

**Measurement of Phytase Activity in a Clymer Forest Soil Using the
TInsP₅ Probe**

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By Zirou Huang

Abstract

Measurement of soil phytase activity (PA) and delineation of the impact of this important phosphomonoesterase on the P-cycling process in soil and sediments suffer from the lack of a reliable assay. A method for measuring PA in soil that promises to be accurate and reliable has been recently published. The method involves the use of a novel chromophoric analog of phytic acid, referred to as T(tethered)InsP₅ (5-*O*-[6-(benzoylamino)hexyl]-*D*-*myo*-inositol-1,2,3,4,6-pentakisphosphate). This study was conducted to measure PA in a Clymer forest soil, which contained over twice the amount of soil organic C as previously tested soils, using the TInsP₅ PA assay. This investigation specifically addresses: (1) the development of a soil dilution technique for determining maximal PA, (2) identification of previously unsubstantiated soil-produced dephosphorylated intermediate probe species, (3) the impact of increasing assay buffer pH on soil PA and (4) testing stability of the probe's amide bond in a highly (bio)active forest soil. PA assays were conducted by measuring dephosphorylation of TInsP₅ in citrate-acetate buffered (pH 4.2) active and autoclaved (Control) soil suspensions. Phosphorylated probe intermediates (i.e., TInsP₄, TInsP₃, TInsP₂ and TInsP₁) and T-*myo*-inositol were extracted from samples of soil suspension following incubation. Probe species were quantified using reversed phase high-performance liquid chromatography (RPHPLC) with UV detection. PA was calculated based on a mass balance approach. A soil dilution technique was developed to address the challenge of determining maximal PA in soils containing higher organic matter content. In the initial report on use of the TInsP₅ method for measuring PA in soil, two 'soil-generated' UV-adsorbing compounds (designated Y and Z) were observed, but never confirmed as probe species. The experimental evidence presented in this report supports inclusion of compound Y as a phosphorylated probe intermediate species (i.e. TInsP_y), based primarily on its UV adsorption spectra (diode-array detection analysis). Compound Z could not be substantiated as a probe species based on the evidence presented in this study. PA of Claymer forest soil decreased with an increase in assay buffer pH. Further, the probe's amide bond linkage was stable in a forest soil exhibiting high PA.

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Measurement of Phytase Activity in a Clymer Forest Soil Using the TInsP₅ Probe

Introduction

Presence of inositol phosphates in soils

Inositol phosphates are dominant form of P esters among the organic P compounds identified from soils, comprising up to 50 to 60% of soil organic P (Anderson, 1980; Stevenson, 1994). Soil inositol phosphates are mainly in the form of hexakisphosphates although considerable amounts of pentakisphosphate and lesser phosphorylated species can also be found in soil (Turner et al., 2002; Turner, 2007). Five of the possible nine stereoisomers of inositol phosphates (*myo*, *scyllo*, *neo* and racemic *D*, *L-chiro* forms) have been isolated from soil samples; among these stereoisomers the *myo*-form predominates and is followed by *scyllo*-, *D-chiro*- and *neo*-inositol phosphate forms (Dalal, 1977; Turner et al., 2002). Most research has centered on the *myo*-inositol hexakisphosphate, better known as phytic acid, InsP₆ (Fig. 1) because it is the most prevalent of the inositol hexakisphosphate (IP₆) species present in soil.

Biogeochemical processes involved in phosphorus (P) cycling in P-burdened manure amended soils play an important role in transport of P to surface and ground water. Given that swine and poultry are unable to utilize a large percentage of the P in grain feed, present mainly in the form of InsP₆, high concentrations of InsP₆ are excreted in their wastes, which are then commonly land applied. Continued land application of these wastes, based on N demand, has resulted in a buildup of soil P in regions with a high density of Confined Animal Feeding Operations. When applied P exceeds the level of crop utilization, 'excess P' is transported to streams, rivers, lakes and estuaries.

Inositol phosphates – chemistry and bioavailability

The phosphoric ester linkages in InsP₆ are chemically stable, particularly in concentrated alkali. The rate of hydrolysis is at maximum near pH 4.0, where the monoanionic form predominates. Below pH 4.0 the rate of hydrolysis decreases, as the charge on the phosphate ester groups become more neutralized (Cosgrove, 1980).

Inositol hexakisphosphate can complex with proteins, lipids and quite probably organic matter (Cosgrove, 1980; Turner et al., 2002). Dissociation of protein-InsP₆ complexes occurs only at a pH less than 2. Inositol hexakisphosphate readily forms soluble complexes, within specific pH ranges, with a number of divalent and trivalent metal ions, e.g., Ca, Mg, Zn, Fe, Al and Mn (Maddaiah et al., 1964; Vohra et al., 1965).

Inositol phosphates are strongly sorbed by clay minerals and sesquioxides (Goring and Bartholomew, 1950; Anderson and Arlidge, 1962; Anderson et al., 1974; McKercher et al., 1986; Shang et al., 1990, 1993; Ognalaga et al., 1994; Celi et al., 1999). As P groups increase, retention of inositol phosphate increases, with IP₆ being preferentially adsorbed (Anderson and Arlidge, 1962; Anderson et al., 1974; Shang et al., 1990; Celi et al., 1999). Anderson et al. (1974) showed that adsorption of InsP₆ by acidic Scottish soils was highly correlated with ammonium oxalate extractable Al and Fe. Shang et al. (1990, 1993) compared the adsorption behaviors of orthophosphate, InsP₁, InsP₆ and glucose 6-phosphate (G6P) on amorphous Al and Fe (hydr)oxides. Shang et al. (1992) demonstrated that sorption was impacted by the molecular structure of the organic P compound along with solution pH. Higher pH decreased the adsorption of organic phosphate compounds with the impact being much more pronounced for InsP₆.

Experimental results suggest that orthophosphate and inositol phosphates adsorb to the same active sites on mineral surfaces (McKercher and Anderson, 1968; Anderson et al., 1974; Evans, 1985; Shang et al., 1990, 1992; Celi et al., 1999). Furthermore, FTIR studies showed that the orthophosphate groups of InsP₆ versus orthophosphate form similar types of surface complexes (e.g., binuclear), with goethite and amorphous Fe(III)(hydr)oxide (Shang, 1989; Ognalaga et al., 1994).

Ligands such as the hydroxyl ion and organic anions (citrate, oxalate and tartrate) can compete with InsP₆ for adsorption sites on mineral surfaces through ligand exchange. Not surprisingly, surface complexed InsP₆ is far more stable than surface complexed orthophosphate or glucose-6-phosphate (G6P), owing to the fact that InsP₆ has multiple oxygen donor groups, which were capable of forming covalent bonds at multiple sites on the mineral surface (Shang, 1989; Celi et al., 1999). The relative abundance of InsP₆ in soil can be explained at least partly on the fact that this compound is very strongly sorbed to soil with a very low rate of desorption.

Despite its predominance, not a great deal is known about the bioavailability of InsP₆ in soil and natural water/sediment environments (Turner et al., 2002). In one of the few studies available on the subject, Richardson et al. (2001) found that the P-nutrition of pasture grass seedlings was significantly improved when InsP₆, along with microorganisms capable of releasing orthophosphate from this phosphate ester, were added to a sand-vermiculite potting media. Presumably, the microorganisms that Richardson and his coworkers added to the soil were capable of producing enzymes that exhibited “phytase-like” activity. The finding that microbial phytases participate in increasing plant available P is not surprising in light of the fact that microbes serve as the primary source of most soil enzymes (Tabatabai, 1994).

Shang et al. (1996) found that bioavailability of adsorbed InsP₆ was much lower compared to the bioavailability of sorbed orthophosphate or G6P. The amount of adsorbed InsP₆ required to support microbial growth was at least 30 times higher than that observed for orthophosphate (i.e., 30 to 1 cmol P kg⁻¹ adsorbent), although, sorbed InsP₆ became more bioavailable with higher surface coverage levels (Shang et al., 1996). These investigators concluded that the bioavailability of adsorbed organic P was primarily determined by the stability of phosphate-surface complexes, rather than the total amount of P adsorbed.

Under natural or undisturbed conditions, accumulation of inositol phosphates in soil likely results from pedogenic processes. It appears that the pool size of inositol phosphates is relatively large in organic and virgin prairie soils, but smaller in agricultural soils where chemical fertilizers are regularly applied (Turner et al., 2002). Pool size of inositol phosphates in soil is regulated by factors such as climate, land use/management and soil properties (Turner et al., 2002).

Phytase biochemistry

Phytases, reported to be highly specific for InsP₆, catalyze hydrolysis of this substrate, forming orthophosphate and a series of partially dephosphorylated phosphoric esters of *myo*-inositol (Cosgrove, 1980; Mitchell et al., 1997). In some cases hydrolysis may go to completion, yielding the parent compound *myo*-inositol.

Based primarily on differences in their catalytic mechanism, four classes of phosphatases, each containing members with phytate-degrading capability, are currently recognized: (i) histidine acid phosphatases (HAPs), (ii) β -propeller phytases (BPP), (iii) cysteine phosphatases (CP) and (iv) the purple acid phosphatases (PAP) (Mullaney and Ullah, 2007). The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) has classified phytase(s), based on the 1D-numbering system, as 3-phytase (EC 3.1.3.8), 4-phytase (EC 3.1.3.26) or 5-phytase (EC 3.1.3.72) depending upon the specific position of the initial dephosphorylation reaction. While 4-phytase (i.e., 6-phytase based on the 1L-numbering system) appears to be associated mostly with higher plants, microorganisms likely are the primary source of 3-phytase (Cosgrove, 1980; Greiner, 2007). Furthermore, filamentous fungi generally produce extracellular phytases while bacteria, with some notable exceptions (bacteria from the genera *Bacillus* and *Enterobacter*), tend to produce mostly cell associated and/or intracellular phytases (Konietzny and Greiner, 2004).

3-Phytase catalyzes the reaction: *myo*-inositol-1,2,3,4,5,6-hexakisphosphate + H₂O → 1D-*myo*-inositol-1,2,4,5,6-pentakisphosphate + orthophosphate. Likewise, 4-phytase catalyzes the reaction: *myo*-inositol-1,2,3,4,5,6-hexakisphosphate + H₂O → 1D-*myo*-inositol-1,2,3,5,6-pentakisphosphate + orthophosphate. For 4-phytase, the primary site of reaction is the C-4 position; however, other positions are susceptible to P-group removal in the initial reaction, but to a lesser extent (Greiner et al., 1993). Similarly, the P group in the 3-C position is preferentially removed in the initial reaction catalyzed by 3-phytase. In either case, the rate of dephosphorylation slows down as P groups are removed from the inositol moiety, probably as a result of product (i.e., orthophosphate) inhibition (Greiner et al., 1993; Wyss et al., 1999). While phytase is highly efficient in dephosphorylating InsP₆ along with bis-, tris-, tetrakis- and pentakis-phosphate (i.e., InsP₂₋₅), the final dephosphorylation (of InsP₁) can be effectively achieved through the activity of acid (EC 3.1.3.1) or alkaline phosphatases (EC 3.1.3.2) (Turner et al., 2002). Fungal phytases appear to preferentially remove P-groups located at the five equatorial positions of InsP₆ with a tendency for accumulation of *myo*-inositol 2(axial)-monophosphate (Wyss et al., 1999). As it turns out, InsP₆ is resistant to dephosphorylation by either acid or alkaline phosphatases (Meek and Nicoletti, 1986;

Marko-Varga and Gorton, 1990; Shan et al., 1993). It is also important to realize, however, that InsP_{5-2} and even InsP_6 are, to at least some degree, susceptible to dephosphorylation in the presence of nonspecific acid phosphohydrolases (Rossolini et al., 1998).

Divalent and trivalent metal cations can form soluble complexes with InsP_6 , which influences rates of phytase-catalyzed hydrolysis of the P group. Most of the characterized phytases appear to be inhibited by Zn, which is understandable, since this particular metal cation tends to form the most stable metal-phytate complex (Maenz et al., 1999). Manez et al. (1999) determined that the most potent inhibitor Zn^{2+} was followed by $\text{Fe}^{2+} > \text{Mn}^{2+} > \text{Fe}^{3+} > \text{Mg}^{2+}$. Further, a decrease in the pH of the medium from 7 to 4 decreased potency of inhibition for all the divalent metal cations tested. The presence of EDTA and citrate increased efficacy of microbial phytase activity in canola meal suspension, perhaps because these chelating agents shifted the equilibrium towards formation of 'phytase-susceptible' at the expense of 'phytase-resistant' metal-phytate complex forms (Manez, et al., 1999).

Laboratory studies have shown that metal cations such as Cu^{2+} , Zn^{2+} , Cd^{2+} , Al^{3+} , Ca^{2+} or Mg^{2+} and soil minerals (montmorillonite, illite, kaolinite, gibbsite, and goethite) can inhibit phytase-catalyzed dephosphorylation of InsP_6 , either from enzyme inactivation or restriction of substrate activity through formation of metal-phytate complexes and/or sorption to surfaces. (Mortland and Giesecking, 1952; Svenson, 1986; Dao, 2003). Dao (2004) was able to show an increase in PA following addition of organic ligands to soil, presumably because inositol phosphates were liberated from insoluble metal complexes.

Measurement of PA in soil

The soil chemistry of inositol phosphates has been reasonably well investigated over the past quarter century (Cosgrove, 1980; Graf, 1986; Stevenson, 1994; Turner et al., 2002). Just recently, quantification of InsP_6 in soil extracts was achieved using ^{31}P -NMR spectroscopy (Turner, 2007). On the other hand, there has been little, if any, progress in characterizing PA in soil (George et al., 2007).

Up until recently, no reliable methods for measuring PA in soil have been reported. The *p*-nitrophenylphosphate assay for measuring phosphomonoesterase activity in soil (Tabatabai, 1994) is not specific for measuring PA. Further, the use of methods calling for addition of phytic acid to soil with subsequent quantification of released orthophosphate are not reasonable for measuring PA in soil. Many agricultural soils contain either high or moderately high levels of orthophosphate (e.g., manure amended high P soils) making measurements of a small amount of orthophosphate released from the phytase-catalyzed dephosphorylation of phytic acid, over and above extraneous orthophosphate present in soil problematic.

Quantifying soil-generated phosphorylated intermediates (i.e., InsP_{5-1}) as a means of determining soil PA is not realistic as it would require the use of either reversed-phase ion pair chromatography (RPIPC) or high performance ion chromatography (HPIC). While there are RPIPC methods available for separation and quantification of inositol phosphates (Lehrfeld, 1994) they require the use of expensive ion pair reagents and generally suffer from poor resolution. Anion exchange columns (i.e., HPIC) can be used to separate inositol phosphate species, but are expensive and do not tolerate high concentrations of humic substances (i.e., irreversible sorption of humic materials with rapid loss of column resolution). Inositol phosphates can be detected in these chromatography systems with either a conductivity or colorimetric (i.e., by measuring absorbance of metal-phosphate complexes) detector. In each of these instances, response of the detector is dependent upon the number of phosphate groups attached to the *myo*-inositol moiety. Thus, one must have authentic inositol phosphate standards to determine the amount (quantitative analysis) of a particular species produced in a PA assay involving InsP_6 . Inositol phosphate standards are prohibitively expensive and not readily available (to everyone).

If one intends to measure PA in soil by quantifying disappearance of InsP_6 or the appearance of inositol phosphate intermediates (e.g., InsP_5), then there are several fundamental issues to address. A PA assay consisting of one gram of mineral soil and 4 mL aqueous phase buffer solution would likely require > 5 mM phytate (InsP_6) to saturate enzyme active sites and satisfy chemical demand (i.e., sorption and precipitation)(Berry et al., 2009). At this high substrate concentration level, measuring

the disappearance of an extremely small amount of substrate (on the order of nM to μM levels, Berry et al., 2009) from such a large substrate pool (e.g., 5 mM) would be problematic. So, biochemists prefer to measure the product produced in enzyme-catalyzed reactions. Generally speaking, it is much easier to measure a small change in a dilute pool, i.e., quantification of intermediate(s) produced. However, there is a problem if one intends to measure products produced in soil suspension assays containing InsP_6 , because as Turner et al., (2002 and articles cited therein) points out, inositol phosphates (i.e., InsP_6 , InsP_5 , InsP_4 , InsP_3 , InsP_2 , InsP_1 .) are naturally present in soil and, in some cases, the amount of InsP_5 and/or InsP_4 found in soil can be as high as that of InsP_6 (Turner et al., 2007) Thus, if the ‘products’ of PA (e.g., InsP_5) are already present in soil, then it becomes problematic to differentiate between freshly-produced assay product(s) and previously or naturally present chemical species.

In most situations, measurement of enzymatic activity in soil usually means monitoring a ‘single substrate – single product’ reaction (Tabatabai, 1994). Ideally, one would like to measure only a single product for phytase catalyzed dephosphorylation of InsP_6 :



However, in this particular instance the ‘single substrate – single product’ reaction is not a realistic scenario since most phytases are capable of dephosphorylating, InsP_5 , InsP_4 , InsP_3 and/or InsP_2 (Greiner, 2007). Furthermore, phytase-catalyzed dephosphorylation of InsP_6 is not synchronous, nor can synchronization of the reaction(s) be forced. In soil, measuring PA gets even more complicated since one can expect to observe dephosphorylation of inositol phosphate intermediates by phytases and non-phytate-degrading acid phosphatases, i.e., a ‘mixed’ enzyme system.

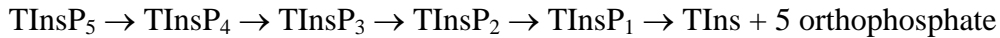
These inherent characteristics of phytase make it difficult to measure PA in soil. To accomplish the task of measuring PA in soil a reliable, traceable and durable chromophoric probe that can react with soil phytases in a specific manner identical to that of the natural substrate, InsP_6 , is necessary. Berry and Berry et al. (2005) has recently reported on the creation of just such a probe, 5-*O*-[6-(benzoylamino)hexyl]-*D*-*myo*-inositol-1,2,3,4,6-pentakisphosphate, i.e., T(tethered) InsP_5 . T InsP_5 (Fig. 1) has a UV

absorbing chromophore attached to the C-5 position of the *myo*-inositol moiety via an amide bond to a C₆-linker. The advantages of this type of analytical approach for measuring PA are: (i) that response of the detector (UV) to the analyte is not dependent upon the number of phosphate groups attached to the *myo*-inositol moiety, thus TInsP₅ can be used as a standard for any phosphorylated probe species produced and (ii) that the TInsP₅ probe is readily differentiated from naturally occurring inositol phosphate chemical species making for a robust quantification procedure, i.e., reproducible and precise. Possible disadvantages of the TInsP₅ probe method for measuring PA in environmental samples include: (i) the fact that the TInsP₅ is a synthetic substrate and may not be dephosphorylated by all the phytases present in soil and (ii) the probe's amide bond may be susceptible to either chemical and/or arylamidase-catalyzed hydrolysis.

Since phytate-degrading enzymes generally exhibit broad substrate specificity (Konietzny and Greiner, 2002), it is anticipated that the majority of the phytases present in soil will be capable of catalyzing the dephosphorylation of TInsP₅ (Berry et al., 2009). Because TInsP₅ serves as a good substrate for the *Aspergillus. ficuum (niger)* phytase, which is characterized as being specific for phytate (Wyss et al., 1999), one can be reasonably confident that a high percentage of the phytate-specific phytases will also be capable of dephosphorylating TInsP₅ (Berry et al., 2009). The probe's amide bond should be resistant to arylamidase-catalyzed hydrolysis because its aryl group is attached to the carbonyl carbon of the amide rather than the nitrogen atom, which is the opposite orientation of that found in the enzyme's natural substrates (Berry et al., 2009).

The probe's phosphate monoester linkages are cleaved by *Aspergillus* 3-phytase and wheat (4)-phytase, forming the readily detectable chromophoric intermediate species TInsP₄, TInsP₃, TInsP₂ and TInsP₁ (Berry and Berry, 2005; Berry et al., 2007). Berry et al. (2007) designated, TInsP₃, a key intermediate because it tended to accumulate during 3- and 4-phytase catalyzed reactions. Further, Berry et al. (2007) has presented evidence supporting phytase specificity for TInsP₅. These researchers found that TInsP₅ was not hydrolyzed to any appreciable extent by either potato or wheat-germ acid phosphatases. Interestingly, both phytic acid (control) and TInsP₅ were slightly susceptible to cleavage by wheat-germ acid phosphatase, suggesting that this particular crude phosphatase preparation contained at least some phytate-degrading capability (Berry et al., 2007).

Recently, Berry et al. (2009) found that PA in soil can be ascertained by quantifying production of dephosphorylated probe species for a given time period:



provided that production of the dephosphorylated probe species resulted from phytase-catalyzed dephosphorylation of TInsP₅ either directly or indirectly. Further, these investigators also found that an accurate measure of PA was possible in soil, which represents a ‘mixed’ enzyme system, if all of the dephosphorylated species produced via enzymatic reaction were quantified, i.e., the mass balance approach. Perhaps most importantly, Berry et al. (2009) noted that higher than expected levels of substrate (> 5 mM TInsP₅) were required to obtain maximal rates of PA in soil suspension assays and that very low concentration levels of product were produced. These researchers postulated that a large amount of TInsP₅/assay unit was required because much of the added probe formed ‘phytase-resistant’ metal-phytate complexes involving divalent and trivalent cations such as Al and Fe.

The paucity of information on the transformation of InsP₆ to plant nutrient P (i.e., orthophosphate) presents the biggest challenge to a thorough understanding of the P-cycling process in P-burdened manure amended soils and in soils where organic pools dominate (e.g., tropical rain forests). In soil, phytase catalyzes hydrolysis of the phosphate ester bond(s) of InsP₆, releasing orthophosphate. In high P soils, rates of ortho-P release in excess of crop uptake will lead to an increase in P transport to nearby surface waters and/or groundwater. Over the long term, increased P release (from phytase-catalyzed dephosphorylation of phytic acid) will ultimately influence the amount of P transported from high P soils to open waterways accelerating eutrophication. Research directed at how phytase impacts the fate of InsP₆ will not advance until an accurate/reproducible and user friendly measure of PA is developed. This point was emphasized in a recent review article by Turner et al (2002) who noted that: “...there has been little research on phytase in soils and waters because no artificial substrates are presently available that can be used for convenient measurement of activity.” The

artificial probe, TInsP₅, represents a promising new tool that can be used to measure PA in soil.

In this study, I used the TInsP₅ molecular probe for determining PA in a Clymer forest soil. My research specifically addressed: (i) development of a soil dilution technique for determining maximal soil PA, (ii) identification of previously unsubstantiated soil-produced dephosphorylated intermediate probe species, (iii) the impact of increasing assay buffer pH on soil PA and (iv) testing the stability of the probe's amide bond in a highly (bio)active forest soil.

Materials and Methods

Chemicals

All reagents were analytical grade. TInsP₅ (decaammonium salt form, purity 97%) and TIns (purity 97 %) were obtained from Berry & Associates, Inc., Dexter, MI. Wheat- and *Aspergillus ficuum (niger)* 3-phytase, along with dodecasodium phytate were purchased from Sigma Chemical Co., St. Louis, MO. Buffer and reagent solutions were prepared with ultra-pure water (18.2 MΩcm⁻¹).

Soil

Samples of the well-drained Clymer series, classified as a fine-loamy, mixed Typic Hapludults were collected, to a depth of 8 cm, from 5 sites located within the boundaries of a forest (primarily oak deciduous forest). Soil samples were collected from the O and (upper) A1 horizons. Composite soil samples were placed into a plastic bag and transported to the laboratory where they were thoroughly mixed and sieved, using a 2 mm sieve, and then placed into a 1 L wide-mouth polypropylene jar (250 g field moist soil) Polypropylene linerless screw closers were placed loosely on the containers and the soils maintained at room temperature with a moisture of 32 ± 2%. Soil characteristics are listed in Table 1.

PA measurements

The following procedures for: (1) preparing probe standards, (2) HPLC analysis of the probe species, (3) calculating soil PA and (4) setting up the PA assay, were adapted from Berry et al. 2009.

Preparation of TInsP₅, TIns and phosphorylated probe intermediate(s) analytical standards

TInsP₅ and the TIns standard(s) were prepared in 0.1 M potassium phosphate buffer (pH 6.0)-methanol solution (9:1; v/v, ratio buffer to methanol) and H₂O-methanol solution (3:1; v/v), respectively. Refrigerated TInsP₅ and TIns standard(s) were stable for up to 8 and 16 weeks, respectively.

The phosphorylated probe intermediate species were prepared by reacting TInsP₅ with either wheat- or 3-phytase. These preparations served as stock-standard solutions.

The wheat-phytase catalyzed reaction was initiated by adding 0.135 U of wheat-phytase to 3 mL of a 200 mM acetate buffer (pH 5.2) containing TInsP₅ (1.2 mM). The reaction mixture was incubated on a rotary shaker (150 rev min⁻¹) for 80 min at 45°C. Samples of the wheat-phytase stock standard reaction mixture, sampled at 60 and 80 min, were boiled for 20 min (enzyme deactivation) and then filtered using a 13 mm Millipore Duropore Millex GV PVDF 0.22 µm membrane syringe filter.

The 3-phytase catalyzed reaction was initiated by adding 0.069 U of 3-phytase to 3 mL of a 50 mM glycine-HCl buffer (pH 2.6) containing TInsP₅. The reaction mixture, containing 1.2 mM TInsP₅, was incubated on a rotary shaker (150 rev min⁻¹) for 90 min at 37°C. After incubation, the 3-phytase generated stock-standard was boiled for 20 min and then filtered using a 0.22 µm syringe filter.

The wheat- and 3-phytase generated stock-standard solutions were stored frozen. Aliquot samples were diluted with 0.1 M phosphate buffer (pH 6.0)-methanol solution (9:1; v/v) in preparation for HPLC analysis.

Preparation of phytase was accomplished by adding freeze-dried wheat- and 3-phytase (1.38 and 1.35 U mL⁻¹ buffer, respectively) to the appropriate buffer. The preparations were mixed for 15 min on a stir plate then placed on ice. Just prior to use, the ice-chilled enzyme preparations were mixed for two minutes on a stir plate.

HPLC analysis

Samples were chromatographed on an Agilent Technologies 1200 Series Binary HPLC system with vacuum degasser and thermostatted column compartment. The Agilent binary pump was connected to either the LDC Analytical Spectromonitor 3100 or an Agilent Series 1200 UV-Visible variable wavelength detector, which was set to 226 nm. A Model 7725i Rheodyne valve, fitted with either a 20 μL PEEK flex loop, was used to deliver samples onto the column. PEEK tubing connected the Rheodyne valve, column and detector used in the analysis of phosphorylated probe species. An Agilent Technologies 3395 integrator or Agilent Technologies LC 2D Chemstation was used to analyze detector output.

For identification purposes, selected samples were chromatographed on an Agilent Technologies 1100 Series Quaternary HPLC system, which included a diode array detector (DAD). The DAD was used to generate UV spectra for probe species and unidentified UV-absorbing compounds. Detector output was collected using the LC 3D C Chemstation software.

Isocratic separation of TInsP₅ and phosphorylated probe intermediate(s) was achieved on a Supelcosil LC-18-T column (3 μm , 15 cm x 4.6 mm, Sigma-Aldrich Co.) operated at 32 °C using a mobile phase consisting of 0.1 M phosphate buffer (pH 6.0)-methanol at a flow rate of 1.0 mL min⁻¹. The ratio of phosphate buffer to methanol in the mobile phase ranged from 11.5:1 to 13.3:1. The phosphate buffered mobile phase was prepared by adding the appropriate amount of methanol to a 1000-mL volumetric flask followed by incremental additions of 0.1 M phosphate buffer (pH 6.0) with intermittent mixing. Components of the mobile phase were filtered separately. Millipore 0.20 μm nylon membranes were used to filter phosphate buffer and methanol.

Isocratic separation of TIns was achieved on a heated (28°C) Supelcosil LC-18-T or Agilent Technologies ZORBAX Eclipse XDB-C18 column (5 μm , 15 cm x 4.6 mm) using an H₂O-methanol (3:1) mobile phase at a flow rate of 1.0 mL min⁻¹.

Isocratic separation of benzoate was achieved on a Supelcosil LC-18-T column (3 μm , 15 cm x 4.6 mm, Sigma-Aldrich Co.) operated at 32 °C using a mobile phase

consisting of 0.1 M phosphate buffer (pH 6.0)-methanol at a flow rate of 1.0 mL min⁻¹. The ratio of phosphate buffer to methanol was 8.5:1.5.

PA calculations

Based on the mass balance approach, Equation [1] was used to calculate PA given that production of any dephosphorylated probe species results from phytase-catalyzed dephosphorylation of TInsP₅ either directly or indirectly:

$$PA = \left(\sum_{i=4}^0 [TInsP_i] / t \right)_{\text{Active}} - \left(\sum_{i=4}^0 [TInsP_i] / t \right)_{\text{Control}} \quad [1]$$

where Δt = incubation time interval, TInsP₀ = TIns and [TInsP_{*i*}]_{Control} represents any dephosphorylated probe species formed primarily through non-enzymatic processes, e.g., chemical hydrolysis (Berry et al., 2009). The units for PA are number of mol of TInsP₅ dephosphorylated/g soil/h.

Measurement of soil PA at pH 4.2

Experiment 1—Soil dilution technique

Moist soil (1.25 g oven dry wt. basis) was added to each of three 50 mL flasks. One of the flasks, designated autoclaved control, was covered with aluminum foil and autoclaved for 30 min. After receiving 2.5 mL of citrate (75 mM)-acetate (50 mM) buffer (pH 4.2) the Active and Control soil suspensions were tightly sealed with rubber lined screw caps and then equilibrated for 30 min at 37°C on a rotary shaker (150 rev min⁻¹). Following equilibration, One Active and the autoclaved control soil suspensions received 2.5 mL of 10 mM TInsP₅ citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.5 mL ethanol, i.e., 20 μmol TInsP₅/g soil. The one remaining Active soil suspension received 2.5 mL of 10 mM citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.5 mL ethanol. The Active and Control flasks were tightly sealed and incubated at 37°C on a rotary shaker (150 rev min⁻¹). Contents of the flasks were sampled at 16 and 40h.

In preparation for HPLC analysis of phosphorylated probe intermediates, 720 μL of soil slurry suspension was placed in a 1.5 mL microcentrifuge tube containing 180 μL of a 100 mM citrate buffered phytate-EDTA (50 mM) solution (pH 6.0). The contents were mixed for 30 s using a vortex mixer and tubes then shaken, on their side, on a

reciprocating shaker (200 rev min^{-1}) at room temperature for 30 min. The tubes were centrifuged at $10,000 \text{ g}$ for 10 min. An aliquot sample of the clarified soil extract $700 \mu\text{L}$ was diluted with $1400 \mu\text{L}$ of a 0.1 M phosphate buffer (pH 6.0)-methanol solution (9:1; v/v). The diluted sample was filtered ($0.22 \mu\text{m}$ syringe filter) and then analyzed. Any remaining supernatant was removed before proceeding with the second extraction, which was carried out by adding 0.9 mL of phytate-EDTA (50 mM) extracting reagent to the soil pellet followed by 30 s of vortex mixing then 30 min on a reciprocating shaker (200 rev min^{-1} at room temperature). The tubes were centrifuged at $10,000 \text{ g}$ for 10 min. Supernatant from the second extract was filtered, using a $0.22 \mu\text{m}$ syringe filter, and then analyzed.

In preparation for HPLC analysis of TIns, a 0.4 mL aliquot sample of soil slurry were placed in a 1.5 mL microcentrifuge tube containing 0.6 mL of 200 mM acetate-buffered (pH 4.2) *myo*-inositol (20 mM) – methanol solution (5:1; v/v) and then mixed for 30 s using a vortex mixer. Tubes were laid on their side and shaken at 200 rev min^{-1} for 30 min at room temperature and then centrifuged at $10,000 \text{ g}$ for 10 min. An aliquot sample of the clarified extract was filtered using a $0.22 \mu\text{m}$ syringe filter, and then analyzed.

In preparation for HPLC analysis of TInsP₅ concentration, $200 \mu\text{L}$ of soil suspension was placed in 1.5 mL microcentrifuge tube. The soil suspension was clarified by centrifugation ($10,000 \text{ g}$ for 10 min), diluted, and filtered using a $0.22 \mu\text{m}$ syringe filter.

Samples of the soil slurry suspension were obtained using a volume precalibrated single-channel pipetter fitted with modified $101\text{-}1000 \mu\text{L}$ pipet tips. Tips were cut to a 2-mm orifice. Flasks were shaken prior to sampling to ensure that the aliquot sample contained the same solid to liquid phase ratio as the remaining slurry suspension. Solid phase volume displacement was taken into consideration when calculating probe concentration.

The concentration of TInsP₄ in soil suspension was determined using the external standard procedure with TInsP₅ serving as the analytical standard. 3-Phytase generated TInsP₃ that had been standardized using TInsP₅, served as an analytical standard for determining the concentrations of TInsP₃ and TInsP₂ in soil suspensions.

Experiment 2—Soil dilution technique and identification of phosphorylated probe intermediates

Moist soil (0.625 g oven dry wt. basis) was added to each of three 50 mL flasks. One of the flasks, designated autoclaved Control, was covered with aluminum foil and autoclaved for 30 min. After receiving 2.5 mL of citrate (75 mM)-acetate (50 mM) buffer (pH 4.2) the Active and Control soil suspensions were tightly sealed with rubber lined screw caps and then equilibrated for 30 min at 37°C on a rotary shaker (150 rev min⁻¹). Following equilibration, one of the Active and the autoclaved Control soil suspensions received 2.5 mL of 10 mM TInsP₅ citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.5 mL ethanol, i.e., 40 µmol TInsP₅/g soil. The one remaining Active soil suspension received 2.5 mL of 10 mM citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.5 mL ethanol. The Active and Control flasks were tightly sealed and incubated at 37°C on a rotary shaker (150 rev min⁻¹). Contents of the flasks were sampled at 16 and 40h.

In preparation for HPLC analysis of phosphorylated probe intermediates, 720 µL of soil slurry suspension was placed in a 1.5 mL microcentrifuge tube containing 180 µL of a 100 mM citrate buffered phytate-EDTA (50 mM) solution (pH 6.0). The contents were mixed for 30 s using a vortex mixer and tubes then shaken, on their side, on a reciprocating shaker (200 rev min⁻¹) at room temperature for 30 min. The tubes were centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified soil extract 700 µL was diluted with 1400 µL of a 0.1 M phosphate buffer (pH 6.0)-methanol solution (9:1; v/v). The diluted sample was filtered (0.22 µm syringe filter) and then analyzed. Any remaining supernatant was removed before proceeding with the second extraction, which was carried out by adding 0.9 mL of phytate-EDTA (50 mM) extracting reagent to the soil pellet followed by 30 s of vortex mixing then 30 min on a reciprocating shaker (200 rev min⁻¹ at room temperature). The tubes were centrifuged at 10,000 g for 10 min. Supernatant from the second extract was filtered, using a 0.22 µm syringe filter and then analyzed.

In preparation for HPLC analysis of TIns, a 0.4 mL aliquot sample of soil slurry was placed in 1.5 mL microcentrifuge tube containing 0.6 mL of 200 mM acetate-

buffered (pH 4.2) *myo*-inositol (20 mM) – methanol solution (5:1; v/v) and then mixed for 30 s using a vortex mixer. Tubes were laid on their side and shaken at 200 rev min⁻¹ for 30 min at room temperature and then centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified extract was filtered using a 0.22 µm syringe filter and then analyzed.

In preparation for HPLC analysis of TInsP₅ concentration, 200 µL of soil suspension was placed in 1.5 mL microcentrifuge tube. The soil suspension was clarified by centrifugation (10,000 g for 10 min), diluted and filtered using a 0.22 µm syringe filter.

Experiment 3—Soil dilution technique

Moist soil (0.250 g oven dry wt. basis) was added to each of four 25 mL flasks. Two of the flasks, designated autoclaved Control, were covered with aluminum foil and autoclaved for 30 min. After receiving 1.6 mL of citrate (75 mM)-acetate (50 mM) buffer (pH 4.2) the Active and Control soil suspensions were sealed tightly with leak-proof polyethylene screw caps and then equilibrated for 30 min at 37°C on a rotary shaker (150 rev min⁻¹). Following equilibration, The Active and the autoclaved Control soil suspensions received 2.0 mL of 10 mM TInsP₅ citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.4 mL ethanol, i.e., 80 µmol TInsP₅/g soil. The Active and Control flasks were sealed tightly and incubated at 37°C on a rotary shaker (150 rev min⁻¹). Contents of Active (1a) and Control (1b) flasks were sampled at 7 h and 24 h (Replicate 1) while Active (2a) and Control (2b) flasks were sampled at 16h and 24h (Replicate 2).

In preparation for HPLC analysis of phosphorylated probe intermediates, 720 µL of soil slurry suspension was placed in a 1.5 mL microcentrifuge tube containing 180 µL of a 100 mM citrate buffered phytate-EDTA (50 mM) solution (pH 6.0). The contents were mixed for 30 s using a vortex mixer and tubes then shaken, on their side, on a reciprocating shaker (200 rev min⁻¹) at room temperature for 30 min. The tubes were centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified soil extract 700 µL was diluted with 1400 µL of a 0.1 M phosphate buffer (pH 6.0)-methanol solution (9:1;

v/v). The diluted sample was filtered (0.22 μm syringe filter) and then analyzed. Any remaining supernatant was removed before proceeding with the second extraction, which was carried out by adding 0.9 mL of phytate-EDTA (50 mM) extracting reagent to the soil pellet followed by 30 s of vortex mixing then 30 min on a reciprocating shaker (200 rev min^{-1} at room temperature). The tubes were centrifuged at 10,000 g for 10 min. Supernatant from the second extract was filtered, using a 0.22 μm syringe filter before analysis.

In preparation for HPLC analysis of TIns, a 0.4 mL aliquot sample of soil slurry was placed in 1.5 mL microcentrifuge tube containing 0.6 mL of 200 mM acetate-buffered (pH 4.2) *myo*-inositol (20 mM) – methanol solution (5:1; v/v) and then mixed for 30 s using a vortex mixer. Tubes were laid on their side and shaken at 200 rev min^{-1} for 30 min at room temperature and then centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified extract was filtered using a 0.22 μm syringe filter and then analyzed.

In preparation for HPLC analysis of TInsP₅ concentration (Active soil suspensions only), 200 μL of soil suspension was placed in 1.5 mL microcentrifuge tube. The soil suspension was clarified by centrifugation (10,000 g for 10 min), diluted, and filtered using a 0.22 μm syringe filter.

Measurement of soil PA at pH 5.4

Moist soil (0.250 g oven dry wt. basis) was added to each of two 25 mL flasks. One of the flasks, designated autoclaved Control, was covered with aluminum foil and autoclaved for 30 min. After receiving 1.6 mL of citrate (75 mM)-acetate (50 mM) buffer (pH 5.4) the Active and Control soil suspensions were sealed tightly with leak-proof polyethylene screw caps and then equilibrated for 30 min at 37°C on a rotary shaker (150 rev min^{-1}). Following equilibration, The Active and the autoclaved Control soil suspensions received 2.0 mL of 10 mM TInsP₅ citrate (75 mM)-acetate (50 mM) buffered (pH 5.4) solution and 0.4 mL ethanol. The Active and Control flasks were sealed tightly and incubated at 37°C on a rotary shaker (150 rev min^{-1}). Contents of Active and Control flasks were sampled after 7h incubation.

In preparation for HPLC analysis of phosphorylated probe intermediates, 720 μL of soil slurry suspension was placed in a 1.5 mL microcentrifuge tube containing 180 μL of a 100 mM citrate buffered phytate-EDTA (50 mM) solution (pH 6.0). The contents of were mixed for 30 s using a vortex mixer and tubes then shaken, on their side, on a reciprocating shaker (200 rev min^{-1}) at room temperature for 30 min. The tubes were centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified soil extract 700 μL was diluted with 1400 μL of a 0.1 M phosphate buffer (pH 6.0)-methanol solution (9:1; v/v). The diluted sample was filtered (0.22 μm syringe filter) and then analyzed. Any remaining supernatant was removed before proceeding with the second extraction, which was carried out by adding 0.9 mL of phytate-EDTA (50 mM) extracting reagent to the soil pellet followed by 30 s of vortex mixing then 30 min on a reciprocating shaker (200 rev min^{-1} at room temperature). The tubes were centrifuged at 10,000 g for 10 min. Supernatant from the second extract was filtered, using a 0.22 μm syringe filter, and then analyzed.

In preparation for HPLC analysis of TIns, a 0.4 mL aliquot sample of soil slurry was placed in a 1.5 mL microcentrifuge tube containing 0.6 mL of 200 mM acetate-buffered (pH 4.2) *myo*-inositol (20 mM) – methanol solution (5:1; v/v) and then mixed for 30 s using a vortex mixer. Tubes were laid on their side and shaken at 200 rev min^{-1} for 30 min at room temperature and then centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified extract was filtered using a 0.22 μm syringe filter, and then analyzed.

In preparation for HPLC analysis of TInsP₅ concentration (Active soil suspension only), 200 μL of soil suspension was placed in 1.5 mL microcentrifuge tube. The soil suspension was clarified by centrifugation (10,000 g for 10 min), diluted, and filtered using a 0.22 μm syringe filter.

Chemical stability of probe's amide bond in soil suspension

Moist soil (1.25 g oven dry wt. basis) was added to each of three 50 mL flasks. One of the flasks, designated Autoclaved Control, was covered with aluminum foil and autoclaved for 30 min. After receiving 2.5 mL of citrate (75 mM)-acetate (50 mM) buffer (pH 4.2) the Active and Control soil suspensions were sealed tightly with leak-proof

rubber-lined screw caps and then equilibrated for 30 min at 37°C on a rotary shaker (150 rev min⁻¹). Following equilibration, the Active and the autoclaved Control soil suspensions received 2.0 mL of 2.5 mM TIns citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.5 mL ethanol. An Active Control flask received 2.0 mL of citrate (75 mM)-acetate (50 mM) buffered (pH 4.2) solution and 0.5 mL ethanol. The Active, Active Control (background determination) and autoclaved Control flasks were sealed and incubated at 37°C on a rotary shaker (150 rev min⁻¹). Contents of Active and Control flasks were sampled after 24h incubation.

In preparation for HPLC analysis of TIns, a 0.4 mL aliquot sample of soil slurry was placed in 1.5 mL microcentrifuge tube containing 0.6 mL of 200 mM acetate-buffered (pH 4.2) *myo*-inositol (20 mM) – methanol solution (5:1; v/v) and then mixed for 30 s using a vortex mixer. Tubes were laid on their side and shaken at 200 rev min⁻¹ for 30 min at room temperature and then centrifuged at 10,000 g for 10 min. An aliquot sample of the clarified extract was filtered using a 0.22 µm syringe filter and diluted.

In preparation for benzoate analysis, a 0.9-mL aliquot sample of soil slurry was placed in a 1.5-mL microcentrifuge tube and centrifuged at 10,000 g for 10 min. The clarified sample was diluted 1:1 with 0.1 M phosphate buffer (pH 6.0)-methanol solution and filtered (Millipore Duropore Millex GV PVDF 0.22 µm membrane).

Results and Discussion

Measurement of soil PA at pH 4.2

Experiments 1, 2 and 3 were conducted for the purpose of maximizing the rate of PA in forest soil suspension assays—soil dilution technique and analyzing UV spectra of putative phosphorylated intermediate probe species.

Experiment 1—Soil dilution technique

The ‘chromatographic or elution profile’ of phosphorylated probe intermediates produced by the Clymer forest soil (Figures 2 and 3) is similar to that previously observed for the Frederick agricultural and forest soils (Berry et al., 2009). This observation is relevant because it shows consistency in the PA measurement process

across soils. Berry et al. (2009) observed dephosphorylation of the TInsP₅ probe in a Frederick agricultural and forest soil with measurable production of TInsP₃ and the putative probe species, TInsP₄ and TInsP₂ (forest soil only). In addition to the UV-absorbing phosphorylated probe species previously mentioned, these investigators also observed the presence of two unidentified UV-absorbing chemical species, referred to as compounds X and Y, in both the Frederick agricultural and forest soils. A third unidentified UV-absorbing chemical species, compound Z, was produced only in the Frederick agricultural soil.

Purified or partially purified phytases are used to produce phosphorylated probe intermediate species (Berry et al., 2009 and articles referenced therein). Partially purified wheat phytase catalyzes hydrolysis of TInsP₅ producing small amounts of TInsP₂, TInsP₁ and compound Y, relative to TInsP₄ and TInsP₃ (Figs. 2A or 3A). Partially purified *Aspergillus*-phytase (3-phytase) generates primarily TInsP₃ (2B and 3B). The identity of wheat- and 3-phytase generated TInsP₃, the key intermediate, was confirmed in a previous investigation (Berry et al., 2007). Neither wheat- nor 3-phytase generates any measurable amounts of compounds X or Z.

The 'soil generated' probe species, TInsP₄, TInsP₃ and TInsP₂ along with compound Y were tentatively identified by comparing their HPLC chromatographic retention times (t_R) (Figs. 2C or 3C) with t_R of wheat- and 3-phytase generated phosphorylated probe intermediate standards (Figs. 2A and B or 3A and B). The soil generated probe species present in the autoclaved Controls (Figures 2D and 3D) were produced primarily as a result of abiotic chemical hydrolysis reactions. The chromatographic profile of phosphorylated probe intermediates did not change with prolonged incubation times (compare Figs. 2C and D with Figs. 3C and D).

The dephosphorylated probe species, TInsP₄, TInsP₃, TInsP₂ and compound Y were used to determine PA in Clymer forest soil (Table 2). Examination of the results in Table 2 reveals two unsettling pieces of information: first, the overall percent of TInsP₅ in solution phase of the Active soil suspension was substantially less than 95% (see Berry et al., 2009) and second, there was a measurable decrease in PA with increased incubation time. Taken together, these results indicate that maximal PA was not achieved

over the incubation time period. PA was very likely influenced by sorption and/or precipitation reactions involving the substrate, TInsP₅.

Berry et al. (2009) used the standard enzymatic procedure for determining maximum velocity of PA, which calls for fixing the enzyme concentration (i.e., soil) while increasing substrate concentration over a series of individual reactions. While this approach worked well for determining the concentration of TInsP₅ at which maximal reaction velocity could be achieved, the process consumed a large quantity of probe material. Given that the probe is expensive, it makes sense to investigate alternative approaches for determining soil PA maxima that conserves probe material. The alternative approach chosen in this investigation was basically a reversal of the standard enzymatic procedure outlined above. Given that the substrate concentration is held constant, conventional wisdom dictates that the rates of substrate conversion will decrease with decreasing enzyme concentration (Tinoco et al., 1978), but mitigating circumstances could change the expected outcome in this particular instance.

Experiment 2—Soil dilution technique and identification of phosphorylated probe intermediates

Comparison of t_R of the wheat phytase generated phosphorylated probe intermediate standards (Fig. 4A) with t_R of the UV-adsorbing compounds in Active soil suspensions (Fig. 4B) indicated the presence of the soil generated probe species, TInsP₄, TInsP₃ and TInsP₂ along with compound Y.

The high degree of similarity in the absorption characteristics (e.g., λ_{max}) between the UV spectra of the TInsP₅ (Fig. 5A) and that of wheat-phytase generated compound Y (Fig. 5C) substantiate the identification of compound Y as a probe species, i.e., TInsP_y. As expected, there was a high degree of similarity in the absorption characteristics between the UV spectra of TInsP₅ (Fig. 5A) and that of wheat-phytase generated TInsP₄ (Fig. 5B), TInsP₃ (Fig. 6A), TInsP₂ (Fig. 6B) and TInsP₁ (Fig. 6C).

The UV-adsorbing compounds in Active soil suspensions, tentatively identified as TInsP₄, TInsP₃, TInsP₂ and compound Y based on t_R , were substantiated by comparing the UV absorption characteristics of their respective UV spectra (Figs. 7, 8 and 9) with that of the wheat-phytase generated phosphorylated probe intermediate standards (Figs. 5

and 6). The identity of soil-generated TIns was substantiated by comparing its t_R with that of authentic TIns (data not shown).

Two UV-absorbing compounds, X and Z were not used in calculating PA. As expected, compound X was present in both the TInsP₅-containing Active (Fig. 4B) and Control (Fig. 4C) soil suspensions since this compound is an impurity in the TInsP₅ preparation (Fig. 10), and was not included in calculation of PA because it is not a probe species based on its UV spectral characteristics (compare Figs. 7C and 11B with Fig. 7A).

It is important to point out that the small quantity of compound Y, present as impurity in the TInsP₅ preparation (Figs. 10 and 11A) or generated as a result of abiotic chemical reaction(s), did not interfere with PA calculations because ‘background’ phosphorylated probe was taken into account (see Eq. [1]). Berry et al. (2009) tentatively identified another impurity in the TInsP₅ preparation as TInsP₄ based on a t_R comparison with wheat-phytase generated TInsP₄. Furthermore, comparison of the UV absorption characteristics of the TInsP₄ impurity (Fig. 11C) with that of the TInsP₅ probe (Fig. 7A) further substantiates its identity. Berry et al. (2009) pointed out that the small amount of TInsP₄ present as an impurity does not interfere with PA calculations because ‘background’ phosphorylated probe is taken into account (see Eq. [1]). The TInsP₅ preparation also contained an unidentified impurity with a t_R of about 37 min (Fig. 10), which was readily identifiable in chromatograms of the samples from the TInsP₅-containing Active PA and autoclaved Control assays.

The PA assays in Experiments 1 and 2 contained the same amount of assay buffer (5 mL) and TInsP₅ (i.e., 25 μ mol), but differed in the amount of soil by a factor of 2x. Given this particular scenario, conventional wisdom suggests that the rate of phytase-catalyzed dephosphorylation should be slower for Experiment 2 assays, which contained half the amount of phytase as did the PA assays in Experiment 1. This was clearly not the case however, as Experiment 2 assays actually exhibited greater PA (Fig. 12). The reason for this apparent anomaly has more to do with the [bioavailable TInsP₅] to [phytase] ratio than it does with the [total TInsP₅] to [phytase] ratio. The concentration of ‘phytase-susceptible’ substrate (Berry et al., 2009) increased with a decrease in the assay buffer:soil ratio, which presumably offset the decrease in phytase concentration. Thus,

PA increased when the concentration of TInsP₅ (on a per gram soil basis) increased from 20 μmol TInsP₅/g soil to 40 μmole TInsP₅/g soil, which was relative to the increase in the amount of substrate recovered from the solution phase increased (Fig. 13) with the corresponding reduction in the amount of (the sorptive-complexing component of) soil added to the reaction.

Experiment 3—Soil dilution technique

PA appeared to be approaching a maximal value at a substrate concentration of 80 μmol TInsP₅/g soil (Fig. 12). Further, PA is passably stable over a 24 h incubation time (Fig. 14). The information obtained from Experiment 3 established that PA assays involving the Clymer forest soil should be conducted with a substrate concentration of 80 μmol TInsP₅/g soil over a 16 h incubation time period. Furthermore, the amount of TInsP₅ recovered from the solution phase of the assay was over 95% (Fig. 13).

PA in the Clymer forest soil (determined using Eq. [1] w/o TInsP_y) was much greater than that observed for the Frederick agricultural or forest soils (compare data from Figure 14 – this study with data from Table 4 – Berry et al., 2009). One likely reason for this observed difference in PA is soil sampling depth. Soil sampling depth for the Frederick was 15 cm, as opposed to 8 cm for the Clymer soil. Sampling to a lower depth resulted in collecting a soil sample with less microbiological activity.

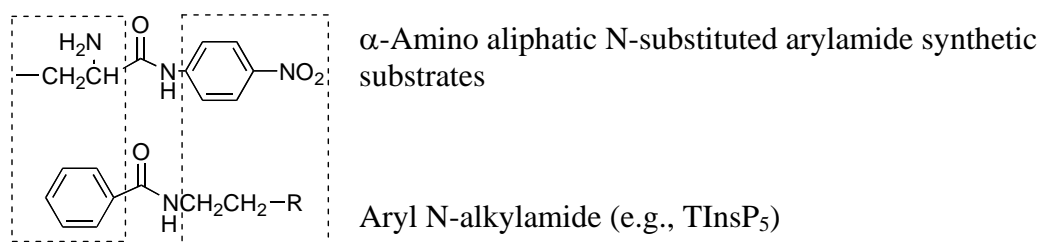
Measurement of soil PA at pH 5.4

Soil pH is likely an important factor when it comes to the inherent biochemical characteristics of microbial-produced phytases. Given that most fungal and bacterial phytases are classified as HAPs (Oh et al., 2004) it is likely that a competent microbe's response to acidic soil (i.e., pH < 7) would be to produce phytate-degrading enzyme(s) belonging to the HAPs family. The pH optimum for the acid phytate-degrading enzymes ranges from 4.5 to 6.0 (Konietzny and Greiner, 2002). It is reasonable to hypothesize that the general pH optimum of the HAPs produced by competent phytase-producing microbes in a given soil would tend to match the soil pH. Furthermore, it is reasonable to conclude that, the larger the difference between pH of the soil and assay buffer, the lower the measured value of PA. The results in Figure 15 appear to support this hypothesis.

The PA value for the Clymer soil (pH 3.7) measured at an assay pH of 4.2 was about 1.8 x greater than that measured at an assay pH of 5.4.

Chemical stability of probe's amide bond in Clymer soil suspension

The probe's amide bond should be resistant to arylamidase-catalyzed hydrolysis because the aryl group present in the probe is attached to the carbonyl carbon of the amide, not the nitrogen of the amide group, that is to say, the opposite orientation of that found in the enzyme's natural substrate and in the α -amino aliphatic N-substituted arylamide synthetic substrates, β -naphthylamide- and *p*-nitroanilide-derivatized L-amino acids (Berry et al., 2009) as illustrated below:



Further, arylamidase activity is expected to be low in soil slurry suspensions buffered at $\text{pH} < 6.0$ since this pH is well below the observed pH optimum for soil arylamidase activity, which is 8.0 (Tabatabai et al., 2002).

The TInsP₅ probe should be highly resistant to soil amidase (EC 3.5.1.4) activity because this enzyme, which exhibits activity against aliphatic amides (acetamide, propionamide), does not hydrolyze aryl amide bonds (e.g., benzamide) (Frankenberger and Tabatabai, 1980 and references therein; Jakoby and Fredericks, 1964).

Berry et al. (2009) established the use of TIns as a means to determine the fate of the probe's amide bond. Hydrolytic cleavage of probe's amide bond would result in the release of benzoic acid (Fig. 16). Addition of TIns directly to the soil suspension simplifies the analysis process since the fate of this non-phosphorylated form of the probe can readily be monitored using HPLC to measure benzoate production (Fig. 17).

The results in Table 3 support the hypothesis that the probe's amide bond is stable in highly active forest soil. A small amount of the TIns did disappear from the Active soil

suspensions (40 nmol/g soil) over the 24 h incubation time period. Based on the difference in benzoate content following 24 h of incubation, about half of the amount of the TIns that disappeared (i.e., 23 nmol/g soil) could be attributed to hydrolytic cleavage. A somewhat larger amount of TIns disappeared from the Active soil suspension following 24 h of incubation (650 nmol/g soil). The reason for the disappearance of TIns in the Control is unknown since there was no difference in benzoate content (Table 3). There was no benzoate detected in the background Controls.

Summary

Production of TInsP₃, the key intermediate (Berry et al., 2009), TInsP₄, and compound Y have been observed in all soils tested to date. The relevance of this observation is that PA comparisons across different soils would be facilitated given similar chromatographic profiles.

The experimental evidence supports inclusion of compound Y as a phosphorylated probe intermediate species (i.e. TInsP_y) based primarily on its UV adsorption characteristics. Identification of compound Z as a probe species could not be substantiated based on the evidence presented in this study.

Because the Clymer soil contained about twice the amount of organic C compared to Frederick soils tested by Berry et al. (2009), sorption of TInsP₅ was much greater, resulting in a lower solution phase concentration, which negatively impacted PA. The soil dilution technique was successfully applied to determine the appropriate substrate-to-soil ratio for achieving maximal PA.

The hypothesis stating that a substantial change in pH of the PA assay buffer compared to that of the soil pH, which would be considered optimum of microbial-produced phytate-degrading phosphatase, would result in a decrease in the observed PA, was apparently supported in this study.

The results from this study support the hypothesis that the probe's amide bond was stable in a forest soil exhibiting high PA.

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Tables

Table 1. Chemical and physical properties of Clymer forest soil.

Parameter	Forest Soil
Total P (mg kg ⁻¹) ¹	619 ± 2
Total N (mg kg ⁻¹) ²	0.35
Organic C (%)	7.29% ²
	6.18% ³
Sand (%)	16.5
Silt (%)	78.3
Clay (%)	5.2
Soil pH ⁴	3.72

¹Digestion with sulfuric acid-hydrogen peroxide-hydrofluoric acid (Bowman, 1988).

²Varil Max CNS Macro Elemental Analyzer (Elementar Analysensysteme, Germany).

³Walkley-Black method.

⁴1:1 Soil:Water.

Table 2. Determination of phosphorylated probe intermediate concentrations in Active and autoclaved Control soil suspensions – Experiment 1.

<u>Assay</u>	<u>Probe Concentration</u>	
	<u>16 h</u>	<u>40 h</u>
Active	<p>[TInsP₅]¹ = 3.52 mM Percent of starting [TInsP₅] = 80% [TInsP₄]_{Ext1} = 489.1 nmol/g soil [TInsP₄]_{Ext2} = 64.8 nmol/g soil Total [TInsP₄] = 553.9 nmol/g soil Δ[TInsP₄][§] = 365.4 nmol/g soil [TInsP₃]_{Ext1} = 442.9 nmol/g soil [TInsP₃]_{Ext2} = 59.4 nmol/g soil Total [TInsP₃] = 502.3 nmol/g soil [TInsP₂]_{Ext1} = 148.8 nmol/g soil [TInsP₂]_{Ext2} = 15.6 nmol/g soil Total [TInsP₂] = 164.4 nmol/g soil [TInsP₁]_{Ext1} = ND [TInsP_y]_{Ext1} = 210 nmol/g soil [TInsP_y]_{Ext2} = 7.4 nmol/g soil Total [TInsP_y] = 217.4 nmol/g soil Δ[TInsP_y] = 140.9 nmol/g soil [TIns] = 55.2 nmol/g soil</p>	<p>[TInsP₅][†] = 3.40 mM Percent of starting [TInsP₅] = 77% [TInsP₄]_{Ext1} = 526.7 nmol/g soil [TInsP₄]_{Ext2} = 61.2 nmol/g soil Total [TInsP₄] = 587.9 nmol/g soil Δ[TInsP₄][§] = 370.2 nmol/g soil [TInsP₃]_{Ext1} = 757.5 nmol/g soil [TInsP₃]_{Ext2} = 84 nmol/g soil Total [TInsP₃] = 841.5 nmol/g soil [TInsP₂]_{Ext1} = 369.4 nmol/g soil [TInsP₂]_{Ext2} = 36 nmol/g soil Total [TInsP₂] = 405.4 nmol/g soil [TInsP₁]_{Ext1} = 85.5 nmol/g soil [TInsP_y]_{Ext1} = 284 nmol/g soil [TInsP_y]_{Ext2} = 29.4 nmol/g soil Total [TInsP_y] = 313.4 nmol/g soil Δ[TInsP_y] = 233.2 nmol/g soil [TIns] = 324.2 nmol/g soil</p>
Control	<p>[TInsP₅][†] = 4.11 mM Percent of starting [TInsP₅] = 93% [TInsP₄]_{Ext1} = 169.3 nmol/g soil [TInsP₄]_{Ext2} = 19.2 nmol/g soil Total [TInsP₄] = 188.5 nmol/g soil [TInsP_y]_{Ext1} = 69.3 nmol/g soil [TInsP_y]_{Ext2} = 7.2 nmol/g soil Total [TInsP_y] = 76.5 nmol/g soil [TIns] = ND nmol/g soil</p>	<p>[TInsP₅][†] = 4.10 mM Percent of starting [TInsP₅] = 93% [TInsP₄]_{Ext1} = 194.9 nmol/g soil [TInsP₄]_{Ext2} = 22.8 nmol/g soil Total [TInsP₄] = 217.7 nmol/g soil [TInsP_y]_{Ext1} = 71.8 nmol/g soil [TInsP_y]_{Ext2} = 8.4 nmol/g soil Total [TInsP_y] = 80.2 nmol/g soil [TIns] = ND nmol/g soil</p>
Calculated PA	<p>PA (w/o TInsP_y) = 68 nmol/g soil/h PA (w/ TInsP_y) = 90 nmol/g soil/h</p>	<p>PA (w/o TInsP_y) = 50.7 nmol/g soil/h PA (w/ TInsP_y) = 57 nmol/g soil/h</p>

¹Nominal starting concentration of solution phase TInsP₅ was 5.0 mM. Actual starting concentration was 4.43 mM considering soil water content.

Table 3. HPLC analysis of benzoate in Active and autoclaved Control soil suspensions containing TIns.

<u>Sample</u>	<u>nmol Benzoic acid/g soil</u>	<u>μmol TIns/g soil¹</u>
T = 0, Active	--	4.43
T = 0, Active	7.14	--
T = 24h, Active	--	4.39
T = 24h Active	30.0	--
T = 0, Control	--	5.13
T = 0, Control	15.4	--
T = 24h, Control	--	4.48
T = 24h, Control	11.5	--
T = 0, Background Control	--	--
T = 0, Background Control	ND	--
T = 24h, Background Control	--	--
Control		
T = 24h, Background Control	Trace	--
Control		

¹Nominal starting concentration 4.0 μmol TIns/g soil.

Figures

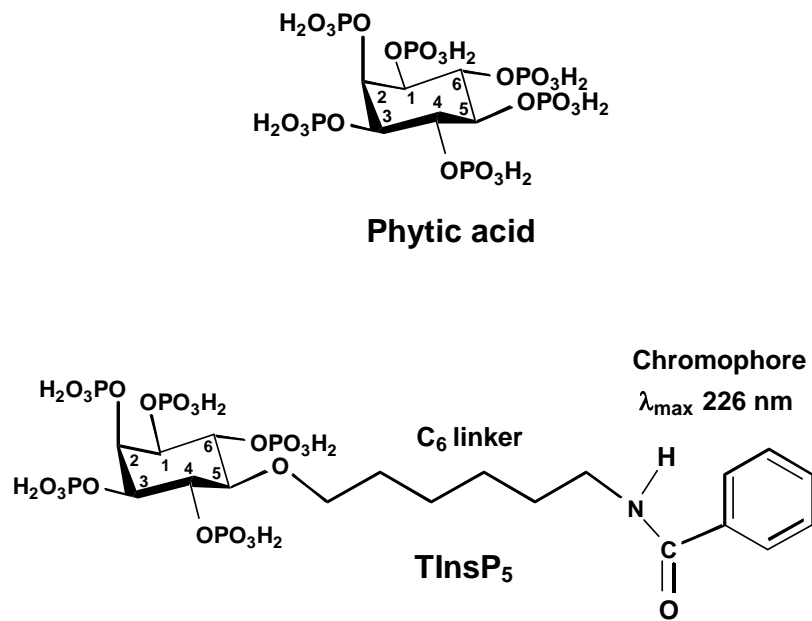


Figure 1. Molecular structures of phytic acid and the TInsP₅ probe.

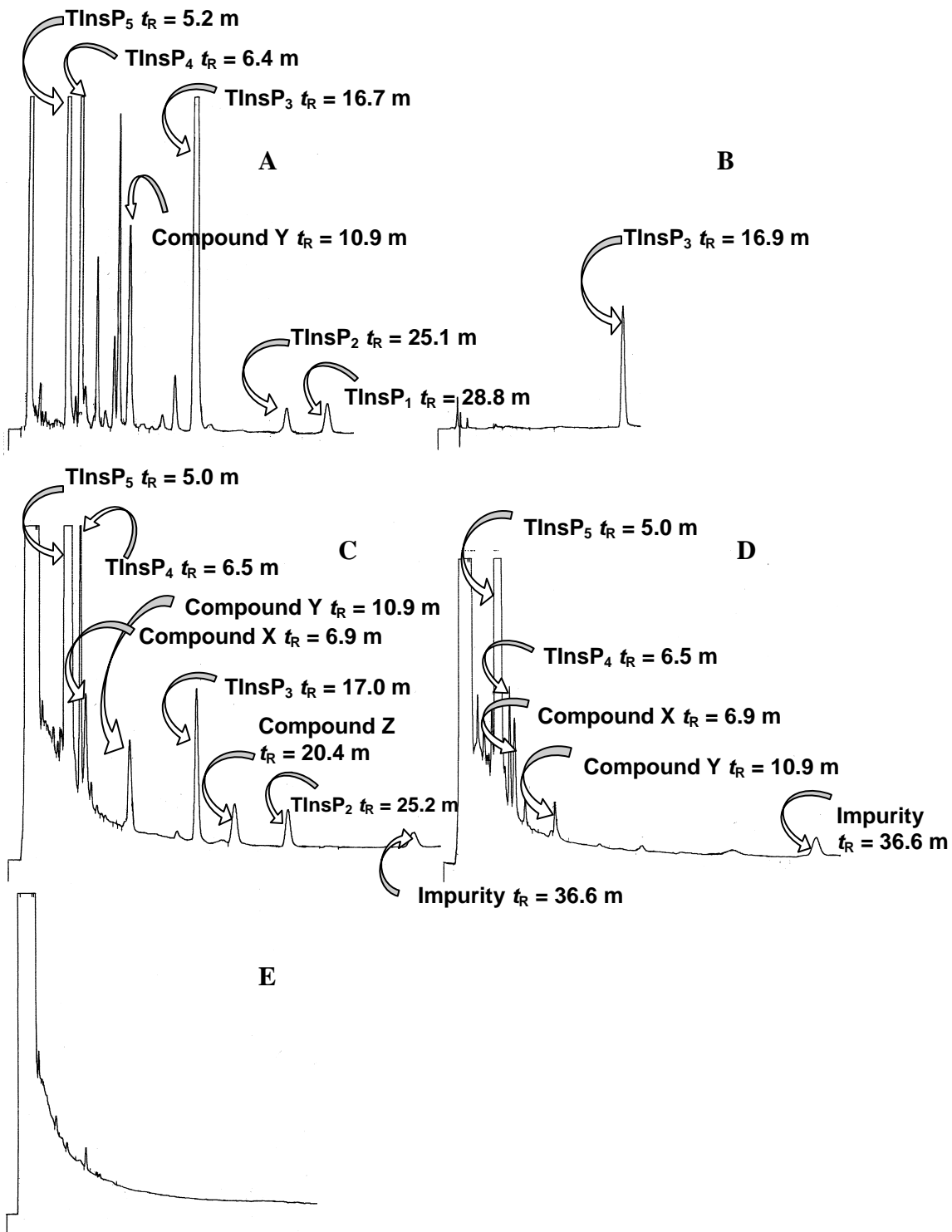


Figure 2. Chromatographic analysis of: a 1:2 dilution of the wheat-phytase stock standard – 80 min incubation time (A), 3-phytase generated TInSP₃ standard (20 μ M) (B), Active soil slurry following 16 h of incubation (C), Control TInSP₅-containing soil slurry following 16 h of incubation (D) and Active InsP₆-containing Control soil slurry following 16 h of incubation (E).

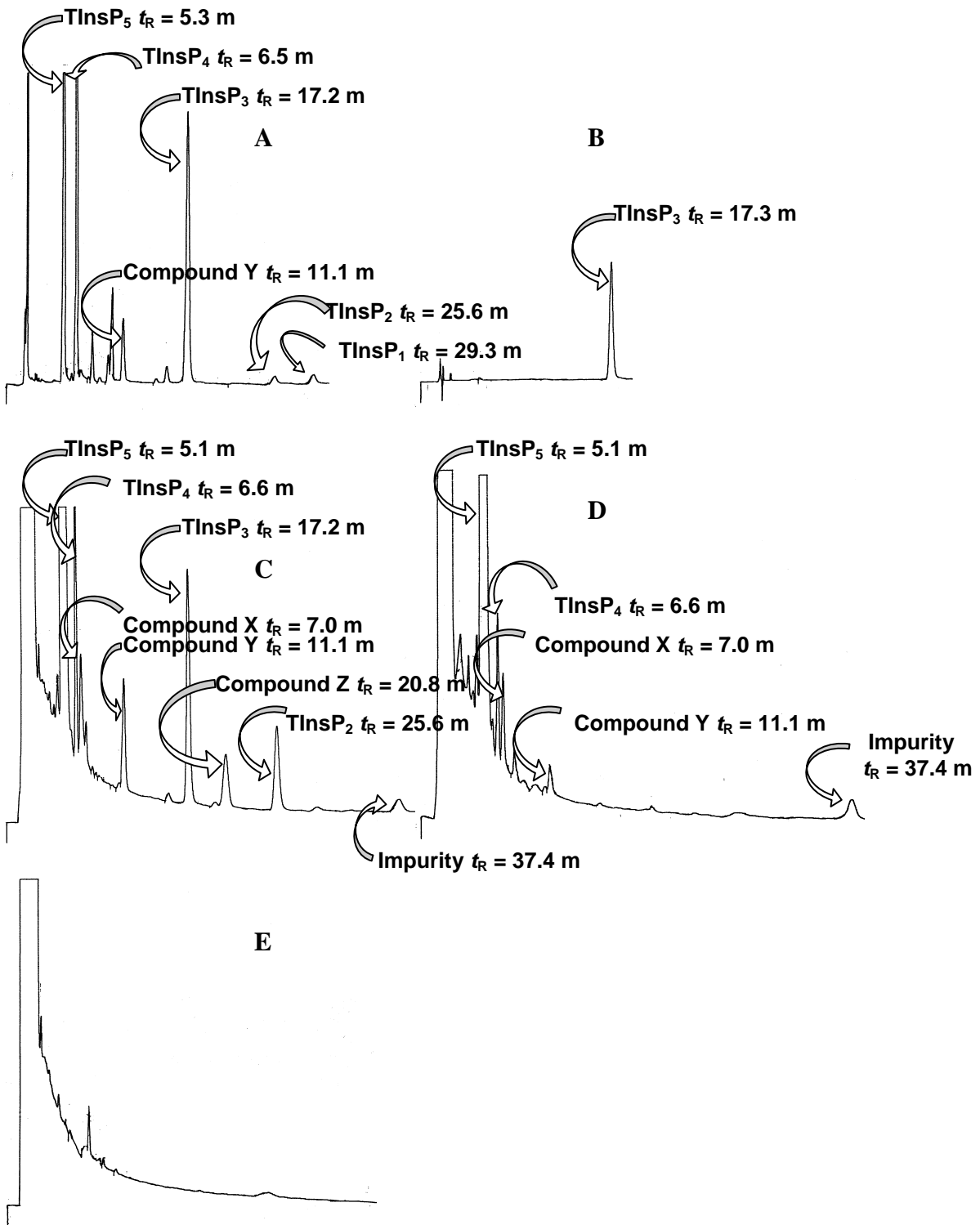


Figure 3. Chromatographic analysis of: a 1:4 dilution of the wheat-phytase standard – 80 min incubation time (A), 3-phytase generated standard (20 μ M) (B), Active TInSP₅ soil slurry following 40 h of incubation (C), Control TInSP₅-containing soil slurry following 40 h of incubation (D) and the Active InsP₆-containing Control soil slurry following 40 h incubation (E).

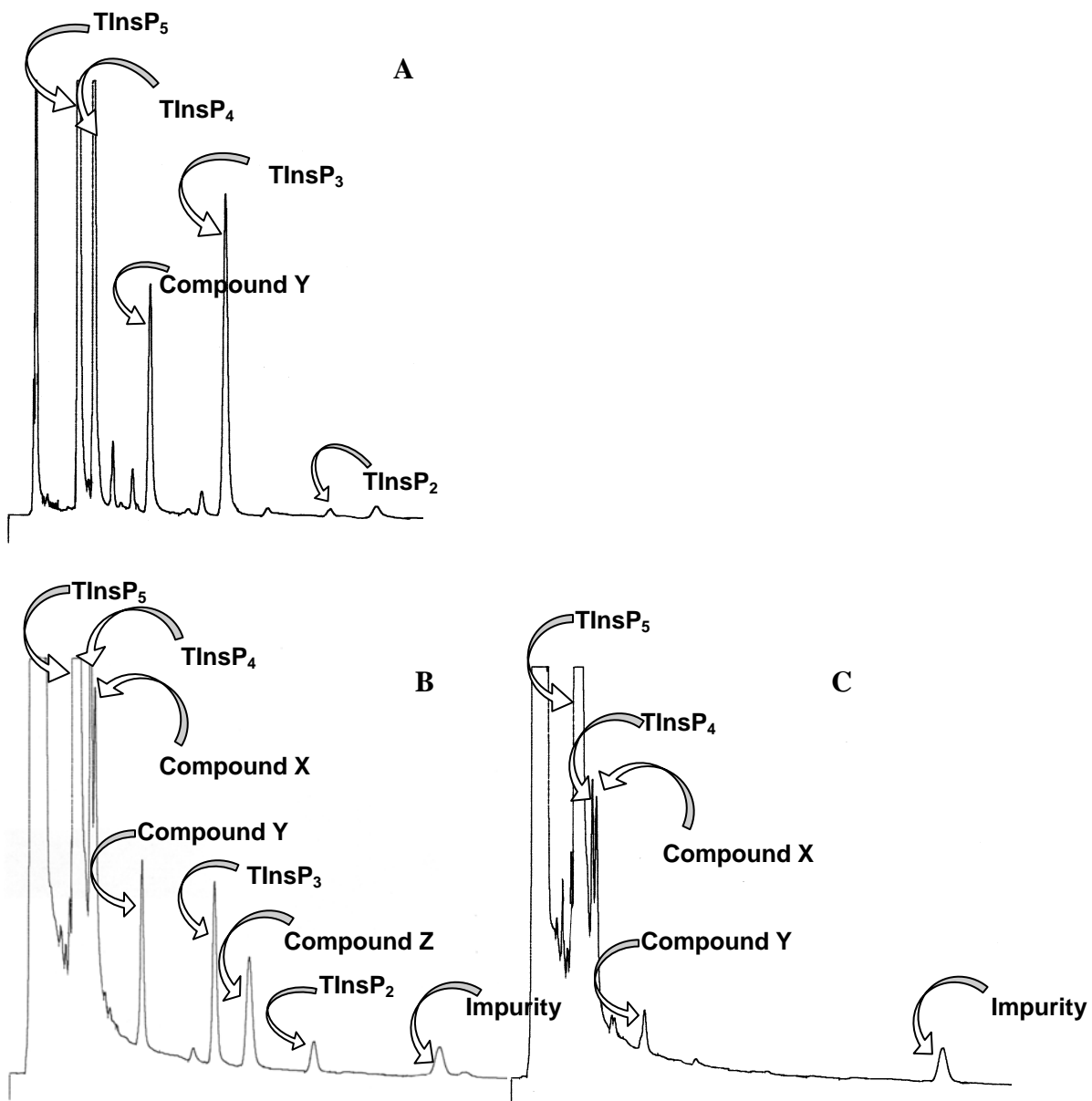


Figure 4. Chromatographic analysis of: wheat phytase generated standard (A) – 60 min incubation time, Active soil suspension (B) and autoclaved Control soil suspension (C).

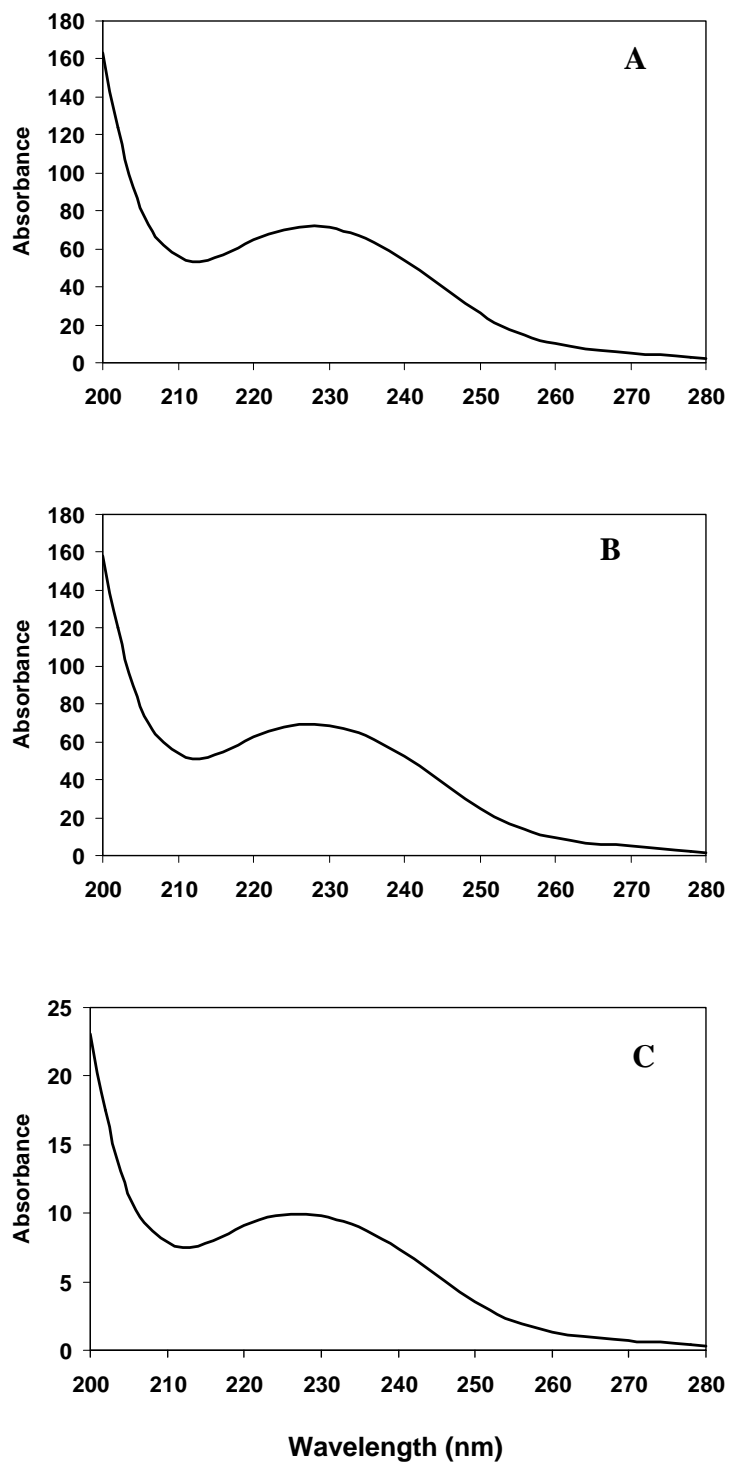
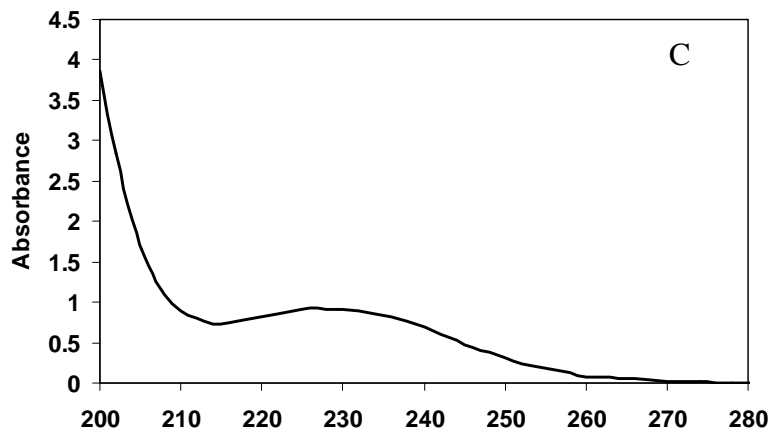
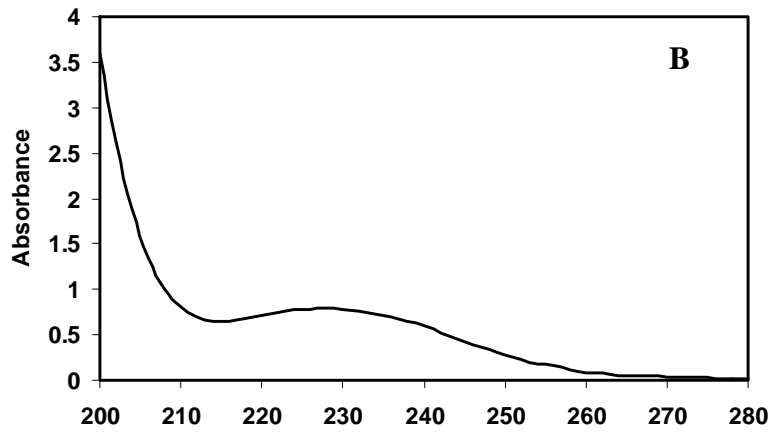
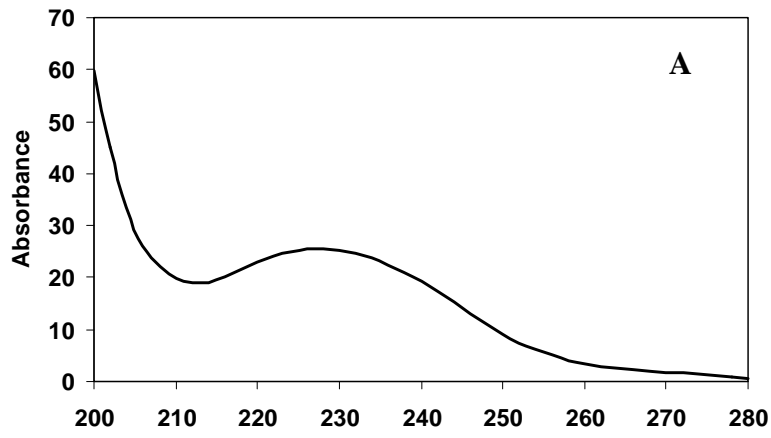


Figure 5. UV spectra recorded for TInsP₅ (A), wheat phytase generated TInsP₄ (B) and compound Y – TInsP_y (C).



Wavelength (nm)

Figure 6. UV spectra recorded for wheat phytase generated TInsP₃ (A), TInsP₂ (B) and TInsP₁ (C).

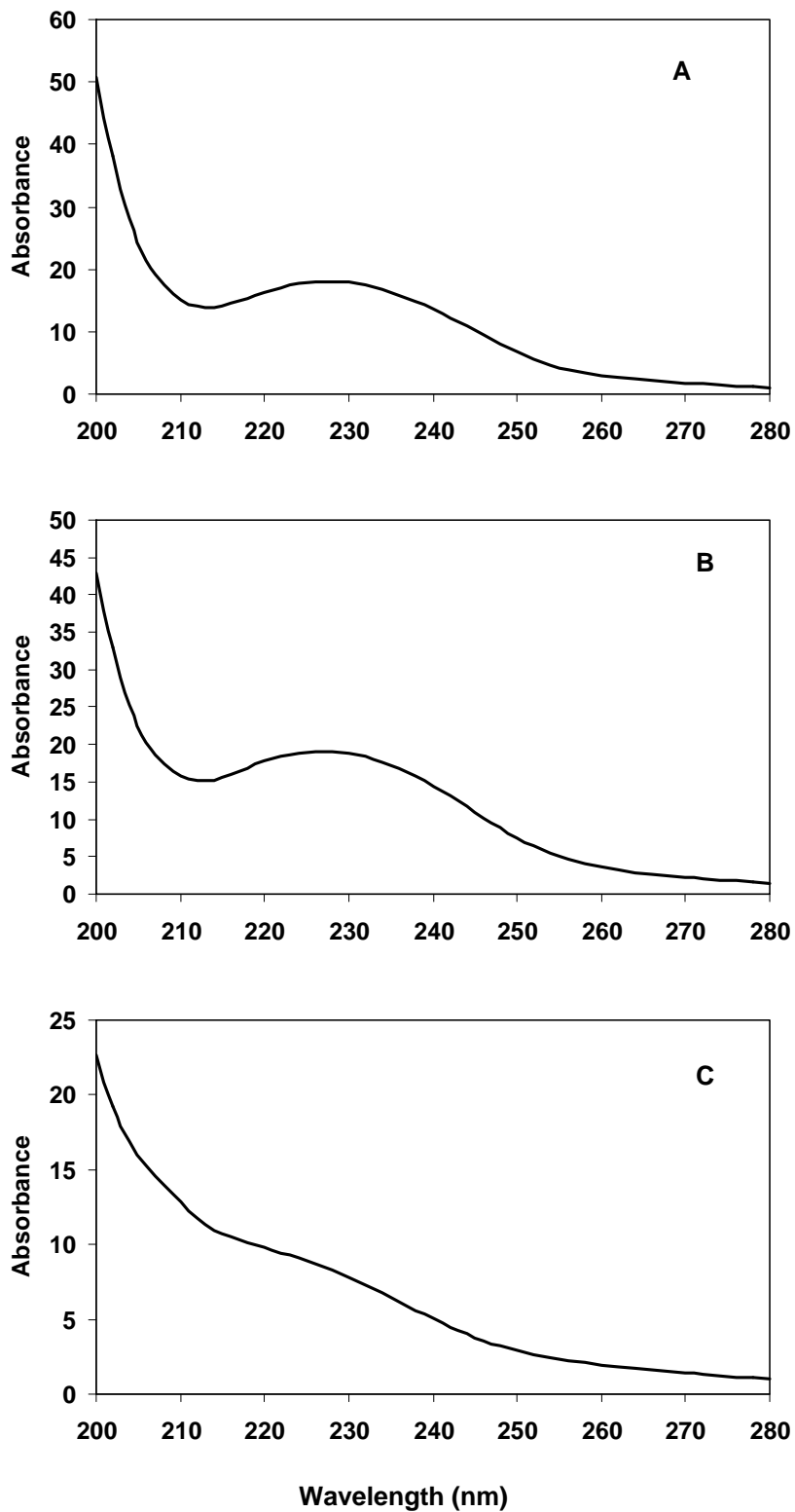


Figure 7. Results of HPLC-DAD analysis. The UV spectra recorded for the TIInsP₅ standard (A) and for the putative intermediate probe species, TIInsP₄ (B) and Compound X (C) from the Active soil suspensions.

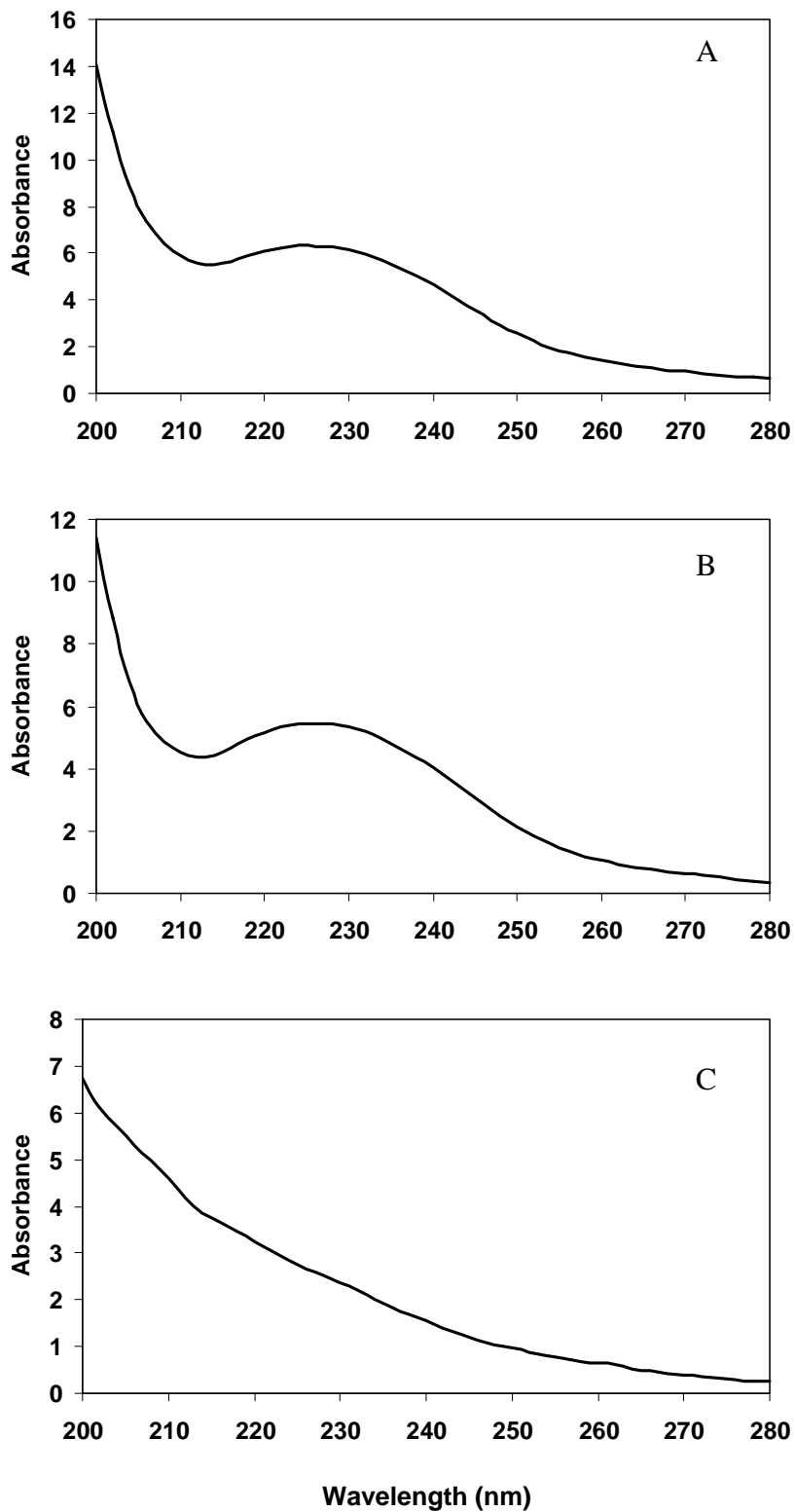


Figure 8. Results of HPLC-DAD analysis. The UV spectra recorded for putative intermediate probe species TInsP_γ (A), TInsP₃ (B) and Compound Z (C) from the Active soil suspensions.

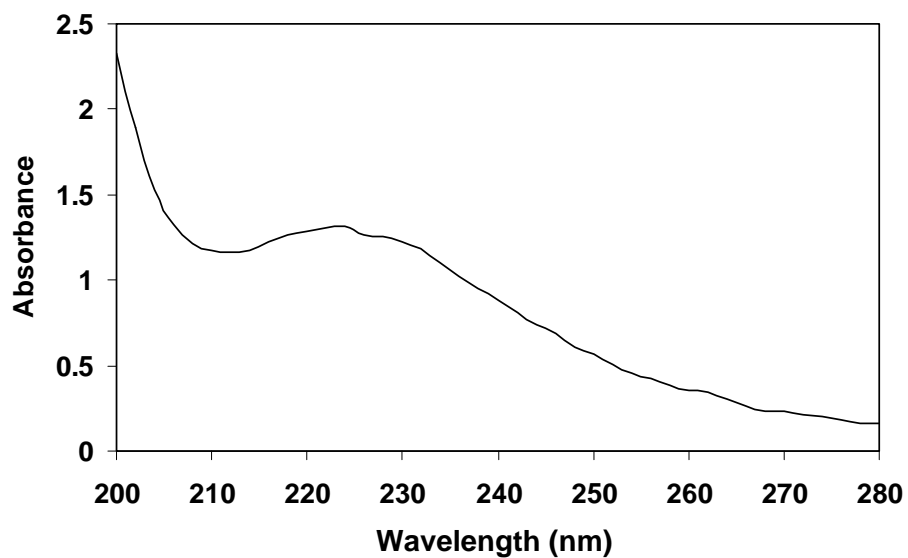


Figure 9. Results of HPLC-DAD analysis. The UV spectra recorded for putative intermediate probe species, TInsP₂ from the Active soil suspension.

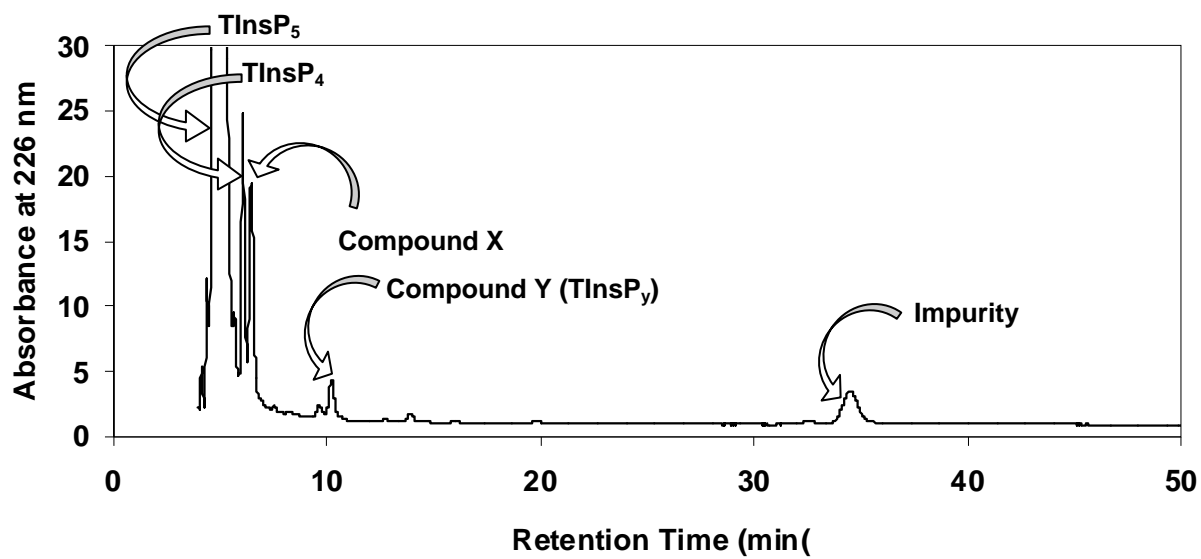


Figure 10. HPLC chromatogram of the TInsP₅ preparation (2.5 mM).

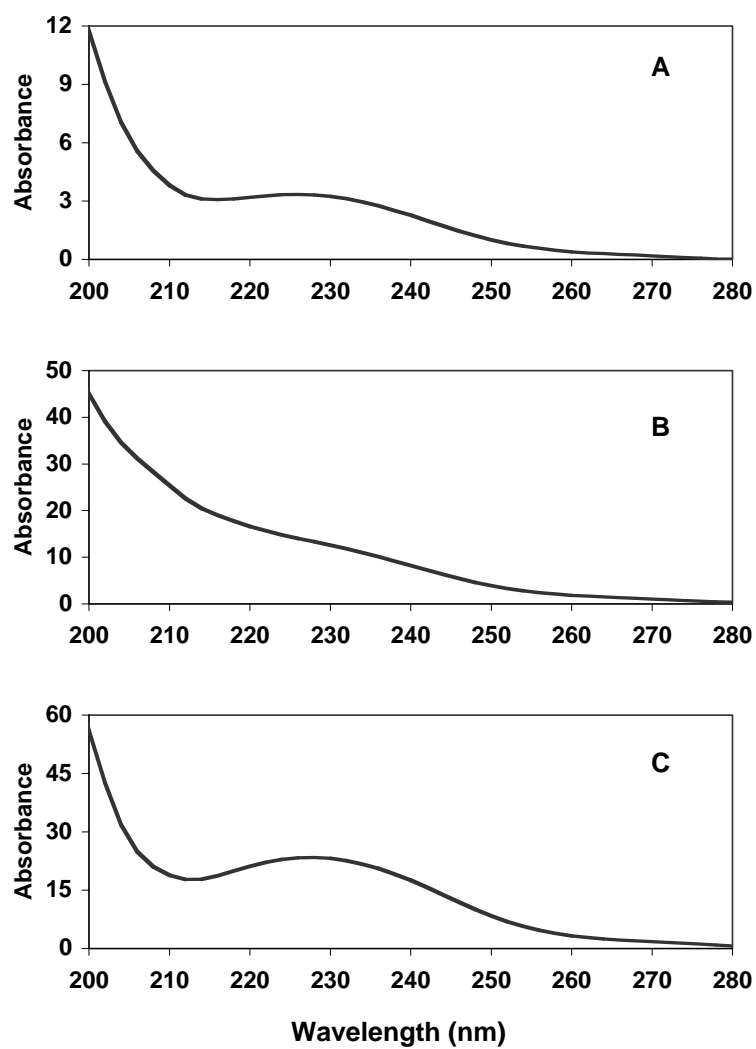


Figure 11. Recorded UV spectra for TInsP₅ preparation impurities: compound Y – TInsP_y (A), compound X (B) and TInsP₄ (C).

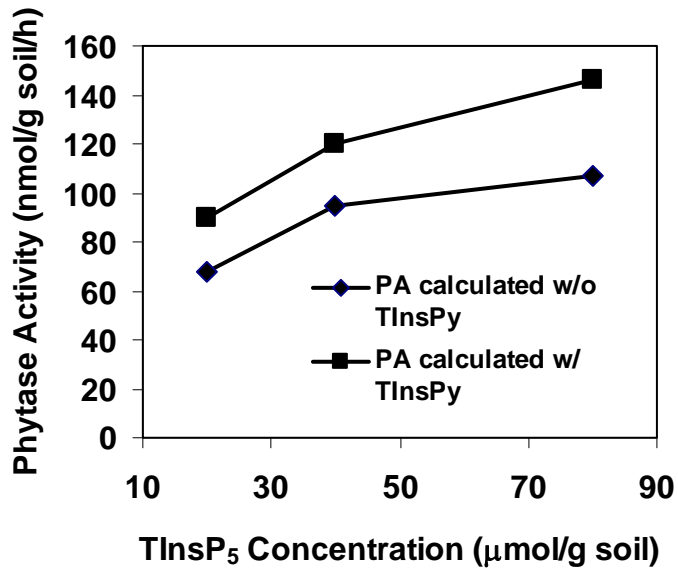


Figure 12. Results of the soil dilution investigation. The graph was developed by combining PA versus starting TInsP₅ concentration data obtained from Experiments 1, 2 and 3 (16 h incubations).

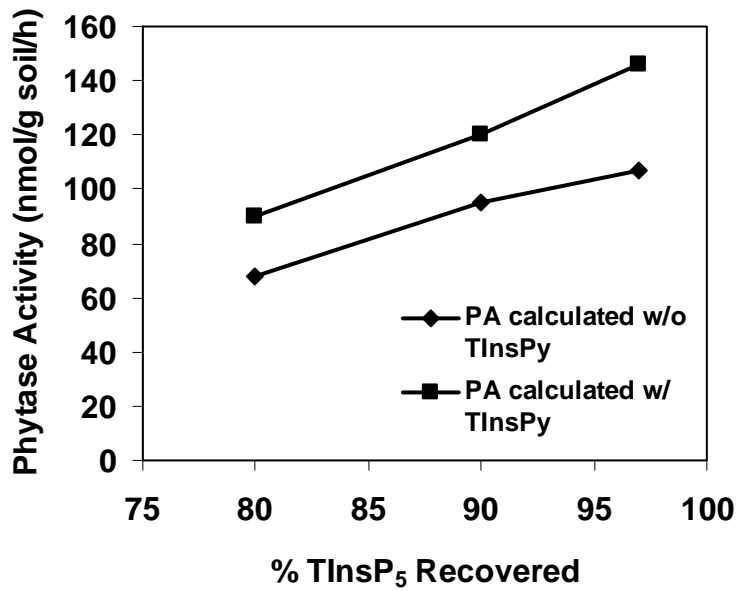


Figure 13. Results of the soil dilution investigation. The graph was developed by combining data obtained from Experiments 1, 2 and 3 (16 h incubations). Percent TInsP₅ recovered = (Solution phase [TInsP₅]_{T=16h}/Solution phase [TInsP₅]_{T=0h}) x 100.

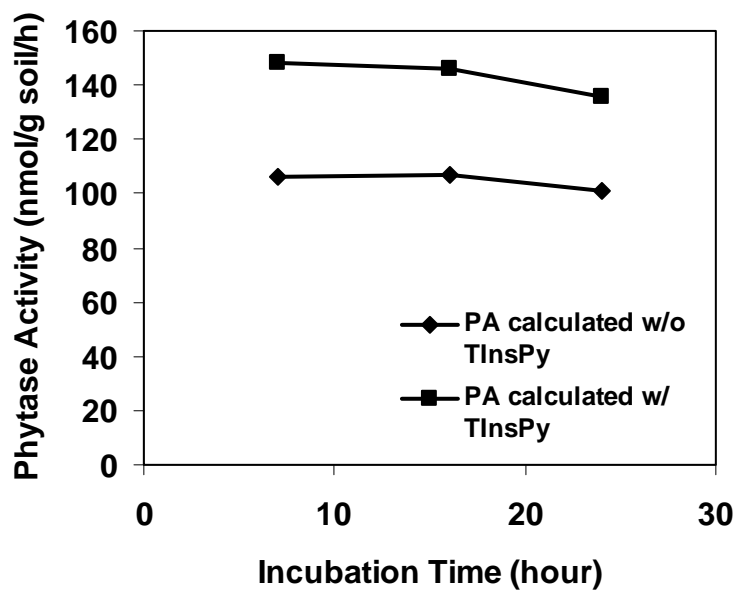


Figure 14. Influence of incubation time on PA.

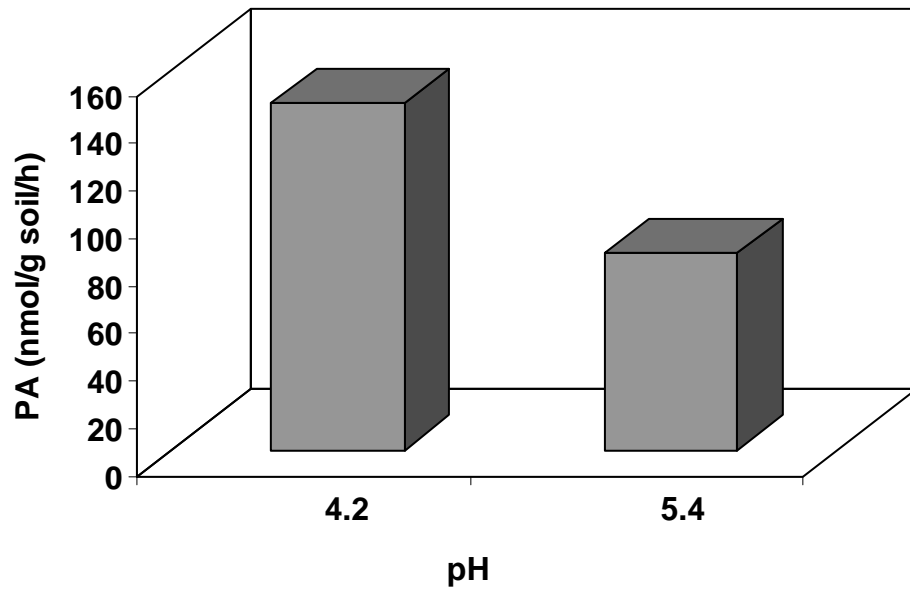


Figure 15. Influence of the assay buffer pH on PA.

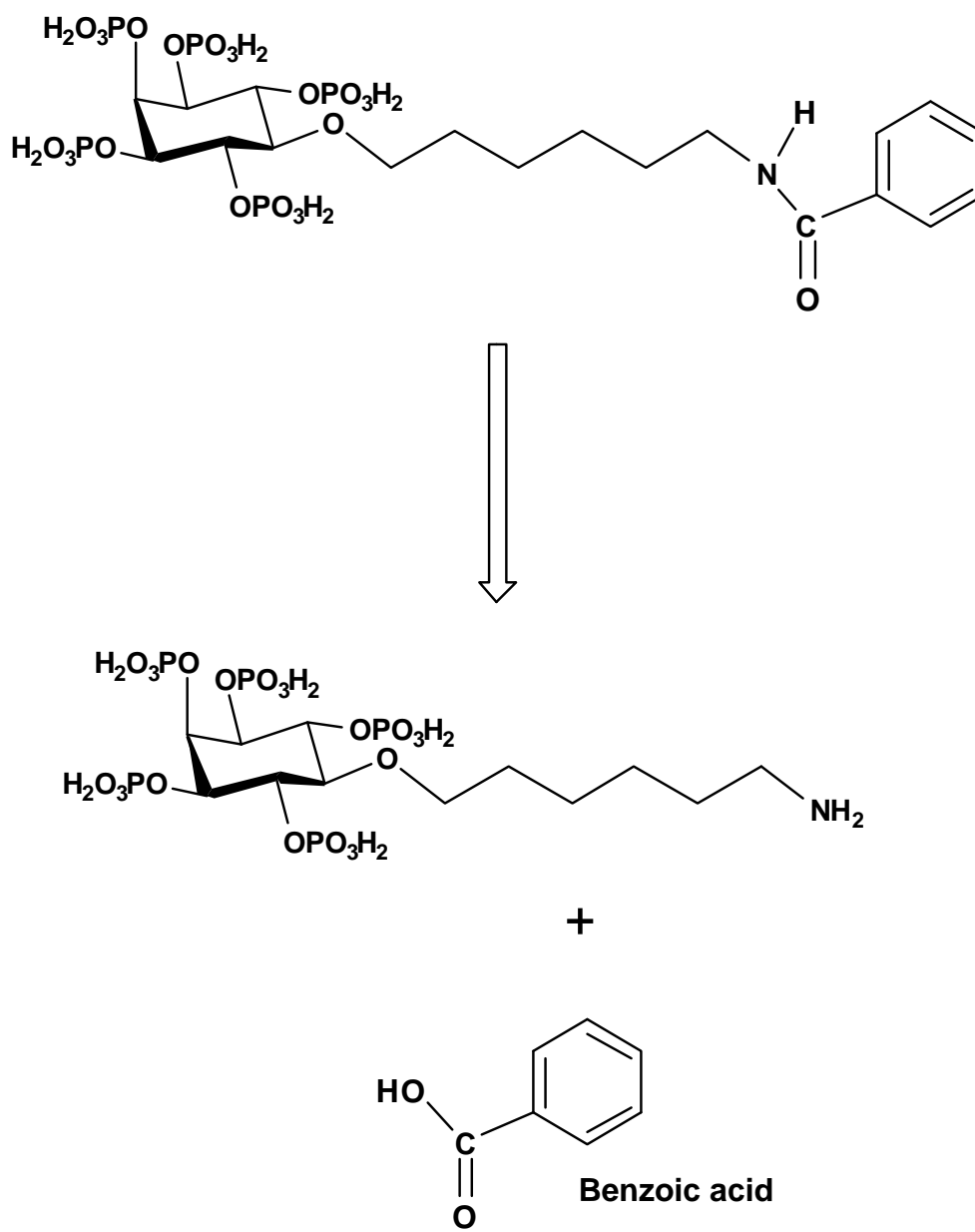


Figure 16. Hydrolytic cleavage of the TInSP₅ probe's amide bond with subsequent production of benzoic acid.

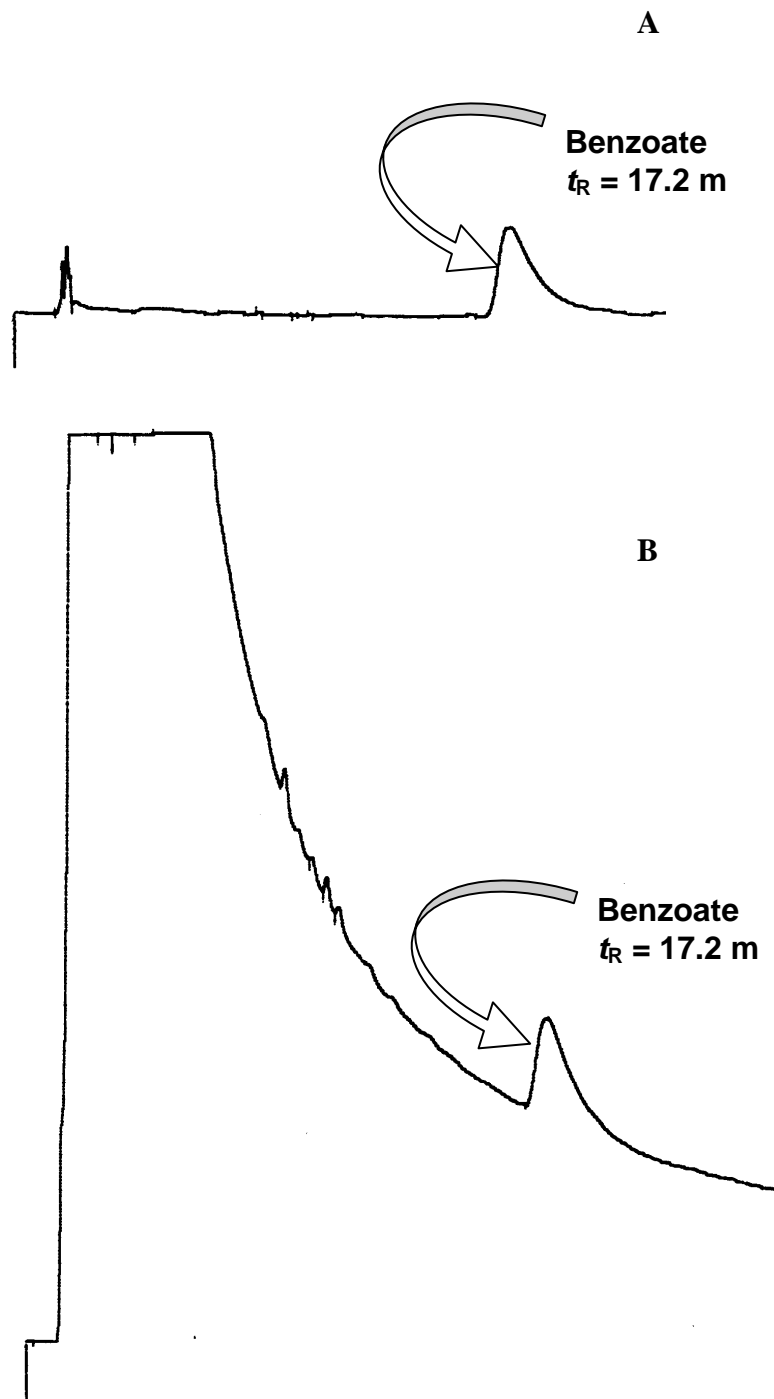


Figure 17. HPLC analysis of 5 μ M benzoate standard (A) and the Active soil suspension following 24 h incubation (B).

Appendix

TInsP₅ Probe Applications

The technique of using TInsP₅ as a probe is now reliable to measure phytase activity in soils. It is important to point out that the TInsP₅ molecular probe can also be used to measure phytase activity in other environmental samples such as water, sediments, and biosolids. It is also highly likely that the TInsP₅ probe could also be used to measure phytase activity associated with animal feed, rumen, intestinal contents and manure.

Phytase amended animal feed was prompted by the need to make more of the naturally occurring organic-P present in feed, primarily in the form of phytate-P, available to the animal and thus reduce P excretion and subsequent contamination of soil and open water, given that swine and poultry are unable to utilize phytate-P. The extent to which the added phytase generates improvements in P-availability in swine, poultry and cattle remains largely unsubstantiated. Measurement of phytase activity the TInsP₅ probe may be able to shed some light on this issue by: (i) serving as an inert marker which can be used in digestibility assays, (ii) tell the differences between ingredient types and (iii) show the dietary levels and effects of Ca-phytate-P versus non-phytate organophosphates.

Divalent and trivalent cations complexes with InsP₆ can influence the rate of phytase-catalyzed hydrolysis of the P group. Metal cations like Cu²⁺, Zn²⁺, Al³⁺ and Mg²⁺ tend to form 'phytase-resistant' species, while metal cations such as Mn²⁺ have a tendency to form 'phytase-susceptible' complex species. Issues relating to bioavailability of phytase can be more rapidly discerned using the TInsP₅ method for measuring phytases activity.

In most situations, feed grain for cattle is supplemented with inorganic P (i.e., orthophosphate) in order to ensure that the animal is receiving an ample supply of nutrient P. This practice usually results in release of excess nutrient P in manure. If the manure is land applied, which it commonly is, the question of whether or not amending soils with manure containing an excess of orthophosphate impacts phytase production by soil microorganisms. It is reasonable to conclude that the TInsP₅ method for measuring phytase activity can provide the answer to this question.