DETERMINATION OF *MYO*-INOSITOL PHOSPHATES IN MAJOR TREE NUTS BY HPLC/ESI/MS

by

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(Under the Direction of Ronald B. Pegg)

ABSTRACT

In plant foods, inositol phosphates (InsPs) are commonly found in highest abundance in seeds, but knowledge on the InsPs deposition of tree nuts has been lacking. Utilizing HPLC/ESI/MS, an InsPs extraction and analysis method was refined specifically for the complex matrices of almond meal and almond brown skins. This assay was used to evaluate six major California sweet almond cultivars, as well as the meal of seven major tree nuts (cashews, Brazil nuts, macadamias, walnuts, pecans, pistachios, hazelnuts) and three grain components allegedly rich in phosphorus (wheat aleurone, rice bran, corn germ). InsPs composition was found to vary strongly among different almond cultivars, among different tree nuts, and between tree nuts and grain components. Possible influences from growing and processing factors were also observed. These results suggest that further investigation on the InsPs composition of tree nuts, as affected by cultivation and processing conditions, highly desirable.

INDEX WORDS: Inositol phosphates; Phytic acid; Almonds; Cashews; Brazil nuts; Macadamias; Walnuts; Pecans; Pistachios; Hazelnuts; Wheat aleurone; Rice bran; Corn germ; HPLC/ESI/MS

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DEDICATION

To my parents and uncles, brother and cousin – my guides, my protectors, my inspirations.

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CHAPTER 1

INTRODUCTION

Phytic acid (*myo*-inositol hexakisphosphate - $InsP_6$), also frequently referred to by its salt form phytate and phytin, is an important component of plant seeds and a controversial constituent in the human diet. The compound is commonly consumed in high amounts from staple cereals and legumes, in which it serves as the main phosphorus reservoir and one of the major binders of minerals for the seeds (Kornegay, 2000). While plant seeds possess endogenous phytases to hydrolyze $InsP_6$ and its salts upon germination, humans produce very minimal amount of this enzyme group endogenously and must rely on microbial phytases, which are often insufficient for the complete digestion of the $InsP_6$ quantity present in foods (Lopez, Leenhardt, Coudray, & Remesy, 2002). At the pH levels of the digestive tract, $InsP_6$ can form insoluble salts and complexes with minerals and macronutrients, rendering these nutrients unavailable for absoprtion or other activities (Konietzny, Jany, & Greiner, 2006; Kumar, Sinha, Makkar, & Becker, 2010). As a result, this compound has been implied to be an antinutrient even though its negative effects have not been well described *in vivo.* On the other hands, emerging research is showing that $InsP_6$ as well as lower forms of inositol phosphates (InsPs), including *myo*-inositol mono-, bis-, tris-, tetrakis-, and pentakisphosphate ($InsP_{1-5}$), are widely present in eukaryote cells and may have important physiological functions (Irvine & Schell, 2001; Sauer & Cooke, 2010; Shears, 1998). $InsP_6$ has also been found to

have anti-inflammatory and anticancer effects in various types of cell lines (Vucenik et al., 2006). At the same time, it is still unclear whether dietary InsPs can be absorbed in the human digestive tract and mobilized for metabolic activities, as no carrier for these compounds has been detected, but some studies have reported intracellular InsPs to be dependent on extracellular InsPs (Grases et al., 2002). Overall, there remains many questions pertaining to the role of dietary InsPs in human health and disease prevention.

One of the challenges in studying InsPs has been the lack of information of their occurrence in some common foods. In recent years, nut consumption has been shown to have an inverse relationship with chronic diseases in humans. This effect has been attributed to antioxidant and anti-inflammatory components in these products. As a result, tree nuts are steadily rising in popularity and may soon become a significant source of InsPs in the average human diet (Topper, 2016; USDA Foreign Agricultural Service, 2016). Meanwhile, knowledge of the InsPs composition in tree nuts is scarce and fragmented, as existing data focus mainly on $InsP_6$ and most survey studies of InsPs in different plant cultivars only target cereals and legumes. Additionally, the contribution of InsPs to the organic phosphorus and total phosphorus contents of these products is rarely reported. Apart from InsPs, organic phosphorus may come from sugar phosphates, nucleotides, phospholipids, and thiamine phosphate derivatives. Inorganic phosphorus salts, together with organic phosphates, contribute to the total phosphorus content in a food component. Because tree nut matrices can be very different from those of other seed types, a method for the specific determination of InsPs in this food group is also in demand.

The goals of the present study are to build upon existing analysis methods and data pertaining to InsPs in commonly consumed tree nuts and to contribute to the understanding of InsPs occurrence in plant foods. The specific objectives include the following:

1. To refine an existing method and utilize high-pressure liquid chromatography coupled with electrospray ionization mass spectrometry for the evaluation of InsPs in the complex matrices of almond meal and almond brown skins.

2. To extract and evaluate the InsPs composition of the meal and brown skins of six major California sweet almond (*Prunus dulcis* [Mill.] D.A. Webb) cultivars, and to compare these values with the organic phosphorus and total phosphorus contents of these samples.

3. To evaluate the application of this method on seven economically-important tree nuts as well as the phosphorus-rich component of three common grains, and to compare the InsPs values with the organic phosphorus and total phosphorus contents of these samples, as well as to those in existing literature

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CHAPTER 2

LITERATURE REVIEW

2.1. Chemical properties of inositol phosphates (InsPs)

Inositol phosphates (InsPs) refer to the phosphorylated compounds of inositol (cyclohexane-1,2,3,4,5,6-hexol), in which one or more hydroxyl moieties on the six-carbon ring of inositol is replaced with a phosphate group. Inositol exists in nine possible stereoisomers, of which *myo*-inositol (Figure 2.1) is the most abundant species found in nature. As a result, "inositol phosphate" commonly refers to a phosphorylated *myo*-inositol. Depending on the number of phosphate groups on the inositol ring, a compound in this family can be referred to as *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis, or hexakisphosphate ($InsP_{1-6}$). All lower inositol phosphates ($InsP_{1-5}$) display isomerism.

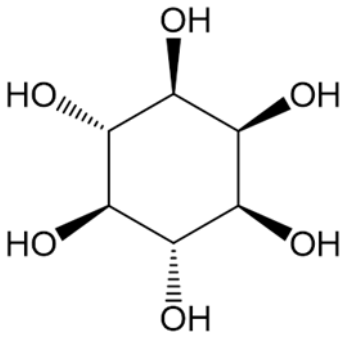

Figure 2.1. *myo*-inositol

To this day, *myo*-inositol hexakisphosphate ($InsP_6$ – Figure 2.2), also commonly referred to as phytic acid, has garnered the most attention due to its capability to chelate mineral cations in the digestive system and its high apparent concentration in plant seeds. The compound is

sterically stable at pH 0.5-10.5, with one phosphate in the axial position and five phosphates in the equatorial position (Bohn, Meyer, & Rasmussen, 2008). Under the effect of heat, acid treatment, or enzymatic activity by the enzyme group phytase, $InsP_6$ can be dephosphorylated into $InsP_{1-5}$ as well as inositol. While the complete acid dissociation constant profile of all InsPs is not known, $InsP_6$ has been found to have eight acid dissociation constants, ranging from 1.9 to 9.5 (Heighton, Schmidt, & Siefert, 2008). Phytic acid is fully deprotonated at pH 13.

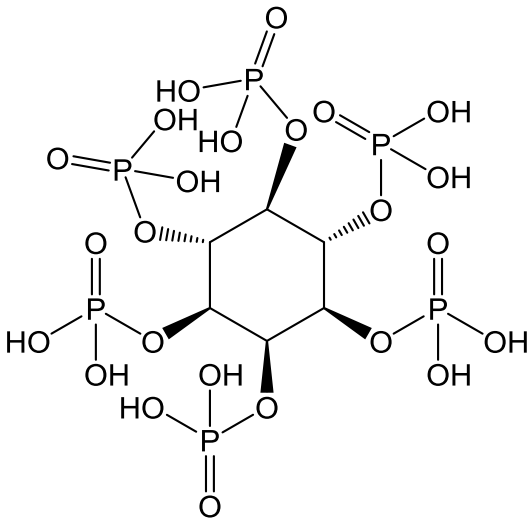

Figure 2.2. *myo*-inositol hexakisphosphate ($InsP_6$)

As indicated by their prefixes, the phosphate groups of $InsP_{2-6}$ are not internally connected and these compounds are polydentate ligands (Bohn et al., 2008). InsPs are capable of binding mineral cations such as calcium, zinc, copper, cobalt, manganese, iron, magnesium, *etc.*, forming the salt phytate (phytin if it is a mixed salt). Binding capability varies among InsPs and is dependent on the type of mineral. For example, *in vitro* studies at different pH in the range of 3 to 8 have found that InsPs typically have greater affinity with copper, zinc, and nickel than with other mineral cations (Persson, Tuerk, Nyman, & Sandberg, 1998; Tsao, Zheng, Lu, & Gong, 1997; Vasca et al., 2002).

$InsP_6$ and $InsP_5$ have been remarked to have higher binding strength than $InsP_3$ and $InsP_4$, and the distribution of the phosphate groups on the inositol ring also differentiates binding affinity among stereoisomers. InsPs and their salts with monovalent cations, such as sodium and potassium, are typically water soluble, while salts with multivalent cations have varying solubility depending on the pH of the environment and the presence of competing cations.

At low pH levels, $InsP_6$ can bind to the cationic groups of proteins and form insoluble complexes (Ellis & Morris, 1983). This might include interactions with the α-NH_2 terminal group, the ε-NH_2 group of lysine, the imidazole group of histidine, and the guanidyl group of arginine. At pH levels higher than these proteins' isoelectric points, soluble protein–mineral–phytate complexes may form (Gemede, 2014).

2.2. The physiological roles of extraneous inositol phosphate (InsP)

InsPs are ubiquitous in seeds, of which cereals, legumes, and nuts are frequent components in human and animal diets. $InsP_6$ is said to typically comprise more than 85% of InsPs composition and serves as a mineral binder, phosphorus source, and energy source for the seed (Kornegay, 2000). During germination, phosphorus and minerals will be released from phytate by phytases. Many animals, especially ruminant types, have been found to depend on microbial phytases in the gut for inositol phosphate (InsP) catabolism. In humans, phytase secretion appears to be limited and microbial phytases in the large intestine are likely to be important to the digestion of phytates (Iqbal, Lewis, & Cooper, 1994; Lopez, Leenhardt, Coudray, & Remesy, 2002; Schlemmer, Frólich, Prieto, & Grases, 2009).

2.2.1 Inositol phosphates as an antinutrient

Dietary InsPs are often viewed as undesirable due to their capability to chelate mineral cations and to form complexes with protein, carbohydrate, and lipid, which reduce the availability of these nutrients in the body (Kumar, Sinha, Makkar, & Becker, 2010). $InsP_6$ and $InsP_5$ are the most abundant InsPs in foods and also the InsPs with the strongest binding capabilities. In many countries where whole grain cereals and legumes are staples, InsPs, along with other components such as inorganic phosphates, polyphenols, and nondigestible fiber, may exacerbate on-going nutrient deficiency problems (Schlemmer et al., 2009). Of most concern are the potential adverse effects on the absorption of zinc and iron - two minerals with prevalent deficiencies around the world - as well as calcium and magnesium. Furthermore, the formation of insoluble phytates/phytins and complexes also render phosphorus unavailable for the body (Konietzny, 2006).

Zinc is strongly sequestered by $InsP_6$ and zinc phytate forms readily within the pH range 3 to 7, which includes the pH range of the duodenum, where the absorption of this mineral occurs (Persson et al., 1998). Among $InsP_{3-6}$, only $InsP_6$ and $InsP_5$ have been found to exert a significant inhibitory effect on zinc absorption (Lönnerdal, Sandberg, Sandström, & Kunz, 1989). The level of inhibition also depends on the molar ratio between InsPs and Zn^{2+} and the presence of secondary cations such as calcium and magnesium ions, as secondary cations can either displace Zn^{2+} from InsPs' binding sites or form insoluble phytins in conjunction with Zn^{2+} (Forbes, Parker, & Erdman Jr, 1984; Gemede, 2014; Walter, Rimbach, Most, & Pallauf, 2000).

Between two types of dietary iron, heme and non-heme iron, dietary $InsP_6$ and $InsP_5$ have been linked to decreased non-heme iron absorption (Reddy, Hurrell, Juillerat, & Cook, 1996; Sandberg et al., 1999). $InsP_4$ and $InsP_3$ did not exhibit an inhibitory effect on iron absorption when they were the only InsPs in foods, but when present in combination with $InsP_5$ and $InsP_6$, the inhibitory efficacy was stronger than when only $InsP_5$ and $InsP_6$ were in the samples (Sandberg et al., 1999). This suggested that there could be a synergistic effect between $InsP_{5-6}$ and lower InsPs, where an iron ion at a high degree of covalency could bind with two or three different InsPmolecules simultaneoulsy, some of which could be $InsP_{3-4}$. The authors also remarked that different isomeric forms of $InsP_{3-4}$ could have exerted varying levels of effect in this case, as *myo*-1,2,6-inositol trisphosphate and InsPs with the 1,2,3-trisphophate grouping can form more stable complexes with Fe^{3+}. On the other hand, the negative effect of InsPs on iron absorption was not found in all studies, showing that this effect can be affected by factors other than the levels of InsPs and iron (House & Welch, 1987; Simpson, Morris, & Cook, 1981). Hallberg and others (1987) have observed that iron phytate in foods can be present in notable quantities as monoferric phytate, which is well absorbed. However, when sodium phytate or a mixture of magnesium phytate and potassium phytate were added to the samples, iron absorption decreased, likely due to the formation of insoluble phytins.

Similarly, the inhibitory effect of phytate on calcium and magnesium ion absorption can be suggested, but experimental values have been inconclusive (Crea, de Robertis, de Stefano, & Sammartano, 2006). Miyazawa and others (1996) reported a negative effect on the apparent absorption of magnesium but not that of calcium in rats. Lönnerdal and others (1989) observed inhibition of calcium ion uptake by $InsP_6$ and

$InsP_5$, but not by $InsP_4$ and $InsP_3$. It is also unclear whether lowered absorption of these minerals actually translates in to health concerns. For example, a survey study by López-González and others (2008) have assessed that dietary phytate is not a risk factor of osteoporosis, and increasing phytate consumption can even be linked with higher bone mineral density.

In pig and poultry, phytase supplementation to the fodder has been demonstrated to improve amino acid digestibility, and $InsP_6$ can be implied to impede amino acid absorption in the gut (Selle, Rvindran, Caldwell, & Bryden, 2000). The form of InsP can influence this effect, as the magnesium–potassium salt of $InsP_6$ showed a higher inhibitory strength than free $InsP_6$ (Onyango, Asern, & Adeola, 2008). $InsP_6$ has also been shown to inhibit several digestive enzymes such as α-amylase, lipase, and proteinases (e.g. pepsin, trypsin, chymotrypsin) in fish and poultry (Denstadli, Skrede, Krogdahl, Sahlstrøm, & Storebakken, 2006; Khan & Ghosh, 2013; Liu, Ru, Wang, & Xu, 2010). These effects may be explained by the non-specific bindings of InsPs with cationic moieties of amino acids and proteins, followed by the precipitation of these complexes at low pH levels. In additional, ternary protein–mineral–phytate complexes may form, with a multivalent mineral cation serving as the cationic bridge (Selle et al., 2000). The solubility of these complexes is also dependent on pH. Among soluble mineral–phytate complexes and ternary protein–mineral–phytate complexes, an equilibrium may exist. Preference for either type of complex is influenced by ion concentrations as well as the degree of strain placed on the inositol ring by each type of complex (Champagne, Fisher, & Hinojosa, 1990). For example, the equilibrium involving calcium ion can be illustrated by the following:

$$\text{Protein} + \text{Ca} + \text{Phytate} \rightleftharpoons [\text{Protein–Ca–Phytate}] \rightleftharpoons [\text{Ca–Phytate}] + \text{Protein}.$$

From these perspectives, InsPs appear to be generally antinutritional. Breeding, genetic modification, germination, soaking, heat treatment, and fermentation are methods that have been investigated and recommended to reduce InsP levels in plant foods. On the other hand, it must be noted that InsPs are only some among many dynamic factors that can interact with each other in the digestive tract to affect mineral absorption. For example, the inhibitory effect of InsPs on zinc ion absorption can be reduced with dietary protein, while administration of ascorbic acid with a phytate-containing meal has been demonstrated to improve iron absorption (Davidsson et al., 1994; Sandström, Almgren, Kivistö, & Cederblad, 1989; Siegenberg et al., 1991). Regarding magnesium, many unrefined plant foods rich in phytate are also rich in this mineral, and it has been argued that the eventual amount of magnesium absorbed from these foods may be equal to or higher than when low-phytate foods are consumed (Levrat-Verny et al, 1999). In rats, resistant starch has been shown to be able to negate the effects of phytic acid on the absorption of iron, zinc, and copper (Lopez et al, 1998). A potential mechanism behind this effect is the production of short-chain fatty acids (SCFA) during the fermentation process, which adjust the pH of the environment. Additionally, these SCFA can compete with InsPs to form complexes with mineral cations, which may be low in charge and can enter the enterocytes. Taking these effects into account, improving mineral absorption by maintaining a diverse diet might be a more positive approach to avoid mineral deficiency than reducing InsP contents in foods. Some amount of dietary InsPs may be important, because InsPs' general capability to chelate mineral ions may help reduce the absorption of toxic trace elements, such as cadmium and lead (Schlemmer et al., 2009). Furthermore,

new findings on the roles of InsPs in normal body functions and disease states propose that InsPs may be essential and beneficial to human health.

2.2.2 The roles of inositol phosphates in normal cell functions

In recent decades, insights on the importance of InsPs to eukaryote cells have emerged. *Myo*-inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$), *myo*-inositol-1,3,4-trisphosphate, *myo*-inositol-1,3,4,5-tetrakisphosphate, and *myo*-inositol-1,3,4,5,6-pentakisphosphate have been found to work dynamically in different Ca^{2+} signaling pathways (Irvine & Schell, 2001; Lückhoff & Clapham, 1992; Streb, Irvine, Berridge, & Schulz, 1983; Tsubokawa, Oguro, Robinson, Masuzawa, & Kawai, 1996; Vajanaphanich et al., 1994). Both *myo*-inositol-1,3,4,5,6-pentakisphosphate and $InsP_6$ have important roles in mRNA export, genomic stability, and apoptosis (Loss, Wu, Riccio, & Saiardi, 2013; Majerus, Zou, Marjanovic, Kisseleva, & Wilson, 2008; Monserrate & York, 2010). More than half of the 63 possible InsPs isomers have been identified in various types of cell (Irvine & Schell, 2001; Shears, 1998). Different reactions by InsP pools to specific stimulations imply that these compounds may actually have many more functions to be investigated (Irvine & Schell, 2001; Otto, Kelly, Chiou, & York, 2007).

Many different pathways for the synthesis of InsPs by cells have been discovered in plants, moulds, and animals. Phosphatidylinositol 4,5-bisphosphate can be hydrolyzed into $Ins(1,4,5)P_3$, which, together with inositol, can be rapidly converted by plant and slime mould cells into other InsPs through a series of positionally selective and sequential kinase reactions. In mammalian cells, glucose-6-phosphate is believed to serve as the precursor for $Ins(1,4,5)P_3$, providing a second pathway that is independent of inositol

lipid metabolism (Irvine & Schell, 2001; Sasakawa, Sharif, & Hanley, 1995). However, both pathways have been found to occur at extremely slow rates, despite some evidences indicating that the conversion of Ins(1,4,5)P_3 into InsP_6 in cells can be very fast. Hence, the synthesis of InsPs in animals remains as an ongoing topic for examination.

At this point, it is also worthwhile to mention a class of molecules closely related to InsPs – inositol pyrophosphates (PPx-InsPs). Using InsP_6 as precursor, these compounds are synthesized by linking additional phosphate(s) to an existing phosphate group on the inositol ring via pyrophosphate bond(s) (Williams, Gillaspy, & Perera, 2015). So far, a few members of this family have been described, including several stereoisomers of triphosphoinositol pentakisphosphate (PPP-InsP_5), diphosphoinositol pentakisphosphate (PP-InsP_5), bis-disphosphoinositol trisphosphate ($(PP)_2$-InsP_3), and bisdiphosphoinositol tetrakisphosphate ($(PP)_2$-InsP_4). These compounds are sometimes abbreviated as InsP_7 and InsP_8, although these names fail to signify the important presence of the high-energy diphosphate or triphosphate chain(s). Just like InsPs, PPx-InsPs have also been found to be ubiquitous in eukaryote cells, and have been implied to participate in many cell functions, including energy sensing, inorganic phosphate sensing, and immune response (Williams et al., 2015; Wilson, Livermore, & Saiardi, 2013). Because PPx-InsPs are present in very low abundances, dietary PPx-InsPs are unlikely to be absorbed at significant levels by the human body. Consequently, the body may have to rely on the synthesis of these compounds from InsP_6.

Meanwhile, the contribution of exogenous sources to the maintenance of InsP pools in our body is also not fully understood. Depending on the pH, InsPs can be negatively charged, which hinder their absorption in the gut. The small size of the inositol

ring also leads to a high-charge density. So far, no carrier has been detected for InsPs in the human digestive track. Nevertheless, existing studies suggest that cellular intake of these compounds are possible and that InsP levels in the body are influenced by dietary intakes.

Grasses and others have demonstrated that in rats fed with different levels of $InsP_6$, the concentration of extracellular $InsP_6$ (in plasma and interestitial fluid) increases with dietary intake, while the concentration of extracellular $InsP_3$ is not significantly affected (Grases et al., 2002). In the same study, human epithelial and mesenchymal cells grown in culture media with inositol reached an $InsP_6$ concentration of approximately 16 μmol/kg regardless of whether $InsP_6$ was supplied in the media or not, indicating the cells' capability to synthesize $InsP_6$ from inositol. When these cells were treated with 1.8 mM $InsP_6$ for 1 hour, the intracellular $InsP_6$ content did not change, wherea the intracellular $InsP_3$ content doubled. This effect was attributed to dephosphorylation activity by the cell, which varies in rate depending on cell type (Vucenik & Shamsuddin, 1994). The concentration of $InsP_3$ and $InsP_6$ in plasma fluid and in cells were thus concluded to have partial links to exogenous $InsP_6$.

By monitoring $InsP_6$ intake through diet and administration of oral doses, Grases and others showed that the plasma level and urinary excretion of $InsP_6$ in humans are affected by oral ingestion in both the short and long term (Grases et al., 2001). A positive correlation was detected between these two measurements. Under the tested conditions, the urinary excretion level was always much lower than the intake level and would plateau at a certain $InsP_6$ dose, indicating that humans have a limited ability to absorb and/or excrete $InsP_6$. Another study also found that $InsP_6$ absorption is independent of the

condition of the stomach (whether empty, empty with an alkalinizer, or full), suggesting that absorption may occur during intestinal transit (Grases et al., 2006).

Using the ileostomy model, Agte and others (2005) were able to describe the apparent absorption of all six InsPs from traditional Indian vegetarian meals. The age range of the subjects was within 35 and 58 years old, and the weight range was limited from 52 to 61 kg. The level of apparent phosphorus absorption was found to increase from $InsP_6$ to $InsP_1$, but it is notable that higher InsPs contributed more phosphorus than lower forms. $InsP_1$, which was consumed in minimal amount, was completely degraded, while absorption for phosphorus from $InsP_2$ only reached 64%. For $InsP_{3-6}$, which were responsible for roughly 96% of the phosphorus amount bound by InsPs in these meals, the % phosphorus absorption varied between 29 and 39. The net absorption of InsP phosphorus from these meals was 35%. As only three subjects were able to complete the trial (two men and one woman), this study was limited in sample size. For $InsP_{1-5}$ output results, it was not possible to distinguish between the amount of InsPs retained from the original meals and the quantity of InsPs generated by the degradation of higher forms. Nevertheless, these results still suggest that InsPs might be degraded and absorbed at a significant degree in the human digestive system.

In a controlled trial with Korean women, Joung and others found that the degradation of dietary $InsP_5$ and $InsP_6$ is superior in elderly women compared to that in young women (Joung et al., 2007). Additionally, a rise in dietary phytate level induced a significant rise in phytate excretion in young women, but not in elderly women. In both subject groups, phosphorus absorption was not affected when phytate became the main dietary phosphorus source. These results suggest that the human body may have a

mechanism to alter phytate degradation activity in the digestive tract according to its needs.

2.2.3. Inositol phosphates' disease fighting abilities

Recently, many findings have highlighted InsPs' disease fighting capabilities. These include the prevention of pathological calcification, antioxidative activities, and anticancer effects. $InsP_6$ was first considered as an agent against pathological calcification in hypercalcuria patients due to its possible interference with calcium absorption (Nassim & Higgins, 1965). Later, it was found to have strong crystallization inhibiting property against these unwanted calcium salts, which allows it to be effective even at a relatively low concentration. Experiment with synthetic urine has demonstrated that phytic acid can completely stop the growth of calcium oxalate stones (Costa-Bauzá, Isern, Perelló, Sanchis, & Grases, 2005). In animal models, $InsP_6$ has been shown to reduce calcium deposition in kidneys and related organs, as well as slow down the growth of dystrophic calcification plaques in the cardiovascular system (Grases, García-Ferragut, & Costa-Bauzá, 1996; Grases, Perelló, Prieto, Simonet, & Torres, 2004). In humans, lower urinary concentration of $InsP_6$ has been associated with calcium oxalate active stone-formers, and high intake of dietary $InsP_6$ has been suggested to decrease the risk of kidney stones in these subjects (Conte et al., 1999; Curhan, Willett, Knight, & Stampfer, 2004; Grases et al., 2000). Additionally, as a crystallization inhibitor, $InsP_6$ may contribute to the prevention of sialolithiasis and dental calculus formation (Grases et al., 2009; Grases, Santiago, Simonet, & Costa-Bauzá, 2003).

InsPs may exert their antioxidative property via generic as well as specific chelating activities. Other than binding iron and copper ions in general, some InsPs has the 1,2,3-trisphosphate arrangement which, upon interacting with Fe^{3+}, would occupy all six co-ordination sites of this ion with hydroxyl groups (Phillippy & Graf, 1997). As a result, these compounds inhibit iron's catalytic action on lipid peroxidation and hydroxyl radical formation in the Fenton reaction. A similar effect has been observed with ferrous ion. Phytate can be used to prevent myoglobin oxidation in meat processing, which changes the color of homogenized meat from red to brown (Park et al., 2004; Stodolak, Starzyńska, Czyszczoń, & Żyła, 2007). In studies with rat livers, however, dietary phytate has not demonstrated the capability to reduce oxidation markers (Rimbach & Pallauf, 1998). Intravenously-injected phytate appears to provide some degree of myocardial protection to rats subjected to cardiac excision, but not to rat hearts that were removed from hypothermic storage (modeling a heart transplant) (Kazimoglu et al., 2004; Rao, Liu, Das, Weinstein, & Tyras, 1991). These results suggest that InsPs' performance as antioxidants *in vivo* may be limited to specific working conditions and routes of administration (Iemma et al., 2007).

$InsP_6$ has demonstrated a remarkable preventive effect on tumor development in different cancer cell lines in blood, colon, liver, lung, mammary, melanoma, pancreas, prostate, skin, soft tissue, and uterine cervix (Vucenik & Shamsuddin, 2006). Multiple effects beyond antioxidative activity have been recognized, such as the inhibition of cell proliferation, metastasis, angiogenesis, and inflammation (Gu, Raina, Agarwal, & Agarwal, 2010; Norazalina, Norhaizan, Hairuszah, & Norashareena, 2010; Raina et al., 2013; Kumar et al., 2004). $InsP_6$ has been shown to support apoptosis as well as the

differentiation and maturation of malignant cells, which can recover the normal phenotype (Deliliers et al., 2002; Shamsuddin & Yang, 1995; Singh, Agarwal, & Agarwal, 2003; Vucenik, Passaniti, Vitolo, Tantivejkul, Eggleton, & Shamsuddin 2004). Some studies observed that $InsP_6$ enhances the activity of natural killer cells and reduces the depression of these cells by carcinogens (Baten, Ullah, Tomazic, & Shamsuddin, 1989; Zhang, Song, & Wang, 2005). These activities vary depending on dose, time, and the origin of the cells. A synergistic effect upon combination with inositol has also been suggested in a number of animal studies on different cancer types (Shamsuddin, Ullah, & Chakravarthy, 1989; Vucenik, Yang, & Shamsuddin, 1995).

Dietary phytate exhibits an antineoplastic effect in non-ruminant animal models equally well whether consumed in a fluid or non-fluid system, highlighting the possibility of effective absorption mechanisms for extraneous InsPs that do not depend on microbial phytases. However, a human's ability to absorb InsPs cannot be directly extrapolated from rodent models because phytase activity in our tissue is approximately 30 times lower than that of rat tissues (Iqbal, Lewis, & Cooper, 1994). On the other hand, it has been hypothesized that $InsP_6$'s anticancer activities depend on the compound's dephosphorylation to lower InsPs, even though the exact metabolism pathways and mechanisms of action still need to be elucidated (Vucenik & Shamsuddin, 2006). From this point of view, extraneous $InsP_{1\text{-}5}$ may also have important roles against cancer.

In mouse, $InsP_6$ has demonstrated a protective effect against Alzheimer's disease by decreasing lipidic peroxidation and increasing cytochrome oxidase levels in the brain (Anekonda et al., 2011). In the same study, the compound was found to prevent the apoptosis of neurons by protecting MC65 cells against cytotoxicity by amyloid-β peptide.

This action is opposite to what $InsP_6$ exerts on malignant cells, which has been described above, implying that $InsP_6$ is likely to serve as an apoptosis modulator. Similar protective activities have also been observed in other cell conditions. In studying the rat mesencephalic dopaminergic cell line (N27), Xu and others (2008) reported $InsP_6$ to decrease pathological apoptosis induced by 1-methyl-4-phenylpyridinium and increase cell viability, suggesting that $InsP_6$ may offer protective therapy for Parkinson's disease. In pig small intestines, $InsP_6$ was found to reduced cell proliferation, apoptosis, and cyclooxygenase-2 expression, which are associated with inflammation (Silva, Cerez, & Bracarense, 2014). The compound also protected cells against hypoxia-induced morphological changes. These effects were attributed to $InsP_6$'s antioxidant property, which may have inhibited the production of cytosolic reactive oxygen species. Notably, $InsP_6$ from rice was found to be more efficient than $InsP_6$ from corn, which was hypothesized to be due to the different numbers of sodium and water moieties attached to each $InsP_6$ molecule between the two sources (i.e., $InsP_6 \cdot 12Na \cdot 8H_2O$ from rice versus $InsP_6 \cdot 11Na \cdot 7H_2O$ from corn).

Some positive effects of phytate on diabetes mellitus have been suggested, as phytate intake has been found to negatively correlate with blood glucose response. In mice fed a high-fat diet, $InsP_6$ exerted an antihyperglycemic effect by regulating the activities of glucose-6-phosphatase, glucokinase, and phosphoenolpyruvate carboxykinase (Kim, Roco, Lee, & Kang, 2010). Larsson and others (1997) demonstrated that $InsP_6$ may play a role in regulating insulin secretion, possibly by inhibiting the activity of serine–threonine protein phosphatases, thus opening intracellular calcium channels and leading to insulin release. $InsP_5$ could exert similar effects on calcium ion

channels, but required a higher amount than $InsP_6$, while $InsP_4$ was not able to provide any action of the type.

Jariwalla and others (1990) reported phytate to significantly lower serum cholesterol and triglyceride levels. A lower serum zinc level and zinc–copper ratio were observed in conjunction with these effects. Zinc and copper ions share the same mucosal carrier systems, and excess zinc in the diet can lead to reduced copper absorption, which has been hypothesized to cause coronary heart disease (Klevay, 1975). Because $InsP_6$ has a greater binding affinity for zinc than copper ions, its presence may have helped in lowering the zinc–copper ratio (Peson et al., 1998). However, in experimenting with rats fed a high-sucrose diet, Onomi and others (2004) noted that the hepatic concentrations of total lipids and triglycerides, as well as the hepatic activity of glucose-6-phosphate dehydrogenase, were reduced when the sodium phytate level in the feed was as low as 0.02%. Meanwhile, the serum zinc level was not affected until the sodium phytate content reached 2.5%. These authors also detected reductions in cholesterol level and fatty acid synthetase activity, but the effects did not show a clear dose-dependent relationship. As a result, the mechanisms behind the reductive effects of dietary phytate on serum cholesterol still need to be investigated.

Several *in vitro* studies have been conducted on $InsP_6$'s antiviral potential on the human immunodeficiency virus (HIV). The compound was found to inhibit the cytopathic effect of this virus and the expression of the HIV-specific antigen (Kumar et al., 2010). In a T-cell line, $InsP_6$ was also shown to be able to suppress the replication of HIV-1 (Otake et al., 1999). Recently, Tateishi and others (2014) found $InsP_6$ to be able to tightly bind the matrix domain of the $Pr55^{Gag}$ protein, which is a critical component for

the assembly of the HIV-1 virus. Subsequently, these authors coupled $InsP_6$ with diacylglycerol moieties to synthesize *myo*-phosphatidylinositol 2,3,4,5,6-pentakisphosphate derivatives. These derivatives were found to have an even stronger binding capacity with the $Pr55^{Gag}$ protein's matrix domain, suggesting a potential molecular design for anti-HIV agents.

The effects of $InsP_6$ against mycotoxins have been investigated with some results. $InsP_6$ has been shown to impede the histopathological alterations caused by aflatoxin B1 in albino rats, which include heightened oxidative stress and decreased reproductive function (El-Saad & Mahmoud, 2009). In swine, $InsP_6$ exhibited protective effects on the cytoplasmic membrane of intestinal cells when these cells were exposed to deoxynivalenol and fumonisin B1 (Silva, Gerez, Drape, & Bracarense, 2014).

Along with new understandings on the physiological functions of InsPs, the need to separate and quantify InsPs in food have arisen. While accurate and sensitive methods are becoming more available, the diversity of food and biomedical systems require that modifications be investigated for each sample type.

2.3. Methods of separation and quantification for InsPs

Although $InsP_6$ was identified as far back as 1903, the detection and quantification of InsPs have been of continuous interest for researchers to this day. These compounds do not possess a characteristic spectrophotometric absorbance maximum and are available only at relatively low concentrations. At the moment, an optimal method for

the complete separation and measurement of all InsPs with their isomers has yet to be developed.

2.3.1. Non-specific methods

In 1914, Heubner and Stadler introduced a non-specific method for the determination of phytic acid in ground cereal powder (Rather, 1917). In this method, phytic acid is extracted with HCl, then titrated with a ferric chloride solution in the presence of ammonium thiocyanate to form a pink-colored chromogen. The quantity of Fe^{3+} that reacts is used to calculate the phytic acid content. Because ferric phytate forms as a white precipitate, the exact end point of this titration is difficult to detect. Furthermore, the molar ratio between Fe^{3+} and $InsP_6$ in the complex is inconsistent. Using the assumption that the total phosphorous content of the sample would originate from phytate, McCance and Widdowson later modified this method by quantifying the phosphorus content of the ferric phytate precipitate instead of Fe^{3+} (McCance & Widdowson, 1935). A major disadvantage of assays that depend on ferric phytate, however, is the overestimation of phytic acid in samples with high contents of inorganic phosphorus and/or non-InsPs organic phosphates, as these compounds can also precipitate with the ferric ion.

To eliminate inorganic phosphorus from samples, researchers have attempted to develop quantitative methods based on ion-exchange interactions. Most notable is a method by Harland and Oberleas, in which the supernatant from an HCl extraction of the sample is placed on an AG 1-X8 chloride form anion-exchange column (Harland & Oberleas, 1977). The column is rinsed with water and 0.05 M NaCl to elute inorganic

phosphates. Organic phosphates trapped on the resin are then eluted with 0.7 M NaCl. Assuming that phytates accounts for all forms of organic phosphates in the sample, an acid digestion of the final fraction followed by the measurement of inorganic phosphates via the Fiske-Subbarow colorimetric method can be used to infer the phytate content. In the Fiske-Subbarow assay, the sample is treated with ammonium molybdate to form phosphomolybdate, which can be reduced to molybdenum blue in the presence of a reductant, such as aminonaphthosulfonic acid (Goldenberg & Fernandez, 1966). The intensity of the blue-colored chromogen, with maximum absorbance at $\lambda = 640$ nm, is directly correlated to the concentration of phosphate in the sample. In an improvement of this method, Ellis and Morris added EDTA and NaOH to prevent the binding of $InsP_6$ to proteins and metal ions in the extraction (Ellis & Morris, 1983). This version was later adopted as the Official Method 986.11 for Phytate in Foods by AOAC International (1986).

Due to the non-specificity of this chromatographic method, $InsP_6$ concentration can still be heavily overestimated in samples where $InsP_{1-5}$ make up more than 20% of the InsP content (Lehrfeld & Morris, 1992). Additionally, sugar phosphates, phospholipids, nucleotides, and phosphate derivatives of thiamine also contribute to the organic phosphate levels in foods. Overestimation problems are of particular importance, because foods and diet patterns reported to have high phytic acid contents would often be associated with mineral deficiency concerns.

2.3.2. Specific methods

In 1952, while working with soil, Smith and Clark were able to extract $InsP_{3-6}$ from hydrolyzed sodium phytate via anion-exchange chromatography, utilizing a weak-base exchange resin and stepwise elution with increasing HCl concentration (Cosgrove 1963a). The content of each InsP was deduced via the phosphorus–inositol ratio in each collected fraction. Later, Cosgrove demonstrated that utilizing the strong anion exchange resin AG 1 for this method allowed for separation of different $InsP_5$ isomers (Cosgrove, 1963b). The author, however, did not perform a quantification of InsPs in food samples.

To achieve a shorter elution time, Tangendjaja and others (1980) then applied high pressure liquid chromatography (HPLC), particularly reversed-phase chromatography, using a μBondapak C18 column and sodium acetate as mobile phase. Graf and Dintzis (1982) later improved this method by adding a purification step with the AG 1 resin after extraction. With both methods, however, the retention time of $InsP_6$ was less than 2 min and other InsPs could not be identified. In a modification, Sandberg and Ahderinne (1986) used formic acid/methanol as the mobile phase and added tetrabutylammonium hydroxide as the ion-pair reagent, thus affording an increase in the retention time and differentiation between $InsP_{3-6}$, although their stereoisomers were still indiscriminable.

Around this time, the application of gas chromatography (GC) in InsP analysis was also explored. Heathers and others derivatized InsP fractions collected from HPLC separation into hexatrimethylsilyl derivatives for quantification by GC, and successfully applied this method to isomers of $InsP_{1-3}$ (Heathers, Juehne, Rubin, Corr, & Evers, 1989).

The need for a derivatization process and the inability to measure $InsP_{4-6}$ are considered drawbacks for this method.

Overall, HPLC is still the most common separation technique for InsPs due to its versatility. While ion-pair reverse-phase HPLC has been succesfully used to separate InsPs, anion-exchange HPLC has become more popular due to its easy of use. In recent decades, many different mobile phase gradients and anion-exchangers for HPLC have been used to separate InsPs down to their isomers. Nevertheless, it remains that $InsP_{3-6}$ are better eluted with an acidic mobile phase while $InsP_{1-2}$ prefer an alkaline mobile phase (Schlemmer et al., 2009). Good separation of all InsPs and their isomers is unlikely to be achieved in the same run, while time-effective separation of $InsP_{1-6}$ in one run will sacrifice information on isomers. Noteworthy is that a high pH level may reduce the life time of a silica-based stationary phase, and hence if an alkaline mobile phase is utilized, a polymer-based column is preferable. Taking this into consideration, there might be some limitations to the choice of column for the sensitive detection of $InsP_{1-2}$ by HPLC (Sjöberg, Theline, & Rydin, 2016).

Taking advantage of the exchange of Fe^{3+} from the ferric sulfosalicylic acid complex to phytate, Latta and Eskin (1980) developed a colorimetric detection method using a modified Wade reagent. Reaction with phytate in a sample extract would result in a loss in the absorbance at $\lambda = 500$ nm for this purple reagent. Rounds and Nielsen then modified this assay by replacing the AG 1 resin in the preceding method with a strong anion-exchanger equipped on HPLC. In this case, $InsP_{2-6}$ could be separated within 30 min (Rounds& Nielsen, 1993).

In 1988, another post-column dye-detection method was developed based on the exchange of yttrium ion from the Y^{3+}-4-(2-pyridylazo)resorcinol complex to InsPs, which reduces the absorbance at λ = 546 nm of this colored complex (Mayr, 1988). In a modified method utilizing the same approach, Guse and others (1993) were able to quantify $InsP_{3-6}$ in cell extracts with high sensitivity: the detection limits were approximately 15 pmol for $InsP_3$, 10 pmol for $InsP_4$, 5 pmol for $InsP_5$, and 1-3 pmol for $InsP_6$. Yet, this method requires great care to avoid contamination of the eluent with ions such as Fe^{3+} and Zn^{2+}, as these ions can complex with both InsPs and 4-(2-pyridylazo)resorcinol.

Ligand exchange reactions between InsPs and fluorescent complexes have also been introduced, including the use of a ferric methylcalcein blue complex, copper(II) gelatin complex, and copper(II) 2,2′-bipyridine complex (Cao, Dong, & Chen, 2011; Chen, Chen, Ma, Cao, & Chen, 2007; Irth et al., 1990). Interestingly, $InsP_6$ has been found to activate the oxidation of 1,1′-dipyridyl ketone hydrazone (DPKH) in the presence of Cu^{2+}, resulting in an intense fluorescent product (March, Simonet, & Grases, 1999). The authors suggest that the copper(II)–phytate complex may have heightened catalytic activity compared with Cu^{2+} alone on the reaction between DPKH and oxygen. This method is useful for the detection of phytic acid in foods and urine.

A method for on-line detection of InsPs has always been in demand. In qualitative studies on cells and tissue, a number of authors have chosen to radiolabel their samples with myo-[^{3}H]inositol or [^{31}P]PO_4^{3-}. In working on several isomers of $InsP_{1-4}$, Taylor, Garcia, Dukes, and English (1990) coupled this technique with separation via HPLC for the quantitation of InsPs. They successfully recorded changes in the distribution of the

analytes overtime, proposing a potential approach for the study of InsPs in biological processes, such as cellular signaling. Due to the lack of absolute concentration data, this method is considered unsuitable for many research purposes. Furthermore, it is laborious and less sensitive than some off-line analyses. Instead, several studies have investigated the application of chemically–suppressed conductivity and the researchers were able to separate different phosphorylated organic compounds, including InsPs, in both physiological and food samples (Smith & MacQuarrie, 1988; Talamond, Doulbeau, Rochette, Guyot, & Treche, 2000). Notable is the coupling of isotachophoresis and zone electrophoresis with contact conductivity, as demonstrated by Kvasnička and others (2011).

When multiple InsPs are being identified or when matrix effects can cause the elution times of InsPs to deviate from those of standard compounds, reliance on retention time for detection in HPLC methods can also risk inaccurate interpretation. Nuclear magnetic resonance (NMR) spectroscopy, atomic emission spectroscopy (AES), and mass spectrometry (MS) are among detection techniques that can reduce this risk in susceptible samples.

^{31}P-NMR spectroscopy is prized as a noninvasive and highly specific method for InsPs determination. Because it can be applied on intact tissues and cell suspensions, it is especially suitable for the study of these compounds' metabolism, binding activities, and degradation (Heighton et al., 2008; Zhuang, Vishnivetskiy, Gurevich, & Sanders, 2010). Alternatively, some authors have used ^{1}H-NMR to study the interactions between $InsP_6$ and paramagnetic metal ions, such as ferric and ferrous ions (Heighton et al., 2008). NMR can be used to identify and confirm stereoisomers that often coelute during

chromatographic separation (Phillippy, 1989). The disadvantages of NMR, however, are that the technique is relatively insensitive, cost-prohibitive, and spectra can be very complex (Kemme et al., 1999; Schlemmer et al., 2009).

In 1996, a method using ICP coupled with AES for $InsP_6$ determination in urine was reported, but the sample treatment process is long, complicate, and requires a large amount of sample (Grases & Llobera, 1996). Later, a more efficient and sensitive method was developed, attaining a limit of detection of 64 μg/L and limit of quantification of 213 μg/L (Grases, Perelló, Isern, & Prieto, 2004). Although still less sensitive than ICP coupled with MS, this method is considered suitable for routine determination. A preliminary study by Amaro and others suggested that coupling HPLC with ICP-AES can improve sensitivity (Amaro, Escalona, & Murillo, 2004).

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are powerful approaches to attain high sensitivity and can be coupled with HPLC, GC, and ICP for different purposes. ICP-MS is suitable for biomedical samples such as urine (Muñoz & Valiente, 2003). For food samples, the use of sector field ICP-MS has been investigated with initial success (Helfrich & Bettmer, 2004). GC-MS has also been applied for food and biological samples, such as rat organs, human plasma, urine and kidney stones (de Koning, 1994; March, Simonet, & Grases, 2001). However, this method still requires a derivatization step of InsPs to trimethysilyl derivatives. Meanwhile, HPLC-MS can achieve comparable results without calling for derivatization of the sample (Perelló, Isern, Muñoz, Valiente, & Grases, 2004). Further modification may even eliminate all together the need for a separate chromatographic extraction step preceding detection. Using negative mode electrospray ionization (ESI) with HPLC–MS/MS, Liu and others

were able to simultaneously separate and quantify all six InsPs from various foods and cell samples, with sensitivity reaching as low as the pmol range and elution time of less than 30 min (Liu, Villalta, & Sturla, 2009). This can be considered one of the most efficient methods for measuring InsPs in samples without regard to stereoisomers. A similar method was later successfully applied for the qualitative detection of InsPs from environmental matrices, including soil, manure, and aquatic sediments (Sjöberg et al., 2016).

An option for the distinction of positional isomers by MS/MS has also been investigated: utilizing positive-ion mode ESI with low-energy collisional-activated dissociation MS/MS, Hsu and others (2003) were able to differentiate amount the stereoisomers of $InsP_1$, $InsP_2$, and $InsP_3$ in bovine brain extract. This method was unfortunately unable to identify the stereoisomers of $InsP_4$ and $InsP_5$, however, and how it can be applied for the quantification of $InsP_{1-3}$ isomers still needs to be examined.

2.4. Iositol phosphates in tree nuts

Compared with other groups of plant food such as cereals and legumes, tree nuts have received less attention because their consumption is often lower in volume and frequency. In recent years, however, tree nuts' richness in unsaturated lipids, proteins, minerals, and other healthy components have increased their popularity. According to Mintel, up to 66% of U.S. households currently purchase loose nuts (peanuts excluded), and the number is expected to continue to grow, especially as this product group is most popular among Millenials (Topper, 2016). At the same time, many products which contain tree nuts are also being marketed. As a result, it can be inferred that a large

portion of the public are consuming tree nuts on a regular basis. Tree nut consumption around the world has also been steadily increasing over the past decades, as indicated by growing trade volumes and demand in all key markets (USDA Foreign Agricultural Service, 2016). With this outlook, tree nuts can become a significant source of InsPs for many people. As a result, accurate information on InsP contents in these products will be important for researches on the impact of InsPs on health, especially in cohort studies.

The distribution of InsPs in plant seeds varies greatly, with the major concentration compartments being the bran, the germ, and the cotyledon. Toluidine blue staining can be used to identify phosphate depositions in these food matrices, where these compounds would appear red-violet under an optical microscope. In almond meal and hazelnuts, these depositions have been found to be small pockets densely surrounded by proteins, suggesting the storage location of InsPs in these foods (Andriotis, Smith, & Ross, 2005; Dourado, Barros, Mota, Coimbra, & Gama, 2004).

At the moment, information on the InsP profile of common tree nuts is lacking, especially on how much these compounds contribute to the organic phosphate and total phosphate contents of the products. Existing data have mainly reported only $InsP_6$ and the values have shown significant variations, as the highest $InsP_6$ value for a nut is often found to be 6 to 10 folds higher than the lowest value in the range (Table 2.1). Drawing a general consensus on the occurrence of InsPs in these products is also difficult because not all studies utilized specific methods and some studies have estimated the $InsP_6$ content by assuming that a certain percentage of the total phosphorus content is made up by phytic acid, which disregards possible deviations due to cultivation and processing factors.

Table 2.1. Content of *myo*–inositol hexakisphosphate in common tree nuts

Tree nuts	Taxonomic names	$InsP_6$ content (µmol/g)
Almonds	*Prunus dulcis* [Mill.] D.A. Webb	5.3 – 32.0[1-7]
English walnuts	*Juglans regia* L.	2.7 – 21.0[1,2,5,7]
Cashews	*Anacardium occidentale* L.	3.3 – 29.8[1,2,5,7]
Brazil nuts	*Bertholletia excelsa* Humb. & Bonpl.: Lecythidaceae	2.9 – 27.3[5,7]
Macadamias	*Macadamia integrifolia* Maiden & Betche	2.3 – 14.3[1,2,7]
Pistachios	*Pistachia vera* L.	3.0 – 43.0[2,5,7]
Pecans	*Carya illinoinensis* (Wangenh.) K. Koch.	4.4 – 28.9[2,7]
Hazelnuts	*Corylus avellana* L.	2.2 – 35.5[2-7]

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While phytate phosphorus can make up 60-90% of the total phosphorus content in some cereal and oil plant seeds, earlier studies using non-specific methods have suggested that this value can vary widely for tree nuts, ranging from as low as 25% in walnut and as high as 75% in pistachio (Deshpande, 2002). To accurately assess the contribution of InsPs to phosphates in tree nuts, it is necessary to measure individual InsPs by a specific method and to compare the results with the total phosphate contents in these products. In comparison with other types of plant seeds, tree nuts also have much higher lipid, protein, and minerals contents, which are factors that can interfere with the extraction and analysis of InsPs. Such complex matrices call for the development of a method specifically refined for this food group.

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CHAPTER 3

QUANTIFICATION OF INOSITOL PHOSPHATES IN ALMOND MEAL AND ALMOND BROWN SKINS BY HPLC/ESI/MS

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Abstract

The extraction and measurement of all 6 forms of inositol phosphates (InsPs) in almond meal and brown skins were improved from existing methods by pH adjustment, supplementation of EDTA, and rapid analysis via anion-exchange high-performance liquid chromatography coupled with electrospray ionization mass spectrometry. The quantity of InsPs in 6 major almond cultivars ranged from 8-12 μmol/g in the meal and 5-14 μmol/g in the brown skins. $InsP_6$ was the dominant form, but lower forms still accounted for ~20% of the total InsPs molar concentration in a majority of the samples. InsPs contributed 32-55% of the organic phosphorus content and 20-38% of the total phosphorus content in the meal. In brown skins, these ranges were 44-77% and 30-52%, respectively. The successful application of this analytical method with almonds demonstrates its potential use for re-examination of the reported phytic acid contents in many other tree nuts, legumes, grains, and complex foods.

3.1. Introduction

Inositol hexakisphosphate ($InsP_6$), also commonly called by the name phytic acid, is one of the most prominent phosphate-containing compounds in plant seeds. Comprising of a *myo*-inositol ring with six phosphate groups, it provides an efficient form of phosphate storage for the seed before sprouting begins (Figure A.1). Furthermore, $InsP_6$ is capable of gaining a negative charge by donating up to 12 protons,

with eight acid dissociation constants ranging from 1.9 to 9.5 (Heighton, Schmidt, Rice, & Siefert, 2008). This characteristic affords $InsP_6$ the capacity to bind with selected mineral ions and cationic moieties of proteins over a wide pH range. On the one hand, these interactions are highly important to the storage of nutrients in seeds, but on the other, they have been reported to possess inhibitive potentials on the absorption of nutrients essential for human nutrition (Konietzny, Jany, & Greiner, 2006). At the pH levels present in our digestive system, salts and complexes formed with phytic acid may be insoluble, and the low level of endogenous phytase available is insufficient for the digestion of those salts and complexes. Consequently, $InsP_6$ is often viewed as an undesirable component in human diets. *In vivo* studies of this topic are difficult to conduct; besides, the negative implications of phytic acid have yet to be firmly established (Lopez, Leenhardt, Coudray, & Remesy, 2002). Furthermore, knowledge of the accurate $InsP_6$ content in foodstuffs is incomplete. Due to the assumption that $InsP_6$ makes up the majority, if not all, of the organic phosphate content of foods, the most common assay for phytic acid measurements has been to quantitatively determine the total organic phosphate content and then attribute it to $InsP_6$. This assumption risks the overestimation of $InsP_6$ when other phosphate-containing compounds are available at significant levels in the sample (Lehrfeld & Morris, 1992). An assay for phytic acid alone is also not especially desirable when there is significant content of lower InsPs. *Myo*-inositol mono-, bis-, tris-, tetrakis-, and pentakisphosphate ($InsP_{1-5}$) may behave similarly

to $InsP_6$ in non-specific organic phosphate quantification assays, but actually these have lower binding affinity than $InsP_6$ for cations, and therefore different nutritional effects in the digestive system.

In recent decades, the need for accurate measurement of all six inositol phosphates (InsPs) has been accentuated by new insights into the beneficial and possibly essential roles of these compounds in human health. $InsP_{3-6}$ have been found to be ubiquitous in eukaryote cells, where they participate in many different cell functions, including several Ca^{2+}-signaling pathways, mRNA exportation, maintenance of genomic stability, and apoptosis (Majerus, Zou, Marjanovic, Kisseleva, & Wilson, 2008; Monserrate & York 2010; Loss, Wu, Riccio, & Saiardi, 2013). InsPs' capability to chelate cations provides them with general antioxidative properties. Additionally, those with the specific 1,2,3-trisphosphate arrangement can also inhibit the Fenton reaction by interfering with lipid peroxidation and hydroxyl-radical formation processes (Phillippy & Graf, 1997). Other disease-preventive effects by $InsP_6$ have been recognized, including the inhibition of pathological calcification, cell proliferation, metastasis, angiogenesis, and inflammation (Costa-Bauzá, Isern, Perelló, Sanchis, & Grases, 2005; Grases, Santiago, Simonet, & Costa-Bauzá, 2003; Gu, Raina, Agarwal, & Agarwal, 2010; Kumar, Sinha, Makkar, & Becker, 2010; Norazalina, Norhaizan, Hairuszah, & Norashareena, 2010; Raina et al., 2013). Some of these activities have been hypothesized to be dependent on the dephosphorylation of $InsP_6$ into lower InsPs, implying that

extraneous $InsP_{1-5}$ may also afford some of the same benefits (Vucenik & Shamsuddin, 2006).

The measurement of individual InsPs in foods has been a challenge, because these compounds have relatively low abundances and lack characteristic absorbance maxima spectrophotometrically. Among different separation methods for InsPs, anion-exchange high pressure liquid chromatography (HPLC) is often considered superior over thin-layer chromatography, gas chromatography, and electrophoresis, because it can deliver high efficiency and sensitivity while not requiring cumbersome preparation steps such as derivatization, where losses will occur. Mass spectrometry has also become an excellent choice for the simultaneous on-line detection of multiple InsPs. Ion-pairing chromatography paired with inductively coupled plasma-sector field-mass spectrometry has been successfully applied to measure the contents of $InsP_{4-6}$ in several types of fruits, vegetables, cereals, and oil seeds (Helfrich & Bettmer, 2004). Recently, Liu and others were able to couple HPLC with tandem mass spectrometry to measure *myo*-inositol, along with InsPs, in multiple types of samples, including foods and human cells (Liu, Villalta, & Sturla, 2009).

Most research on the InsPs contents in plant seeds has focused on cereals and legumes, due to the high volume and frequency in which they are consumed. However, tree nuts are rapidly gaining popularity around the world for their high nutrient density and health benefits. It is notable that the high abundances of lipid, minerals, and protein

in this food group pose as possible interferences to the extraction and measurement of InsPs, requiring the refinement of existing techniques in order to maximize/optimize recovery.

Among tree nuts, sweet almonds (*Prunus dulcis* [Mill.] D.A. Webb) standout for having significant antioxidant properties, which have been attributed to vitamin E (i.e., α-tocopherol) and many polyphenols in the meal and the skins (Chen & Blumberg, 2008; Jenkins et al., 2006; Li et al., 2007). Some studies have shown almonds to contain marked amounts of InsPs, which might also contribute to these effects (Chen, 2004; Liu et al., 2009; Simonet, Ríos, Grases, & Valcárcel, 2003). Information on InsPs in the brown skins – a component commonly included in some almond products – is also lacking. In this study, we report an analytical method utilizing high-pressure anion-exchange liquid chromatography/mass spectrometry (HPLC/MS) with electrospray ionization (ESI) for the quantification of InsPs in almond meal and almond brown skins. The method has been refined for the complex matrix of almonds, and has potential application for other types of tree nuts, legumes, grains and complex foods. Measurement of InsPs in the meal and brown skins was also performed on six prominent almond cultivars grown in California and compared to the organic phosphate and total phosphate contents present in these samples.

3.2. Materials & Method

3.2.1. Chemicals and supplies

Adenosine 5′-monophosphate (AMP), phytic acid sodium salt from rice, ammonium carbonate for HPLC, Dowex® 1X4 chloride form ion-exchange resin (100-200 mesh), 1-amino-2-naphthol-4-sulfonic acid with ≥ 95.0% purity, and HPLC-grade methanol were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Analytical standards with >98% purity included D-*myo*-inositol-1-phosphate monosodium salt, D-*myo*-inositol 1,4-diphosphate disodium salt, D-*myo*-inositol-1,4,5-triphosphate trisodium salt, and D-*myo*-inositol-1,3,4,5-tetraphosphate octasodium salt; these were acquired from the Cayman Chemical Company (Ann Arbor, MI, USA). D-*myo*-Inositol 1,3,4,5,6-pentakisphosphate pentapotassium salt and D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate dodecasodium salt were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and EMD Millipore Corporation (Billerica, MA, USA), respectively.

Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), ACS-grade sodium hydroxide, glacial acetic acid, hydrochloric acid, and hexanes as well as HPLC-grade water were purchased from the Fisher Scientific Company (Suwanee, GA, USA). Sodium sulfite and sodium bisulfite were obtained from Aqua Solution, Inc. (Deer Park,

TX, USA). Potassium dihydrogen phosphate and ammonium molybdate were acquired from J.T. Baker (Avantor Performance Materials, Center Valley, PA, USA).

A commercial raw almond product with brown skins intact was used to develop the method. Whole, untreated Nonpareil, Butte, Monterey, Mission, Aldrich, and Price almonds were generously supplied by the Almond Board of California for the measurement of InsPs in the different almond cultivars. To provide independent replicates for the analysis, each cultivar was sampled from three different farms. The samples were stored at -40 °C until they were processed.

3.2.2. Preparation of reference and analytical standards

The preparation of analytical standards was adapted from a method by Liu and others with some modifications (Liu et al., 2009). Briefly, standard solutions of individual InsPs were prepared in CH_3OH:H_2O (5:95, v/v). The solutions were combined with the AMP internal standard to provide a series of working standard solutions with analyte concentrations of 5, 20, 30, 40, 50, 60, 80, and 100 μM. Additional standard solutions with concentrations of 150, 200, 300, 350, and 450 μM were prepared for $InsP_6$. The final concentration of AMP in each solution was 25 μM. The ratio of the analyte's peak area over AMP's peak area was plotted against the amount of analyte in the injection volume (pmole) to construct the standard curve in linear regression for each inositol phosphate (InsP).

To prepare a reference standard solution containing all six InsPs, 0.09 g of phytic acid sodium salt was dissolved in 30 mL of 3.2 M acetic acid. The solution was transferred to a glass vial, flushed with N_2, sealed with a rubber septum cap, and heated at 140 °C for 3 h, then at 70 °C for 13 h. After having cooled to room temperature, the solution was subjected to a N_2 evaporator (N-EVAP™ 111 with an aluminum bead dry bath set at ~50 °C, Organomation Associates, Inc., Berlin, MA, USA). The solid residue was reconstituted in 15-mL CH_3OH:H_2O (5:95, v/v) and stored at -20 °C until used.

3.2.3. Preparation of samples

To avoid losses of InsPs due to hot water blanching, raw almonds were blanched with liquid nitrogen 3×, 10 min apart, and hand-peeled to separate the brown skins from the meal. The samples were frozen at -80 °C and then finely ground in a coffee grinder (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ) to a very fine powder using an intermittent pulsing technique. Because high-lipid contents will interfere with the extraction of hydrophilic bioactives, the ground almond meal was defatted with hexanes in a Sohxlet apparatus. The defatted sample was then left in a fume hood for several hours to ensure removal of hexanes before being stored at -80 °C until further analyzed.

On the day of analysis, prepared samples were removed from the freezer and reground. For InsPs extraction, 0.5 g of a sample was mixed with 3 mL of 3.2 M acetic acid and blended using a PT-3100 Polytron™ homogenizer (Brinkmann Instruments,

Westbury, NY, USA) at 15,000 rpm for 60 s. Afterward, the sample was shaken for 3 h at 22 °C and centrifuged (3,345 × *g*, 20 min, 22 °C). Half a milliliter of the supernatant was combined with 1 mL of 0.11 M Na_2EDTA dissolved in 0.75 M NaOH. The sample was evaporated to dryness in a RT400 Speed Vac System (Savant, Holbrook, NY, USA), reconstituted in 0.5 mL CH_3OH:H_2O (5:95, v/v) and sonicated to facilitate dissolution. The sample was then filtered through a 0.22-µm Phenex™-RC nylon syringe filter (Phenomenex, Torrance, CA, USA) and combined with the AMP standard solution to attain a final concentration of 25 µM AMP. All samples were tested in triplicate.

3.2.4. Separation and quantification of the InsPs

The separation of InsPs was achieved with an Agilent 1100 HPLC system equipped with a BioBasic™ AX anion-exchange column (2.1 × 150 mm, 5-µm particle size, Thermo Fisher Scientific, Waltham, MA, USA). Analytes were detected by a Waters Micromass Quadrupole Time-of-Flight (ToF) mass spectrometer with an electrospray ionization (ESI) source (Waters Corporation, Milford, MA, USA). Spectra were acquired and analyzed using MassLynx software (Version 4.1, Waters Corporation). Each sample was injected manually using a 25-µL Hamilton syringe. Analyses were performed at 22 °C.

Inositol phosphates were eluted using two mobile phases: (A) CH_3OH:H_2O (5:95, v/v) and (B) 200 mM aqueous $(NH_4)_2CO_3$. The target compounds (i.e., the different InsP

forms) were eluted at 200 µL/min with a 20-min gradient program beginning with mobile phase A for 4 min, followed by 0 to 20% B over 2 min, and finally 20 to 55% B over 14 min. At the end of each run, the column was washed with 100% mobile phase A for 25 min.

The ESI source was set in negative-ion mode, with spray voltage of 2500 V, ion-transfer capillary temperature at 300 °C, and a N_2 flow at 450 L/h. These parameters were optimized for the simultaneous detection of AMP and all six InsPs.

3.2.5. Matrix effects

After the addition of the 3.2 M acetic acid solution to either dry almond meal or brown skins, the test samples were spiked with an aliquot of the reference standard. The samples were then extracted and analyzed according to the procedure described above. Recovery experiments were determined according to AOAC guidelines (AOAC, 2016). The percent recovery was calculated by the following equation:

$$\text{Recovery (\%)} = [(C_s - C_o)/C_s] \times 100\%$$

where R (%) is the percent recovery of the added standard, C_o is the concentration of each analyte in the non-spiked sample, and Cs is the concentration of each analyte in the spiked sample.

3.2.6. Organic phosphorus and total phosphorus contents

The AOAC Official Method 986.11 for Phytate in Foods, originally adapted from a chromatographic method by Harland & Oberleas (1986), is a non-specific assay which employs the assumption that $InsP_6$ comprises the 'organic' phosphate content of foods. Hence, this technique was used for the measurement of organic phosphorus in the present study with some modifications. Briefly, a 2-g sample was mixed with 40 mL of 2.4% (*v/v*) HCl and shaken for 3 h at 22 °C. The resultant test solution was filtered *in vacuo* using a small Büchner funnel and Whatman No. 1 paper. A 1-mL aliquot of the filtrate was pipetted into a 25-mL volumetric flask, combined with 1 mL of 0.11 M Na_2EDTA in 0.75 M NaOH, and then diluted to mark with deionized water. Meanwhile, a 3-mL Luer-lock Monoject™ syringe (Covidien Ltd., Dublin, Ireland) was placed on top of a solid-phase extraction manifold. A small piece of glass wool was inserted at the bottom of the syringe and 0.5 g of Dowex® 1X4 ion-exchange resin, in the form of an aqueous slurry, was poured in. After the resin bed was established, the column was washed with 15 mL of 0.7 M NaCl and then 15 mL of deionized H_2O. The test sample was loaded quantitatively onto the column. Inorganic phosphates were eluted from the column with 15 mL of deionized H_2O, followed by 15 mL of 0.1 M NaCl, and then discarded. Organic phosphates were recovered from the resin with 15 mL of 0.7 M NaCl. The resin was regenerated with 15 mL of H_2O and used for up to 3 samples before its replacement. The collected organic phosphate eluent was transferred to a Kjeldahl digestion tube and

combined with 0.5 mL of concentrated H_2SO_4, 3 mL of concentrated HNO_3, and a couple of glass beads. The sample was digested in a BD-20 Tecator™ Kjeldahl digestion system (FOSS, Eden Prairie, Minnesota, USA). The digestion program began with heating to 100 °C and a hold period of 14 min followed by heating to 200 °C, holding there for 10 min, and finally heating to 300 °C with a hold period of 30 min. After the tube had cooled, 10 mL of deionized H_2O was added to dissolve the salts. The sample was heated for another 10 min at 200 °C and then left to cool. The solution was quantitatively transferred to a 50-mL volumetric flask, mixed with 2 mL of 2.5% (*w/v*) ammonium molybdate in 0.5 M H_2SO_4, 1 mL of sulfonic acid reagent (*i.e.*, 0.92 M $NaHSO_3$, 0.15 M Na_2SO_3, and 6.69 mM 1-amino-2-naphthol-4-sulfonic acid), and then diluted to mark with deionized water. The sample was held for 30 min to allow for maximum color development before reading its absorbance at a λ = 640 nm using an Agilent 8453 diode-array spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA). To prepare a series of phosphate standard solutions, appropriate volumes of an 80 mM KH_2PO_4 solution were pipetted into 50-mL volumetric flasks along with 2 and 1 mL of the molybdate and sulfonic acid reagents, respectively; each flask was filled to mark with deionized water. A phosphate standard curve was constructed by plotting the quantity of phosphate in the standard solutions against the absorbance readings at λ = 640 nm.

To prepare the samples for total phosphorus analysis, a 500-mg sample was ashed in a porcelain crucible in a tabletop, 1.6-ft^3 muffle furnace (Thermo Scientific

Thermolyne, Fisher Scientific) at 525 °C for ~18 h. The resultant ash was dissolved in 10 mL of aqua regia ($HCl:HNO_3:H_2O$ 30:10:60, *v/v/v*), and the sample was centrifuged (3,345×*g*, 2 min, 22 °C). Analysis of the phosphorus content in the supernatant was carried out by inductively coupled plasma-optical emission spectroscopy (ICP-OES) via the Chemical Analysis Laboratory, a part of the University of Georgia Center for Applied Isotope Studies. A peanut butter standard reference material with known phosphorus content was used to calibrate the system (SRM 2387, National Institute of Standards and Technology, Gaithersburg, MD, USA). All analyses were performed in triplicate.

3.2.7. Method characterization

The limits of detection (LODs) and limits of quantification (LOQs) for the InsPs were calculated on the basis of a minimal accepted value of the signal-to-noise ratio of 3 and 10, respectively. The interday variability of the HPLC/ESI/MS analysis was evaluated at five concentrations of each InsP standard across three consecutive days. The interday variability of the AOAC Official Method 986.11 for Phytate in Foods was also determined by measuring five concentrations of a KH_2PO_4 standard over three consecutive days. Relative standard deviation (% RSD_r) was calculated as follows:

$$\% \, RSD_r = \text{standard deviation/mean} \times 100$$

The interday precision, accuracy, and bias of the ICP-OES method were evaluated by assaying the peanut butter standard SRM 2387 and comparing the results with the

certified value. Five replications were performed. Bias and percent accepted value were calculated as follows:

$$\text{bias} = \text{expected value} - \text{analytical value}$$

$$\%\ \text{accepted value} = (\text{analytical value} \times 100)/\text{accepted value}$$

3.2.8. Statistical analysis

All values were reported as mean ± standard deviation (n=3). Differences in the means of each InsP form were determined with the means procedure and a two-way Student's *t*-test with $P < 0.05$ using the JMP Pro software, Version 12 (SAS Institute Inc., Cary, NC, USA).

3.3. Results & Discussion

3.3.1. Method development

A challenge often encountered in utilizing anion-exchange HPLC for separating InsPs is poor resolution of peaks. A gradient that enable all six compounds to be completely separated is often too gradual to avoid band broadening in some of the peaks. This problem becomes even more complicated when different stereoisomers of one or more InsPs are present in the sample, as the binding strength of stereoisomers can vary

according to the position of the phosphate group(s) on the *myo*-inositol ring. By coupling HPLC with mass spectrometry, the mass-to-charge ratio can be used as the primary identification means, and the dependency on retention time (t_r) is lowered. Additionally, tolerance of some co-elution also allows for a shorter analysis time. This approach is highly suitable when the goal is to quantify InsPs without regard to their stereoisomers. With the gradient chosen for this study, the six InsPs were eluted between 10 and 20 min after the sample injection (Figure 3.1). This range of time was sufficient to produce sharp peaks while ensuring that other compounds with similar mass-to-charge ratios as those of the InsPs eluted very early from the column. The calibration curves constructed using the various InsP standards and AMP achieved excellent linearity ($R^2 > 0.99$) across the concentration ranges of interest (Table 3.1 & Figure A.2). The interday $\%RSD_r$ values were within 10% for all InsPs concentrations tested (Table A.1). The limit of detection was 25 pmole and the limit of quantification was 50 pmole for each InsP form.

pH plays a critical role in the recovery of InsPs. At low pH levels, InsPs can form insoluble complexes with proteins. In almond meal, InsPs have been found to occur as globoid inclusions in the protein bodies of cotyledon cells (Douradou, Barros, Mota, Coimbra, & Gama, 2004). Hence, precipitation of InsP–protein complexes is highly probable when the sample is suspended in the acid extracting solvent. To avoid this effect, a pH > 5 is generally required (Ellis & Morris, 1983). In almond meal, our preliminary experiments with $InsP_{4\text{-}6}$, which have the highest binding affinity for mineral

cations among the six InsPs, showed that the highest recovery across a pH range of 4.5 to 13.5 was attained at pH=6 (Figure 3.2). Nevertheless, InsPs at this pH can still form insoluble salts with divalent cations such as calcium and magnesium. This effect can be reduced by the addition of EDTA. A minimum quantity of 1 mmol of EDTA, added for each gram of original sample, has been reported to increase organic phosphate recovery in general (Ellis & Morris, 1983). Applying this knowledge in the present study, 1 mL of a mixed solution containing 0.11 M Na_2EDTA in 0.75 M NaOH was added to each 0.5 mL of acid extract before the evaporation of the acid using the Speed Vac system.

In the past, HCl was found effective in extracting phosphates from food samples, leading to the application of 2.4% HCl as the extraction solvent in the AOAC Official Method for Phytate in Food. However, other researchers have commented that HCl can have a hydrolyzing effect on InsPs, and consequently recommended using 3.2 M acetic acid (Liu et al., 2009). In a preliminary study, we determined 3.2 M acetic acid to be less efficient than 2.4% HCl in extracting total phosphates. As a result, both solutions were tested as extracting solvents for InsPs from the model almond samples. It was found that HCl significantly hydrolyzed $InsP_4$ in almond meal and $InsP_1$ in both samples. The levels of other InsPs were comparable among the two extraction methods. This demonstrated that even though acetic acid might be less efficient than HCl in total phosphate extraction, it is the superior extracting solvent for InsPs (Table A.2).

The matrix effects of almond meal and brown skins on extraction efficiency were evaluated by spiking these samples with a mixture of the reference standard. Recoveries were satisfactory, ranging from 94 to ~107% (Table 3.2). No significant shift in t_r was detected in any of the samples (Figure 3.1).

Validation of the ICP-OES method was conducted with the peanut butter SRM 2378. The average phosphorus content was determined to be 3463 ± 226 mg/kg, compared to the certified value of 3378 ± 92 mg/kg. Bias, % acceptance value, and $\%RSD_r$ value were 85 mg/kg, 102.5%, and 6.52%, respectively, indicating close agreement between the analytical and true values for the sample. The calibration curve constructed using the KH_2PO_4 standards for the AOAC Official Method 986.11 for Phytate in Foods also achieved outstanding linearity (R^2 = 0.9997), and the interday $\%RSD_r$ values were less than 4% for all concentrations tested (Figure A.3 & Table A.3).

3.3.2. InsP contents in the meal and brown skins of major almond cultivars

The total amount of InsPs in the tested cultivars ranged from 8 to 12 µmol/g for almond meal and 5 to 14 µmol/g for almond brown skins (Table 3.3). Across all samples, $InsP_6$ was the dominant form, followed by $InsP_5$ and then $InsP_4$. $InsP_{1\text{-}5}$ typically comprised at least 20% of the total molar concentration of all six InsPs combined.

To our knowledge this is the first reporting of the InsP levels in almond brown skins. Previously, this component of almonds was declared to possess more InsPs than its meal counterpart; yet, no data is reported to support this hypothesis (Liu et al. 2009). In the current study, such a pattern was observed in some, but not all of the almond cultivars tested. In the California almond cultivars, the levels of $InsP_{1-3}$ were generally lower in the brown skins than in the meal. As the outer layer, the brown skins may have more interaction with phytases produced by microorganisms than that of the meal, resulting in earlier loss of lower InsPs. On the other hand, it is also possible that the main functions of InsPs differ between the brown skins and the meal. In the skins, high amounts of $InsP_{4-6}$ can serve as efficient phosphorus reserves and effective binders of mineral cations such as Ca^{2+} and Mg^{2+}, ready to release these nutrients for use upon seed germination. Meanwhile, InsPs in the meal may have additional roles as active participants in cell functions in a germinated seed. In plant cells, $InsP_6$ and several stereoisomers of $InsP_{1-5}$ have been found to take part in different metabolic pathways, most of which are central around $InsP(1,4,5)P_3$, which is a well-known second messenger in the Ca^{2+} cell-signaling pathway (Irvine & Schell, 2001). Thus, more $InsP_{1-3}$ might have been produced in the meal to serve these pathways.

Although almonds have been referred to as being high in $InsP_6$, data found in the literature constitutes a wide range from 5 to 32 μmol $InsP_6$/g for this particular tree nut (Chen, 2004; Harland, Smikle-Williams, & Oberleas, 2004; Helfrich & Bettmer, 2004;

Liu et al., 2009; Lott et al., 2000; Simonet et al., 2003; Venkatachalam & Sathe, 2006; Yada & Lapsley, 2012). Reaching a general conclusion about the $InsP_6$ content in almonds is difficult, because specific methods have not always been employed and some studies have made errors when employing reference values to calculate the $InsP_6$ concentration, as seen in the data reported by Schlemmer and others (2009). Compared to investigations that measured multiple InsPs, the concentration of each InsP in the meal of the tested cultivars for this work was comparable with that of several published results, but lower than some others (Chen, 2004; Helfrich & Bettmer, 2004; Liu et al., 2009, Simonet et al., 2003). Nevertheless, these studies all reported a similar trend in the distribution of InsPs, where InP_6 and InP_5 were present as the most and second-most abundant InsPs, respectively. The variations in InsP levels might be attributed to differences in cultivars, growing conditions, and processing parameters. In many cases, it was unclear whether the brown skins were kept intact with the meal during sample extraction. In this study, considerable variation was observed among the replicates of Aldrich brown skins, Nonpareil meal, and Butte meal. As replicates for each cultivar were harvested from different farms, it can be inferred that farming conditions had significant impacts on the InsP levels of these cultivars.

3.3.3. Contribution by InsPs to organic phosphorus and total phosphorus levels in almond meal and brown skins

Apropos the tested cultivars: InsPs represented 32 to 55% of the organic phosphorus content and 20 to 38% of the total phosphorus content in the meal (Table 3.4). These values are in agreement with earlier findings reported for almonds (Frank, 2013). Yet in some of the existing data on almond, the $InsP_6$ content was estimated by assuming that $InsP_6$ contributes 82% of the total phosphorus in the food group (Lott et al., 2000). It is clear that this assumption is not accurate for all almond products, as the percent contribution of $InsP_6$ to total phosphorus determined in this work was not only smaller than 82%, but also showed significant variation amongst different cultivars.

In this study, InsPs accounted for 44 to 77% of organic phosphorus and 30 to 52% of total phosphorus levels in the brown skins. It is discernible that although InsPs were significant sources of phosphorus in both the meal and the brown skins, other organic phosphates as well as inorganic phosphates were highly abundant in these samples. This is very different from the profile of common cereals, cereal by-products, and oil seeds, where InsPs are often reported to account for much higher portions of phosphorus (Frank, 2013; Kornegay, 2000). The assumption that InsPs or $InsP_6$ makes up the organic phosphate content in seeds would heavily overestimate the concentrations of these compounds in almonds. For example, when $InsP_6$ is considered to be the sole contributor of organic phosphorus in foods, such as assumed by the AOAC Official Method 986.11

for Phytate in Foods, the abundance of $InsP_6$ in the tested cultivars was overestimated by 125 to 281% in the meal and 60 to 322% in the brown skins (Table A.4). If the total phosphate content is assumed to comprise only of $InsP_6$, the degree of overestimation could be as high as 480% in the meal and 582% in the brown skins.

Similar to a pattern observed on InsPs levels, some cultivars, such as Aldrich and Butte, showed higher variation in organic phosphorus content among replicates than others, indicating the influences of growing and harvesting conditions. Because the precursors of InsPs in plants have been identified to be a different type of phosphate – the phospholipid phosphatidylinositol-4,5-bisphosphate – it would be of interest to further investigate how different factors that affect the synthesis of InsPs as well as other organic phosphates during seed formation may vary in influences among different almond cultivars.

3.4. Conclusions

Recovery and method of detection are two major challenges in the measurement of InsPs, particularly in complex matrices such as almonds. This study introduces a method specifically refined for the extraction and on-line analysis of almond meal and almond brown skins, with potential application to other types of tree nuts and samples. The use of ion-exchange HPLC coupled with mass spectrometry presented significant advantages for rapid separation and on-line detection of all six InsPs.

Concerning the meal and brown skins of six major almond cultivars: InsPs were found to account for a marked proportion of the organic and total phosphorus contents. Yet, they cannot be assumed to constitute all of the organic phosphates of these samples. While $InsP_6$ was the dominant form of the InsPs, lower InsPs were also presented at considerable levels. These results underline the importance of using a specific method for the quantification of InsPs in food or biological matrices.

Acknowledgments

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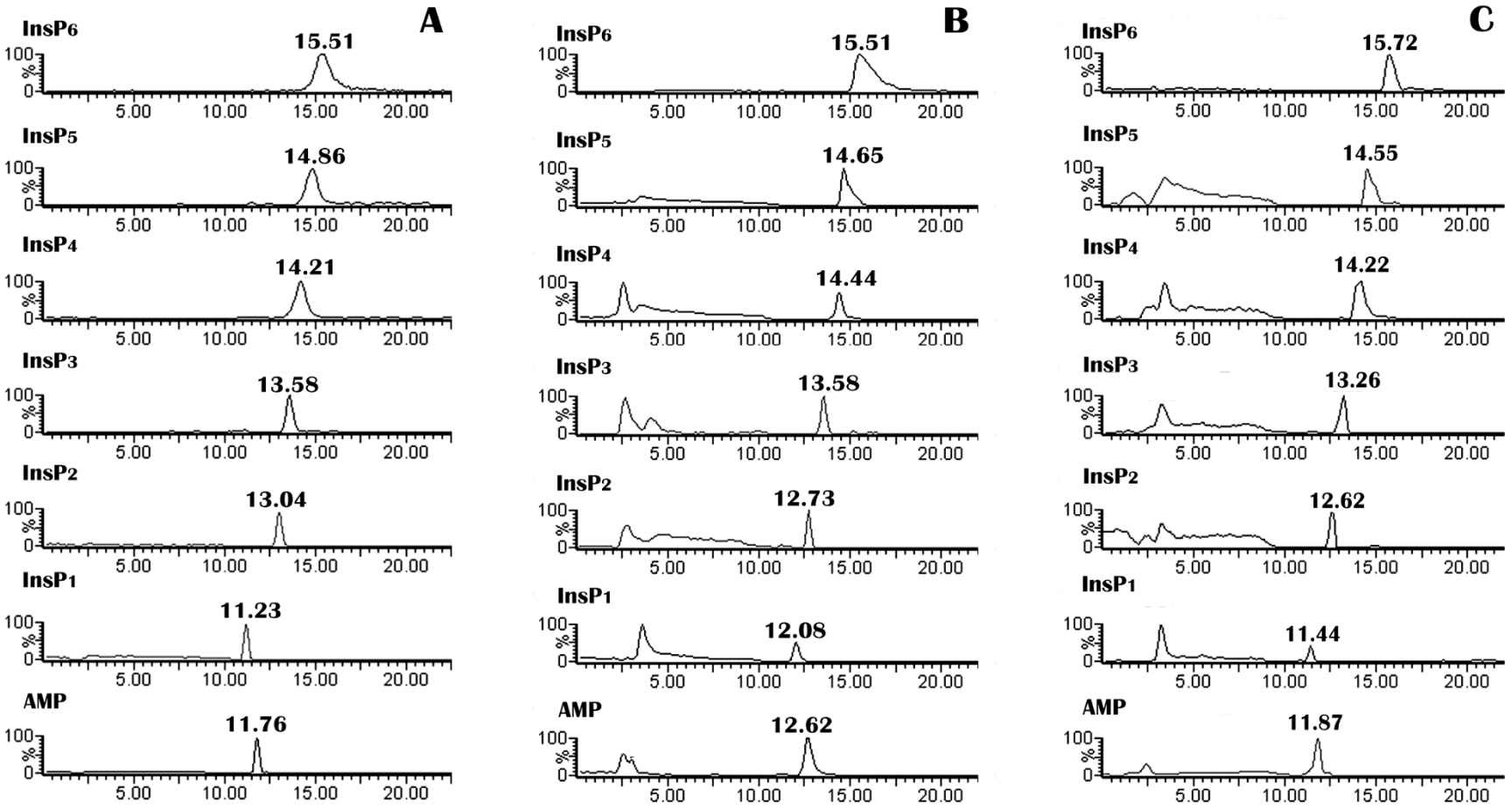

Figure 3.1. Representative elution peaks of $InsP_{1\text{-}6}$ and adenosine 5′-monophosphate detected in **(A)** commercial standards; **(B)** almond meal; and **(C)** almond brown skins.

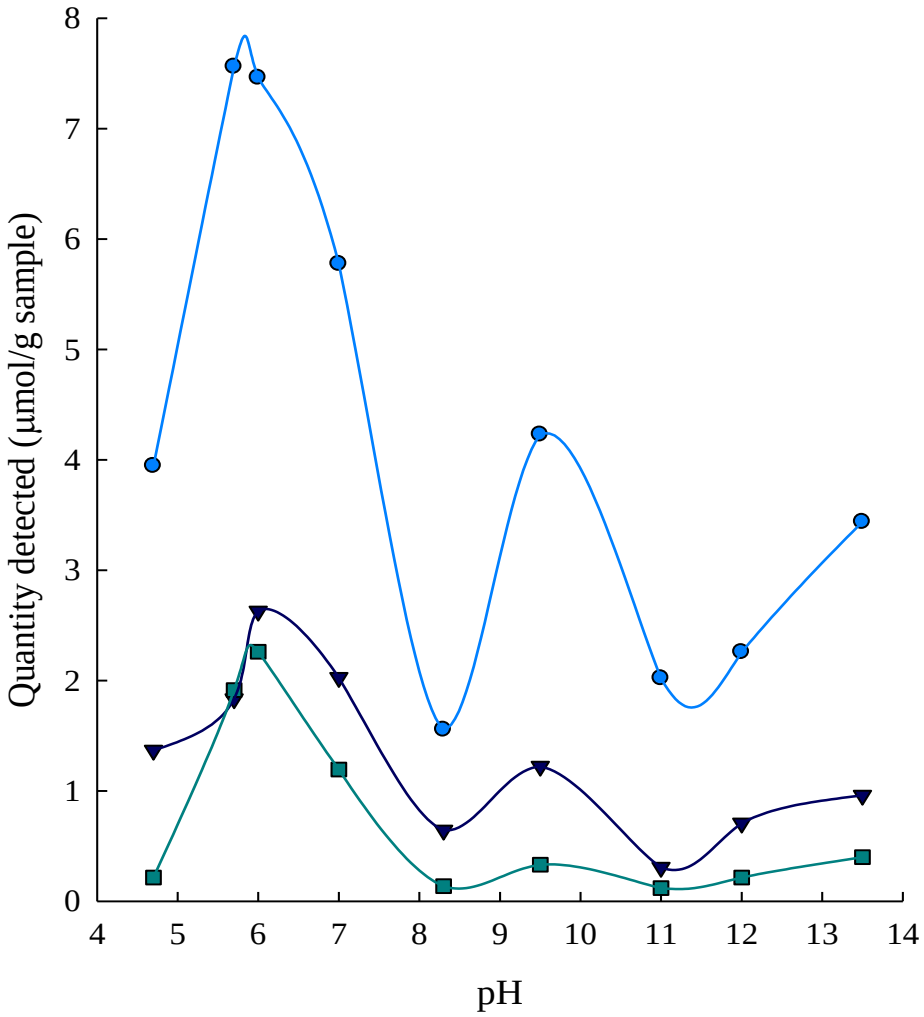

Figure 3.2. Quantities (µmol/g sample) of $InsP_6$ (circle), $InsP_5$ (triangle down), and $InsP_4$ (square) detected in a model almond meal sample cross a pH range of 4.5 to 13.5[a]

[a]Values represent means of duplicates.

Table 3.1. Parent mass, slopes, intercepts, correlation coefficients, and concentration ranges of interest of individual inositol phosphates.

Analyte[a]	Parent mass (*m/z*)[b]	Slope ($\times 10^{-2}$)	Intercept	R^2	Concentration range (μM)
$InsP_6$	659	0.17	-0.0805	0.9964	5 to 450
$InsP_5$	579	0.24	-0.0522	0.9929	5 to 100
$InsP_4$	499	0.23	-0.0295	0.9941	5 to 100
$InsP_3$	419	0.16	-0.0113	0.9967	5 to 100
$InsP_2$	339	0.10	0.0004	0.9959	5 to 100
$InsP_1$	259	0.08	0.0109	0.9952	5 to 100

[a]Abbreviations: $InsP_{1\text{-}6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]*m/z* is the mass-to-charge ratio.

Table 3.2. Recovery of $InsP_{1-6}$ in model almond meal and brown skin samples spiked with a mixture of standards with known concentrations.

Inositol Phosphate Species	Amount added (nmol/g sample)	Almond meal		Almond brown skins	
		Original amount (μmol/g)[b]	Recovery (%)	Original amount (μmol/g)[b]	Recovery (%)
$InsP_6$	136.9	5.66 ± 0.23	98.3	7.34 ± 0.66	106.6
$InsP_5$	171.9	1.61 ± 0.12	104.2	3.02 ± 0.23	94.0
$InsP_4$	153.4	0.43 ± 0.06	95.2	0.82 ± 0.18	98.0
$InsP_3$	115.8	0.17 ± 0.02	102.3	0.18 ± 0.05	99.1
$InsP_2$	74.9	0.13 ± 0.02	104.7	0.08 ± 0.01	95.3
$InsP_1$	51.4	0.07 ± 0.01	97.5	0.09 ± 0.02	94.1

[a]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]Values (mean ± SD) are based on triplicate analyses.

Table 3.3. Levels of inositol phosphates[a,b] (μmol/g) in the meal and brown skins of six major almond cultivars

Almond meal

	Mission		**Aldrich**		**Nonpareil**	
	μmol/g	% Total	μmol/g	% Total	μmol/g	% Total
$InsP_6$	7.41 ± 1.14	72.3	6.45 ± 1.62	70.4	7.98 ± 2.30	71.2
$InsP_5$	1.99 ± 0.46	19.5	1.69 ± 0.38	18.5	1.28 ± 0.60	11.4
$InsP_4$	0.31 ± 0.01	3.0	0.53 ± 0.05	5.8	1.16 ± 0.38	10.3
$InsP_3$	0.26 ± 0.16	2.6	0.26 ± 0.09	2.8	0.45 ± 0.19	4.0
$InsP_2$	0.18 ± 0.13	1.8	0.19 ± 0.05	2.1	0.24 ± 0.12	2.7
$InsP_1$	0.09 ± 0.09	0.9	0.04 ± 0.03	0.5	0.10 ± 0.03	0.9
Total	**10.24**		**9.17**		**11.21**	
	Price		**Butte**		**Monterey**	
	μmol/g	% Total	μmol/g	% Total	μmol/g	% Total
$InsP_6$	5.92 ± 0.78	71.0	7.22 ± 2.25	73.4	9.44 ± 1.29	76.1
$InsP_5$	1.59 ± 0.12	19.1	1.61 ± 0.26	16.4	2.21 ± 0.53	17.8
$InsP_4$	0.31 ± 0.06	3.8	0.43 ± 0.05	4.4	0.41 ± 0.001	3.3
$InsP_3$	0.22 ± 0.11	2.6	0.26 ± 0.03	2.7	0.18 ± 0.02	1.4
$InsP_2$	0.14 ± 0.06	1.7	0.22 ± 0.04	2.2	0.17 ± 0.02	1.4
$InsP_1$	0.15 ± 0.12	1.8	0.09 ± 0.02	0.9	ND[c]	–
Total	**8.34**		**9.84**		**12.41**	

[a]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]Values (mean ± SD) are based on triplicate analyses.
[c]ND: Not detected (<2 nmol/g sample)

Table 3.3. (cont.) Levels of inositol phosphates[a,b] (μmol/g) in the meal and brown skins of six major almond cultivars

Almond brown skins

	Mission		**Aldrich**		**Nonpareil**	
	μmol/g	% Total	μmol/g	% Total	μmol/g	% Total
$InsP_6$	5.43 ± 0.26	74.5	7.79 ± 4.76	74.9	3.32 ± 0.75	66.9
$InsP_5$	0.93 ± 0.27	12.7	1.95 ± 0.91	18.7	0.99 ± 0.26	20.0
$InsP_4$	0.77 ± 0.39	10.6	0.45 ± 0.03	4.3	0.58 ± 0.27	11.7
$InsP_3$	0.16 ± 0.04	2.2	0.12 ± 0.09	1.2	0.03 ± 0.002	0.7
$InsP_2$	ND[c]	–	ND[c]	–	ND[c]	–
$InsP_1$	ND[c]	–	0.09 ± 0.04	0.9	0.04 ± 0.02	0.7
Total	**7.29**		**10.40**		**4.96**	

	Price		**Butte**		**Monterey**	
	μmol/g	% Total	μmol/g	% Total	μmol/g	% Total
$InsP_6$	9.03 ± 0.98	77.2	11.10 ± 1.04	81.6	6.09 ± 1.20	77.6
$InsP_5$	2.31 ± 0.31	19.8	2.06 ± 0.75	15.1	1.41 ± 0.42	18.0
$InsP_4$	0.13 ± 0.01	1.1	0.37 ± 0.14	2.7	0.29 ± 0.25	3.7
$InsP_3$	0.15 ± 0.03	1.3	0.03 ± 0.03	0.2	0.05 ± 0.05	0.7
$InsP_2$	0.04 ± 0.01	0.3	0.05 ± 0.05	0.3	ND[c]	–
$InsP_1$	0.03 ± 0.01	0.3	ND[c]	–	ND[c]	–
Total	**11.69**		**13.60**		**7.84**	

[a]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]Values (mean ± SD) are based on triplicate analyses.
[c]ND: Not detected (<2 nmol/g sample)

Table 3.4. Levels of organic phosphorus and total phosphorus in the meal and brown skins of six major almond cultivars[a]

Sample		Organic P		Total P		
		μmol/g	%P from InsPs[b]	μmol/g	% from organic P	%P from InsPs[b]
Mission	Meal	148.3 ± 5.5	38.4	229.7 ± 13.0	68.4	24.8
	Brown skins	85.3 ± 3.4	47.8	126.8 ± 15.5	67.6	32.2
Aldrich	Meal	121.9 ± 19.4	41.4	183.0 ± 37.0	66.6	27.6
	Brown skins	101.4 ± 23.3	58.0	158.8 ± 25.2	63.8	37.0
Nonpareil	Meal	118.2 ± 9.4	32.6	160.8 ± 7.8	74.1	20.1
	Brown skins	84.0 ± 3.9	51.1	135.8 ± 8.1	61.9	37.9
Price	Meal	136.4 ± 5.9	39.9	203.9 ± 3.6	65.6	26.7
	Brown skins	112.8 ± 4.7	69.7	152.4 ± 3.8	60.0	51.6
Butte	Meal	135.4 ± 20.2	33.9	206.3 ± 32.4	66.9	22.2
	Brown skins	86.9 ± 9.3	76.9	144.8 ± 24.5	74.0	46.1
Monterey	Meal	127.5 ± 11.6	55.1	186.4 ± 4.6	64.6	37.7
	Brown skins	101.7 ± 8.8	44.1	150.6 ± 16.5	67.4	29.8

[a]Values (mean ± SD) are based on triplicate analyses.
[b]Abbreviation: InsPs stands for inositol phosphates.

Appendix A. Supplementary Figures & Tables

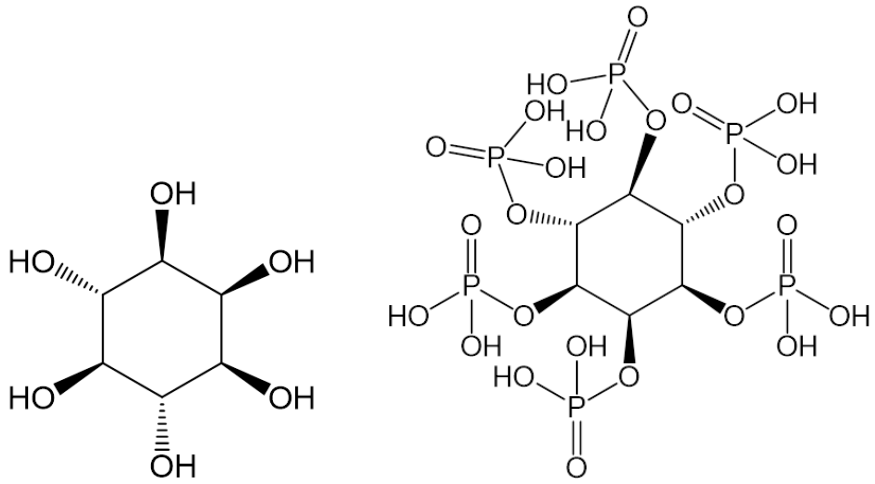

Figure A.1. *myo*-inositol (Ins) and *myo*-inositol hexakisphosphate ($InsP_6$)

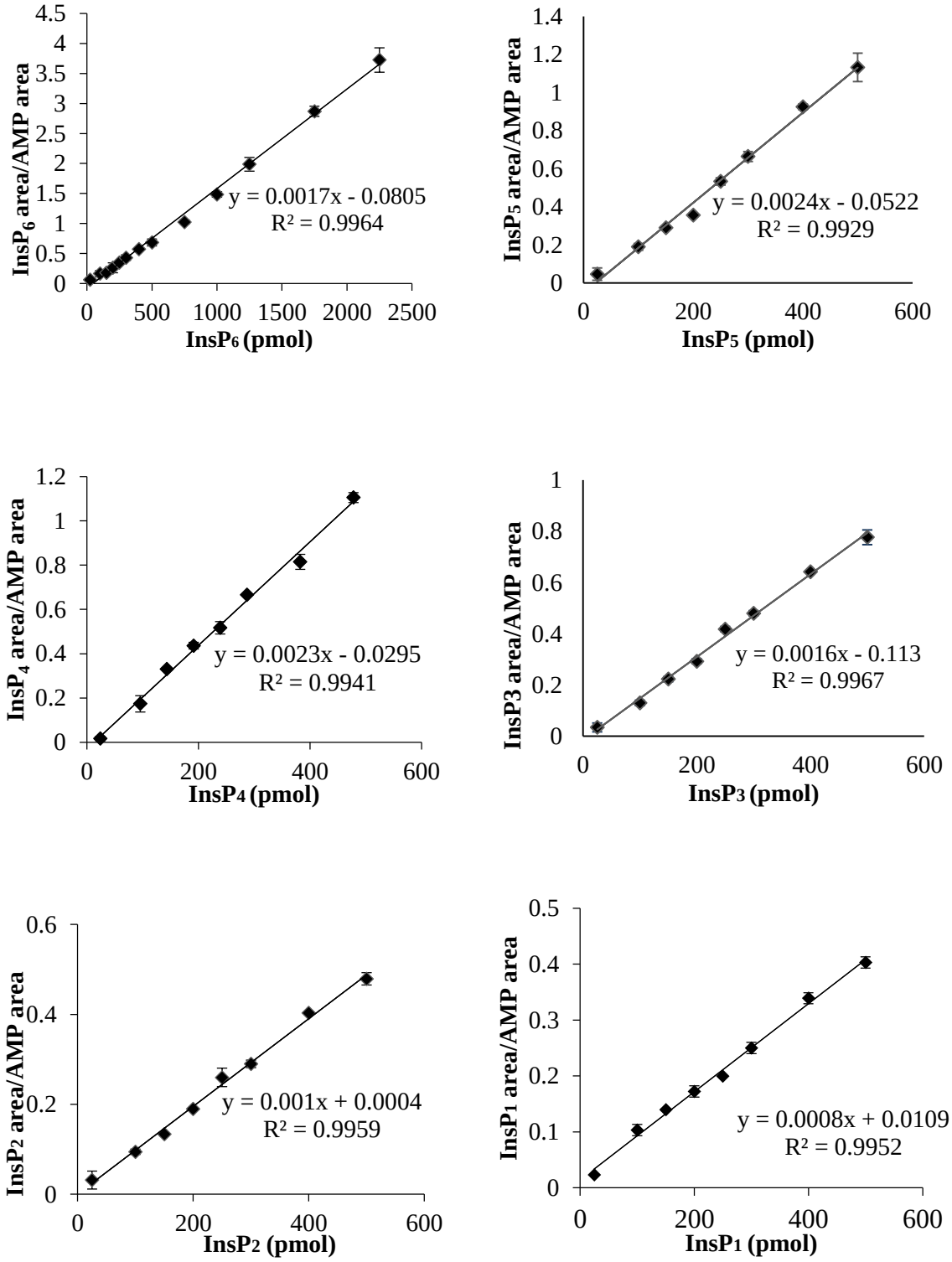

Figure A.2. Calibration curves of inositol phosphate standards

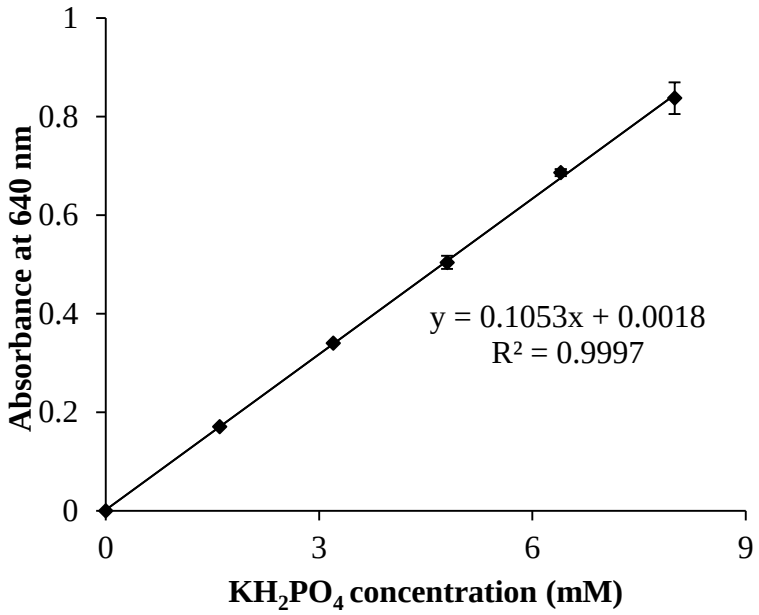

Figure A.3. Calibration curve of phosphate standard solution.

Table A.1. Interday precision for analysis of inositol phosphate standards at five concentrations.[a]

Analyte[b]	20 µM RSDr (%)	40 µM RSDr (%)	60 µM RSDr (%)	80 µM RSDr (%)	100 µM RSDr (%)
$InsP_6$	8.6	6.2	5.8	3.3	8.3
$InsP_5$	9.0	1.3	3.9	1.5	6.6
$InsP_4$	7.8	3.5	0.2	4.2	2.1
$InsP_3$	7.5	0.5	2.7	0.7	3.7
$InsP_2$	6.2	4.3	2.9	2.7	2.9
$InsP_1$	2.9	4.5	5.8	5.1	4.5

[a]Values are based on three analyses over three days.
[b]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.

Table A.2. Inositol phosphate levels in almond meal and brown skin samples (µmol/g) extracted by two different types of acids[a]

Inositol Phosphate[b]	Almond meal		Almond brown skins	
	3.2 M acetic acid	2.4 % HCl	3.2 M acetic acid	2.4 % HCl
$InsP_6$	5.66 ± 0.23a	5.82 ± 0.59a	7.34 ± 0.66a	6.32 ± 0.92a
$InsP_5$	1.61 ± 0.12a	1.62 ± 0.10a	3.02 ± 0.23a	3.45 ± 0.28a
$InsP_4$	0.43 ± 0.06a	0.29 ± 0.09b	0.82 ± 0.18a	0.54 ± 0.21a
$InsP_3$	0.17 ± 0.02a	0.14 ± 0.03a	0.18 ± 0.05a	0.11 ± 0.03a
$InsP_2$	0.13 ± 0.02a	0.10 ± 0.03a	0.08 ± 0.01a	0.08 ± 0.07a
$InsP_1$	0.07 ± 0.01	ND[c]	0.09 ± 0.02	ND[c]

[a] Different letters correspond to values with significant difference according to two-tailed t-test. The level of significance was $P < 0.05$. Calculations were done using the software JMP Pro (Version 12, SAS Institute Inc., Cary, NC).
[b]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[c]ND: Not detected (< 2 nmol/g sample).

Table A.3. Interday precision for absorbance reading at 640 nm of phosphate standards at five concentrations.[a]

Phosphate concentration (mM)	RSD_r (%)
1.6	0.3
3.2	0.2
4.8	2.7
6.4	1.0
8.0	3.8

[a]Values are based on three analyses over three days.

Table A.4. Comparison of $InsP_6$[a] concentration as measured by HPLC/ESI/MS[a] to $InsP_6$ equivalence as calculated based on organic phosphorus content (AOAC 986.11)[b] and total phosphorus content (ICP–OES)[c]

Almond meal

Cultivar	Method				
	HPLC/ESI/MS	Based on organic phosphorus		Based on total phosphorus	
	$InsP_6$ (µmol/g)	$InsP_6$ eq. (µmol/g)[d]	% overestimate	$InsP_6$ eq. (µmol/g)[d]	% overestimate
Mission	7.41 ± 1.14	24.7 ± 0.9	234	38.3 ± 2.2	417
Aldrich	6.45 ± 1.62	20.3 ± 3.2	215	30.5 ± 6.2	373
Nonpareil	7.98 ± 2.30	19.9 ± 1.6	149	26.8 ± 1.3	236
Price	5.92 ± 0.78	22.6 ± 3.4	281	34.4 ± 5.4	480
Butte	7.22 ± 2.25	22.7 ± 1.0	215	34.0 ± 0.6	370
Monterey	9.44 ± 1.29	21.2 ± 1.9	125	31.1 ± 0.8	229

Almond brown skins

Cultivar	Method				
	HPLC/ESI/MS	Based on organic phosphorus		Based on total phosphorus	
	$InsP_6$ (µmol/g)	$InsP_6$ eq. (µmol/g)[d]	% overestimate	$InsP_6$ eq. (µmol/g)[d]	% overestimate
Mission	5.43 ± 0.26	14.2 ± 0.6	162	21.1 ± 2.6	289
Aldrich	7.79 ± 4.76	16.9 ± 3.9	117	26.5 ± 4.2	240
Nonpareil	3.32 ± 0.75	14.0 ± 0.6	322	22.6 ± 1.4	582
Price	9.03 ± 0.98	14.5 ± 1.5	60	24.1 ± 4.1	167
Butte	11.10 ± 1.04	18.8 ± 0.8	69	25.4 ± 0.6	129
Monterey	6.09 ± 1.20	17.0 ± 1.5	179	25.1 ± 2.7	312

[a]Abbreviations: $InsP_6$ is *myo*-inositol hexakisphosphate; HPLC/ESI/MS is high-pressure liquid chromatography coupled with electronspray ionization mass spectrometry.

[b]Association of Official Analytical Chemists official method 986.11 for phytate in foods.

[c]Analyzed by inductively coupled plasma optical emission spectroscopy.

[d]Molar concentraiton of $InsP_6$ equivalence was calculated by dividing the phosphorus molar concentration by 6.

CHAPTER 4

DETERMINATION OF *MYO*-INOSITOL PHOSPHATES IN TREE NUTS AND GRAIN FRACTIONS BY HPLC/ESI/MS

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A**bstract**

High-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) was utilized for the rapid, on-line quantification of all six forms of inositol phosphate (InsP) in seven major tree nuts (i.e., cashews, Brazil nuts, macadamias, walnuts, pecans, pistachios, hazelnuts) and three grain components that are allegedly rich in phosphorus (wheat aleurone, rice bran, corn germ). The total InsP levels ranged from 3 to 20 µmol/g in the tree nuts and from 10 to 97 µmol/g in the grain components. While inositol hexakisphosphate was the predominant form in all samples, at least 20% of the InsP molar concentration comprised lower forms of InsPs. In tree nuts, InsPs accounted for 18 to 59% of the organic phosphorus content and 12 to 46% of the total phosphorus content. For grain samples, these values ranged from 66 to 97% and 58 to 80%, respectively. Significant differences in InsP levels among tree nuts underline the need for further investigation of InsPs in this food group, particularly with regard to different cultivars, growing conditions, and processing conditions. HPLC-ESI-MS offered a sensitive and time-efficient detection approach for InsPs in various complex nut and grain matrices, highlighting its potential application for many other sample types.

4.1. Introduction

Myo-inositol hexakisphosphate (phytic acid, $InsP_6$ – Figure 4.1) is a common component in mature plant seeds, often reported to make up more than 60% of the total phosphorus content, and is regarded as the principle phosphate reserve of common cereal, cereal by-products, and oil seeds (Kornegay, 2000). Additionally, due to its capability to donate up to twelve protons and bind with mineral cations, $InsP_6$ can also function as a

versatile form of nutrient storage for the seeds (Heighton et al., 2008). Nonetheless, the chelating potential of this biocompound has been suggested to have negative nutritional effects for humans. In seeds, endogenous phytases activated during germination can dephosphorylate the salts of phytic acid (commonly referred to as phytate for single salts and phytin for mixed salts) to mobilize phosphorus and nutrients. In humans, the phytase concentration in the digestive tract is dependent on phytase-producing microorganisms in the gut, and hence is often insufficient for the complete breakdown of phytates and phytins. This is critical because at physiological pH levels, the salts of $InsP_6$ with multivalent cations, such as zinc, calcium, or iron, can be insoluble. Under these conditions, $InsP_6$ can also form insoluble complexes when binding with cationic moieties of proteins, lipids, and carbohydrates. Consequently, $InsP_6$ has been implicated in impeding the absorption or hindering the activity of these nutrients. Lower InsPs, which include *myo*-inositol mono-, bis-, tris-, tetrakis-, and pentakisphosphate ($InsP_{1\text{-}5}$), may also be present in foods and exert similar effects, albeit to a lesser extent, but $InsP_6$ garners the most attention due to its higher abundance and binding affinity. The compound is often referred to as an anti-nutrient in the human diet, even though the link between dietary $InsP_6$ and nutrient deficiency has not been adequately confirmed (Lopez et al., 2002).

Emerging research on the physiological functions of inositol phosphates (InsPs) is suggesting that these compounds may be necessary to cell functions and one's general overall health. Originally thought to be present mainly in plant cells, InsPs have now been found in mammalian cells and are speculated to be present in all eukaryote cells. $InsP_6$ and several isomers of $InP_{3\text{-}5}$ have been observed to participate in several Ca^{2+} and

Cl^- channels, vesicle recycling, DNA and mRNA activities, and protein activities (Irvine and Schell, 2001; Monserrate and York, 2010; Sauer and Cooke, 2010). Furthermore, InsPs possess antioxidative potential through their chelating capability, and $InsP_6$ has been found to help prevent inflammation, pathological calcification, and neoplasm (Grases et al., 2004; Kumar et al., 2004; Raina et al., 2013; Vucenik et al., 2004). With these findings, the need to closely examine the role of dietary InsPs in health and disease prevention has been renewed.

Whereas many studies have focused on the abundance of InsPs in major cereals and legumes, there is currently an information gap on the level of these compounds in tree nuts. In recent years, tree nuts have seen a strong rise in popularity due to their nutritional and health benefits. Many tree nut types have developed beyond the role of an ingredient in traditional and seasonal foods, reaching a status where they are enjoyed as stand-alone snacks and regarded as healthy ingredients. Together with the growing trend of snacking, the consumption of tree nuts is predicted to continue to increase in the future. Around the world, the most commercially-important edible tree nuts include sweet almonds (*Prunus dulcis* [Mill.] D.A. Webb), cashews (*Anacardium occidentale* L.), Brazil nuts (*Bertholletia excelsa* Humb. & Bonpl.: Lecythidaceae), hazelnuts (*Corylus avellana* L.), macadamias (*Macadamia integrifolia* Maiden & Betche), pecans (*Carya illinoinensis* (Wangenh.) K. Koch.), pistachios (*Pistachia vera* L.), and English walnuts (*Juglans regia* L). At present, knowledge on the InsPs composition of these nuts is severely fragmented, mostly focusing on the $InsP_6$ concentration alone and lacking in the relationships between InsPs and levels of organic and total phosphates in these foods.

In foodstuffs, the total phosphorus content includes phosphorus from organic as well as inorganic forms. To illustrate, major classes of naturally occurring organic phosphate in plant foods would include inositol phosphates, nucleotides, sugar phosphates, phospholipids, and phosphate derivatives of thiamine (Frank, 2013). Inorganic phosphates, on the other hand, refer to phosphate salts with mineral ions and inorganic groups (e.g., ammonium, calcium, sodium, magnesium). In many products, inorganic and organic phosphate sources might also be added as flavor enhancers and texture modifiers, such as disodium guanylate, sodium hexametaphosphate, and tricalciumphosphate (Lehrfeld and Morris, 1992; Watanabe et al., 2016). Some of the most popular methods for $InsP_6$ determination in foods, such as the Association of Official Analytical Chemists (AOAC) Official Method 986.11 for phytate in foods, carries the assumption that $InsP_6$ comprises all of the organic phosphate content of the food in question. This non-specific approach disregards all other phosphate-containing organic compounds, including $InsP_{1\text{-}5}$, and may result in an overestimation of the true $InsP_6$ content. There is a great diversity of phosphorus-containing organic compounds in plant foods. Similarly, estimation of $InsP_6$ or total InsP content based on the total phosphorus level of a food product can lead to inaccurate results. So to better understand the physiological effects of InsPs, a specific analytical method for their quantification is essential.

In surveying the InsP profile of the meal and brown skins of six major California almond cultivars using high pressure anion-exchange liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS), we observed that the amount of phosphorus accounted for by InsPs was significantly ($p < 0.05$) lower than the organic

phosphorus and total phosphorus levels in these samples (Duong et al., 2016). These results suggest that the relationship between phosphorus concentration and InsP concentrations in tree nuts may be very different in comparison with those in cereals and legumes. Consequently, the measurement of individual InsPs is highly important for this food group.

In this study, we report on the application of a method, previously refined for the analysis of InsPs in almonds, for seven commercially-available tree nut types found in the market: cashews, Brazil nuts, hazelnuts, macadamias, pecans, pistachios, and English walnuts. With the use of HPLC-MS-ESI, all six InsPs were separated in each nut type and detected within 20 min of sample injection. The contribution of InsPs to the organic phosphorus and total phosphorus contents of each sample was then determined. For comparative purposes and to demonstrate the robustness of the analytical technique, we also evaluated three grain components typically reported to be rich in phosphorus: rice bran, wheat aleurone, and corn germ.

4.2. Materials & Method

4.2.1. Reagents and materials

Adenosine 5′-monophosphate (AMP), HPLC-grade ammonium carbonate and methanol, phytic acid sodium salt from rice, Dowex® 1X4 chloride form ion-exchange resin (100-200 mesh), and 1-amino-2-naphthol-4-sulfonic acid with ≥95.0% purity were acquired from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Inositol phosphate analytical standards with >98% purity included D-*myo*-inositol-1-phosphate

monosodium salt, D-*myo*-inositol-1,4-diphosphate disodium salt, D-*myo*-inositol-1,4,5-triphosphate trisodium salt, and D-*myo*-inositol-1,3,4,5-tetraphosphate octasodium salt; these were purchased from the Cayman Chemical Company (Ann Arbor, MI, USA). D-*myo*-Inositol-1,3,4,5,6-pentakisphosphate pentapotassium salt and D-*myo*-inositol-1,2,3,4,5,6-hexakisphosphate dodecasodium salt were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and EMD Millipore Corporation (Billerica, MA, USA), respectively.

Ethylenediaminetetraacetic acid disodium salt dehydrate (Na_2EDTA), ACS-grade sodium hydroxide, glacial acetic acid, hydrochloric acid, sulfuric acid, nitric acid, hexanes, and HPLC-grade water were purchased from the Fisher Scientific Company (Suwanee, GA, USA). Sodium sulfite and sodium bisulfite were obtained from Aqua Solution, Inc. (Deer Park, TX, USA). Potassium dihydrogen phosphate and ammonium molybdate were acquired from J.T Baker (Avantor Performance Materials, Center Valley, PA, USA).

Whole, unsalted, commercial tree nut products including macadamias, cashews, Brazil nuts, hazelnuts, pecans, walnuts, and roasted pistachios were obtained in three independent lots from local grocery stores. Commercial samples of wheat aleurone, corn germ, and rice bran were also bought in three independent lots. To provide independent replicates, each tree nut or grain product was sampled from three different grocery stores.

4.2.2. Preparation of reference and analytical standards

Analytical standards of InsPs were prepared according to a method by Liu et al. (2009) with some modifications. Briefly, standard solutions of individual InsPs were prepared in methanol:water (5:95, *v/v*), then combined with the AMP internal standard to provide a series of working standard solutions with analyte concentrations of 5, 20, 30, 40, 50, 60, 80, and 100 μM. For $InsP_6$, concentration points at 150, 200, 300, 350, and 450 μM were also made. The concentration of AMP in each solution was 25 μM. A standard curve for each InsP was constructed by plotting the ratio of each analyte's peak area over AMP's peak area against the quantity of analyte in the injection volume (pmol).

An in-house reference standard solution containing all six InsPs was formulated by dissolving 90 mg of phytic acid sodium salt in 30 mL of 3.2 M acetic acid in a glass vial. The vial was flushed with nitrogen, sealed with a rubber septum cap, and heated at 140 °C for 3 h, then at 70 °C for 13 h. After having cooled to room temperature, the solution was subjected to a nitrogen evaporator (N-EVAP™ 111 with an aluminum bead dry bath set at ~50 °C, Organomation Associates, Inc., Berlin, MA, USA). The solid residue was reconstituted in 15-mL methanol:water (5:95, v/v) and stored at -20 °C until used.

4.2.3. Preparation of samples

The purchased tree nut samples were first frozen in liquid nitrogen and then finely ground in a household coffee grinder (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ, USA) to a very fine powder using an intermittent pulsing technique.

Because high lipid contents will interfere with the extraction of hydrophilic bioactives, the ground tree nut samples were defatted with hexanes in a Soxhlet apparatus. Each defatted sample was then left in a fumehood for several hours to ensure removal of hexanes before being stored at -80 °C until further analysis. Grain samples were also ground using the coffee grinder, but only the corn germ samples were defatted.

On the day of analysis, each ground tree nut sample was removed from the freezer and reground in the coffee mill. For InsP extraction, 500 mg of a sample were mixed with 3 mL of 3.2 M acetic acid and blended at 15,000 rpm for 60 s using a PT-3100 Polytron™ homogenizer (Brinkmann Instruments, Westbury, NY, USA). The sample was then shaken for 3 h at 22 °C followed by centrifugation (3,345 × *g*, 20 min, 22 °C). Half a milliliter of the supernatant was combined with 1 mL of 0.11 M Na_2EDTA dissolved in 0.75 M sodium hydroxide. The sample was evaporated to dryness in a RT400 Speed Vac System (Savant, Holbrook, NY, USA). The residue was reconstituted in 500 µL methanol:water (5:95, *v/v*) and sonicated to facilitate dissolution. Each sample was then filtered through a 0.22-µm Phenex™-RC nylon syringe filter (Phenomenex, Torrance, CA, USA) and combined with the AMP standard solution to attain a final concentration of 25 µM AMP. All tree nut types were analyzed in triplicate.

4.2.4. Separation and quantification of InsPs

Inositol phosphates were separated on a Bio Basic anion-exchange column (2.1 × 150 mm, 5-µm particle size, Thermo Fisher Scientific, Waltham, MA, USA) using an Agilent 1100 HPLC system. Five microliters of sample were manually injected for each

run using a 25-µL Hamilton syringe. The flow rate was 200 µL/min and a gradient elution was employed with two mobile phases: (A) methanol:water (5:95, *v/v*) and (B) 200 mM ammonium carbonate. The program consisted of 4 min of mobile phase A, followed by an increase of B from 0 to 20% over 2 min, and lastly an increase of B from 20 to 55% over 14 min. A 25-min regeneration period with 100% A was employed at the end of each run. The analyses were performed at 22 °C.

Detection of InsPs was carried out by a Waters Micromass Quadrupole Time-of-Flight mass spectrometer with an electrospray ionization (ESI) source (Waters Corporation, Milford, MA, USA). Spectra were acquired and analyzed using MassLynx software (Version 4.1, Waters Corporation). The ESI source was set in the negative-ion mode with a spray voltage of 2500 V, ion-transfer capillary temperature at 300 °C, and nitrogen gas flow at 450 L/h. These parameters were optimized for the simultaneous detection of AMP and all six InsPs.

4.2.5. Evaluation of matrix effects

After the samples were combined with 3.2 M acetic acid at the beginning of the extraction process, they were spiked with a portion of the in-house reference standard. The samples were then extracted and analyzed according to the procedure described above. Recovery experiments were performed according to AOAC guidelines (AOAC, 2016). The percent recovery was calculated by the following equation:

$$\text{Recovery (\%)} = [(C_s - C_o)/C_s] \times 100$$

where R (%) is the percent recovery of the added standard; C_o is the concentration of each analyte in the non-spiked sample; and C_s is the concentration of each analyte in the spiked sample.

4.2.6. Evaluation of organic phosphorus and total phosphorus contents

Using the assumption that $InsP_6$ comprises all of the organic phosphate content in foods, Harland and Oberleas (1986) proposed a chromatographic method, which was later adopted as the AOAC Official Method 986.11 for phytate determination in foods. In the present study, this method was applied for the evaluation of organic phosphate levels in the prepared tree nut samples with some modification. Briefly, 2 g of a ground sample was combined with 40 mL of 2.4% HCl and shaken for 3 h (22 ^oC). The sample was filtered *in vacuo* through Whatman No. 1 paper using a small Büchner funnel. One milliliter of the filtrate was added to a 25-mL volumetric flask together with 1 mL of 0.11 M Na_2EDTA dissolved in 0.75 M NaOH, and then diluted to mark with deionized water. To prepare an anion-exchange column, a 3-mL Luer-lock Monoject™ syringe (Covidien Ltd., Dublin, Ireland) was assembled on a solid-phase extraction manifold, and a small piece of glass wool was placed at the bottom of the syringe. In the form of aqueous slurry, 500 mg of Dowex® 1X4 ion-exchange resin were poured into the syringe. After the resin bed had formed, the column was washed with 15 mL of 0.7 M sodium chloride and then with 15 mL of deionized water. The sample was quantitatively transferred to this ion-exchange column and the eluate was discarded. To elute inorganic phosphates, 15 mL of deionized water and 15 mL of a 0.1 M sodium chloride solution were passed through the column; this portion was discarded. Organic phosphates were then recovered

from the resin bed with 15 mL of a 0.7 M sodium chloride solution. The resin was regenerated with 15 mL of deionized water and used for up to three samples before its replacement. The collected organic phosphate fraction was transferred to a Kjeldahl digestion tube and combined with 500 μL of concentrated sulfuric acid, 3 mL of concentrated nitric acid, and three glass beads. The sample was then digested in a BD-20 Tecator™ Kjeldahl digestion system (FOSS, Eden Prairie, MN, USA). The digestion program began with raising the temperature to 100 °C and a hold period of 14 min, followed by heating to 200 °C, hold for 10 min, and finally heating to 300 °C and hold for 30 min. The tube was left to cool, after which 10 mL of deionized water were added to dissolve the salts. The sample was heated for another 10 min at 200 °C. After the solution had cooled down, it was quantitatively transferred to a 50-mL volumetric flask. Two milliliters of 2.5% ammonium molybdate dissolved in 0.5 M sulfuric acid and 1 mL of sulfonic acid reagent (containing 0.92 M $NaHSO_3$, 0.15 M Na_2SO_3, and 6.69 mM 1-amino-2-naphthol-4-sulfonic acid) were added to flask, and the solution was diluted to mark with deionized water. The sample was held for 30 min to achieve maximum color development before its absorbance was read at a λ of 640 nm using an Agilent 8453 diode-array spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA). To prepare a series of phosphate standard solutions, appropriate volumes of an 80 mM KH_2PO_4 solution were pipetted into 50-mL volumetric flasks, together with 2 mL of the molybdate solution and 1 mL of the sulfonic acid reagent described above. The contents were then diluted to mark with deionized water. A phosphate standard curve was constructed by plotting the quantity of phosphate in the standard solutions against the absorbance readings at $\lambda = 640$ nm. All test concentrations were prepared in triplicate.

To prepare the samples for total phosphorus analysis, 500 mg of ground sample were ashed in a porcelain crucible in a tabletop, 1.6-ft^3 muffle furnace (Thermo Scientific Thermolyne, Fisher Scientific) at 525 °C for 18 h. The ash was dissolved in 10 mL of aqua regia (hydrochloric acid:nitric acid:deionized water, 30:10:60, *v/v/v*) and the sample was centrifuged (3,345×*g*, 2 min, 22 °C). The supernatant extracted was analyzed for its phosphorus content by inductively coupled plasma-optical emission spectroscopy (ICP-OES), performed by the Chemical Analysis Laboratory at UGA's Center for Applied Isotope Studies. The system was calibrated with a peanut butter standard reference material containing a known level of phosphorus (SRM 2387, National Institute of Standards and Technology, Gaithersburg, MD, USA). All analyses were performed in triplicate.

4.2.7. Method validation

The limits of detection (LOD) and limits of quantification (LOQ) for each InsP form in the HPLC-ESI-MS analysis were determined on the basis of a minimal accepted value of the signal-to-noise ratio of 3 and 10, respectively. The interday variability of the analysis was evaluated at five concentrations of each InsP standard across three consecutive days. For the AOAC Official Method 986.11 assay (for phytate in foods), the interday variability was evaluated by measuring five concentrations of the phosphate standard solutions over three days. Relative standard deviation (RSD) was calculated as follows:

$$\text{RSD (\%)} = \text{standard deviation/mean} \times 100$$

To determine the interday precision (RSD), accuracy, and bias of the ICP-OES method, the phosphorus content of the NIST peanut butter SRM 2387 was evaluated and compared against the certified value. Five replications were performed. Bias and percent accepted value were calculated as follows:

$$\text{Bias} = \text{expected value} - \text{analytical value}$$

$$\text{Percent accepted value} = (\text{analytical value} \times 100)/\text{accepted value}$$

4.2.8. Statistical analysis

All values were reported as mean ± standard deviation (n=3). The mean procedure was carried out with the JMP Pro software, Version 12 (SAS Institute Inc., Cary, NC, USA).

4.3. Results and discussion

4.3.1. Method characteristics

With the HPLC-ESI-MS analysis, all six InsPs were detected within 20 min of sample injection. Excellent linearity was observed for the calibration curves between individual InsPs and the AMP internal standard (R^2 > 0.99) over the measured concentration ranges (Table 4.1 and Figure B.1). The limit of detection and limit of quantification for each InsP were 25 pmol and 50 pmol, respectively. The interday (RSD) values ranged between 0.5 and 9.0% for all standard concentrations examined (Table 4.2).

For the AOAC Official Method 986.11 assay (for phytate in foods), the calibration curve between KH_2PO_4 and absorbance reading at λ =640 nm attained outstanding linearity (R^2 = 0.9997) (Figure B.2). Small interday RSD values were achieved across the range of interest (Table 4.3).

Validation of the ICP-OES method conducted with the NIST peanut butter SRM 2378 reported an average phosphorus content of 3463 ± 226 mg/kg, compared with the certified value of 3378 ± 92 mg/kg. Bias, percent accepted value, and RSD were 85 mg/kg, 102.5%, and 6.52%, respectively. These results indicate that there was close agreement between the analytical and true values for this sample.

4.3.2. Inositol phosphate levels in tree nuts and grain components

Among the different types of tree nuts, the InsP composition was found to vary considerably (Table 4.4). Brazil nuts stood out for having a total InsP molar concentration of 20.08 µmol/g, which is especially high compared with the range of 2.63 to 6.51 µmol/g measured in the other tree nuts. Among the samples tested, the $InsP_{1-3}$ levels were similar for the most part, hence the molar concentration differences are attributed to variations in the $InsP_{4-6}$ levels. The most abundant InsP generally was $InsP_6$, followed by $InsP_5$ and $InsP_4$. While $InsP_6$ made up >60% of InsPs in most samples, it accounted for <50% of the InsP molar concentration in cashews.

In a previous study by Chen (2004), the levels of $InsP_6$ and $InsP_5$ (sum of different isomers) in cashews were found to be 7.56 µmol/g and 6.55 µmol/g, respectively. While these values are higher than those found in the samples tested in the present study, it is

important to note that they shared a similar pattern: $InsP_6$ and $InsP_5$ were relatively close in molar concentration, as opposed to $InsP_6$ being 2 to 3 times more abundant than $InsP_5$, as observed in other nuts. This pattern might result from the natural accumulation of these InsPs in cashews or from degradation of InsPs during processing. In cashew processing, the removal of the outer shell from raw nuts is facilitated by heat treatment such as steam boiling, pan roasting, drum roasting, and oil roasting. Consequently, InsPs might be dephosphorylated, and the degree of degradation is dependent on the parameters of the heating step employed.

Compared to the values reported for hazelnuts in this study, Simonet et al. (2003) detected a comparable level of $InsP_5$ but more $InsP_4$ and $InsP_6$, whereas Helfrich and Bettmer (2004) observed considerably more $InsP_6$ but less $InsP_5$, and did not detect any $InsP_4$. Uniquely, Liu et al. (2009) reported a hazelnut sample with $InsP_4$, $InsP_5$, and $InsP_6$ values of 1.06 ± 0.73, 0.36 ± 0.05, and 2.21 ± 0.53 μmol/g, respectively, so the $InsP_4$ content greatly exceeded that of $InsP_5$. Together with the values detected in this study, these results reflect a high degree of variation in the InsP profile of hazelnuts available to consumers.

For Brazil nuts, pistachios, macadamias, pecans, and walnuts, values reported in the existing literature are mostly available only for $InsP_6$. Despite this limitation, these studies have demonstrated great diversity among these nuts, as the $InsP_6$ concentration was shown to range from 2.9 to 27 μmol/g for Brazil nuts, 4.4 to 43 μmol/g for pistachios, 2.3 to 14 μmol/g for macadamias, 2.7 to 29 μmol/g for pecans, and 3.0 to 20.98 μmol/g for walnuts (Chen, 2004; Harland et al., 2004; Lott et al., 2000; Simonet et al., 2003; Venkatachalam and Sathe, 2006). For walnuts, the samples in these studies

were assumed to be English walnuts, unless stated otherwise. This is important, as different types of walnuts (e.g., English vs black) can have markedly different nutrient compositions. For example, Harland et al. (2004) found the level of $InsP_6$ in black walnuts to be 3 times higher than in English walnuts. It is also notable that some of the data pertaining to the $InsP_6$ level in these nuts has been erroneously reported when the study used reference values for $InsP_6$ estimation, as seen in the values reported for Brazil nuts by Schlemmer et al. (2009). Overall, while the values reported in the current study are within the ranges found in literature, it is apparent that tree nuts available in the market can have very different $InsP_6$ contents and potentially different InsP profiles. This accentuates the need of a specific method for the rapid quantification of all six InsP forms in a sample, as well as the need for an evaluation of InsPs in different cultivars and product types for each tree nut.

In corn, the germ possesses the greatest share of the InsP reserve in the grain, while for wheat and rice, $InsP_6$ is deposited chiefly in the aleurone and the bran, respectively (O'Dell et al., 1972). In this study, the method proposed for tree nuts was applied and shown to work equally well for commercial samples of these grain components. Of particular interest, very high contents of $InsP_{4\text{-}6}$ were detected in the wheat aleurone and rice bran samples (Table 4.5). Compared with the tree nut samples, the grain samples also had higher $InsP_{1\text{-}3}$ contents. Regarding distribution, a similar trend to nut samples was observed, in that $InsP_6$ was the predominant form, followed by $InsP_5$ and $InsP_4$.

In this study, $InsP_{3\text{-}6}$ values for wheat aleurone and rice bran were in agreement with findings reported in other literature; however, the level of $InsP_6$ reported in corn

germ (Kasim and Edwards, 1998; O'Dell et al., 1972) was lower than existing values. It is notable that in corn germ, $InsP_{1-3}$ each comprised between 3.4 to 5.2% of the total InsP molar concentration, compared with less than 1.5% in other samples. The ratio of $InsP_6$ in this sample was also relatively low, only 52% of the total InsP molar concentration. In maize, $InsP_6$ has been demonstrated to be nearly the sole InsP form produced, and natural accumulation of lower InsPs might be indicative of a genetic mutation (Raboy et al., 2000). In the case of the commercial product tested in this study, dephosphorylation of $InsP_6$ might have occurred due to processing or exposure to extraneous phytases, such as those from microorganisms.

In the ten tree nut and grain samples examined in this work, $InsP_{1-5}$ comprised 24.7 to 53.4% of the total InsP molar concentration. Consequently, $InsP_6$ should not be assumed to be the single contributor of InsP in these foods. In order to assess the occurrence of InsPs in foods and their health effects, analysis of individual InsPs would be essential, especially because these compounds may have different *in vivo* activities from one another.

To evaluate the matrix effects on the extraction and analysis of InsPs by the HPLC-ESI-MS protocol (Duong et al., 2016), a portion of the in-house reference standard containing all six InsPs with known concentrations were spiked into samples. Percent recoveries ranged from 92 to 110% for the samples, suggesting little to no matrix effect on quantification (Table 4.6). While most samples exhibited no significant change in retention time t_r for the InsPs, there were noticeable shifts in the *tr*s of $InsP_5$ and $InsP_6$ for corn samples (Figure 4.2 and Figure B.3&4). In this case, utilizing mass spectrometry as the primary detection method for InsPs helped to avoid the risk of misidentification

due to t_r alteration. Furthermore, peak identification based on elution times can be challenging in the presence of stereoisomers, as it might be unclear whether co-eluting peaks are indicative of different compounds or stereoisomers of the same InsP. With the use of mass spectrometry, this difficulty can be mitigated. In the rice bran sample, two compounds that co-eluted at 13.8 min shared a mass-to-charge ratio of m/z = 419. As a result, both could be tentatively identified as stereoisomers of $InsP_3$ and were included in the quantification of $InsP_3$ in this sample.

4.3.3. Contribution by inositol phosphates to organic phosphorus and total phosphorus contents

In the seven types of tree nuts examined, phosphorus from InsPs contributed between 18 and 59% of the organic phosphorus content (Table 4.7). These values were significantly lower than those of the grain components, especially in comparison to the wheat aleurone and rice bran samples, which had >90% of organic phosphorus coming from InsPs. In a complementary study, analysis of six California almond cultivars also found InsPs to account for only 32 to 55% of the organic phosphorus level in the meal and 44 to 77% of this level in the brown skins (Duong et al., 2016). These findings signify that the "assumption" that $InsP_6$ accounts for all of the organic phosphate content in foodstuffs is particularly unsuitable for tree nuts. When $InsP_6$ was measured by the AOAC Official Method 986.11, which employs this assumption, the degree of overestimation ranged from 149 to 691% for the tree nut samples, compared with 44 to 129% for the grain fractions (Table 4.8). The higher percent error obtained for tree nut samples may be due to significant abundance in other naturally occurring organic

phosphates such as sugar phosphates, phospholipids, nucleotides, and phosphate derivatives of thiamine. Furthermore, the percent error would be exacerbated by assuming that $InsP_6$ contributes all of the total phosphorus content, as shown by the degree of overestimation of up to 1,119% in tree nut samples and 134% in grain samples (Table 4.8).

Concerning the total phosphorus content, all samples demonstrated >60% of the total phosphorus originating from organic phosphates, but the ratio accounted for by InsPs varied widely between 12 and 46% for tree nuts and between 58 and 80% for grain components. Previously, contribution of InsPs to the total phosphorus content in almonds was also found to be relatively low, ranging from 20 to 38% in the meal and 30 to 52% in the brown skins (Duong et al., 2016). These results demonstrate the differences in phosphorus deposition among tree nut types and between tree nuts and grains. In a previous study, Lott et al. (2000) estimated the $InsP_6$ concentration of various tree nuts by employing the assumption that $InsP_6$ contributes only a certain percentage of the total phosphorus content in the nut, in particular 70% for pistachios and hazelnuts, 86% for Brazil nuts, and 42% for walnuts. In this investigation, the percent contribution of InsPs to total phosphorus content in the corresponding nut types was found to be lower than those employed by these authors, showing that such assumptions heavily overestimate the true $InsP_6$ content for some products. In future studies, it would be of interest to further characterize of the relationship between InsP concentrations as well as organic phosphorus and total phosphorus contents in these foods, especially as factored by cultivar, growing conditions, and processing conditions.

4.4. Conclusions

The combination of HPLC with ESI-MS allowed for the rapid quantification of all six forms of InsPs in all types of tree nuts and phosphorus-rich grain components examined in this study. High sensitivity is especially valuable for the evaluation of $InsP_{1-3}$, which are typically present in small amounts in plant seeds but may play important roles in seed development. With the advantages of time-efficiency and detection limits as low as 25 pmol (i.e., ~2 nmol/g sample), this method shows promising uses for many other food types and for large sample sets, such as in studies on the effects of cultivars, growing factors, and processing factors on InsP content of foods.

Myo-inositol hexakisphosphate was present at significant level in all samples, but the compound cannot be assumed to comprise the total InsP content or organic phosphate content. Distinct differences were also observed in the InsP composition of tree nuts and grain components. The diversity demonstrated by tree nut samples calls for further investigation into the occurrence of individual InsPs in this food type.

Acknowledgement

Financial support for Ms. Quynh H. Duong's research by The Graduate School of UGA and the Almond Board of California are gratefully acknowledged. Use of the Proteomics and Mass Spectrometry (PAMS) core facility under the direction of Dr. Dennis Phillips of UGA's Department of Chemistry, Athens, GA, is greatly appreciated. Thanks are extended to Dr. Kathy Loftis of UGA's Center for Applied Isotope Studies

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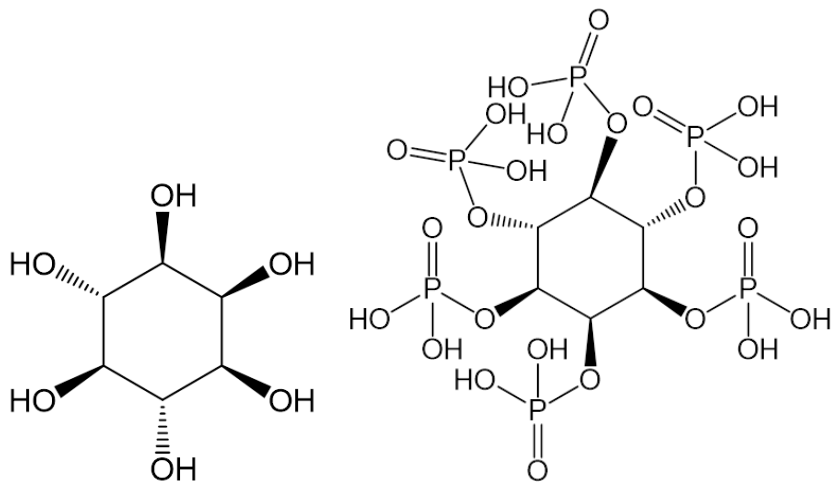

Figure 4.1. Chemical structures of *myo*-inositol (Ins) and *myo*-inositol hexakisphosphate ($InsP_6$).

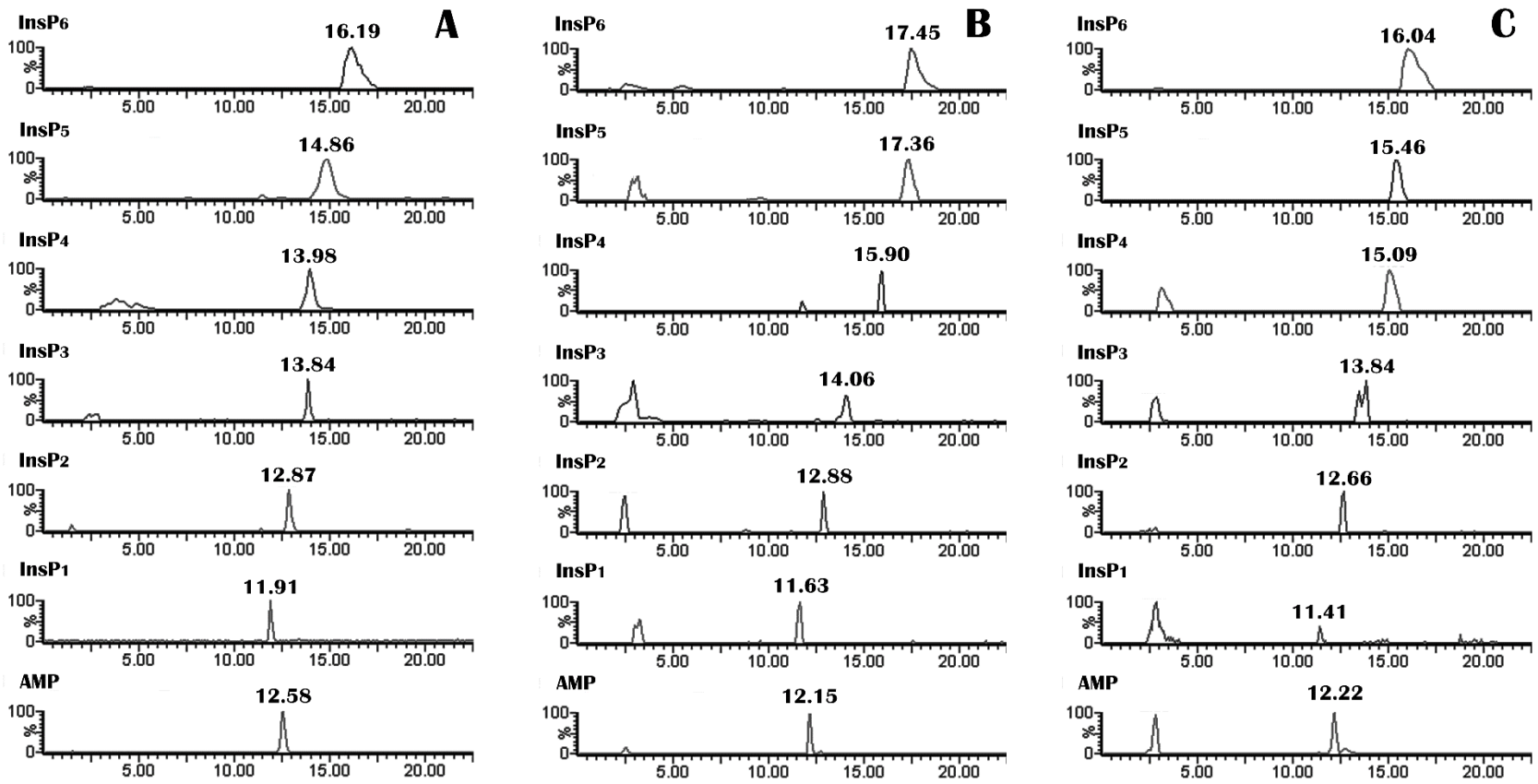

Figure 4.2. Representative elution peaks of $InsP_{1-6}$ and adenosine 5′-monophosphate detected in **(A)** commercial standards; **(B)** corn germ; and **(C)** rice bran.

Table 4.1. Parent mass, slopes, intercepts, correlation coefficients, and concentration ranges of interest of individual inositol phosphates

Analyte[a]	Parent mass (*m/z*)[b]	Slope (x 10^{-2})	Intercept	R^2	Concentration range (μM)
$InsP_6$	659	0.17	-0.0805	0.9964	5 to 450
$InsP_5$	579	0.24	-0.0522	0.9929	5 to 100
$InsP_4$	499	0.23	-0.0295	0.9941	5 to 100
$InsP_3$	419	0.16	-0.0113	0.9967	5 to 100
$InsP_2$	339	0.10	0.0004	0.9959	5 to 100
$InsP_1$	259	0.08	0.0109	0.9952	5 to 100

[a]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively
[b]*m/z* is mass-to-charge ratio

Table 4.2. Interday precision (RSD) of inositol phosphate standards at five different concentrations[a]

Inositol Phosphate Species[b]	20 μM RSD (%)	40 μM RSD (%)	60 μM RSD (%)	80 μM RSD (%)	100 μM RSD (%)
$InsP_6$	8.6	6.2	5.8	3.3	8.3
$InsP_5$	9.0	1.3	3.9	1.5	6.6
$InsP_4$	7.8	3.5	0.2	4.2	2.1
$InsP_3$	7.5	0.5	2.7	0.7	3.7
$InsP_2$	6.2	4.3	2.9	2.7	2.9
$InsP_1$	2.9	4.5	5.8	5.1	4.5

[a] Relative standard deviation (RSD) values are based on three analyses across three days
[b]Abbreviations: $InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.

Table 4.3. Interday precision for absorbance reading at λ = 640 nm of phosphate standards at five different concentrations[a]

KH_2PO_4 concentration (mM)	RSD (%)
1.6	0.3
3.2	0.2
4.8	2.7
6.4	1.0
8.0	3.8

[a]Relative standard deviation (RSD) values are based on three analyses over three days

Table 4.4. Inositol phosphate (InsP) concentrartions[a,b] (µmol/g) in selected tree nut species.

	Macadamias		**Pecans**		**Hazelnuts**		**Brazil nuts**	
	µmol/g	% Total	µmol/g	% Total	µmol/g	% Total	µmol/g	% Total
$InsP_6$	2.67 ± 0.22	75.3	1.79 ± 0.13	68.1	3.66 ± 0.17	71.1	12.62 ± 0.82	62.8
$InsP_5$	0.64 ± 0.11	17.9	0.53 ± 0.07	20.0	1.12 ± 0.04	21.7	6.31 ± 0.10	31.4
$InsP_4$	0.16 ± 0.03	4.5	0.18 ± 0.01	6.9	0.23 ± 0.03	4.4	1.04 ± 0.06	5.2
$InsP_3$	0.02 ± 0.01	0.7	0.08 ± 0.004	2.9	0.05 ± 0.01	1.0	0.05 ± 0.02	0.3
$InsP_2$	ND[c]	–	0.02 ± 0.003	0.7	0.04 ± 0.01	0.8	0.05 ± 0.01	0.2
$InsP_1$	0.06 ± 0.02	1.6	0.04 ± 0.01	1.4	0.05 ± 0.01	1.1	0.02 ± 0.004	0.1
Total	**3.55**		**2.63**		**5.15**		**20.08**	

	Pistachios		**Walnuts**		**Cashews**	
	µmol/g	% Total	µmol/g	% Total	µmol/g	% Total
$InsP_6$	4.17 ± 0.44	64.1	4.80 ± 0.60	72.3	2.34 ± 0.06	46.6
$InsP_5$	1.93 ± 0.48	29.7	1.55 ± 0.33	23.3	1.79 ± 0.19	35.7
$InsP_4$	0.14 ±0.08	2.1	0.20 ± 0.09	3.0	0.60 ± 0.07	12.0
$InsP_3$	0.05 ± 0.02	0.8	0.02 ± 0.01	0.3	0.17 ± 0.05	3.5
$InsP_2$	0.06 ± 0.02	1.0	ND[c]	–	0.07 ± 0.06	1.4
$InsP_1$	0.14 ± 0.07	2.2	0.07 ± 0.05	1.1	0.04 ± 0.02	0.9
Total	**6.51**		**6.64**		**5.02**	

[a]$InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]Values (mean ± SD) are based on triplicate analyses
[c]ND: Not detected (<2 nmol/g sample).

Table 4.5. Inositol phosphate (InsP) concentrations[a,b] (μmol/g) in grain components.

	Wheat aleurone		**Corn germ**		**Rice bran**	
	μmol/g	% Total	μmol/g	% Total	μmol/g	% Total
$InsP_6$	44.14 ± 0.95	69.1	5.46 ± 0.21	56.0	63.36 ± 0.82	65.1
$InsP_5$	15.03 ± 0.70	23.5	2.10 ± 0.09	21.5	28.16 ± 1.77	28.9
$InsP_4$	3.65 ± 0.22	5.7	0.90 ± 0.04	9.3	5.18 ± 0.17	5.3
$InsP_3$	0.41 ± 0.04	0.7	0.51 ± 0.01	5.2	0.43 ± 0.01	0.4
$InsP_2$	0.35 ± 0.16	0.6	0.45 ± 0.02	4.6	0.12 ± 0.03	0.1
$InsP_1$	0.27 ± 0.09	0.4	0.33 ± 0.03	3.4	0.11 ± 0.04	0.1
Total	**63.85**		**9.75**		**97.36**	

[a]$InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]Values (mean ± SD) are based on triplicate analyses

Table 4.6. Recovery of $InsP_{1-6}$ in samples spiked with a mixture of standards with known concentrations[a,b].

	Amount added (µmol/g)	Recovery (%)				
		Macadamias	Pecans	Hazelnuts	Brazil nuts	Pistachios
$InsP_6$	0.97	98.5	98.5	102.1	95.2	99.7
$InsP_5$	1.34	104.2	106.7	92.1	97.4	100.9
$InsP_4$	1.30	101.4	97.3	101.7	99.4	95.7
$InsP_3$	1.21	96.2	96.5	104.3	99.8	92.1
$InsP_2$	0.81	95.1	106.9	104.9	97.0	96.6
$InsP_1$	0.39	99.6	101.9	103.1	97.2	94.7

	Amount added (µmol/g)	Walnuts	Cashews	Wheat aleurone	Corn germ	Rice bran
$InsP_6$	0.97	102.4	95.1	106.8	96.9	97.8
$InsP_5$	1.34	100.7	94.2	92.8	107.9	96.9
$InsP_4$	1.30	102.0	98.6	100.1	103.3	102.9
$InsP_3$	1.21	95.6	95.4	101.1	107.9	109.8
$InsP_2$	0.81	98.1	96.5	102.0	102.1	93.1
$InsP_1$	0.39	103.2	94.0	103.7	95.4	107.4

[a]$InsP_{1-6}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, and *myo*-inositol hexakisphosphate, respectively.
[b]Values (mean ± SD) are based on triplicate analyses

Table 4.7. Levels of organic phosphorus and total phosphorus in selected tree nuts and grain fractions[a]

	Organic P		Total P		
	μmol/g	%P from InsPs[b]	μmol/g	% from organic P	%P from InsPs[b]
Macadamias	45.6 ± 0.83	43.8	65.11 ± 2.9	70.0	30.7
Pecans	78.6 ± 5.81	18.3	116.5 ± 4.6	67.5	12.4
Hazelnuts	86.8 ± 3.14	33.1	117.7 ± 8.5	73.8	24.4
Brazil nuts	188.3 ± 6.46	59.3	245.3 ± 33.7	76.8	45.5
Pistachios	113.2 ± 2.92	31.5	165.4 ± 4.8	68.5	21.6
Walnuts	94.1 ± 4.45	39.8	127.4 ± 10.2	73.9	29.4
Cashews	110.9 ± 1.17	23.5	171.1 ± 13.8	64.9	15.3
Wheat aleurone	382.1 ± 17.1	93.4	611.3 ± 77.0	62.5	58.4
Corn germ	74.9 ± 0.9	66.2	76.8 ± 7.4	97.6	64.6
Rice bran	562.1 ± 25.4	96.7	677.1 ± 17.4	83.0	80.3

[a]Values (mean ± SD) are based on triplicate analyses.
[b]InsPs, inositol phosphates.

Table 4.8. Comparison of $InsP_6$[a] concentration as measured by HPLC/ESI/MS[b] to concentration of $InsP_6$ equivalence as calculated based on organic phosphorus content (AOAC 986.11)[b] and total phosphorus content (ICP–OES).[c]

Sample	Method				
	HPLC/ESI/MS	Based on organic phosphorus content		Based on total phosphorus content	
	$InsP_6$ (μmol/g)	$InsP_6$ eq.[d] (μmol/g)	% overestimate	$InsP_6$ eq.[d] (μmol/g)	% overestimate
Macadamias	2.67 ± 0.22	7.6 ± 0.1	184	10.9 ± 0.5	306
Pecans	1.79 ± 0.13	13.1 ± 1.0	632	19.4 ± 0.8	985
Hazelnuts	3.66 ± 0.17	14.5 ± 0.5	296	19.6 ± 1.4	436
Brazil nuts	12.62 ± 0.82	31.4 ± 1.1	149	40.9 ± 5.6	224
Pistachios	4.17 ± 0.44	18.9 ± 0.5	352	27.6 ± 0.8	561
Walnuts	4.80 ± 0.60	15.7 ± 0.7	227	21.2 ± 1.7	343
Cashews	2.34 ± 0.06	18.5 ± 0.2	691	28.5 ± 2.3	1119
Wheat aleurone	44.14 ± 0.95	63.7 ± 2.8	44	101.9 ± 12.8	131
Corn germ	5.46 ± 0.21	12.5 ± 0.1	129	12.8 ± 1.2	134
Rice bran	63.36 ± 0.82	93.7 ± 4.2	48	112.8 ± 2.9	78

[a]$InsP_6$, *myo*-inositol hexakisphosphate.
[b]HPLC/ESI/MS, high-pressure liquid chromatography coupled with electrospray ionization mass spectrometry.
[c]Calculation of $InsP_6$ equivalence was based on analysis of organic phosphorus content by Association of Official Analytical Chemists official method 986.11 for phytate in foods (AOCS 986.11) and analysis of total phosphorus content by inductively coupled plasma optical emission spectroscopy (ICP–OES).
[d]Molar concentration of $InsP_6$ equivalence ($InsP_6$ eq.) was calculated by dividing the phosphorus molar concentration by 6; values (meant ± SD) are based on triplicate analyses.

Appendix B. Supplementary Figures & Tables

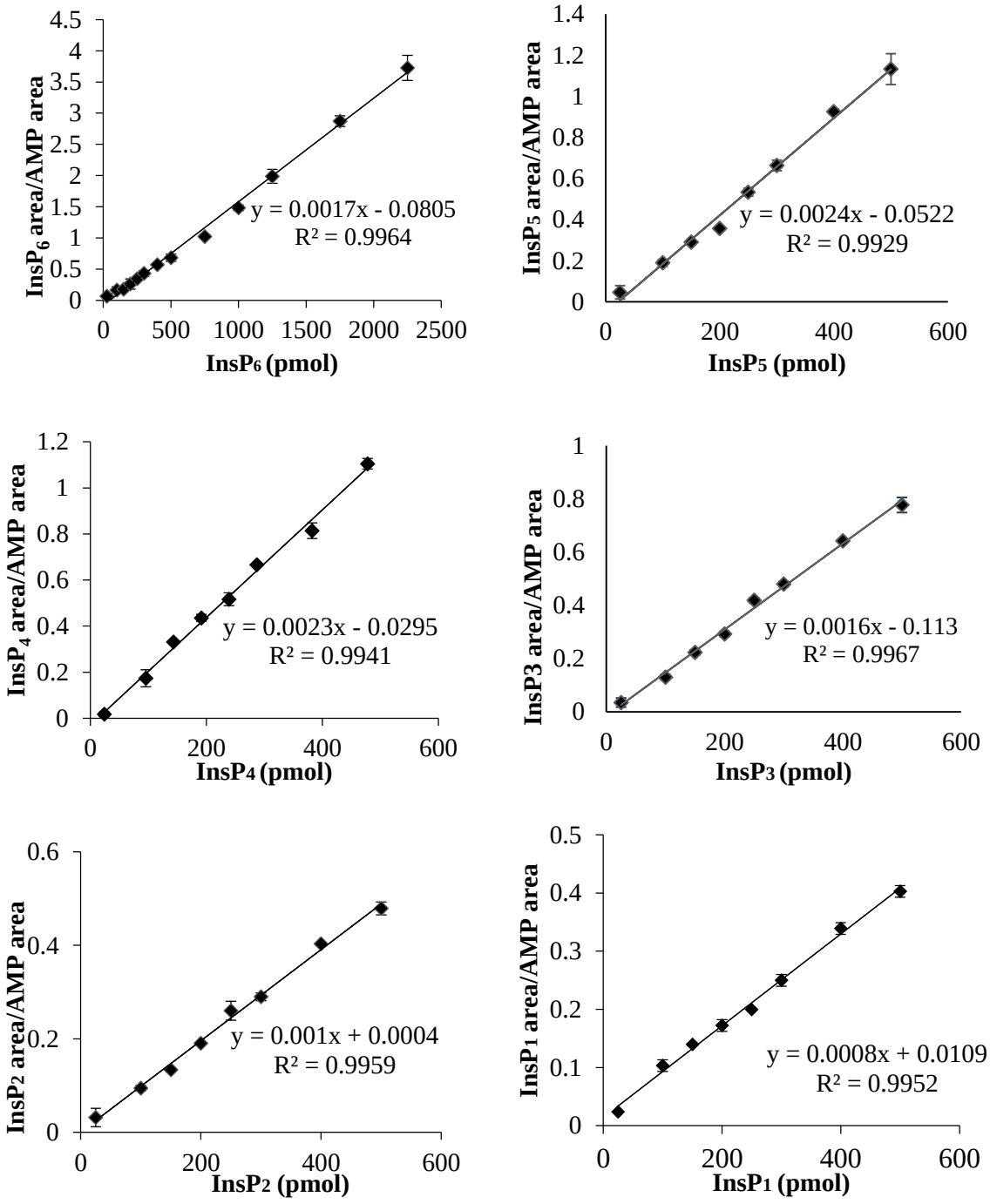

Figure B.1. Calibration curves of inositol phosphate standards

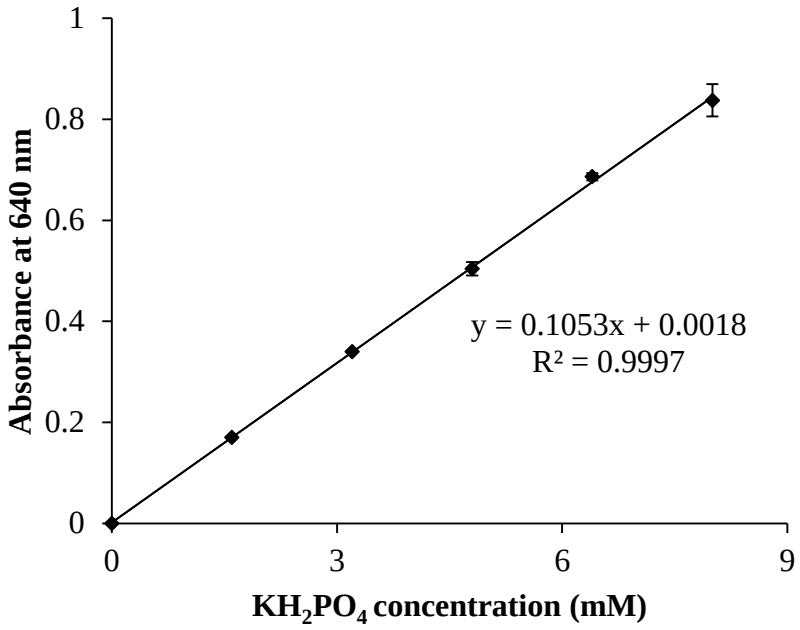

Figure B.2. Calibration curve of phosphate standard solution

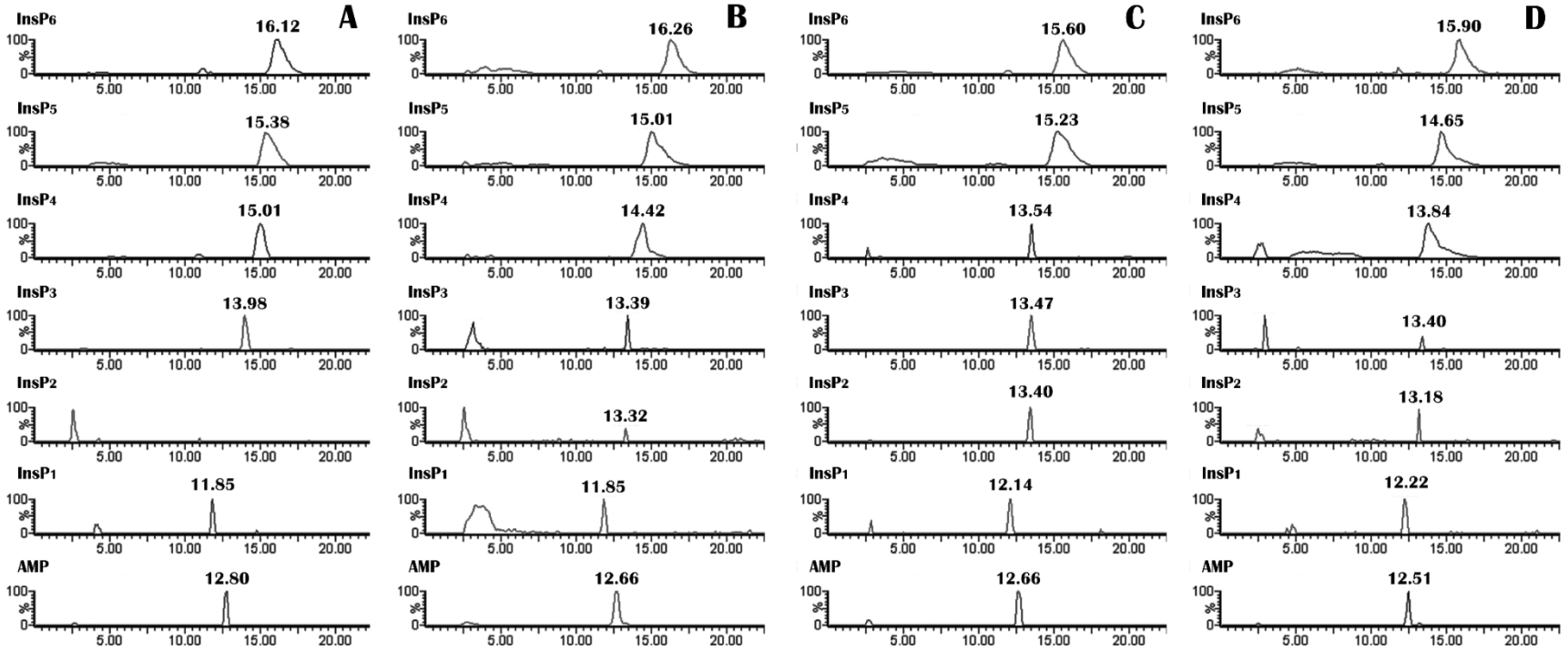

Figure B.3. Representative elution peaks of $InsP_{1\text{-}6}$ and AMP detected in **(A)** macadamias; **(B)** pecans; **(C)** hazelnuts, and **(D)** Brazil nuts.

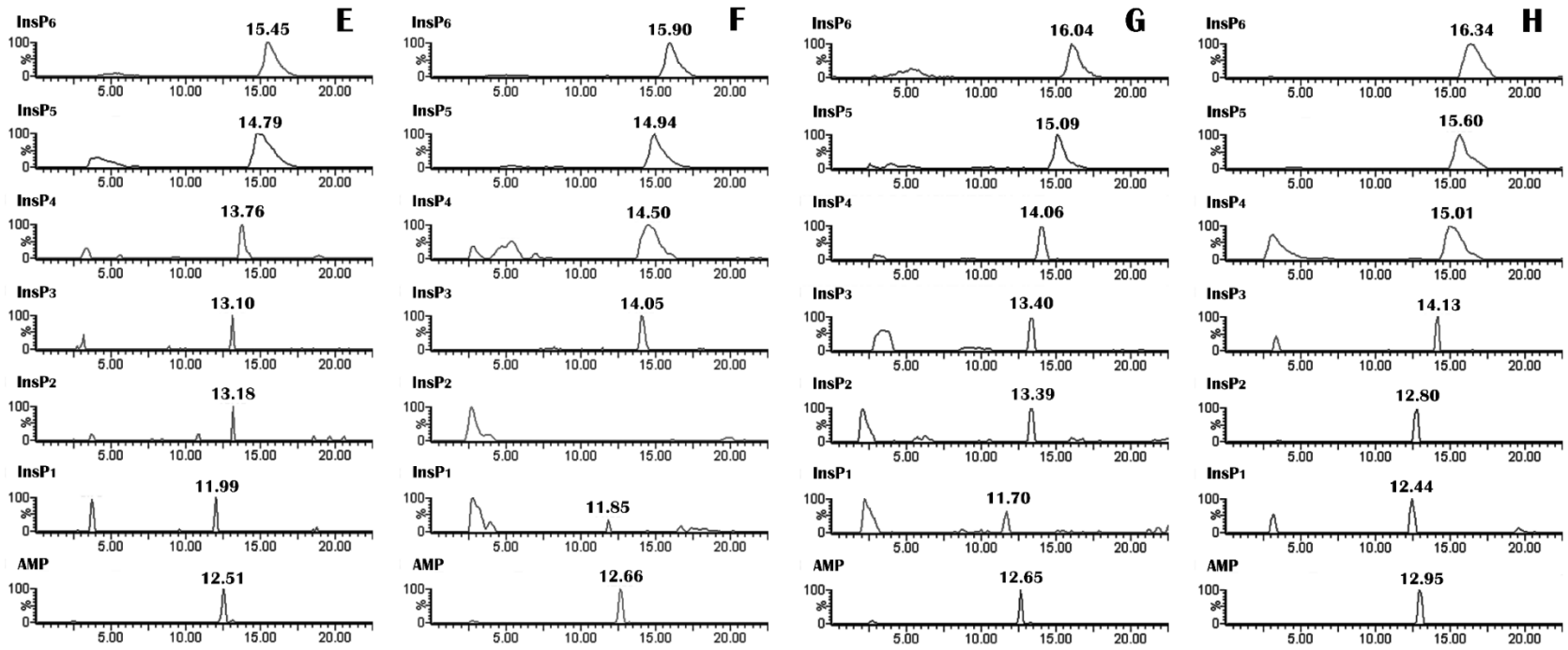

Figure B.4. Representative elution peaks of $InsP_{1\text{-}6}$ and AMP detected in **(E)** pistachios; **(F)** walnuts; **(G)** cashews, and **(H)** wheat aleurone.

CHAPTER 5

CONCLUSIONS

Interest in the potential benefits of inositol phosphates (InsPs) to human health has called for the examination of the inositol phosphate (InsP) composition in common foods, particularly in foods with rapidly growing popularity such as tree nuts. While some existing data have suggested that these products may contain high amount of InsPs, information on the specific content of each InsP in most tree nuts is scarce. One of the challenges of measuring the six forms of InsP has been the lack of a rapid method with on-line detection and high sensitivity, especially because these compounds do not possess characteristic spectrophotometric absorbance maxima. Additionally, an extraction method specifically refined for complex matrices such as those of tree nuts still needs to be investigated. This study represents an attempt to address these concerns and to apply the improved method to tree nuts products that hold economic importance to the U.S. and global markets. While most of these objectives were satisfied, the lack of cultivar and crop year samples for some sample types limited the scope of conclusion that could be made regarding the InsP profiles of these foods.

As almonds has previously been reported to be rich in InsPs, this nut was used as the model sample for developing a quantification method for InsPs in tree nuts. The recovery of InsPs during extraction process was determined to be strongly influenced by the formation of insoluble complexes between InsPs and proteins or minerals. It was

determined that pH adjustment and supplementation of EDTA can help to alleviate this problem. Acetic acid (3.2 M) was found to be superior to 2.4% HCl for InsP extraction. The advantages of utilizing mass spectrometry as the primary detection method of InsPs were also recognized, as mass-to-charge ratio combined with retention time allowed for the rapid and accurate detection of all 6 InsPs within 20 minutes of sample injection, with the limit of detections for all InsPs reaching as low as 2 nmol/g of sample.

The refined method was applied on six major California almond cultivars. The total InsP content was found to range from 8.3 to 12.4 μmol/g in the meal, and from 5.0 to 13.6 μmol/g in the brown skins. Inositol hexakisphosphate ($InsP_6$) was the dominant form, but lower forms still contributed about 20% of the total InsP molar concentration in a majority of the samples. InsPs contributed between 32 and 55% of the organic phosphorus content and between 20 and 38% of the total phosphorus content in the meal. In brown skins, these ranges were 44 to 77% and 30 to 52%, respectively. These values signified that neither the organic phosphate content nor the total phosphate content could be assumed to comprise solely of InsPs or $InsP_6$ in these samples. Several cultivars demonstrated significant variation in InsP composition and organic phosphorus level, suggesting the influence of growing and harvesting conditions. It is notable that the InsP contents of almond brown skins have not been found in previous literature, and results from this study may be useful for the characterization of phytochemicals in this component.

The developed method was found to be readily applicable for samples of 7 other prominent tree nuts, which included cashews, Brazil nuts, macadamias, walnuts, pecans, pistachios, and hazelnuts. Wheat aleurone, rice bran, and corn germ were also

successfully analyzed, demonstrating the versatility of this method. Pecans was found to contain the lowest amount of InsPs – only 2.6 μmol/g in total, which contributed 18% of the organic phosphorus content and 12% of the total phosphorus content in this sample. Among tree nut samples, InsPs were most abundant in Brazil nuts, at 20.1 μmol/g, contributing 49% of the organic phosphorus and 46% of the total phosphorus levels. These data demonstrated that InsP composition can vary very strongly among different tree nuts. The InsP profiles of tree nuts were also very different from those of the grain fractions examined. Wheat aleurone and rice bran contained 63.9 and 97.4 μmol total InsPs/g, respectively, and InsPs contributed to more than 90% of the organic phosphorus content of these samples. In corn germ, more than 65% of the organic phosphorus also came from InsPs, despite these compounds being less abundant in this sample. Nevertheless, all tree nut and grain samples showed a similar InsP distribution pattern to almond samples, in which $InsP_6$ accounted for less than 80% of the total InsPs molar concentration. These results further underline the necessity of using specific methods to measure the $InsP_6$ and/or InsP levels in foodstuffs.

Tree nuts have been recognized for their richness in phytochemicals, some of which have been well documented, while others, such as InsPs, require more extensive study. It is believed that the method described in this study represents a useful approach to the quantification of InsPs in this food group. In additional, the evaluation of InsPs in several tree nuts, especially in the meal and brown skins of important California almond cultivars, can contribute to the understanding of InsP occurrence in foods and the level of InsPs commonly present in the average human diet. As the variety of a few samples was limited, in is hoped that the effects of cultivation and processing factors on InsPs in tree

nuts will be further explored in future studies. Finally, it would also be of interest to evaluate the application of the developed method on biological and environmental samples.