PHYTIC ACID DEGRADATION BY PHYTASE – AS VIEWED BY ^{31}P NMR AND MULTIVARIATE CURVE RESOLUTION

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1 INTRODUCTION

There is a growing interest amongst consumers and industry to ensure that our food is safe, healthy and nutritious. To achieve high standards this will require rapid, sensitive and reliable analytical methods. Spectroscopy is very well suited for this purpose and different types of spectroscopy have already been utilized in industry, such as fluorescence, infrared and near-infrared spectroscopy, including low-field NMR relaxometry. So far there has not been great tradition to utilize high-resolution (HR) NMR spectroscopy within food science and food industry. The fact that NMR spectroscopy is non-destructive and provides quantitative and structural information as well as information on water mobility and distribution about the entire sample volume makes this method particularly useful for quality control in the food industry.

In order to enhance interpretation and the quantitative information for measurement of complex biological/food systems it is often necessary to combine the spectroscopic results with advanced multivariate (chemometric) methods which open up for a great unutilized potential for spectroscopic quality control of food by exploring food systems using unsupervised data technologies which require no *a priori* information. Chemometrics is also referred to as "statistics without tears", because it facilitates handling of large data sets and deals efficiently with real-world multivariate data, taking advantage of the previously feared co-linearity of spectral data and for providing the possibility of projecting the data into a few dimensions via a graphical representation[1]. Multivariate data visualization of real-life data sets is perhaps the single most important feature of chemometrics. It is important to underline that there are NO univariate real-world problems – this has been most definitively stated by the traditionally conservative FDA in its PAT (Process Analytical Technology) guidance for the Pharmaceutical Industry: "*Traditional one-factor-at-a-time experiments do not effectively address interactions between products and process variables*".

With direct reference to metabonomics[2] we have introduced the term *bromatonomics* which covers "quantitative measurements of functional quality parameters of food as studied by NMR on intact food systems and evaluated by multivariate data analysis". In this paper we will exemplify the application of simple unsupervised chemometric methods to ^{31}P NMR spectra in order to study the dynamics of phytic acid being degraded by phytase – an important reaction for the mineral availability in food and feed. Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, IP6), which is shown in Figure 1, forms complexes with different divalent and trivalent cations as well as

with proteins and starch, and the solubility of the complexes is pH-dependent[3]. Most phytic acid-mineral complexes, called phytate, are soluble at a pH less than 3.5 with maximum insolubility occurring between pH 4 and 7[4, 5]. The complexes depend on the numbers of phosphate groups on the inositol ring. The fewer phosphate groups on the ring, the weaker the complexes become. The insolubility of the phytate complexes is probably the major reason why phytic acid historically is considered an anti-nutrient because it binds the essential dietary minerals and thus decreases their bioavailability[6]. Phosphorus in the form of phytate is generally not bioavailable to non-ruminant animals, because they lack the digestive enzyme phytase which is required to separate phosphorus from the phytate molecule. The approximate pH of the intestine, where absorption of metal ions occurs, coincides with the pH at which these complexes precipitate.

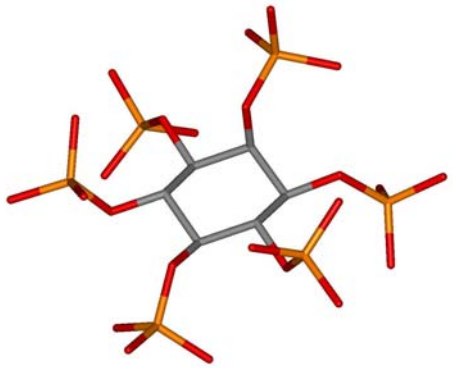

Figure 1 *The heavy atom structure of phytic acid. Only the phosphate group on C-2 is axial.*

Phytase is the enzyme that catalyses the stepwise hydrolysis of phytic acid to lower inositol phosphates (myo-inositol pentaphosphate, IP5 to myo-inositol mono phosphate, IP1, Figure 2).

IP6 → IP5 → IP4 → IP3 → IP2 → IP1 → *myo*-inositol

Figure 2 *Stepwise dephosphorylation of IP6 (phytate)*

There are two international classified phytases: 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). The enzymes are named after the position of the first phosphorester bond of the phytate to be hydrolyzed. 3-phytase seems to be of microbial origin, while 6-phytase is synthesized by plants[7]. A 4-phytase seems to be present in cereals as well[8, 9]. This enzymatic activity produces available phosphates that are considered important in upgrading the nutritional quality of phytate-rich foods and feeds[10]. In modern agriculture, non-ruminant livestock such as swine and poultry are fed mainly grains like soybeans and maize. Because phytate from these grains is unavailable for absorption, the unabsorbed phytate passes through the gastrointestinal tract, elevating the amount of phosphorus in the manure. Excess phosphorus excretion can lead to environmental phosphorus pollution.

2 METHODS

2.1 Samples

The ^{31}P NMR model experiment samples were prepared by dissolving a commercial phytase enzyme isolated from wheat (Biofeed, Novozymes) with a sodium phytate solution (7.5mg/ml, 30mg/ml EDTA) adjusted to pH 5.3 with sodium acetate buffer (0.25M). The enzyme had an activity of 0.01 U/ml in the solution (1 unit of enzyme activity liberates 1 µmol of inorganic phosphorus from a 1.5 mM phytate solution per min. at pH 5.15 at 55°C).

2.2 ^{31}P NMR

^{31}P NMR spectra were recorded to study the phytase reaction every 9 min. over a period of 14 hours. The spectra were acquired on a Bruker Avance Ultra Shield 400 spectrometer (Bruker Biospin Gmbh, Rheinstetten, Germany) operating at 161.98 MHz using a broad band inverse probe head equipped with 5 mm (o.d.) NMR sample tubes. Data were accumulated at 298.5 K using the zgpg30 pulse sequence with an acquisition time of 10.1 s., a recycle delay of 10 s, 32 scans and a sweep width of 3238.34 Hz, resulting in 64 k complex data points. In order to secure quantitative measurements the receiver gain was set constant for all consecutive NMR measurements.

2.3 Unsupervised exploratory analysis

One of the main advantages of applying multivariate chemometric data analysis to collinear spectroscopic data is the possibility of carrying out an exploratory inductive investigation[11]. The universal basic chemometric algorithm principal component analysis (PCA)[12] is a most useful tool for this purpose. PCA is based on the calculation of underlying latent data structures using a two-dimensional data strategy, i.e. measuring a series of samples and finding common latent data structures and individual scores or concentrations. Common to such bilinear models is that an entire matrix, with each row being the measurement from one sample, must be acquired. PCA finds the main variation in a multidimensional data set by creating new linear combinations (orthogonal) of the raw data that approximate the original data set in a least squares sense, a method which is especially well suited to highly collinear data, as is the case in most spectroscopic or instrumental techniques. The model to be solved is $X = T*P^t$ in which the data matrix (samples × spectra) **X** is decomposed into a lower dimensional score matrix (**T**) and a loading matrix (**P**). In this way the information in **X** is projected onto a lower dimensional subspace where the loading vectors for the principal components (PC) can be regarded as pure mathematical spectra that are common to all the measured spectra. What makes the individual raw spectra different are the amounts (scores) of hidden spectra (loadings). PCA can be considered as the first amendment in exploratory data analysis due to its extraordinarily robust data reduction, its low level of *a priori* assumptions and its data presentation capabilities.

2.4 Constrained Alternating RegreSsion (CARS)

Alternating regression, also called multivariate curve resolution (MCR)[13], is a mathematical method for curve resolution that does not impose the orthogonality constraint as utilized in the PCA algorithm. Analogous to PCA, the input to the algorithm is a matrix X with spectral measurements of the relevant mixtures to be resolved, and the output is two matrices: C that contains the estimated concentrations of the mixture components, and S that contains the pure spectra of the mixture components. In this study CARS is implemented with non-negative constraints[14] on both the concentrations and the spectra. The model to be solved is $X = C*S^t$, where t is transposed and dimensions are: X (n samples, m wavelengths), C (n samples, p pure components), S (m wavelengths, p pure components).

The pseudo code for the implemented method is as follows:

```
While changes in S observed
  for i = 1 to number of samples
    Solve C = X/St, with non-negativity constraints
  end
  for i = 1 to number of pure components
    Solve S = X/C, with non-negativity constraints
  end
end
```

In contrast to PCA, CARS require a guess of S in order to initiate the algorithm, but with the presented data the algorithm proved rather reproducible regardless of the initial guess.

The basic chemometric data analysis and visualization of spectra was made using the data workbench and PCA algorithm from the public domain chemometric software LatentiX version 1.05 (www.latentix.com). The CARS algorithm (www.kvl.models.dk) was implemented under MatLab Version 13 (MathWorks Inc., Natic, USA).

3 RESULTS AND DISCUSSION

The degradation of phytic acid in a model system in which phytase was added to an aqueous solution was investigated by a time series of ^{31}P NMR spectra. Figure 3 shows the NMR spectra as a function of time. In the beginning before the phytase action (light blue) four ^{31}P peaks are present (0.14, 0.47, 1.06 and 1.76 ppm) with the intensity ratios 1:2:2:1 resulting from the six P atoms in the phytic acid molecule (IP6) in which 2 pairs of phosphate atoms are degenerated due to the symmetry[15]. However, after addition of phytase the pattern and intensity of the peaks change as a consequence of degradation of IP6 to lower inositol phosphates. First, five new peaks are observed in the spectra which may be due to the formation of one specific IP5, because only five equally strong peaks are formed. This is because it is an asymmetric molecule which is formed where carbons 2 and 5 still are phosphorylated and in good agreement with the fact that we have been using a wheat phytase that is specific for cleavage of carbon 6. The enzymatic process gets more and more complex, but in agreement with earlier studies, one dominating isomer is formed for the penta-, tetra-, tri-, di- and monophosphates, which is outlined more clearly in Figure

6[15]. The phytase process continues for 14 hours and ends with four peaks (0.38, 0.42, 2.45 and 2.77 ppm) shown by the magenta line in the spectra. The four peaks remaining at the end corresponds to the three isomers of IP1 that remain after the hydrolysis and to inorganic phosphate (0.38 ppm).

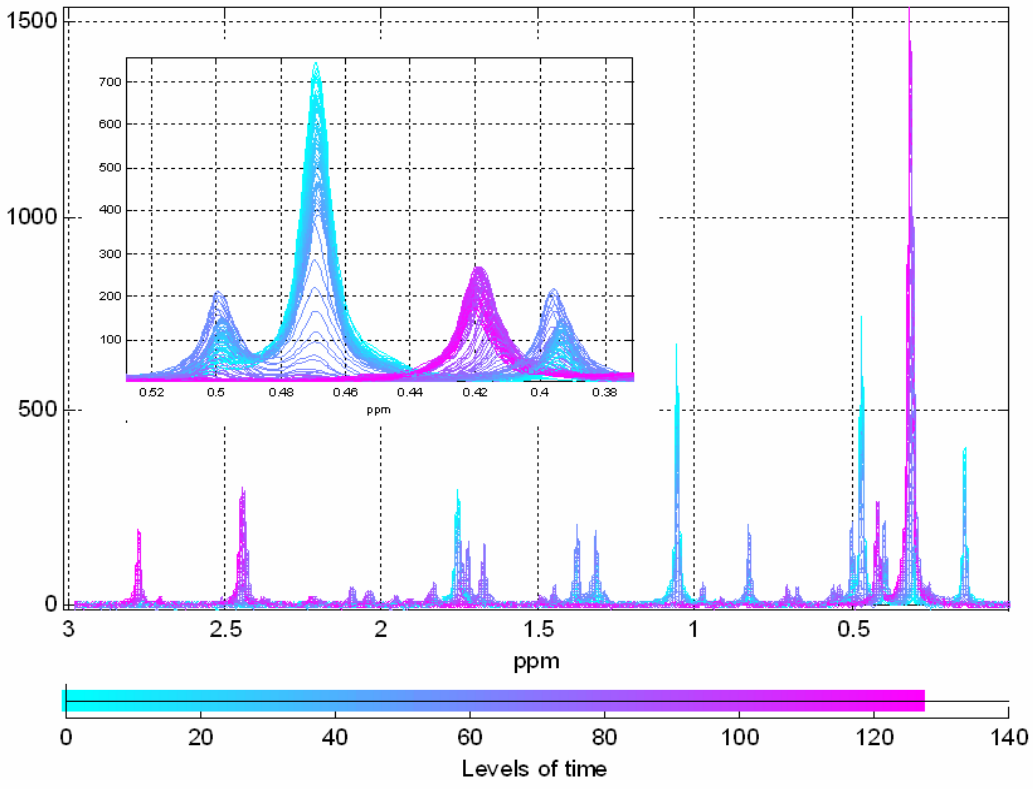

Figure 3 *^{31}P NMR spectra showing the hydrolysis of pure sodium phytate with phytase for 19 hours.*

To illustrate the changes in the ^{31}P spectra over time the area from 0.37 to 0.57 ppm is zoomed. From the figure it is apparent that three peaks decrease (including IP6 at 0.47 ppm) while one increase. This pattern of changes continues as IP5 degrades to IP4 and IP4 to IP3 and so on until only IP1 and inorganic phosphorus remain.

Figure 4 displays the results from a PCA of the time series ^{31}P spectra. The score plot, which sums up 94% of the total variation in the spectral data, has a typical horseshoe shape common to (mean-centered) sample reactions. This simplicity is not anticipated for the IP6 to IP1 complex reactions, which is already reflected if we add a third PC in a 3D score plot (see Figure 5). The additional 3% variation described by PC3 gives a complex loop pattern of the scores, indicating a rather complex score pattern.

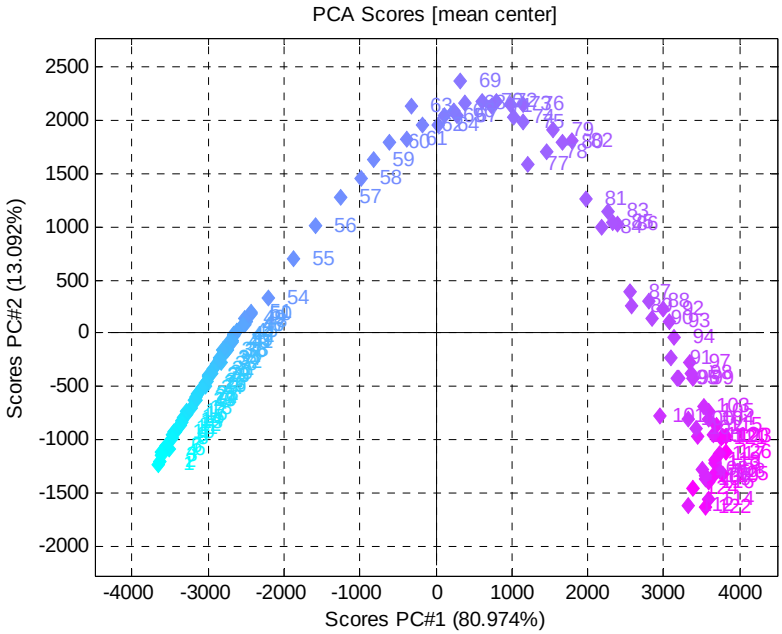

Figure 4 *PCA score plot of the mean-centered ^{31}P spectra. PC1 and PC2 together explain 94% of the variation in the NMR spectra.*

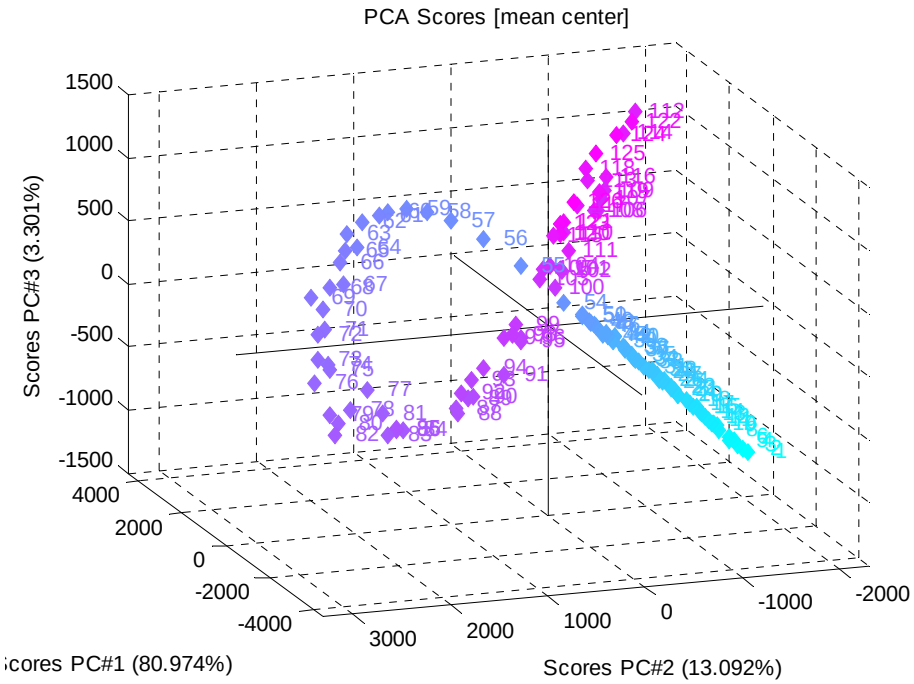

Figure 5 *3D PCA score plot of the mean-centered ^{31}P spectra. PC1, PC2 and PC3 together explain 98% of the variation in the NMR spectra.*

Instead of modeling the ^{31}P NMR spectra by PCA a more realistic model may result from the CARS or multivariate curve resolution method which does not impose the orthogonality constraint. Figure 6 displays the result of a six component CARS analysis of the ^{31}P NMR time series of the phytic acid degradation.

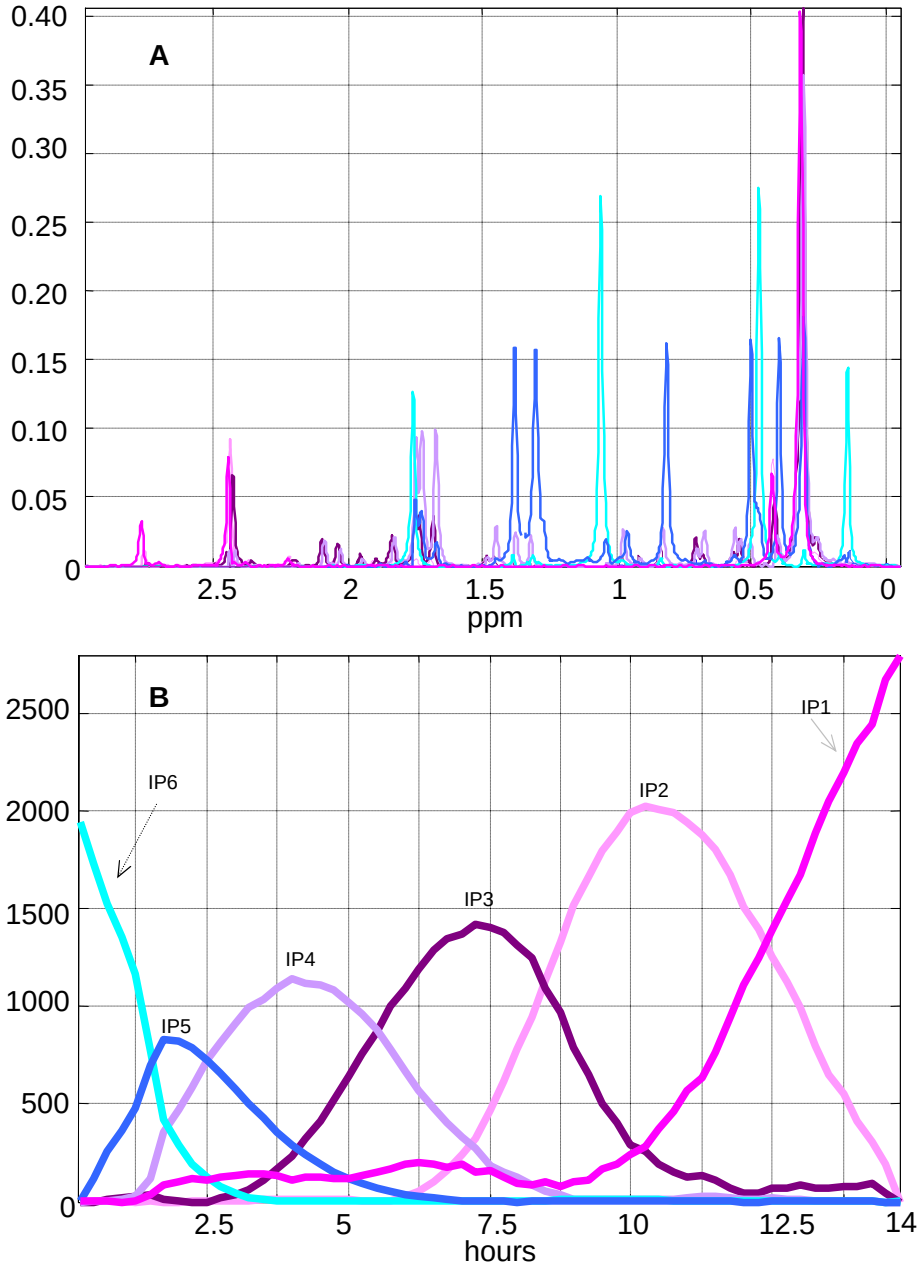

Figure 6 *(A) Pure ^{31}P NMR spectra from the CARS model. Cyan line=IP6, blue line=IP5, light purple line=IP4, purple line=IP3, pink line=IP2 and magenta line=IP1. (B) Plot of the relative intensity as a function of time. The same colors are used as identification for the different inositol phosphates as in (A)*

In the case of these NMR data the CARS method proved very reproducible independent of the initial guesses (random or spectra) of S. The model using six components gave the best description of the time series NMR spectra. A five-component model described less of the total variance and the seven-component model began to divide obvious single components. Figure 6 shows the result of the optimal six-component CARS model.

The six spectra of the compounds resolved from the time series of the phytase reaction using CARS is displayed in Figure 6A. The cyan line showing four peaks at ppm 0.14, 0.47, 1.06 and 1.76 represents IP6. IP5 is represented by five main peaks at 0.30, 0.38, 0.48, 0.83, 1.32 and 1.37 ppm. From then on the reaction becomes more complex because of the many possible configurations of phytate. In the case of IP4 3 peaks located at 1.68, 1.73 and 1.75 ppm dominate, but also many small peaks also coexist in the areas around 1.4, 2.1 and 2.4 ppm. These patterns are in good agreement with the rare literature found about ^{31}P NMR on pure phytate model systems[15-17]. In the case of IP3 and lower inositol phosphates it becomes very difficult to separate the peaks without use of chemometrics; however, the multivariate curve resolution program CARS makes it possible to separate all the components.

Perhaps most important is the result in Figure 6b which shows the complete degradation pattern of the phytase reaction using real multivariate experimental data. From the figure it is evident that already after 2.5 hours all IP6 is degraded. After approximately 1.5 hours the formation of IP5 (blue line) reaches a maximum and for the next six hours undergoes degradation to IP4 which reaches a maximum after four hours and is fully degraded after ten hours. This is in agreement with Frølich and coworkers[15] who did a similar experiment and found that IP4 was degraded after approx. 10 hours. According to the literature, phytic acid should be degraded to at least IP3 before the complex bound minerals are released and thus become bioavailable[18,19]. This implies that it takes approximately 10 hours (at the given concentration and pH) to degrade phytate to a form where the mineral complexes are no longer so strong and the minerals thus bioavailable for human and animals. IP3 has a lifetime of approximately 10 hours; for IP2 it is approximately 8 hours. IP1 is formed during the entire hydrolysis, but increases most after 10 hours.

4 CONCLUSIONS

The ^{31}P NMR method is a most direct and useful method to describe the degradation of phytic acid to lower inositol phosphates by the action of the enzyme phytase. The use of chemometric and CARS visualizes and helps in the interpretation of the results. By means of LatentiX it has been possible to visualize the time-dependent hydrolysis of phytic acid and by PCA the complexity of the phytic acid is shown in the score plots. By modeling the spectra in CARS it is possible to identify and quantify each of the inositol phosphates.

5 REFERENCES

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