Research Article

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Determination of D-*myo*-inositol phosphates in 'activated' raw almonds using anion-exchange chromatography coupled with tandem mass spectrometry

Lianna Y Lee and Alyson E Mitchell*

Abstract

BACKGROUND: Activated almonds are raw almonds that have been soaked in water for 12–24 h at room temperature, sometimes followed by a 24 h drying period at low temperature (50 ± 5 °C). This treatment is thought to enhance the nutrient bioavailability of almonds by degrading nutrient inhibitors, such as phytic acid or D-*myo*-inositol hexaphosphate ($InsP_6$), through the release of phytase or passive diffusion of $InsP_6$ into the soaking water. Over a wide pH range, $InsP_6$ is a negatively charged compound that limits the absorption of essential nutrients by forming insoluble complexes with minerals such as iron and zinc. It is hypothesized that hydrating the seed during soaking triggers $InsP_6$ degradation into lower *myo*-inositol phosphates with less binding capacity.

RESULTS: Anion-exchange chromatography coupled with tandem mass spectrometry was used to quantify *myo*-inositol mono-, di-, tris-, tetra-, penta-, and hexaphosphates ($InsP_{1-6}$) in raw pasteurized activated almonds. At least 24 h of soaking at ambient temperature was required to reduce $InsP_6$ content from 14.71 to 14.01 μmol g^{-1}.

CONCLUSIONS: The reduction in $InsP_6$ is statistically significant ($P < 0.05$) after 24 h of activation, but only represents a 4.75% decrease from the unsoaked almonds.

Keywords: activated almonds; inositol phosphate (InsP); phytic acid; anion exchange chromatography; LC–MS/MS

INTRODUCTION

Mineral deficiency is an international public health concern that affects more than one-third of the world's population, particularly in developing countries.[1] Adequate consumption of iron (Fe) and zinc (Zn) during childhood is necessary to maintain good health, growth, and cognitive development.[2] Low Fe and Zn status has been correlated with impaired immune function, poor pregnancy outcomes, and increased morbidity.[3] In households that rely on a plant-based diet, the low bioavailability of Fe and Zn in unprocessed cereals and legumes contributes to the prevalence of these metabolic disorders.[2] Germinating, fermentation, and soaking seeds in water, prior to consumption, have all been investigated as potential strategies for increasing nutrient bioavailability and improving mineral deficiencies.[4–9] Almonds (*Prunis dulcis*) are interesting in this regard, because they are an excellent source (>20% daily value) of Fe and Zn (both 0.031 g kg^{-1}).[10]

D-*myo*-Inositol hexaphosphate ($InsP_6$), also known as phytic acid, is ubiquitous in plants and is considered an anti-nutrient that reduces the bioavailability of mineral nutrients.[11] $InsP_6$ is the primary form of phosphate storage in seeds, and consists of a *myo*-inositol ring linked with up to six orthophosphate groups ($InsP_{1-6}$) via phosphoester bonds (Fig. 1). $InsP_6$ has 12 acid dissociation constants ranging, from 1.9 to 9.5, and is negatively charged at physiological pH.[11] Phytate, the ionized form of $InsP_6$, reduces mineral bioavailability by forming insoluble salts and complexes with positively charged minerals in pH environments commonly found in food and the human digestive system (pH 4–8).[6] The majority of these complexes remain insoluble during digestion, as the human stomach and small intestine have limited amounts of phytase and microbes capable of hydrolyzing $InsP_6$.[12] While the adverse effects of $InsP_6$ are negligible in households that regularly consume meat, reduced micronutrient bioavailability due to $InsP_6$ becomes significant for individuals that rely on plant-based diets.[2]

One of the most commonly used methods used to remove phytate from cereals, grains, and legumes is to soak them in water.[4–6] Soaking seeds is an ancient practice in many cultures intended to enhance the nutritional value and/or flavor of food.[13,14] In India and Pakistan, raw almonds are soaked overnight and served as a part of a meal to promote cognitive development in children.[13,14] Soaked almond products may be differentiated based on the temperature and pH of the soak water, and the length of soaking time. The most common forms of soaked almonds include sprouted,

* *Correspondence to: AE Mitchell, Department of Food Science and Technology, University of California, Davis, CA 95616, USA. E-mail: aemitchell@ucdavis.edu*

Department of Food Science and Technology, University of California, Davis, CA, USA

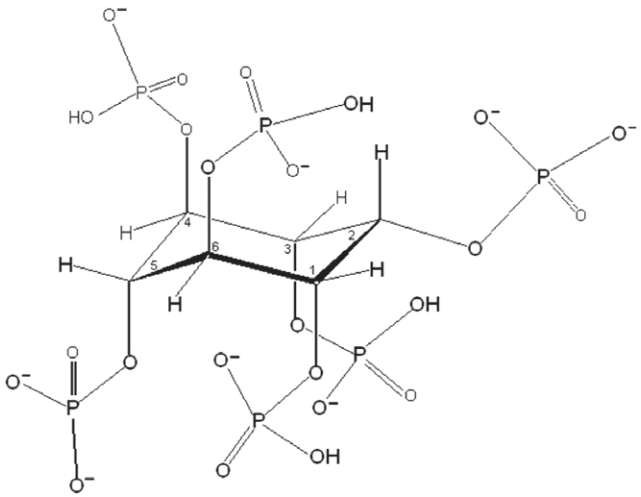

Figure 1. Structure of *myo*-inositol-1,2,3,4,5,6-hexaphosphate ($InsP_6$) at pH 6–7. Under physiological conditions, the negative charges are balanced by metal cations, such as sodium (Na^+). Conformation: 5, axial; 1, equatorial. In $InsP_{1-5}$, hydroxyl groups replace phosphate groups.

fermented, and activated almonds. During sprouting, raw seeds are steeped in water (10–12 h), rinsed, drained, and stored under moist conditions (1–3 days) at ambient temperature until germination occurs and plant sprouts appear.[7] Fermentation uses endogenous bacteria from the seeds to acidify the soaking solution, and typically requires 2–3 days at 25–30 °C to lower the pH.[8,9] Unlike sprouting or fermentation, preparing activated almonds is shorter, requiring 12–24 h of steeping followed by a low-heat drying step (45–55 °C) of 24 h to produce a low-moisture, shelf-stable product.

Activated almonds have drawn significant attention as a simple method for potentially enhancing the nutritional value of almonds, because less total time is required than for sprouting or fermentation. Because phytate is water soluble, the compound can be removed by passive diffusion into soaking water.[15] While several studies have shown that soaking cereals and legumes yields statistically significant reductions in $InsP_6$ (up to 50%) depending on the seed variety and soaking conditions,[5,9,16,17] the effects of soaking on phytate content remain inconclusive, because other investigations do not report large changes in phytate levels due to soaking.[4,18] Seed variety, maturity, structure, and experimental conditions may impact reported changes in phytase activity in response to soaking. The impact of soaking almond kernels has not been studied.

Germination of seeds can also reduce phytate levels by promoting the activity of endogenous phytase (E.C. 3.1.3.8, and 3.1.3.26) through *de novo* synthesis and/or activation of enzyme. Phytase hydrolyzes $InsP_6$ successively into less phosphorylated inositol phosphates (InsPs) – *myo*-inositol penta-, tetra-, tri-, di-, and mono6phosphate ($InsP_5$, $InsP_4$, $InsP_3$, $InsP_2$, and $InsP_1$) – which have less binding capacity and a smaller negative impact on mineral bioavailability.[19] The rate of enzyme activity depends on seed variety, stage of germination, moisture content, temperature, and pH.[4] *In vitro* seed germination introduces an additional incubation period (24–72 h) after seeds have been soaked in water at ambient temperature.[20] These longer soaking periods may also induce $InsP_6$ degradation by initiating the early stages of germination and/or optimizing conditions for phytase activity (45–55 °C; pH 4.5–5.5).[4]

Relative to other strategies for dephytinization, soaking is a simple and inexpensive method that can remove phytic acid without removing other beneficial nutrients through heat or mechanical processing.[15] While the effects of soaking and germination in cereals and legumes have been widely examined, similar possibilities for tree nuts remain relatively unexplored. This study addresses a lack of knowledge concerning dephytinization strategies for edible nuts by evaluating the effect of activation on $InsP_6$ in raw almonds. Levels of $InsP_{1-6}$ were quantified using liquid chromatography with tandem mass spectrometry (LC–MS/MS) and anion-exchange chromatography in almond samples soaked for 0, 4, 6, 8, 10, 14, and 24 h to investigate dephytinization as a function of soaking duration. Forms of InsP with less phosphorylation (i.e. $InsP_{1-5}$) were measured because they are products of $InsP_6$ hydrolysis and could improve understanding of the mechanism behind $InsP_6$ reduction. If activation degrades $InsP_6$ via enzymatic activity, we would observe an increase in hydrolysis by-products, especially $InsP_{1-3,}$ at longer soak times. However, if the main mechanism of $InsP_6$ reduction during activation were by passive diffusion into the water, $InsP_6$ concentration would decrease without increasing the amount of $InsP_{1-5}$ present.

EXPERIMENTAL

Chemicals and supplies

Phytic acid ($InsP_6$) sodium salt hydrate (≥90% phosphorus basis), adenosine 5′-monophosphate (AMP), and pentylamine (PTA) (99% assay) were obtained from the Sigma Aldrich Chemical Company (St. Louis, MO, USA). D-*myo*-Inositol-1,3,4,5,6-pentaphosphate ($InsP_5$) ammonium salt (98% assay), D-*myo*-inositol-1,3,4,6-tetraphosphate ($InsP_4$) ammonium salt (98% assay), D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) sodium salt (98% assay), D-*myo*-inositol-1,5-diphosphate ($InsP_2$) sodium salt (98% assay), and D-*myo*-inositol-1-phosphate ($InsP_1$) sodium salt (98% assay) were acquired from Alfa Aesar™ via Thermo Fisher Scientific (Pittsburg, PA, USA). Deionized water was prepared from a Milli-Q (MQ) system (Millipore, Bedford, MA) to a resistivity of 18 MΩ cm. All other reagents used were of analytical grade and obtained from commercial sources.

Samples

Raw, propylene oxide (PPO)-pasteurized Nonpareil almonds harvested in 2015 were obtained from Blue Diamond Growers (Sacramento California). Raw, whole almond kernels were stored at 4 °C (<3 months) until they were processed.

Moisture content analysis

The moisture content of almonds was determined gravimetrically by drying ground samples (1–2 g) at 90 °C under vacuum until constant weight was achieved (~24 h). Moisture was determined in triplicate samples.

Preparation of reference and analytical standards

The preparation of analytical standards was adapted from a method by Duong *et al.*[21] Individual solutions of $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$ were prepared in methanol–water (5 : 95 v/v) and combined with the AMP internal standard (AMP in methanol–water 5 : 95 v/v). $InsP_1$–$InsP_6$ standards were prepared at concentrations of 1, 5, 10, 20, 30, 40, 50, 60, 80, and 100 μmol L^{-1}. Additional $InsP_6$ standards were prepared at concentrations of

150, 200, 300, 350, and 400 μmol L^{-1}. A mixed standard with 1000 μmol L^{-1} of $InsP_{1-6}$ was prepared and combined with AMP. The final concentration of the internal standard was 75 μmol L^{-1} in each solution. Linear regression was used to construct standard curves for each InsP. All standards were prepared from stock solutions at the beginning of sample injection and stored at 4 °C for up to 1 week between sample injections.

The reference standard solution containing all six InsPs was prepared using a modified procedure based on Duong *et al.*[21] and Chen.[22] Briefly, 0.18 g of phytic acid sodium salt was dissolved in 60 mL of 3.2 mol L^{-1} acetic acid. The solution was transferred to a glass vial, flushed with nitrogen, sealed, and heated at 125 °C for 3 h, and then at 70 °C for 13 h. The solution was cooled to ambient temperature before it was evaporated to dryness using a Speed Vac System (Savant, Holbrook, NY, USA) set to 40 °C. The residue was reconstituted in 2.5 mL methanol–water solution (5 : 95 v/v) and stored at −20 °C until used.

Sample preparation

To prepare control and activated almonds, 100 g of raw, whole kernels were soaked in 500 mL of Milli-Q water in triplicate batches for 0, 4, 6, 8, 10, 16, and 24 h at 24 ± 2 °C. Samples were dried to final moisture content of 3.40–4.06 g kg^{-1} in a convection oven at 50 ± 5 °C for 24 h. Dried almonds were ground and sifted through a 35-mesh (0.417 mm opening) Tyler standard screen (W.S. Tyler, Mentor, OH) to ensure a uniform powder size. Almond powder was stored at −20 °C until analyzed.

Sample preparation was adapted from a method by Liu *et al.* with some modifications.[23] Duplicates of 0.5 g almond powder were extracted with 3 mL of 3.2 mol L^{-1} acetic acid for 3 h (300 rpm, 24 °C), vortex mixed (60 s), and centrifuged (3005 × *g*, 24 min, 20 °C). To prevent formation of insoluble complexes between minerals and phytate, 1 mL of supernatant was combined with 2 mL of 0.11 mol L^{-1} ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) in 0.75 mol L^{-1} sodium hydroxide (NaOH) and vortex mixed for 60 s. To remove fats, the solution was extracted twice with 3 mL aliquots of hexane and vortex mixing for 1.5 min The hexane layer was removed each time after centrifugation (3005 × *g*, 20 min). The final solution was filtered through a 0.22 μm Millex™ nylon syringe filter (EMD Millipore, Pittsburg, PA, USA) and combined with the AMP standard solution to attain a final concentration of 75 μmol L^{-1} AMP (925 μL sample solution and 75 μL of 1 mmol L^{-1} AMP).

LC–MS/MS analysis

$InsP_n$ forms were separated with an Agilent Infinity 1290 high-performance LC (HPLC) system (Wilmington, DE, USA) using a weak anion-exchange column (Hypersil GOLD AX, 100 mm × 2.1 mm, 3 μm) from Thermo Scientific (Pittsburg, PA).[24] The mobile phases for the chromatographic separation were 200 mmol L^{-1} aqueous ammonium carbonate, pH 9.0 (solvent A), and methanol in water, 5 : 95 v/v (solvent B). The injected sample size was 10 μL, and the $InsP_n$ forms were eluted at 200 μL min^{-1} using a 20 min gradient program beginning with solvent B for 4 min, followed by 0–20% A over 2 min, and finally 20–55% A over 14 min. The column was washed with 100% mobile phase B for 20 min at the end of each run. For a total flow rate of 400 μL min^{-1}, the eluents were mixed with 30 mmol L^{-1} PTA in a 5 : 95 v/v methanol–water solution (200 μL min^{-1} flow rate) in a post-column reactor to reduce sodium adduct formation according to the method by Rougemont *et al.*[25]

Table 1. Specifications for multiple reaction monitoring of the parent and product ions of $InsP_n$ and AMP

Analyte	Parent mass (*m/z*)[a]	Product mass (*m/z*)	Collision energy (eV)	Retention time (min)
$InsP_1$	259	79	19	8.77
$InsP_2$	339	241	19	10.22
$InsP_3$	419	321	20	13.03
$InsP_4$	499	401	20	16.37
$InsP_5$	579	481	23	17.47
$InsP_6$	659	561	25	22.16
AMP	346	79	20	9.41

[a] *m/z* is the mass-to-charge ratio.
Retention times displayed for each analyte.

An Agilent 1260 Infinity LC isocratic pump was used for the post-column addition of the PTA solution via a mixing tee (0.12 mm inner diameter, 400 mm length; Agilent Technologies Inc., Santa Clara, CA). Column temperature was maintained at 20 °C. The combined flow rate was 400 μL min^{-1}. Total run time was 45 min, where 25 min was used for compound separation, and 20 min for washing and equilibrating the column with 100% mobile phase B between runs. Retention times of each $InsP_n$ standard and the AMP internal standard are summarized in Table 1.

Electrospray ionization MS

The HPLC instrument was coupled to an Agilent 6460 triple quadrupole tandem mass spectrometer (Agilent Technologies Inc., Santa Clara, CA) with an electrospray ionization (ESI) source. The ESI source was operated in the negative mode to yield higher signal-to-noise (*S/N*) ratios. Data acquisition and analysis were performed with MassHunter software (Version B.06.00, Agilent Technologies Inc.). All samples were transferred into an auto-sampler vial with a 400 μL insert (National Scientific, Rockwood, TN).

Conditions for ESI-MS/MS were as follows: nebulizer pressure 55 psi, nozzle voltage 400 V, sheath gas (nitrogen) temperature 300 °C with flow rate 10 L min^{-1}. Capillary voltage 4000 V, drying gas temperature 300 °C with a flow rate of 10 L min^{-1}. MS^2 full-scan mode was used to identify the precursor ion and the optimal fragmentation voltage of each analyte. Collision energy and product ion(s) were optimized for each $InsP_n$ using product ion scan. Analytes were monitored by multiple reaction monitoring at optimized collision energies (Table 1).

Matrix effects

The influence of almond matrix on $InsP_n$ recovery was examined in solutions spiked with 100 μL and 200 μL of the 1000 μmol L^{-1} mixed $InsP_n$ standards solution ($n = 1, 2, 3, 4, 5$, and 6) at the beginning of sample extraction, such that 50 nmol g^{-1} and 100 nmol g^{-1} sample was added respectively. Recovery (%) was determined according to Association of Official Analytical Chemists:

$$\text{Recovery } (\%) = \frac{C_s - C_u}{C_a} \times 100 \quad (1)$$

where C_s (nmol g^{-1}) is the concentration of analyte measured in the spiked sample, C_u (nmol g^{-1}) is the concentration of analyte measured in non-spiked samples, and C_a (nmol g^{-1}) is the concentration of analyte added to the test sample.

Method validation

The analytical method was evaluated for linearity, precision, and accuracy. The calibration curves were determined in triplicate and were linear over a range of 1–100 μmol L^{-1} for $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$, and $InsP_5$, and 1–400 μmol L^{-1} for $InsP_6$.

Intra-day variability of the method was evaluated by analyzing the 50 μmol L^{-1} concentration of each $InsP_n$ standard at three times on the same day at 7 h intervals. Inter-day variability was assessed by measuring the 50 μmol L^{-1} concentration of each $InsP_n$ standard over three consecutive days. Precision was evaluated by calculating relative standard deviation (% RSD). Accuracy was evaluated by calculating the ratio of the experimental to the nominal concentration of each standard at 10, 50, and 100 μmol L^{-1}.

Quantitation of InsP analytes in almond samples

To determine the amount of $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$ in almond samples (micromoles of analyte per gram dry mass), a standard calibration curve was constructed for each analyte. The regression model was used to determine the concentration (μmol L^{-1}) of each $InsP_n$ in the extract. The amount of $InsP_n$ per dry mass of almonds were calculated using:

$$C = \frac{M \times v \times d_f}{m_d} \quad (2)$$

where C (μmol g^{-1}) is the concentration of analyte ($InsP_n$) per gram dry mass of almonds, M (mol L^{-1}) is the concentration of each analyte ($InsP_n$) in the extract, v is the total volume of extract (0.003 mL), d_f (=3) is the dilution factor to account for the addition of 2 mL ethylenediaminetetraacetic acid–NaOH(aq) solution to 1 mL sample supernatant after acetic acid extraction, and m_d is the mass of almond meal corrected for dry weight.

Statistical analysis

The limit of detection (LOD) and limit of quantification (LOQ) for each $InsP_n$ were calculated from standard solutions using the minimal accepted value of *S/N* ratio of 3 : 1 and 10 : 1 respectively. The effect of soaking time on the average amount of each $InsP_n$ was evaluated by a one-way factorial analysis of variance and followed by the *post hoc* Tukey's honest significant difference test at $P < 0.05$. Analyses were conducted in Microsoft Excel, Version 14.4.3 (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

InsPs are negatively charged compounds suited for separation by anion-exchange chromatography, and they are preferentially ionized by negative-ion electrospray.[21,23,24] Herein, $InsP_{1-6}$ and the internal standard AMP eluted within 25 min. Each $InsP_n$ and the AMP eluted in the same order in commercial standards (Fig. 2(A)) and almond samples (Fig. 2(B)), but retention times of the analytes in sample matrix were slightly delayed. Peak shapes were broader in samples, possibly due to the higher abundance of $InsP_n$ isomers. The calibration curves constructed using the $InsP_n$ standards and AMP achieved strong linearity ($R^2 > 0.99$) across the concentration ranges of interest (Table 2). The LOD (*S/N* of 3 : 1) was 1 pmol for $InsP_{1-5}$ and 3 pmol for $InsP_6$. The LOQ (S/N of 10 : 1) was 3 pmol for $InsP_{1-3}$ and 5 pmol for $InsP_{4-6}$. Intra-day precision for analysis of 80 μmol L^{-1} $InsP_{1-6}$ standards ranged between 1.49% and 2.89%. Inter-day variability of each standard was measured at 80 μmol L^{-1} over three consecutive days and % RSD values ranged between 2.59% and 4.92%.

Because $InsP_n$ has up to 12 ionizable protons, pH plays a critical role in the recovery of InsP. At a low pH, $InsP_n$ can form insoluble complexes with proteins and cations, and precipitate from solution. To avoid this effect, pH can be adjusted to above 4.5–5 following acetic acid extraction.[21] Addition of Na_2EDTA can reduce formation of insoluble salts by acting as strong chelators for cations.[21] In order to prevent precipitation and loss of $InsP_n$ during sample preparation, 0.11 mol L^{-1} Na_2EDTA in 0.75 mol L^{-1} NaOH was mixed with sample extract (2 : 1 v/v) before the solution was defatted using hexane. In addition, the affinity of the negatively charged phosphates to cations in the sample (e.g. sodium) reduced signal sensitivity and suppressed ionization of $InsP_6$. This was resolved with the post-column addition of PTA at a final concentration of 15 mmol L^{-1} in the mobile phase. Polyamines such as PTA remove sodium adducts by creating ion pairs during MS analysis.[25]

To evaluate the matrix effects of almond meal on recovery of the $InsP_n$, the reference standard containing $InsP_{1-6}$ was spiked into almond samples at the beginning of the acetic acid (3.2 mol L^{-1}) extraction. The recovery analysis was conducted at two concentration levels (200 and 400 μL of in-house reference standard per gram dry sample) corresponding to recovery 1 and 2 respectively (Table 3). Recoveries ranged from 73% to 101% (Table 3), which compares with the recoveries determined by previous studies with similar $InsP_n$ extraction methods (68% to 111%).[23] Lower recovery percentages suggest that the almond matrix interferes with ionization of $InsP_n$ compounds, and this is more apparent at lower concentration levels.

The effect of soaking time on $InsP_{1-6}$ concentrations in raw, pasteurized almonds is summarized in Table 4. Results indicate that activating almonds for 24 h reduces $InsP_6$ concentration by 4.75% from the initial value ($P < 0.05$). $InsP_6$ content decreases by 0.21–2.00% between 4 and 14 h of activation, but the difference is not statistically significant ($P > 0.05$) from the control sample (Table 4). $InsP_4$ and $InsP_5$ levels increased after 6 h of soaking ($P < 0.05$), and concentrations continued to increase between 6 and 14 h, but values were not statistically different ($P > 0.05$) (Table 4). After 24 h of soaking, $InsP_4$ and $InsP_5$ demonstrated significant ($P < 0.05$) increases by 38% and 35% respectively from the control (Table 4). $InsP_1$, $InsP_2$, and $InsP_3$ concentrations remain constant, as mean values were not statistically different ($P > 0.05$) between different soaking times. The amount of $InsP_n$ in unprocessed, raw almonds determined in this study is comparable to results reported by Liu *et al.* for $InsP_{1-6}$ analysis using LC–(ESI-)MS/MS) in almonds,[23] demonstrating satisfactory extraction of target analytes.

Our observations are in agreement with previous findings that report a minimal $InsP_6$ reduction after soaking various cereals and legumes in water for 6–24 h at 25 °C.[4,9,16] $InsP_6$ content for most grains and seeds remained between 86% and 100% after soaking.[4] Steeping brown rice in water for 24 h at 25 °C resulted in a minor $InsP_6$ reduction (<20%),[9] and soaking pea cultivars for 6–18 h at ambient temperature decreased $InsP_6$ by 7–12%.[16] Though statistically significant, these reductions in $InsP_6$ will likely be insufficient for influencing mineral bioavailability, because $InsP_6$ is a potent inhibitor even at low levels.[2,9,26,27] $InsP_6$ levels had to be less than 0.3 mg g^{-1} sample before a significant increase of 4.17% in Fe absorption was observed for soy flour ($P < 0.001$).[26] The amount of $InsP_6$ had to be below 0.01 mg g^{-1} isolate, 0.11% of the initial, before Fe absorption increased four- to fivefold.[26]

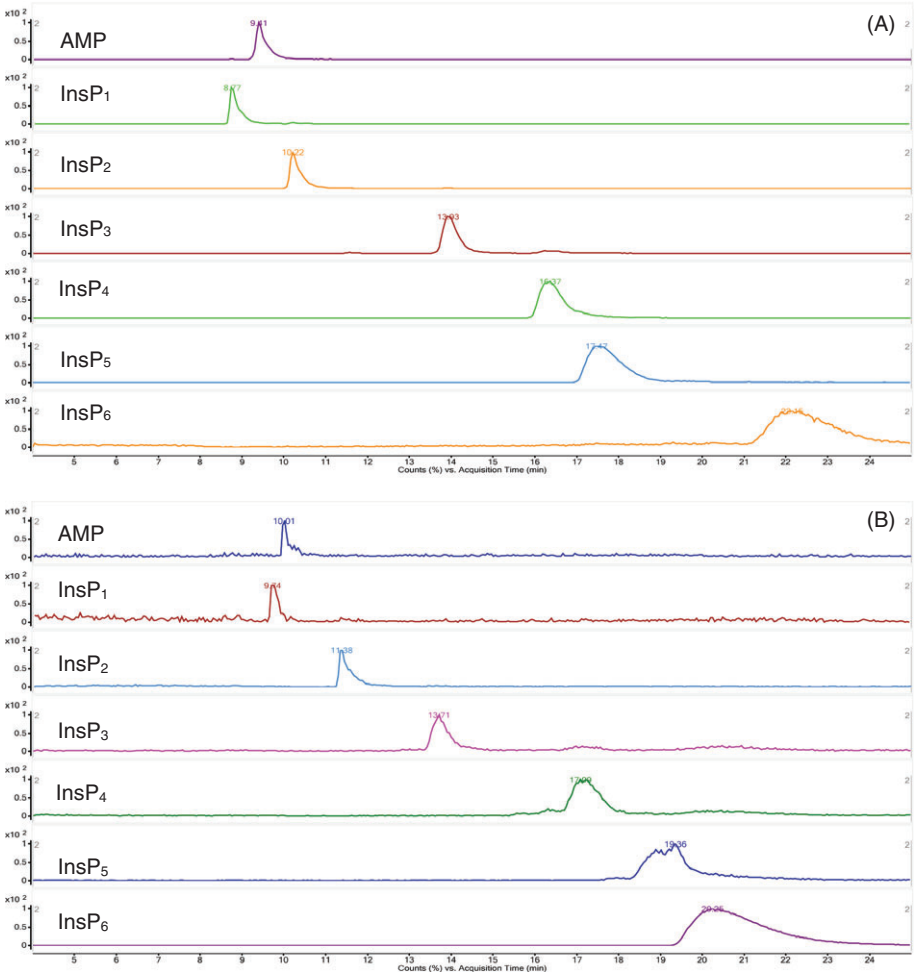

Figure 2. LC–(ESI-)MS/MS chromatograms of $InsP_{1-6}$ and adenosine 5′-monophosphate (AMP) detected in (A) commercial standards and (B) almond powder samples.

Table 2. Linear range, correlation coefficient, slope, intercept, LOD, and LOQ for each $InsP_n$ standard from three replicate analyses

Analyte	Slope ($\times 10^2$)	Intercept ($\times 10^2$)	R^2	Linear range (μmol L^{-1})	LOD (pmol)	LOQ (pmol)
$InsP_1$	0.027	0.1349	0.998	1–100	1	3
$InsP_2$	0.015	0.1301	0.996	1–100	1	3
$InsP_3$	0.012	0.1308	0.996	1–100	1	3
$InsP_4$	0.015	0.1497	0.993	1–100	1	5
$InsP_5$	0.015	0.1171	0.999	1–100	1	5
$InsP_6$	0.004	0.1103	0.998	1–400	3	5

Studies that report large reductions in $InsP_6$ after soaking different cereals and legumes usually combined soaking with other treatments, such as preheating the rice, increasing the time and temperature of soaking, or adjusting pH.[9,16,17] For example, soaking preheated rice at 10 °C led to 42–59% removal.[9] $InsP_6$ content in pea seeds decreased 67–83% after 48 h soaking or sprouting.[16] Significant reductions in $InsP_6$ and $InsP_5$ by 54% were reported in black beans (*Phaseolus vulgaris*) as a result of soaking in water (50–60 °C) at different pH (4.5–8.0).[17] Our findings differ due to dissimilarities in soaking conditions, which would influence the rate of $InsP_6$ hydrolysis by endogenous phytase.

Another explanation for the limited $InsP_6$ reduction observed in our study may be differences in phytase activity and seed matrix composition specific to different plant varieties. In a study by Lestienne *et al.*, identical soaking conditions lowered $InsP_6$ concentration in some cereals and legumes (e.g. millet, maize, rice and soybeans) but not others (e.g. sorghum, cowpeas, and mung beans),[5] which suggests that different seed types have distinctive capacities to hydrolyze $InsP_6$ based on the endogenous phytase activity and/or the seed matrix interactions.[4] Cereals and pseudo-cereals tend to exhibit high phytate-degrading activities, while legumes and oilseeds exhibit a tenfold lower phytate-degrading activity.[28] This information corresponds with another study comparing $InsP_6$ in soaked seeds, which reported 20–30% $InsP_6$ reductions in soaked cereals (e.g. rice, rye, and triticale) compared with 0–14% dephytinization in soaked legumes

Table 3. The effect of almond matrix on recovery. Level of InsPs (μmol g^{-1} sample)[a] and recovery (%) of analytes in 14 h activated almond samples spiked with in-house reference containing $InsP_{1-6}$

Analyte	Amount added (μmol g^{-1})	Amount detected (μmol g^{-1})	Recovery[b] (%)	
			1	2
$InsP_1$	1.25	2.32 ± 0.04	73.5	95.2
	2.01	3.68 ± 0.39		
$InsP_2$	1.76	4.34 ± 0.05	78.8	97.5
	2.82	6.79 ± 0.10		
$InsP_3$	0.41	3.06 ± 0.03	93.2	100.9
	0.63	4.00 ± 0.08		
$InsP_4$	1.43	3.91 ± 0.02	76.4	93.5
	2.29	5.89 ± 0.26		
$InsP_5$	0.73	3.69 ± 0.08	88.3	98.0
	1.17	5.24 ± 0.02		
$InsP_6$	5.56	14.70 ± 0.02	85.7	94.3
	8.90	15.95 ± 0.02		

[a] The mass of almonds has been adjusted for dry mass using moisture content (percetage dry basis).
[b] Recovery 1: 200 μL of reference standard per gram almonds; recovery 2: 400 μL of reference standard per gram almonds.

Table 4. $InsP_{1-6}$ content in raw almonds[a,b] (micromoles analyte per gram dry mass almonds) and percentage loss of $InsP_6$ after 0–24 h of soaking in water at 24 ± 2 °C followed by drying (50 ± 5 °C) for almond activation

Time (h)	$InsP_1$	$InsP_2$	$InsP_3$	$InsP_4$	$InsP_5$	$InsP_6$	$InsP_6$ loss (%)
0	0.66 ± 0.03a	1.87 ± 0.08a	2.22 ± 0.06a	2.34 ± 0.14a	3.54 ± 0.16a	14.71 ± 0.37a	0
4	0.69 ± 0.01a	2.15 ± 0.03a	2.35 ± 0.04a	2.44 ± 0.03a	3.72 ± 0.02a	14.74 ± 0.32a	0.21
6	0.70 ± 0.07a	2.21 ± 0.21a	2.51 ± 0.20a	2.79 ± 0.35b	4.15 ± 0.38b	14.57 ± 0.18a	0.96
8	0.66 ± 0.02a	2.09 ± 0.06a	2.39 ± 0.09a	2.71 ± 0.13ab	4.15 ± 0.10b	14.49 ± 0.17a	1.47
10	0.66 ± 0.05a	2.19 ± 0.17a	2.43 ± 0.15a	2.89 ± 0.20b	4.22 ± 0.31b	14.52 ± 0.49a	1.28
14	0.63 ± 0.03a	2.10 ± 0.11a	2.34 ± 0.13a	2.84 ± 0.14b	4.46 ± 0.25bc	14.42 ± 0.36a	2.00
24	0.66 ± 0.02a	2.11 ± 0.07a	2.39 ± 0.09a	3.27 ± 0.16c	4.82 ± 0.19c	14.01 ± 0.56b	4.75

[a] For each $InsP_n$ form, mean plus/minus standard deviation followed by the same letter is not significantly different across different cultivars according to one-way analysis of variance and Tukey's honest significant difference test ($P > 0.05$).
[b] The mass of almonds with different soaking times has been adjusted for dry mass using the moisture content (percentage dry basis) of each corresponding treatment group.
Values represent mean plus/minus standard deviation of six replicates.

(e.g. chickpeas, lentil, and soybean).[4] With respect to seed matrices, phytase and other enzymes needed during germination are located in the embryo, while metabolized food storage compounds, consisting of proteins, lipids, and starches, are primarily contained in the cotyledons surrounding the seed embryo.[29] The high fat content in almond seed cotyledons (43.36 ± 0.62 g kg^{-1} by weight)[29,30] may interfere with the passive diffusion of $InsP_6$ or activation of phytases by impeding water migration.

The use of pasteurized almond samples may also influence the rate of $InsP_6$ degradation via thermal inactivation of phytase. Pasteurized almonds are considered raw and were used for this study, because previous studies have indicated that neither heat nor fumigant pasteurization diminished the nutritional value and sensory attributes of almonds.[31] However, temperatures during PPO treatment (up to 71 °C) and steam pasteurization (95 °C)[32] can surpass the 60 °C threshold at which phytase begins to denature.[33] If heat exposure were a significant factor that prevented $InsP_6$ hydrolysis, $InsP_6$ reduction after soaking may be more pronounced in unpasteurized almonds. To understand the effects of activation without prior exposure to temperatures above 60 °C, unpasteurized almonds would need to be used. However, these are not available for commercial consumption in the USA and are not the form of almond that people are 'activating'.

Concentrations of $InsP_{1-5}$ were measured in our study to examine the role of phytase in dephytinization in activated almonds. Levels of $InsP_4$ and $InsP_5$, after 24 h of soaking (Table 4), may be attributed to stepwise release of phosphate from $InsP_6$ by phytase.[28,34] The change in $InsP_{1-3}$ is less apparent, as the intrinsic concentration of these compounds in the seed is low (<5.4 μmol g^{-1}), and their quantity may fall below the LOQ as they diffuse into the soaking water. Phytase was likely involved in $InsP_6$ hydrolysis over the 24 h soaking period, but the corresponding rises in $InsP_4$ and $InsP_5$ lead to limited improvements in mineral bioavailability. Additional treatments would be necessary to continue $InsP_{4-6}$ hydrolysis to $InsP_{1-3}$ compounds with fewer negative charges and weaker binding capacity.

CONCLUSION

The results of this study suggest that activation does not enhance the nutritional value of raw pasteurized almonds by significantly reducing phytic acid levels. At least 24 h of activation

was necessary to decrease the amount of $InsP_6$ in almonds by 4.75% to 9.28 mg g^{-1}. This reduction would not represent a significant improvement in mineral absorption, because $InsP_6$ is a strong chelator that must be significantly reduced before mineral absorption would appreciably increase. Activating raw pasteurized almonds is a time-intensive and costly dephytinization method that does not offer the anticipated health benefit of increased mineral bioavailability.

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REFERENCES

1 Gupta RK, Gangoliya SS and Singh NK, Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J Food Sci Technol* **52**:676–684 (2013).
2 Gibson RS, Bailey KB, Gibbs M and Ferguson EL, A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. *Food Nutr Bull* **31**:S134–S146 (2010).
3 Lönnerdal B, Phytic acid–trace element (Zn, Cu, Mn) interactions. *Int J Food Sci Technol* **37**:749–758 (2002).
4 Egli I, Davidsson L, Juillerat MA, Barclay D and Hurrell RF, The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. *J Food Sci* **67**:3484–3488 (2002).
5 Lestienne I, Icard-Vernière C, Mouquet C, Picq C and Trèche S, Effects of soaking whole cereal and legume seeds on iron, zinc and phytate contents. *Food Chem* **89**:421–425 (2005).
6 Greiner R and Konietzny U, Phytase for food application. *Food Technol Biotechnol* **44**:123–140 (2006).
7 Ou K, Cheng Y, Xing Y, Lin L, Nout R and Liang J, Phytase activity in brown rice during steeping and sprouting. *J Food Sci Technol* **48**:598–603 (2011).
8 Obizoba IC and Egbuna HI, Effect of germination and fermentation on the nutritional quality of bambara nut (*Voandzeia subterranea* L. Thouars) and its product (milk). *Plant Foods Hum Nutr* **42**:13–23 (1992).
9 Liang J, Han B-Z, Nout MJR and Hamer RJ, Effects of soaking, germination and fermentation on phytic acid, total and *in vitro* soluble zinc in brown rice. *Food Chem* **110**:821–828 (2008).
10 US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory. *USDA National Nutrient Database for Standard Reference, Legacy*. [Online]. Nutrient Data Laboratory, Beltsville, MD (2018). Available: https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/nutrient-data-laboratory/docs/usda-national-nutrient-database-for-standard-reference/ [30 June 2018].
11 Heighton L, Schmidt WF, Rice C and Siefert R, Electrospray ionization mass spectroscopy shows speciation of phytate to be pH dependent. *Int J Food Agric Environ* **6**:402–407 (2008).
12 Iqbal TH, Lewis KO and Cooper BT, Phytase activity in the human and rat small intestine. *Gut* **35**:1233–1236 (1994).
13 Kamat PS, Mittal N, Vuppu S and Mishra B, A brief study on raw and soaked south Indian almonds, peanuts, raisins and sauerkraut for nutritive value. *Sci J Agric Res Manage* **2012** (2012). https://doi.org/10.7237/sjarm/243
14 Jamshed H, Gilani A-H, Sultan FAT, Amin F, Arslan J, Ghani S *et al.*, Almond supplementation reduces serum uric acid in coronary artery disease patients: a randomized controlled trial. *Nutr J* **15**:77 (2016).
15 Perlas LA and Gibson RS, Use of soaking to enhance the bioavailability of iron and zinc from rice-based complementary foods used in the Philippines. *J Sci Food Agric* **82**:1115–1121 (2002).
16 Bishnoi S, Khetarpaul N and Yadav RK, Effect of domestic processing and cooking methods on phytic acid and polyphenol contents of pea cultivars (*Pisum sativum*). *Plant Foods Hum Nutr* **45**:381–388 (1994).
17 Greiner R and Konietzny U, Improving enzymatic reduction of *myo*-inositol phosphates with inhibitory effects on mineral absorption in black beans (*Phaseolus vulgaris* var. Preto). *J Food Process Preserv* **23**:249–261 (1999).
18 Schlemmer U, Müller H and Jany KD, The degradation of phytic acid in legumes prepared by different methods. *Eur J Clin Nutr* **49**:S207–S210 (1995).
19 Greiner R and Konietzny U, Phytases: biochemistry, enzymology and characteristics relevant to animal feed use, in *Enzymes in Farm Animal Nutrition,* 2nd edn, ed. by Bedford MR and Partridge GG. CAB International, Wallingford, pp. 96–128 (2010).
20 Centeno C, Viveros A, Brenes A, Canales R, Lozano A and de la Cuadra C, Effect of several germination conditions on total P, phytate P, phytase, and acid phosphatase activities and inositol phosphate esters in rye and barley. *J Agric Food Chem* **49**:3208–3215 (2001).
21 Duong QH, Clark KD, Lapsley KG and Pegg RB, Quantification of inositol phosphates in almond meal and almond brown skins by HPLC/ESI/MS. *Food Chem* **229**:84–92 (2017).
22 Chen Q, Determination of phytic acid and inositol pentakisphosphates in foods by high-performance ion chromatography. *J Agric Food Chem* **52**:4604–4613 (2004).
23 Liu X, Villalta PW and Sturla SJ, Simultaneous determination of inositol and inositol phosphates in complex biological matrices: quantitative ion-exchange chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* **23**:705–712 (2009).
24 Paraskova JV, Jørgensen C, Reitzel K, Pettersson J, Rydin E and Sjöberg PJR, Speciation of inositol phosphates in lake sediments by ion-exchange chromatography coupled with mass spectrometry, inductively coupled plasma atomic emission spectroscopy, and ^{31}P NMR spectroscopy. *Anal Chem* **87**:2672–2677 (2015).
25 Rougemont B, Fonbonne C, Lemoine J, Bourgeaux V and Salvador A, Liquid chromatography coupled to tandem mass spectrometry for the analysis of inositol hexaphosphate after solid-phase extraction. *J Liq Chromatogr Relat Technol* **39**:408–414 (2016).
26 Hurrell RF, Juillerat MA, Reddy MB, Lynch SR, Dassenko SA and Cook JD, Soy protein, phytate, and iron absorption in humans. *Am J Clin Nutr* **56**:573–578 (1992).
27 Hurrell R and Egli I, Iron bioavailability and dietary reference values. *Am J Clin Nutr* **91**:1461S–1467S (2010).
28 Konietzny U and Greiner R, Molecular and catalytic properties of phytate-degrading enzymes (phytases). *Int J Food Sci Technol* **37**:791–812 (2002).
29 Dourado F, Barros A, Mota M, Coimbra MA and Gama FM, Anatomy and cell wall polysaccharides of almond (*Prunus dulcis* D. A. Webb) seeds. *J Agric Food Chem* **52**:1364–1370 (2004).
30 Venkatachalam M and Sathe SK, Chemical composition of selected edible nut seeds. *J Agric Food Chem* **54**:4705–4714 (2006).
31 Jeong S, Marks BP, Ryser ET and Harte JB, The effect of X-ray irradiation on *Salmonella* inactivation and sensory quality of almonds and walnuts as a function of water activity. *Int J Food Microbiol* **153**:365–371 (2012).
32 Almond Board of California. *Guidelines for Validation of Propylene Oxide Pasteurization*. [Online]. (2008). Available: https://www.almonds.com/sites/default/files/content/attachments/ppo-validation-guidelines.pdf [30 June 2018].
33 Denstadli V, Vestre R, Svihus B, Skrede A and Storebakken T, Phytate degradation in a mixture of ground wheat and ground defatted soybeans during feed processing: effects of temperature, moisture level, and retention time in small- and medium-scale incubation systems. *J Agric Food Chem* **54**:5887–5893 (2006).
34 Greiner R, Konietzny U and Jany KD, Phytate – an undesirable constituent of plant-based foods? *J Ernährungsmed* **8**:18–28 (2006).

