### Chapter VII

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Abstract

The phytate content in whole-wheat flour is known for its antinutrient activity. The effect of addition of exogenous laboratory purified yeast Phytase to whole wheat flour was analyzed by both chemical and Fourier transform infrared spectroscopy [FTIR] method. The supplementation of phytase promoted an acceleration of the fermentation process and, at the fresh bread level, an improvement in softness were obtained. Simultaneous analysis of IR absorption of phytate was observed from 1400 Cm$^{-1}$-800 Cm$^{-1}$, which is primary range of absorption of P-H, P-H banding, phosphine and phosphoric acid like compound. The measurement of phytate in whole wheat flour can be done rapidly by FT-IR, compare to chemical method. FT-IR analysis gives reproducible values when it replicated, which can be used for the measurement of phytate content directly from the bread.
7.1 Introduction

Currently, health authorities worldwide recommend an increase in the consumption of whole grain cereal products such as breads and breakfast cereals, in order to balance the significant increase in consumption of animal proteins and fats. The dietary guidelines have moved towards the consumption of increasing amounts of fiber during the last decade. Whole wheat flours, besides being a source of fiber, provide complex carbohydrates, proteins, vitamins and minerals. However, simultaneously to the nutritional benefits, whole wheat flours contain some undesirable substances such as phytates. Phytate or myo-inositol hexakisphosphate is a common constituent of most cereals, legumes, some vegetables and fruit. Phytic acid \([\text{IP}_6]\) is a hexaphosphate-substituted inositol ring compound that, in the deprotonated form, has a high affinity for divalent minerals such as calcium, magnesium, zinc, iron, and cobalt \([\text{Phillippy, 2003}].\) The Ca/Mg salt of IP6 is a naturally occurring phosphate storage compound found in plants. Phytic acid binds minerals in the gastrointestinal tract, potentially making them biologically unavailable. As evidence of this, IP6 compromises the zinc status and growth rate of many species \([\text{Harland & Oberleas, 1987}].\)

The importance of IP6 in altering mineral status has led to guidelines for ensuring adequate zinc bioavailability \([\text{Denstadli et.al, 2006; Nair et.al, 1987}].\) Bread making process allows a decrease in the phytate content \([\text{Harlend & Harlend, 1980}].\) Some reports have described different bread making procedures aimed at lowering the phytate content in breads; for example, the phytate content in whole wheat breads was half-reduced by increasing the yeast concentration \([\text{Haros et al., 2001; Tangkongchitr et.al, 1981}].\) Hence, many methods for its measurement and elimination have been investigated. Some method for measurement using ferric precipitation, HPLC or other methods \([\text{Park, 2006}].\) are established but these are too time consuming to use in the bread-making process. Research has shown that Fourier transfer Infrared spectroscopy is simple rapid and reliable method to determine some food component \([\text{Fuller & Griffiths, 1980}].\) The currently used official AOAC method \([\text{Number 986.11}].\) may overestimate the phytic acid content of processed foods. These foods generally contain lower phosphates. These lower phosphates are included in the calculation of phytic acid determined by the AOAC anion-exchange method. The Fourier transform infrared spectroscopy \([\text{FTIR}].\) method quantitates the phytic acid and other inositol phosphates as separate entities. The
phytic acid content was determined according to the chemical method reported by Wheeler and Ferrel [1971] and FTIR method.

The goal of this study was to examine the effect of phytase supplementation on bread-making process. We report here an FTIR analysis method for the measurement of phytate from every stage of the bread making process and the measurement of phytate content in bread.

7.1.1 Objectives of chapter

✔ Evaluation of supplementation of yeast phytase on breadmaking procedure.
✔ Development of novel method for detection of phytic acid during breadmaking process.
7.2 Materials and Methods

7.2.1 Materials

A commercial bland of wheat flour was used in this study. This whole-wheat flour was obtained from local wheat variety Sharbati. Flour characteristics are listed in Table 7.1. A laboratory purified phytase from probiotic yeast *Saccharomyces boulardii* characterized as 3-phytase, containing 0.02 phytase unit per mg was added.

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<tr>
<td>moisture [%]</td>
<td>13.5</td>
</tr>
<tr>
<td>protein [%]*</td>
<td>14.3</td>
</tr>
<tr>
<td>ash [%]*</td>
<td>1.66</td>
</tr>
<tr>
<td>water absorption [%]</td>
<td>60.5</td>
</tr>
<tr>
<td>phytates [mg phytic acid/g]*</td>
<td>9.4</td>
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* Dry matter

7.2.2 Bread making procedure

The bread dough formula consisted of whole wheat flour [200g], salt [2g], dry yeast [5g] and 150 ml water used as control. However in second approach whole dough formula was same as control excepting the presence of phytase [10mg/100gm]. The ingredients were mixed for 15 minute followed by mechanical agitation of dough for removal of air from dough. This stage was referred as before fermentation. The samples were taken for analysis of flour phytic acid by FTIR and chemical method. During the proofing stage, the dough volume increase was followed in a graduated tube, and pH of dough was potentiometrically determined. After 2 hour [optimum volume increment] the one more sample was collected for chemical analysis which was referred as after fermentation. The loves were baked on 180°C for 20 min in microwave convection oven. The prepared bread was further analyzed for its decrease in phytic acid content.
7.2.3 Preparation of raw bread dough, phytate-added bread dough, and dephytate bread dough

For standardizing the measurement method, firstly the raw bread dough[without addition of phytate and phytase] was prepared as follows: as per the composition given above in section 7.2.2 the raw bread dough was prepared. In a preparation of phytate added bread calcium phytate [1 mg%] was added to dough.

50 ml sterile distilled water was added to dough, and slurry was prepared. The extracts of phytate were prepared following the method reported by Latta & Eskin [1980]. The minor modification of extraction procedure was carried out with the use of 2.5 % hydrochloric acid for 30 min \([\text{HCl}]\) as this proved efficient in extracting total phytate. Flour dough and bread ground vigorously in a homogenizer with 25 ml HCl. The homogenate was centrifuged and supernatant was filtered and extracts were collected. The 10% tri-chloro acetic acid \([\text{TCA}]\) was added in the extracts for removal of TCA insoluble protein. Centrifugation \([\text{Sigma 3K30}]\) was carried at 10000 rpm for 10 minute. Supernatants were used for phytate estimation.

7.2.4 Separation of phytate from raw bread dough

100 microliters of 1N NaOH and 30 µl of 1M CaCl \(_2\) were added to 1.5 ml of raw bread dough slurry. The mixture was stirred and centrifuged at 8000g for 5 min. The precipitate containing phytate was separated and drained completely. The precipitate was then dissolved in 0.4 ml of 0.5 M sodium citrate buffer \([\text{pH 6.0}]\). This solution was used as sample for phytate measurement by FT-IR spectroscopy.

7.2.5 Measurement of IR spectra

Sucrose \([0.25 g]\) and stachylose \([0.25 g]\) were mixed and dissolved in 10 ml of water for saccharide solution. IR spectra of raw bread dough slurry and phytate solution were measured from 4000 cm\(^{-1}\) to 500 cm\(^{-1}\) with FTIR spectrophotometer. An overhead attenuated total reflection\([\text{ATR}]\) accessory was equipped as the sample stage for liquid samples. All spectral measurements were done at 1 cm\(^{-1}\) resolution. The single beam ATR spectrum from each sample was corrected using a background spectrum of demonized water, and transformed to absorbance units.
7.2.6 Preparation of standard solution for IR measurement

The IR measurement Standard solutions were made by two methods a) 0.137 g of calcium phytate was dissolved in 10.0 ml of citrate buffer [1.00% solution of phytate], and various concentration solutions were prepared by diluting [0.5 M] citrate buffer. The citrate buffer was used for the blank of these IR measurement. b) phytate added bread dough slurry was then mixed with dephytate bread dough slurry at various ratios. Phytate was separated as described above from each 1.5 ml of these mixed bread dough slurry, and was then dissolved in 0.4 ml of 0.5 M sodium citrate. The citrate solution obtained from dephytate bread dough slurry was used for the blank of these IR measurements.

IR spectra of Calcium Phytate, whole wheat flour, dough before fermentation, dough after fermentation and bread in the presence and absence of phytase were measured from 4000 cm\(^{-1}\) to 800 cm\(^{-1}\) with an FTIR spectrophotometer [Nicolet 6700 FTIR] equipped with a KBr beam splitter and a DIGS KBr [4800-400 cm \(^{-1}\)] detector, an averaging 64 scan. All operations on spectra were performed using original OMNIC software.

7.2.7 Phytate determination by Chemical method

The phytate content of whole wheat flour, dough and bread was measured in the presence and absence of phytase by using the method reported by Wheeler & Ferrel [1971]. Phytic acid was precipitated as ferric salt. The iron content of precipitate was determined calorimetrically at 480 nm and the phytate phosphors content calculated from this value assuming a constant 4 Fe : 6 phosphate molecular ratio in precipitate by using following formula,

Phytate P mg/100g sample = [\(\mu g \text{ Fe} \times 15\)]/Weight of sample [g]

7.2.8 Sample preparation for FTIR analysis

Samples were finely milled [flour, dough and bread, dried in Hot air oven at 100° C for 30 minutes] with potassium bromide [spectroscopic grade] to form very fine powder. This powder was then compressed into a thin dried 1mm pellet. Spectra were recorded in the absorbance mode from mid infrared region. Special effort and care was given to minimize particle size of the powders to give disks that appeared to be clear solid solutions of the samples in KBr. Each spectrum was baseline corrected and the absorbance was normalized between 0
and 1. Standard solid solutions were made for various concentration of calcium phytate in KBr. The KBr disk without calcium phytate was used as blank. Standard phytate calibrations were carried out for Phytate measurement in whole wheat flour [Fig. 7.1].

![Figure 7.1. Calibration of phytic acid standards with FT-IR spectroscopy](image)

### 7.2.9 Processing of spectral data

The absorbance is related to the concentration by the Beer – Lambert law.

\[
K = AC
\]

Where \( K \) is an \( F \times n \) matrix of absorption coefficients for each of the \( n \) components at \( f \) frequencies, Wave number is directly proportional to energy and frequency. By using Multiple Linear Regression model the value for \( K \) was calculated \([\text{data not shown}]\). In the MLR model the data are first calibrated for the elements of the \( K \) matrix using a least squares regression criterion. The \( K \)-matrix method is a precise MLR algorithm that computes the least squares estimate.

\[
K = \text{Ac}^T[cc^T]^{-1}
\]

After calibration, in the validation step, the computed \( K \) values are used to predict the unknown concentrations of components in test samples by the least squares solution \([\text{Marls and Brown,}\]
1983; Griffiths and deHaseth, 1986; Beebe and Kossalski, 1987; Greene, Gordon, Jackson, Bennett, 1992; Gordon, Freer, James, & Greene, 1993].

\[ C = [K^TK]^{-1}K^TA \]
7.3 Results and Discussion

7.3.1 Effect of phytase on bread making

Phytase activity is dependent on pH. The pH of dough was measured during every stage of bread making process. The pH difference on every stage was negligible in presence and in the absence of phytase. We studied the effect of yeast phytase on bread quality along with the quantitative estimation of phytic acid by both chemical and FTIR analysis. The figure 7.2 shows clear difference in softness of breadcrumbs in presence and absence of enzymes, while bread was prepared from whole-wheat flour. The enzyme containing breadcrumbs were more soft, and superior in color and texture.

![Figure 7.2 Breadcrumb quality in presence and absence of enzyme a, without addition of phytase and b, with phytase](image)

7.3.2 IR spectra analysis of whole-wheat flour

In first step analysis of whole-wheat flour was carried out, it contains 4–6% phytate when analyzed by the chemical method. It is problematic to analyze whole wheat flour by IR spectroscopy because the presence of huge number of polysaccharides and proteins, the whole wheat flour in KBr disk was analyzed on mid infrared region of 1200-800 Cm⁻¹[Fig.7.3]. The baseline corrected spectra of whole wheat flour had total 11 peaks, which were at 866 Cm⁻¹, 875 Cm⁻¹, 889 Cm⁻¹, 997 Cm⁻¹, 1002 Cm⁻¹, 1154 Cm⁻¹, 1164 Cm⁻¹, 1361 Cm⁻¹, 1373 Cm⁻¹, 1408 Cm⁻¹ and 1418 Cm⁻¹

being due to C-N, C-O, P-H, P-OR esters-H banding, phosphine oxide, sulfoneand sulfonyl chloride and phosphoric acid
respectively. Matching the peaks with instruments library identified these peaks, the peak, which does not match, were discarded and not considered in further studies.

Figure: 7.3 FT-IR spectrum of whole-wheat flour [for standards please refer Annexure II]

7.3.3 IR spectral analysis of Phytate

IR spectrum of phytate was obtained by using Phytic acid calcium salt [sigma] in 1400 -800 Cm⁻¹ region, which had five peaks, which were at 889 Cm⁻¹, 1002 Cm⁻¹, 1154 Cm⁻¹ 1361 Cm⁻¹ and 1418 Cm⁻¹, because of P-H, P-H banding, phosphine and phosphoric acid respectively [Fig.7.4]. The result demonstrated that 1400 -800 Cm⁻¹ regions were important for analysis of phosphate
compound like phytate. The further analysis of spectral data were conducted in this range, other ranges were not included in analysis.

**Figure 7.4** Calcium phytate analyzed with FT-IR spectrum

### 7.3.4 IR spectral analysis of bread in the presence and absence of phytase

During the bread making there were various steps which decrease the phytic acid content, that shown by step-by step chemical analysis of each stage of process [Fig.7.5]. The IR spectral analysis of vacuum dried bread in KBr with and without phytase shows significant qualitative decrease in phytic acid content [Fig.7.6], and even the similar results were obtained with quantitative analysis by MLR [Multiple Linear Regression, as discussed in Materials and Methods section] model.
Figure: 7.5 Estimation of phytate content at various stages of bread making process by chemical estimation method.

Figure.7.6. IR spectrum analysis of bread in the presence and absence of phytase. Blue arrow indicates the absence of important peak of P-O bond stretch. a. Bread with phytase. b. Bread without phytase. c. Phytic acid in KBr.
7.3.5 standard calibration of phytate for whole-wheat flour by IR method

The two different solutions were analyzed by FT-IR spectroscopy one contains calcium phytate in citrate buffer while other contains whole-wheat flour precipitated in citrate buffer. These solutions then were each measured by a FT-IR instrument equipped with ATR apparatus. IR absorbance at 1154 cm⁻¹ of each solution was used for the measurement of phytate. The absorbance of phytate was converted to the content [%] in wheat flour. The calibration of absorbance against phytate content is shown in Fig. 7.7. They were strongly correlated [coefficient of measurement > 0.98] suggesting that this procedure is available for the measurement of phytate. The plot from the two methods [calcium phytate in citrate buffer, and whole wheat flour precipitate in citrate buffer] gave almost same regression line.

The phytate contents of whole wheat bread were measured by the FTIR method and the chemical method, as shown in Fig. 7.7. The X axis is the value obtained by the chemical method and the Y axis shows the value obtained by the FTIR method. Both values were also correlated with each other [coefficient of determination=0.98].

![Figure 7.7 comparison of FT-IR and chemical method of phytic acid estimation](image)

The previous study of Haros and rosell, 2001 have recently demonstrated that the addition of fungal phytase in bread increase the softness of bread and decrease in proofing time of fermentation, similar result were obtained with yeast phytase.
The FTIR analysis of protein, starch and calcium phytate is presented in Table 7.2.

Table 7.2 FTIR Analysis of Starch-Protein-Calcium phytate Mixtures for validation

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<th>Starch</th>
<th>Protein</th>
<th>Calcium phytate</th>
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<td>known % Calculated %</td>
<td>Known % calculated %</td>
<td>known % calculated %</td>
</tr>
<tr>
<td>0.5</td>
<td>0.42</td>
<td>0.5</td>
</tr>
<tr>
<td>0.4</td>
<td>0.48</td>
<td>0.4</td>
</tr>
<tr>
<td>0.3</td>
<td>0.29</td>
<td>0.3</td>
</tr>
<tr>
<td>0.2</td>
<td>0.18</td>
<td>0.2</td>
</tr>
<tr>
<td>0.1</td>
<td>0.12</td>
<td>0.1</td>
</tr>
<tr>
<td>SEP</td>
<td>SEP</td>
<td>SEP</td>
</tr>
<tr>
<td>2.93</td>
<td>2.73</td>
<td>0.29</td>
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The analytical wavenumber ranges were selected based on knowledge of chemical composition of the phytate. For both priority and empirical reasons, only one wavenumber region was selected for analysis. A broad \([1500-900] \text{ cm}^{-1}\) range that contains the various "fingerprint" absorption patterns and P-O bending frequencies of the phytate. The limits of these wavenumber ranges were not fixed. They were determined empirically during calibration by minimizing the standard error of estimation. The results illustrate the performance of the K-matrix MLR method with data from FTIR spectra of pure calcium phytate mixed with saccharide and protein. Twenty different mixtures were used as the calibration sample. Five mixtures, not included in the calibration set, were tested as the validation samples. The standard error of prediction \([\text{SEP}]\) was lowest for calcium phytate, and highest for protein. The highest SEP was less than 3 %. This precision was better than that using other MLR methods such as the P-matrix method, and the results indicate that with good data accurate analyses are possible without resorting to PCR or PLS.

Direct measurement of phytate from wheat flour, dough and bread by the FTIR method is simple and rapid, but major components such as saccharides and proteins in flour have FTIR
absorption in the same region as that of phytate. The phytate content could be measured in principle by subtracting these absorbencies. The method of Wheeler and Ferrel [1971] has been used for many experiments, but is a very complex procedure and needs much time while the other standard method like AOAC method overestimates the phytate content in food [Lehrfeld & Morris, 1992] compare to all these our method by FTIR measurement can do by using only 1 mg of whole wheat flour and take only 5 min for the preparation of sample. The direct measurement of phytate by FTIR is cost effective in terms of labor and instrumentation, easy to perform and rapid and provides fairly definitive identification of phytate but apart from this very attentive approach and keen observation is needed for finding the significant peaks. To minimize the chances of large error second important step is proper mixing of sample with KBr otherwise due to small concentration of phytate chances of error are more. The experiment also establishes the fact that phytase treatment of wheat flour is beneficial to decrease phytic acid content and it improves bread quality in terms softness of crumb. The comparison of both chemical and FTIR method of phytic acid analysis shows the negligible difference in the value.
Conclusions

The study once again confirm the result which showing the positive effect of phytase supplementation in breadmaking process, additionally, the study also provides the other efficient and rapid method of phytate measurement during bread making process, in minimizes the major steps of traditional reviewed methods, the developed procedure is cheap, rapid and efficient.

From the results, it can also be concluded that phytase supplementation as a bread making additive in bread formulations containing fiber leads to an acceleration of the proofing, an improvement of the bread shape, a slight increase of the specific volume, and also confers softness to the crumb. In addition, from the nutritional point of view, a further hydrolysis of the phytate [considered as anti-nutritional compounds] is reached by adding exogenous yeast phytase. By considering its antinutrient activity we carried out step-by-step measurements of phytate, our data shows that direct measurement of phytate from wheat flour by the FT-IR method is simple and rapid compare with chemical method.

By using this method as prototype it can be employed for estimation of phytic acid content from other food and feed products.

The successful incorporation of phytase in bread may be useful for development of other phytate free products.