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Determination of *myo*-inositol phosphates in tree nuts and grain fractions by HPLC–ESI–MS

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ABSTRACT

High-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC–ESI–MS) was utilized for the rapid, on-line detection 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 18–59% of the organic phosphorus content and 12–46% of the total phosphorus content. For grain samples, these values ranged from 66–97% and 58–80%, 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.

1. Introduction

Myo-inositol hexakisphosphate (phytic acid, $InsP_6$–Fig. 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 (Lopez et al., 2002; Schlemmer et al., 2009). Lower InsPs, which include *myo*-inositol mono-, bis-, tris-, tetrakis-, and pentakisphosphate ($InsP_{1-5}$), 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) suggests that these compounds may be necessary for cell functions and one's general overall health. Originally thought to be present mainly in plant cells, InsPs have

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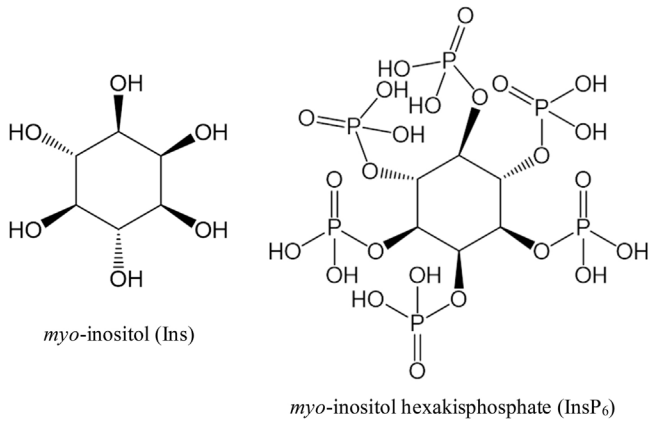

Fig. 1. Chemical structures of *myo*-inositol (Ins) and *myo*-inositol hexakisphosphate ($InsP_6$).

now been found in mammalian cells and are speculated to be present in all eukaryote cells. $InsP_6$ and several isomers of InP_{3-5} 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 health benefits, especially in maintaining nutrient adequacy and cardiovascular health (Bolling et al., 2011; López-Uriarte et al., 2009; O'Neil et al., 2015). 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 tricalcium phosphate (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 Official Method 986.11 (AOAC, 2016) for phytate in foods, carry 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 $InsP_{1-5}$, and may result in an overestimation of the true $InsP_6$ content. Phosphorus-containing organic compounds in plants are quite diverse. 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 (Duong et al., 2016), 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.

2. Materials and methods

2.1. Reagents and materials

Adenosine 5′-monophosphate (AMP), HPLC-grade ammonium carbonate and methanol, phytic acid sodium salt from rice, Dowex® 1 × 4 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). 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). 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) and EMD Millipore Corporation (Billerica, MA), respectively.

Ethylenediaminetetraacetic acid disodium salt dihydrate (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). Sodium sulfite and sodium bisulfite were obtained from Aqua Solution, Inc. (Deer Park, TX). Potassium dihydrogen phosphate and ammonium molybdate were acquired from J.T. Baker (Avantor Performance Materials, Center Valley, PA).

Whole, unsalted, commercial tree nut products, including macadamias, cashews, Brazil nuts, hazelnuts, pecans, walnuts, and roasted pistachios, were obtained in three independent lots in bulk. Commercial samples of wheat aleurone, corn germ, and rice