефтина колориметријска метода за мерење концентрације фитата и неорганског фосфора (P_i) из екстракта семена кукуруза, соје и сунцокрета, комбинујући одређену прецизност и једноставност, идеалне за селекционере заинтересоване за истовремено побољшање односа P_i и фитата у семену. Испитивана екстракциона средства: бидестилована вода (DD H_2O), 2,4 % HCl и 5 % трихлорсирћетна киселина (TCA) су се показале погодним за анализу фитинске киселине и P_i из екстракта семена. Предност 5 % TCA у односу на DD H_2O и 2,4 % HCl се огледа у ниској граници детекције за фитинску киселину и P_i, добром recovery-ју и ниским вредностима одступања (bias). Ниска граница детекције за P_i је важна за узорке са природно ниском P_i концентрацијом, као што је семе соје.

Кључне речи: фитинска киселина, неорганski фосфор, семе, валидација

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