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Authors

Lee, Lianna Y
Mitchell, Alyson E

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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 ($50 \pm 5^\circ\text{C}$). 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 $\mu\text{mol g}^{-1}$.

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.

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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 (*Prunus 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 (InsP₆) 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 InsP₆ (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 InsP₆ successively into less phosphorylated inositol phosphates (InsPs) – *myo*-inositol penta-, tetra-, tri-, di-, and mono-phosphate (InsP₅, InsP₄, InsP₃, InsP₂, and InsP₁) – 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 InsP₆ 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 InsP₆ 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 InsP₆ hydrolysis and could improve understanding of the mechanism behind InsP₆ reduction. If activation degrades InsP₆ 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 InsP₆ reduction during activation were by passive diffusion into the water, InsP₆ concentration would decrease without increasing the amount of InsP_{1–5} present.

EXPERIMENTAL

Chemicals and supplies

Phytic acid (InsP₆) 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 (InsP₅) ammonium salt (98% assay), *D*-*myo*-inositol-1,3,4,6-tetraphosphate (InsP₄) ammonium salt (98% assay), *D*-*myo*-inositol-1,4,5-trisphosphate (InsP₃) sodium salt (98% assay), *D*-*myo*-inositol-1,5-diphosphate (InsP₂) sodium salt (98% assay), and *D*-*myo*-inositol-1-phosphate (InsP₁) 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 InsP₁, InsP₂, InsP₃, InsP₄, InsP₅, and InsP₆ were prepared in methanol–water (5 : 95 v/v) and combined with the AMP internal standard (AMP in methanol–water 5 : 95 v/v). InsP₁–InsP₆ standards were prepared at concentrations of 1, 5, 10, 20, 30, 40, 50, 60, 80, and 100 μmol L⁻¹. Additional InsP₆ standards were prepared at concentrations of

150, 200, 300, 350, and 400 $\mu\text{mol L}^{-1}$. A mixed standard with 1000 $\mu\text{mol L}^{-1}$ of InsP_{1-6} was prepared and combined with AMP. The final concentration of the internal standard was 75 $\mu\text{mol L}^{-1}$ 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 ($3005 \times g$, 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 ($3005 \times g$, 20 min). The final solution was filtered through a 0.22 μm Millex™ nylon syringe filter (EMD Millipore, Pittsburgh, PA, USA) and combined with the AMP standard solution to attain a final concentration of 75 $\mu\text{mol L}^{-1}$ 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 $\times$ 2.1 mm, 3 μm) from Thermo Scientific (Pittsburgh, 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 $\mu\text{L min}^{-1}$ 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 $\mu\text{L min}^{-1}$, the eluents were mixed with 30 mmol L^{-1} PTA in a 5 : 95 v/v methanol–water solution (200 $\mu\text{L min}^{-1}$ 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 $\mu\text{L min}^{-1}$. 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} . MS² 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 $\mu\text{mol L}^{-1}$ 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 $\mu\text{mol L}^{-1}$ for InsP_1 , InsP_2 , InsP_3 , InsP_4 , and InsP_5 , and 1–400 $\mu\text{mol L}^{-1}$ for InsP_6 .

Intra-day variability of the method was evaluated by analyzing the 50 $\mu\text{mol L}^{-1}$ 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 $\mu\text{mol L}^{-1}$ 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 $\mu\text{mol L}^{-1}$.

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 ($\mu\text{mol L}^{-1}$) 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 ($\mu\text{mol g}^{-1}$) 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 $\mu\text{mol L}^{-1}$ InsP_{1-6} standards ranged between 1.49% and 2.89%. Inter-day variability of each standard was measured at 80 $\mu\text{mol L}^{-1}$

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 InsP₁₋₆ 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 ($\mu\text{mol L}^{-1}$)	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 InsP₆ 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 InsP₆ content in pea seeds decreased 67–83% after 48 h soaking or sprouting.¹⁶ Significant reductions in InsP₆ and InsP₅ 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 InsP₆ hydrolysis by endogenous phytase.

Another explanation for the limited InsP₆ 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 InsP₆ 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 InsP₆ 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 InsP₆ in soaked seeds, which reported 20–30% InsP₆ 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 ($\mu\text{mol g}^{-1}$ sample)^a and recovery (%) of analytes in 14 h activated almond samples spiked with in-house reference containing InsP₁₋₆

Analyte	Amount added ($\mu\text{mol g}^{-1}$)	Amount detected ($\mu\text{mol g}^{-1}$)	Recovery ^b (%)	
			1	2
InsP ₁	1.25	2.32 $\pm$ 0.04	73.5	95.2
	2.01	3.68 $\pm$ 0.39		
InsP ₂	1.76	4.34 $\pm$ 0.05	78.8	97.5
	2.82	6.79 $\pm$ 0.10		
InsP ₃	0.41	3.06 $\pm$ 0.03	93.2	100.9
	0.63	4.00 $\pm$ 0.08		
InsP ₄	1.43	3.91 $\pm$ 0.02	76.4	93.5
	2.29	5.89 $\pm$ 0.26		
InsP ₅	0.73	3.69 $\pm$ 0.08	88.3	98.0
	1.17	5.24 $\pm$ 0.02		
InsP ₆	5.56	14.70 $\pm$ 0.02	85.7	94.3
	8.90	15.95 $\pm$ 0.02		

^a The mass of almonds has been adjusted for dry mass using moisture content (percentage 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.** InsP₁₋₆ content in raw almonds^{a,b} (micromoles analyte per gram dry mass almonds) and percentage loss of InsP₆ after 0–24 h of soaking in water at 24 $\pm$ 2 °C followed by drying (50 $\pm$ 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 $\pm$ 0.03a	1.87 $\pm$ 0.08a	2.22 $\pm$ 0.06a	2.34 $\pm$ 0.14a	3.54 $\pm$ 0.16a	14.71 $\pm$ 0.37a	0
4	0.69 $\pm$ 0.01a	2.15 $\pm$ 0.03a	2.35 $\pm$ 0.04a	2.44 $\pm$ 0.03a	3.72 $\pm$ 0.02a	14.74 $\pm$ 0.32a	0.21
6	0.70 $\pm$ 0.07a	2.21 $\pm$ 0.21a	2.51 $\pm$ 0.20a	2.79 $\pm$ 0.35b	4.15 $\pm$ 0.38b	14.57 $\pm$ 0.18a	0.96
8	0.66 $\pm$ 0.02a	2.09 $\pm$ 0.06a	2.39 $\pm$ 0.09a	2.71 $\pm$ 0.13ab	4.15 $\pm$ 0.10b	14.49 $\pm$ 0.17a	1.47
10	0.66 $\pm$ 0.05a	2.19 $\pm$ 0.17a	2.43 $\pm$ 0.15a	2.89 $\pm$ 0.20b	4.22 $\pm$ 0.31b	14.52 $\pm$ 0.49a	1.28
14	0.63 $\pm$ 0.03a	2.10 $\pm$ 0.11a	2.34 $\pm$ 0.13a	2.84 $\pm$ 0.14b	4.46 $\pm$ 0.25bc	14.42 $\pm$ 0.36a	2.00
24	0.66 $\pm$ 0.02a	2.11 $\pm$ 0.07a	2.39 $\pm$ 0.09a	3.27 $\pm$ 0.16c	4.82 $\pm$ 0.19c	14.01 $\pm$ 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 ($43.36 \pm 0.62 \text{ g kg}^{-1}$ by weight)^{29,30} may interfere with the passive diffusion of InsP₆ or activation of phytases by impeding water migration.

The use of pasteurized almond samples may also influence the rate of InsP₆ 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 InsP₆ hydrolysis, InsP₆ 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 InsP₁₋₅ were measured in our study to examine the role of phytase in dephytinization in activated almonds. Levels of InsP₄ and InsP₅, after 24 h of soaking (Table 4), may be attributed to stepwise release of phosphate from InsP₆ by phytase.^{28,34} The change in InsP₁₋₃ is less apparent, as the intrinsic concentration of these compounds in the seed is low ($<5.4 \mu\text{mol g}^{-1}$), and their quantity may fall below the LOQ as they diffuse into the soaking water. Phytase was likely involved in InsP₆ hydrolysis over the 24 h soaking period, but the corresponding rises in InsP₄ and InsP₅ lead to limited improvements in mineral bioavailability. Additional treatments would be necessary to continue InsP₄₋₆ hydrolysis to InsP₁₋₃ 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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