Characterization of Phytic acid in Tempered Canned Red kidney beans (*Phaseolus vulgaris*) using Raman Spectroscopy

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Abstract—**Rapid and non-invasive analytical method for quality control is a priority for the food industry, hence the objective of this study is to use Raman spectroscopy (RS) to identify phytic acid in RKB. Phytic Acid was extracted from treated ground dry RKB, while standard concentration of PA was prepared from a PA solution 50% w/w. Extracted samples and standard solutions were analyzed using the RS. The laser light beams focused on the PA samples generated molecular bond vibrations which resulted to inelastic scattering of photons in the light beam. The result showed that P-O-C, and P=O which are bonds identified with PA emitted light intensities at wavelength 858.13, and 1198.04 nm, respectively. Correlation (0.93) was established between results obtained for the standard RAMAN spectroscopy method and UV spectrophotometric method. Canned BL treatments showed increased concentration with blanched samples with lowest concentration (0.1175 g/mL) for P-O-C. Thus, the correlation established is indicative that the RS has potential applications in the food industry.**

Keywords; Phytic acid; Raman spectroscopy; Tempering; Red Kidney Bean; Canning

I. INTRODUCTION

Phytic acid, myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate (IP_6) (Figure 1) is a principal storage form of phosphorous compound existing naturally in plants. Phytic acid (PA) is indigestible by humans and monogastric animals due to the lack of the enzyme phytase, which dephosphorylates the PA compound. It has been identified as an anti-nutritional factor [1], [2].

Legumes contain significant amount of PA, which exist intrinsically as phytate in the anionic form in plants. It exists as a complex salt of cations (calcium, magnesium and potassium) in matured seeds, and is usually bound to macronutrients such as proteins and starches. Raw whole and cooked red kidney beans contain about 9.56 and 8.09 mmol/kg, respectively [3], [4] reported phytic content of 4.4, 24.3, 57, 39 mg/g, in carrots, hard wheat, hard wheat brand and fermented hard wheat brand bread, respectively.

The Ca and Mg complex salt form of PA and also referred as phytin [5]. Phytin is usually stored in the germ of corn, the
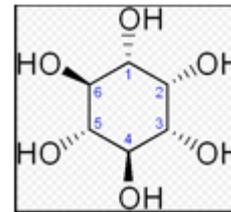
Figure 1. Molecular structure of phytic acid, myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate (IP6)

aleurone layer and outer bran of wheat and rice, and globoids in legumes and oil seeds [6], [7], [5]. In effect, PA inhibits the bioavailability of minerals by forming insoluble complexes with mono-di and trivalent cations, thus impedes the hydrolytic enzymes such as protease and amylase.

Soaking of legumes in water prior to canning is a critical step in reducing the PA concentration [8], [9]. Tempering is a hydration processing without direct submergence in water. It is a method that subjects specimens to a specific environmental condition for a specific period of time to induce chemical and morphological changes. Thapa, Juelich and Kassama [10] reported an equivalent hydration coefficient for conventional soaking to tempering red kidney beans at high relative humidity pretreatments.

The AOAC method 986.11 [11] is one of the traditional spectrophotometry methods for quantifying IP_6 by means of determining inorganic phosphorous. The limitation of traditional method is the capacity to distinguish between IP_6 and inositol pentakisphosphate, IP_5 hence systematically overestimates IP_6 content [3]. Currently high performance liquid chromatograph (HPLC) is one of the common methods used to determine PA in red kidney beans. Chen [3] estimated PA and IP_5 in varieties of beans and nuts to be between 0.1 to 0.2 µg/100 µL (1.5 to 3.4 µM) with HPLC, Dost and Tokul [4] reported 10 to 125 µg/mL in wheat and wheat products. However, there is increase interest in the rapid detection and quantification of organic chemical compounds in foods without complex sample derivatization and preparation protocol.

Surface-enhanced Raman scattering is a novel vibrational spectroscopy for rapid analysis of organic and biochemical compounds within wide range different complex components [12], [13]. The Raman scattering signals of targeted molecules can be largely enhanced through adsorption of analyte molecules onto nanostructured metal surface [12], [14], [15]. Raman spectroscopies quantify antioxidants in vegetables oils, microbial contaminants in foods and in acetylated food proteins [15], [16]. Hampton, Demoin and Glaser [17] and De Gelder, de Gussem, Vandenabeele and Moens [18] reported the P-O-C and P=O stretch has been identified at wavenumbers ranging from $787 - 1088$ cm^{-1} and $1140 - 1320$cm^{-1} using Raman spectroscopy. However, limited studies have been conducted on Raman based detection of PA in beans and other legumes. Therefore the objective of the study is to evaluate the feasibility of Raman spectroscopy as an alternate approach to quantify PA in red kidney beans.

II. MATERIAL AND METHODS

A. Dry Wholesome RKB Kernels

Dry red kidney beans (RKB) were obtained in the courtesy of the Chippewa Valley Beans, Co., Inc. Menomonie, Wisconsin. The RKB kernels were passed through stereoscopic microscope with a digital camera (Model DC5-420th, Cole Palmer, Vernon hills, Illinois) to identify wholesome kernels from cracked or fissured kernels. About 500 g of RKB kernels were tempered in the Environmental chamber (EC) (ESPEC Model BTL 433-4 CFT, Hudsonville, Michigan).

B. Extraction of PA in Red Kidney Beans

The treatment RKB samples were freeze dried using a Freezone freeze dryer (Model 195, Labconco Corporation, Kansas City, MO) and ground with the Thomas-Willey Laboratory mill (Model 4, Arthur H. Thomas Company, Philadelphia, PA), the ground samples were passed through US Standard Testing Sieve Size 20 (Tyler Equivalent 20 Mesh). Phytic acid was extracted from 1g of dry ground samples by adding 0.66 M of hydrochloric acid (HCL) in an Erlenmeyer flask (250 mL). The flasks were placed on a Laboratory Shaker (Eberbach Orbital-60 CV-AC, Eberbach Corporation, Ann Arbor, MI) and shake overnight. The extracts were transferred into microfuge tubes (1.5 mL) and centrifuged at 13,000 rpm for 10 min with the Fisher Scientific Marathon Centrifuges (Model 16km, Fisher Scientific, USA). Supernatant were carefully separated and was neutralized with 0.5 mL of 0.75 M of NaOH solution.

C. Enzymatic Assay of PA

Neutralized solutions of the PA extracts were used in the enzymatic de-phosphorylation reaction assay using Magazine kits [19]. The total phosphorous and free phosphorous concentrations were determined using the colorimetric method. Phytic acid content was computed [19] from the result obtained from the relative absorbance determined with a UV-VIS Spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan).

D. Preparation of Standard Solutions

A stock standard solution of PA (50% solution in water with a concentration of 1.1 mol/mL) purchased from Fisher Scientific, USA was prepared in HCL and stored at 4°C until used. A working standard solution concentrations (0.1, 0.2, 0.3, 0.4, 0.5 mol/L) were prepared by diluting with the stock solution with HCL. Concentration of PA in standard solution was determined using a UV-VIS Spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan).

E. Surface Raman Spectroscopy Determination of Phytic

A Dilor Confocal Laser Raman imaging microscope system (Model Labram, Horiba Jobin Yvon, France) equipped with an Olympus BX40 with 20x microscope objective lens was used to determine the PA content on the standard solutions and the extracts samples solutions. The objective lens was set at 10x with an acquisition time of 20 s at a grating rate of 1800 g/mm. Sample solutions were pipetted into cuvettes and loaded on the stage preceding equipment calibration. The screen was the adjusted to ensure that the laser light beam passes through the solution prior to data acquisition, and the light intensity was subsequently plotted against the wavenumber.

F. Canning of Red Kidney Beans

Wholesome RKB kernels were used for canning after the tempering process and were soaked and blanched (BL) as method used by Thapa [20], and some treatments were not-blanched (UBL). The RKB samples (160 g) were canned (No. 300) in 175 mL of brine solution [12]. The cans were sealed with the Dixie Automatic Can Sealer (Model 23-500, Dixie Canner Co, Athens, GA). Cooking and sterilization was done in All American Electric Pressure Steam sterilizer (Model no 25X, Wisconsin Aluminum Soundry Co. Inc, Manitowoc, WI) at a temperature and pressure of 116°C (240°F) for 40 min and the cans were rapidly cooled after cooking. Raw and non-cooked RKB samples are referred as not-canned (UC).

G. Experimental Design

The experimental treatments applied in this study were conventional soaked beans (SB), tempered no soaking (T_{25}), tempered and soaked for 1 h (T_{S1}) and 3 h (T_{S3}) and the control (WB). Each treatment was canned as described above and phytic acid content was determined before and after canning. All the experiments were conducted in three replications.

III. RESULTS AND DISCUSSION

The effects of the different pretreatment on the PA strength in RKB were determined using Raman vibrational spectroscopy to detect the chemical structure of interest (myo-inositol hexaphosphate). Phytic acid, myo-inositol hexaphosphate (IP$_6$) is a ring of 6-carbon atoms (myo-inositol

or cyclo hexane) connected by single bonds and has 6-phosphate groups attached [21], [22], [23]. Barrientos, Scott and Murthy [23] reported that phytase hydrolysis results to sequential reduction of phosphate ester from PA, hence, structural modification of IP_6 to IP_5 and gradually to IP. Hydrolysis of phosphate esters is indicative of the weakening of the P-O-C and P=O bond, which attaches the phosphate group to the inositol. Molecular bond vibration frequency was used to identify the P-O-C and P=O. The molecular bond Raman spectral frequency of the P-O-C and P=O detected in this experiment and their respective harmonic wave number is shown in Fig. 2.
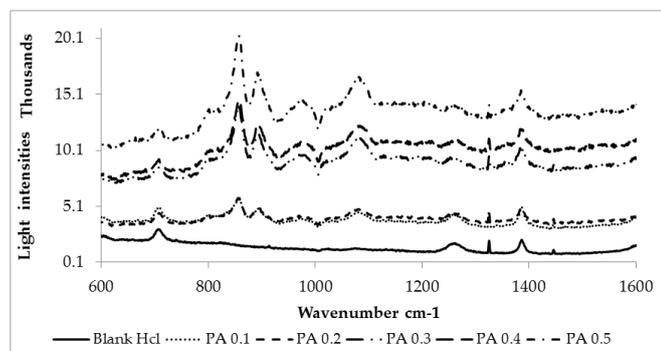
Figure 2. Raman spectra of blank HCl solution in contrast to the spectra of PA in HCl standard solutions at concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 mol/mL).

A. Phytic acid in standard solutions

The Raman vibrational spectra of phytic acid ($C_6H_{18}O_{24}P_6$: inositol hexakisphosphate) standard solutions (0, 0.1, 0.2, 0.3, 0.4, 0.5 mol/mL) was used as the baseline for quantifying PA content in the treatments as shown in Fig. 2. Hydrochloric acid (HCl) was used for the extraction of phytic acid in the RKB samples, hence also used as the blank (Fig. 2). The Raman vibration spectra peaks for the samples were overlaid on the HCl and standard solutions spectra for comparison of the peaks. The Raman vibration spectra peaks of the samples coincided with the peaks of the standard solutions but not the blank, thus indicate the harmonization of the specific compounds that vibrates at the same frequency. The identification of the intense spectra is not repeated in HCl spectrum. These regions marked the point of interest, and were selected as the region of PA molecular bond vibration (Fig. 2).

The Raman vibration peaks of the P-O-C and P=O stretches were identified at the following spectra regions 787 – 1088 cm^{-1} and 1140 – 1320 cm^{-1}, respectively, this was corroborated by the studies of Hampton, Demoin and Glaser [17]; Yang et al. [24]; and De Gelder, de Gussem, Vandenabeele and Moens [18]. The spectra of all the solutions displayed peaks coinciding to the P-O-C and P=O stretches. The highest intensity was observed at the frequency of 858.13 cm^{-1} for the P-O-C stretches. The higher frequency

(1198.04 cm^{-1}) region of the spectrum shows the least contribution based on the localized vibrations within phytic acid observed at P=O stretch shown in Fig. 3. Hampton, Demoin and Glaser [17] reported a medium strength for P=O stretch at this range in Raman spectroscopy. The frequency intensity for standard solutions obtained at the selected P-O-C and P=O stretches indicated a linear correlation with the concentration as shown in Table 1.
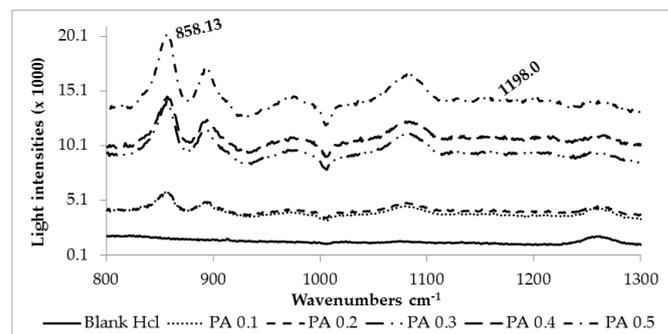
Figure 3. Raman spectra of peak identity of the P-O-C and P=O stretch bond of the blank HCl solution in contrast to the spectra of PA in HCl standard solutions at concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 mol/mL).

Table 1 Raman spectra light intensity of P-O-C (858.13 cm^{-1}) and P=O (1198.04 cm^{-1}) bonds of the standard solution

Wavelength Cm^{-1}	Raman Spectra Light Intensities at different Standard solution Concentration (mol/L)				
	0.1	0.2	0.3	0.4	0.5
858.13	0.0843	0.1086	0.0742	0.1040	0.1060
1198.04	0.1011	0.1183	0.1170	0.1084	0.1124

Table 2 shows the relationship between the UV-Vis and the Raman vibrational spectroscopic data of the standard solutions. A correlation ($R^2 = 0.93$) was established between the intensities of the P-O-C stretch of the standard solution by Raman spectroscopy and standard UV-Vis spectroscopy as shown in Fig. 4. The reliability of the correlation ensures the applications of Raman spectroscopy technique used in analyzing the PA strength of RKB and future uses in other food matrix.
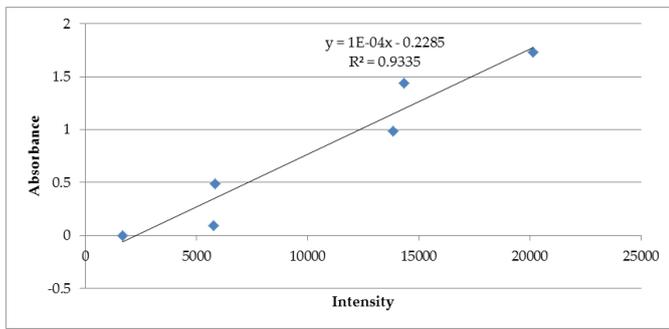
Figure 4 Standard correlations between Raman vs UV spectroscopy

The Raman peaks obtained from the blank and the standard solution at 0.5 mol/mL was compared to the peaks obtained for each of the treatments as shown in Fig. 5. Although, the frequency of the entire 5 band shifted by ca. 40 cm^{-1} observed for the P-O-C stretches in contrast to the standard solution spectrum, but the bond conformation was observed at 902.02 cm^{-1}. The band shift could be the result of the pretreatment that may have affected the molecular bond structure. However, the peak falls within the vibrational frequency region as reported in many studies by Hampton, Demoin and Glaser [17]; Yang et al. [24]; and De Gelder, de Gussem, Vandenabeele and Moens [18]. Isbrandt and Oertel [25] also reported a 10 cm^{-1} band shift for myo-Inositol Hexakisphosphate. Thus, frequency shift was not observed for the P=O band shown in Fig. 5.

Table 2 Correlation between the Raman spectra intensity and the UV spectroscopic absorbance

Spectra Methods	Raman Spectra Light Intensities and UV-Vis Absorbance at different concentrations (mol/L)					
	0	0.1	0.2	0.3	0.4	0.5
Raman	1665.79	5787.41	5841.93	13838.7	14345.8	20157.7
UV-Vis	0	0.098	0.489	0.989	1.438	1.733
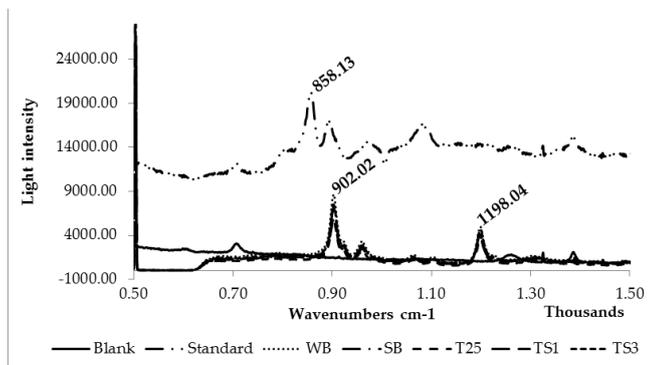
Figure 5. Raman spectra of peak identity of the P-O-C and P=O stretch bond of the standard solutions at concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 mol/mL) compared to the treatments.

The selected frequencies of uncooked RKB samples subjected to different treatments and their spectra intensities are tabulated in Table 3. The intensity values of the treatment spectrums observed for each wave number did not vary significantly ($p > 0.05$). The vibration peaks emitted are closely knitted due to the breakdown of PA, hence the bonds stretch deteriorate as the concentration of PA decreases (Fig. 5).

The lowest intensity values were observed in the T_{25} treatments while the WB treatment registered the highest value at both wavelengths (902.02 and 1198.04 Cm^{-1}). Thus tempering at 25°C seems to weaken the structural bond linking the phosphate group to the inositol and the other treatments also showed the same characteristic feature compared to the WB treatment. The result obtained from the phytic quantification using the method developed by Megazyme [19] corroborated the values obtained by Raman spectroscopy, although no significant ($p > 0.05$) changes were observed between the control and all treated values as shown in Table 4.

Table 3. Raman spectra light intensity of P-O-C (902.02 cm^{-1}) and P=O (1198.04 cm^{-1}) bonds of the treatments at different wave numbers

Compound	Concentration (g/100g)				
	WB	SB	T_{25}	T_{S1}	T_{S3}
Phytic Acid	25.58[a]	25.07[a]	27.87[a]	26.59[a]	29.08[a]

Table 4. Phytic acid concentration of the different treatments determined using the Megazyme enzymatic assay

Compound	Concentration (g/100g)				
	WB	SB	T_{25}	T_{S1}	T_{S3}
Phytic Acid	25.58[a]	25.07[a]	27.87[a]	26.59[a]	29.08[a]

Means with the same letter in the same row are not significantly different ($p > 0.05$). PA: phytic acid. WB: whole beans, SB: soaked beans, T_{25}: tempering only, T_{S1}: tempering and 1 hour soaking, T_{S3}, tempering and 3 hours soaking

Thapa [20] reported (12.4 to 23.6 mg/g) phytic acid concentrations in dry RKB, these values are much less than the values (25 to 29 g/100g) obtained in this research shown in Table 4. The results of Thapa, [20] were based on tempering at 45°C for 12 hours, which is lower than the value obtained for 25°C tempering for 12 hours in this study, the variation could be attributed to the temperature differences.

B. Phytic acid concentration in canned red kidney beans

The correlation of Raman vibration peaks for the P-O-C and P=O stretch to concentration of phytic acid was used to determine the phytic acid concentration in canned RKB as shown in Tables 5 and 6. Whole bean uncooked beans for UC and UBL treatments showed decreased concentration of PA for the P-O-C and P=O stretch. The other treatments had higher concentrations compared to the WB. All canned BL treatments showed increased concentration with T_{S3} BL having the lowest concentration (0.1175 g/mL) for P-O-C. The variation is probably due to the bond strength and liberation of phosphorous during the pretreatment. Tables 5 and 6 shows the data of tempering (T_{25}) without blanching reduced the Raman spectral intensity when compared with the WB, thus shows an indication of reduced PA concentration of 0.074 g/mL for P-O-C and P=C. However, an increased Raman spectral intensity was observed on blanched (T_{25}) treatment and the relative PA concentration (0.1281 g/mL for P-O-C) increased compared to WB. Increased intensity was equally observed after the immersion of tempered samples in water (T_{S1} and T_{S3}). Bond hydration as a result of diffusion might have some effect on the P-O-C and P=O structure. Tempering at high RH environment tend to hydrate without immersing in water have similar effect as soaking in reducing PA strength.

Table 5. Raman spectra light intensity of P-O-C stretch and characterization of phytic acid content in canned RKB

| Processing Methods | Pre-treatments | | | | |
| | Concentration phytic acid (g/mL) | | | | |
	WB	SB	T_{25}	T_{S1}	T_{S3}
Not Canned	Neg.	Neg.	Neg.	Neg.	Neg.
Canned	0.0934	0.1186	0.0735	0.1115	0.1164
Blanched and Canned	0.1124	0.1317	0.1281	0.1183	0.1175

WB: whole beans, SB: soaked beans, T_{25}: tempering only, T_{S1}: tempering and 1 hour soaking, T_{S3}, tempering and 3 hours soaking; Neg.: value negligible.

Table 6. Raman spectra light intensity of P=C stretch and characterization of phytic acid content in canned RKB

| Processing Methods | Pre-treatments | | | | |
| | Concentration P=C stretch g/mL) | | | | |
	WB	SB	T_{25}	T_{S1}	T_{S3}
Not Canned	0.0949	0.0670	0.0580	0.0727	0.0867
Canned	0.0843	0.1086	0.0742	0.1040	0.1060
Blanched and Canned	0.1011	0.1183	0.1170	0.1084	0.1124

WB: whole beans, SB: soaked beans, T_{25}: tempering only, T_{S1}: tempering and 1 hour soaking, T_{S3}, tempering and 3 hours soaking

Megazyme [19] indicated in their studies the ease of de-phosphorylate IP6 to IPs because of the less resistance observed in the region IP2 to IP. Reduction of PA during soaking is proven as a result of partial diffusion of hydrolyzed phosphate group into soaking water [8], [9]. Phytic acid

strength was impaired in all tempered samples as indicated in the results obtained. The increase intensity of Raman Spectra and the corresponding concentrations after immersing in water for the UC, UBL, and BL samples indicates high resistant to de-phosphorylation hence could be classified in the IP2 to IP region due to the high vibration intensity of the P-O-C and P=O bonds.

IV. CONCLUSION

The correlation of Raman vibrational spectra peaked for the P-O-C and P=O stretch to concentration of phytic acid content in RKB was established. The hydration pretreatment have significant ($p < 0.05$) effect on the P-O-C and P=O structure and hence contribute to the final concentration of phytic acids in RKB. The frequency of the entire 5 band shifted by ca. 40 cm^{-1} for the P-O-C stretches in contrast to the standard solution spectrum, thus the bond conformation was observed at 902.02 cm^{-1}. All canned BL treatments showed increased concentration with T_{S3} BL having the lowest concentration (0.1175 g/mL) for P-O-C. The results indicated that the use of Raman molecular vibrational spectroscopy to determine chemical constituents of food have potential applications in the food industry.

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