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TITLE:

Extraction and Quantification of Soluble, Radiolabeled Inositol Polyphosphates from Different Plant Species using SAX-HPLC

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Strong anion exchange chromatography (SAX)-HPLC, radiolabeling, *Arabidopsis thaliana*, inositol polyphosphates (InsPs), inositol pyrophosphates (PP-InsPs), signaling molecules, *Lotus japonicus,* plant defense, jasmonate perception, phosphate starvation response, energy homeostasis

SUMMARY:

Here we describe strong anion exchange high-performance liquid chromatography of [^{3}H]-*myo*-inositol-labeled seedlings which is a highly sensitive method to detect and quantify inositol polyphosphates in plants.

ABSTRACT:

The phosphate esters of *myo*-inositol, also termed inositol phosphates (InsPs), are a class of cellular regulators playing important roles in plant physiology. Due to their negative charge, low abundance and susceptibility to hydrolytic activities, the detection and quantification of these molecules is challenging. This is particularly the case for highly phosphorylated forms containing 'high-energy' diphospho bonds, also termed inositol pyrophosphates (PP-InsPs). Due to its high sensitivity, strong anion exchange high-performance liquid chromatography (SAX-HPLC) of plants labeled with [^{3}H]-*myo*-inositol is currently the method of choice to analyze these molecules. By using [^{3}H]-*myo*-inositol to radiolabel plant seedlings, various InsP species including several non-enantiomeric isomers can be detected and discriminated with high sensitivity. Here, the setup of a suitable SAX-HPLC system is described, as well as the complete workflow from plant cultivation, radiolabeling and InsP extraction to the SAX-HPLC run and subsequent data analysis. The protocol presented here allows the discrimination and quantification of various InsP species, including several non-enantiomeric isomers and of the PP-InsPs, $InsP_7$ and $InsP_8$, and can be easily adapted to other plant species. As examples, SAX-HPLC analyses of *Arabidopsis thaliana* and *Lotus japonicus* seedlings are performed and complete InsP profiles are presented and discussed. The

method described here represents a promising tool to better understand the biological roles of InsPs in plants.

INTRODUCTION:

Almost four decades ago, inositol phosphates (InsPs) emerged as signaling molecules, after Ins(1,4,5)P_3 ($InsP_3$) was identified as a second messenger that activates the receptor-mediated release of Ca^{2+} in animal cells[1,2]. To date, no $InsP_3$ receptor (IP3-R) has been identified in plants, which questions a direct signaling role for $InsP_3$ in plant cells[3]. Regardless, $InsP_3$ serves as a precursor for other InsPs involved in several plant developmental processes, including the regulation of specific signaling pathways[3-8]. For instance, $InsP_3$ can be further phosphorylated to $InsP_6$, also known as "phytic acid", which represents a major source of phosphate, *myo*-inositol and cations, and was shown to play key roles in plant defense against pathogens, mRNA export and phosphate homeostasis[5,9-12].

Inositol pyrophosphates (PP-InsPs) are a class of InsPs that contain at least one high-energy di-phospho bond, initially identified in animal cells, amoeba and yeast, where they play critical roles in various cellular processes[13-15]. Despite seminal work on PP-InsPs in plants [16-26], the biological functions and isomer identity of these molecules still remain largely enigmatic. In the model plant *Arabidopsis thaliana*, cellular $InsP_8$ was proposed to regulate defenses against insect herbivores and necrotrophic fungi via coincidence-detection of $InsP_8$ and active jasmonate by the ASK1-COI1-JAZ receptor complex[17]. Furthermore, roles of $InsP_8$ and other PP-InsPs in energy homeostasis and nutrient sensing, as well as phosphate homeostasis have been proposed[17,23-26].

Regardless of the biological system employed, one major methodological challenge when studying InsPs has been their precise measurement and quantification. Mass spectrometry-based methods have been used to detect InsPs including PP-InsPs from cell extracts, but those studies failed to differentiate distinct isomers[26,27]. Another approach to analyze InsPs employs pull-down of InsPs from cell lysates using TiO_2 beads, followed by polyacrylamide gel electrophoresis (PAGE) of the eluted InsPs. The InsPs can then be stained by either toluidine blue or DAPI[24,28,29]. However, it is so far not possible to reliably detect InsPs lower than $InsP_5$ from plant extracts using this method. Recently, a method using [^{13}C]-*myo*-inositol for nuclear magnetic resonance (NMR) analysis of InsPs was published as an alternative to strong anion exchange high-performance liquid chromatography (SAX-HPLC), the method described here[30]. This technique has been reported to achieve a similar sensitivity compared to SAX-HPLC and to allow the detection of 5-$InsP_7$, as well as the discrimination of different non-enantiomeric $InsP_5$ isomers from cell extracts. To implement this method, however, one requires chemically synthesized and commercially not available [^{13}C]-*myo*-inositol. Therefore, the method employed in most cases is radiolabeling samples with [^{3}H]-*myo*-inositol, followed by SAX-HPLC[31-33]. This technique is based on the uptake of radioactive *myo*-inositol into the plant and its conversion into different InsPs by the combined activity of dedicated cellular kinases and phosphatases.

The [^{3}H]-labeled InsPs are then acid-extracted and fractionated using SAX-HPLC. Because of their negative charge, the InsPs strongly interact with the positively charged stationary phase of the SAX-HPLC column and can be eluted with a buffer gradient containing increasing phosphate

concentrations to outcompete InsPs from the column. Elution times thus depend on charge and geometry of the InsP species to be separated. In the absence of chiral columns, only non-enantiomeric isomers can by separated by this protocol. However, radiolabeled standards can be used to assign the isomeric nature of a specific InsP peak. Multiple efforts in the past by various laboratories to generate labeled and unlabeled standards with (bio)chemical methods or to purify them from various cells and organisms have helped assigning peaks to certain InsP species, and also to narrow down the isomeric identity of individual InsP species[5,7,21,34-43]. Also, the recent elucidation of enzymatic pathways leading to the formation of PP-InsPs in plants, as well as the discovery of a bacterial type III effector with a specific 1-phytase activity, provide information on how to generate useful standards for these analyses[10,17,18,22,23].

The resulting fractions can be measured in a liquid scintillation counter due to the β-decay of tritium (^{3}H). With increasing labeling time, a steady-state isotopic equilibrium is reached, after which the obtained InsP profiles should represent the InsP status of the plant[31]. The major advantage of this protocol in comparison to other available techniques is the high sensitivity achieved by the use of the direct precursor for InsPs and the measurement of a radioactive signal.

SAX-HPLC of samples extracted from [^{3}H]-*myo*-inositol-labeled plants or other organisms is commonly used for the detection and quantification of InsPs ranging from the lower InsP species to the PP-InsPs, representing a valuable tool to better understand the metabolism, function and modes of action of InsPs. So far, this method is also the most appropriate choice for researchers with special interest in lower InsP species. While the basics of this procedure, on which the protocol described here builds on, have been previously described[7,21,31,34], a detailed protocol tailored to the analysis of plant-derived InsPs and especially of PP-InsPs is still missing. Previous publications reported difficulties to reliably detect the low abundant PP-InsPs, especially $InsP_8$, due to their use of the following components: relatively low amounts of plant material, [^{3}H]-*myo*-inositol with low specific activity (> 20 Ci/mmol), different extraction buffers that are either not based on perchloric acid or are less concentrated than 1 M, different neutralizing buffers, as well as sub-optimal gradients or detection of ^{3}H with an on-line detector. In comparison to those studies, the protocol presented here is designed for the reliable detection of PP-InsPs[7,21,34].

Here we present a detailed workflow, starting from the setup of the equipment to plant cultivation and labeling, InsP extraction and the SAX-HPLC run itself. Although the method was optimized to the model plant *A. thaliana*, it can be easily modified to study other plant species, as shown here with the first reported InsP profile of the model legume *Lotus japonicus*. Although the use of a different plant species might require some optimization, we envisage that those will be minor, making this protocol a good starting point for further research in plant InsPs. In order to facilitate possible optimizations, we indicate every step within the protocol in which modifications are possible, as well as all critical steps that may be challenging when establishing the method for the first time. Additionally, we report how data obtained by this method can be used for the quantification of specific InsPs and how different samples can be analyzed and compared.

PROTOCOL:

1. Setting up the HPLC system

1.1. Set up a system consisting of two independent HPLC pumps (binary pump), one for each buffer. Both pumps need to be controlled together via a computer with respective software or by having a master pump. Implement a piston seal wash for both pumps, either via gravitational force or through a third low pressure pump. Designate one pump for buffer A (termed pump A) and one for buffer B (termed pump B).

NOTE: Both have to be able to generate pressures up to 60 bar (6 MPa) and flow rates of at least 0.5 mL/min.

1.2. Connect both pumps to a dynamic mixer.

1.3. Connect the mixer to an injection valve with a sample loop of at least 1 mL capacity.

1.4. Connect the injection valve to the column with a capillary via the corresponding end fittings.

1.5. Connect the column to the fraction collector by using a capillary with an appropriate length.

NOTE: This description is based on our HPLC system (see the **Table of Materials**), which requires more manual steps than newer and more sophisticated systems (e.g., Waters Alliance HPLC systems). Our system allows easy access and modification of all components. Quaternary pumps (with the binary gradient described here) can also be used and will lead to elution profiles and overall quality of the analyses similar to those achieved with binary pumps.

2. Preparation of buffers, column and HPLC system

2.1. Prepare the buffers for the extraction of soluble InsPs: extraction buffer (1 M $HClO_4$) and neutralization buffer (1 M K_2CO_3). Prepare both buffers with ultra-pure deionized water. They are stable at room temperature for several months. Immediately prior to extraction, add EDTA to both solutions to a final concentration of 3 mM (e.g., from a filtered 250 mM EDTA stock solution).

CAUTION: $HClO_4$ (perchloric acid) is strongly corrosive.

2.2. Prepare the buffers for the SAX-HPLC run: buffer A (1 mM EDTA) and buffer B (1 mM EDTA, 1.3 M $(NH_4)_2HPO_4$; pH 3.8 with H_3PO_4). Prepare both using ultra-pure deionized water followed by vacuum filtration with 0.2 µm pore-sized membrane filters. These are stable at room temperature for several months.

NOTE: EDTA should be included in all buffers to prevent the formation of insoluble InsP salt complexes and binding of cations to the column.

2.3. Program the gradient as follows: 0–2 min, 0% buffer B; 2–7 min, up to 10% buffer B; 7–68 min, up to 84% buffer B; 68–82 min, up to 100% buffer B; 82–100 min, 100% buffer B, 100–101 min, down to 0% buffer B; 101–125 min, 0% buffer B. The optimal flow-rate for this gradient is 0.5 mL/min.

2.3.1. During the run, collect fractions every minute, starting from minute 1 to minute 96. The remaining 30 min of the gradient serve to wash the column and the system, and do not have to be collected for scintillation counting.

2.4. If possible, set the maximal reachable pressure before the emergency shutdown of the HPLC pumps to 80 bar (8 MPa). This prevents **critical damage** to the column's resin.

2.5. When using a new SAX HPLC column, **wash** it **thoroughly** (>50 mL) with filtered ultra-pure deionized water before the first use.

NOTE: This will ensure removal of the contained methanol, thus preventing salt precipitation in later steps. If possible, use a separate HPLC pump. If this is not available, make sure that the HPLC has flushed with water before washing the column. The flow-rate should not exceed 2 mL/min. After washing, the column is ready for the analysis and, when properly handled, can be **used for 20–40 runs**. After that, the resolution will successively decrease. Prolonged washing with buffer A (>1 h) and performing step 2.6 can help increase the lifetime of the column. If the decrease in resolution persists, the column needs to be exchanged. The gradient can be adjusted to increase the separation between specific inositol polyphosphate species or to decrease the overall runtime. Using different HPLC systems (with different void volume or different volume of the capillaries) will strongly affect the retention times. Also, column changes have minor effects on the retention times.

2.6. Perform a "mock run". Instead of an extracted sample, inject filtered ultra-pure deionized water in the HPLC system and run the standard gradient. The fractions do not have to be collected.

NOTE: Step 2.6 is optional. However, it should be performed if one of the following situations apply: A new column is installed; The HPLC system has been used for a different method beforehand; The HPLC system has not been used for longer than 3 days.; There was a problem with the preceding run.

3. Plant cultivation and labeling with [^{3}H]-*myo*-inositol

NOTE: The following steps should be performed with **sterile components** and under **sterile conditions**, while wearing gloves to protect hands from contamination with the radiolabel. Plant media, especially when containing sucrose, are prone to microbial contamination.

3.1. Sterilize *A. thaliana* seeds with 1 mL of 1.2% sodium hypochlorite for 3 min followed by 1 mL of 70% ethanol for 3 min. Then add 1 mL of 100% ethanol, pipette the seeds with the ethanol onto a circular filter paper and allow them to air-dry under a laminar-flow on a clean bench.

3.1.1. When using *L. japonicus* seeds, place them in a mortar and scrub seeds with sandpaper before sterilization to ensure a sufficient germination rate.

3.2. Sow out *Arabidopsis* seeds in 1–2 rows on square Petri dishes filled with solid growth media consisting of half-strength Murashige and Skoog (MS) salt solution, 1% sucrose, 0.7% gellan gum in deionized water adjusted to pH 5.7 with KOH and allow them to stratify for at least 1 day at 4 °C in the dark.

3.2.1. For *Lotus* seeds, sow them out in 1 row on square Petri dishes filled with solid growth media consisting of 0.8% bacteriological agar in deionized water and allow them to stratify for at least 3 days at 4 °C in the dark.

3.3. Place the plates vertically in a growth incubator or climate chamber and allow them to grow for 10–12 days under short-day conditions (8 h light at 22 °C, 16 h dark at 20 °C).

3.4. Transfer 10–20 seedlings into one well of a 12-well clear flat-bottomed cell culture plate filled with 2 mL of half-strength MS salt solution supplemented with 1% sucrose and adjusted to pH 5.7.

3.5. Add 45 µCi of [^{3}H]-*myo*-inositol (30–80 Ci/mmol, dissolved in 90% ethanol) and mix by gentle swirling. Cover the plate with the corresponding lid and seal it with microporous surgical tape (e.g., micropore or leucopore tape), placing it back into the growth incubator.

CAUTION: ^{3}H is a low-energy beta emitter that can be a harmful radiation hazard when inhaled, ingested or absorbed through bare skin. **Always** wear gloves when handling radioactive material or equipment that has direct or indirect contact to radioactive material. Also follow the local rules for safe handling of radiochemicals (e.g., wearing additional protective clothes, use of a dosimeter and surveys of surfaces for contaminations on a regular basis).

3.6. After 5 days of labeling, remove seedlings from the media and wash them briefly with deionized water. Dry them with paper towels and transfer them into a 1.5 mL microcentrifuge tube. **Do not overfill** the tube, and place no more than 100 mg FW/tube, which corresponds to approximately 10–20 17-day-old seedlings.

NOTE: An excess of plant material will dilute the acid during the extraction process and will strongly decrease the extraction efficiency.

3.6.1. Snap-freeze the tube in liquid nitrogen and store it at -80 °C until extraction.

NOTE: Samples can be kept at -80 °C for several weeks without compromising sample quality. The growth conditions (media, light, temperature, time) can be modified according to the needs of a specific experiment or plant species. However, care should be taken when diluting the [^{3}H]-*myo*-inositol, in order to ensure quantifiable SAX-HPLC runs of good quality. Therefore, it is recommended to start with the [^{3}H]-*myo*-inositol concentrations stated here and reduce it stepwise if desired. During labeling time, plants can be submitted to different treatments (e.g., environmental stresses or chemical agents) to assess the impact of those conditions on global InsPs. To reach steady-state labeling, we recommend to label plants for at least 5 days.

4. Extraction of soluble InsPs

NOTE: Keep samples and reagents on ice during the whole extraction process. **Always wear gloves and protective glasses** due to the high risk of contact with radioactive material, especially during grinding. Everything that gets in contact with samples is considered as **radioactive waste** and should be disposed of according to the local rules for safe disposal of radioactive material.

4.1. Prepare the working solutions for the extraction and neutralization buffer as in step 2.1. Each sample will require 600 µL of extraction buffer and 400 µL of neutralization buffer. Store the buffers on ice.

4.2. Take the samples from -80 °C freezer and keep them in liquid nitrogen until further processing. Grind the samples with a microcentrifuge tube pestle until they start thawing and add 500 µL of ice-cold extraction buffer. Continue grinding until sample is completely homogenized and the solution has a deep green color (when leaves are present in the sample).

4.3. Centrifuge the samples for 10 min at 4 °C at ≥ 18000 x *g*. Transfer the supernatant into a fresh 1.5 mL tube. Keep in mind that the tubes used for extraction are considered solid radioactive waste and need to be disposed of accordingly.

4.4. **Carefully** add 300 µL of neutralization buffer to the extract. Precipitation of proteins and bubbling will start immediately. Mix by swirling with a pipette tip after a minute and wait for a seconds before pipetting a small amount (5 µL) on pH paper (ideally range of pH 6–9). The pH should be between pH 7 and 8 in the end.

4.4.1. If necessary, add small amounts (typically 10–20 µL) of either neutralization buffer or extraction buffer until the desired pH is reached. Let the samples rest on ice for at least 1 h with an open lid.

4.5. Centrifuge the samples for 10 min at 4 °C at ≥ 18,000 x *g*. Transfer the supernatant into a fresh 1.5 mL tube.

NOTE: The samples can be either directly used in a SAX-HPLC run or kept on ice (if used later on the same day) or frozen in liquid nitrogen and stored at -80 °C for 2–4 weeks. To ensure a high reproducibility and comparability, it is recommended to always freeze the samples in liquid

nitrogen for 5 min, even if they will be directly used afterwards. Longer term storage of extracted samples at -80 °C is possible as long as samples are only thawed once. If frozen samples are used for the analysis, make sure that **no particles** are visible after thawing. Otherwise, centrifuge again for 10 min at 4 °C at ≥ 18,000 x *g* and transfer the supernatant into a fresh 1.5 mL tube.

5. Performing the HPLC run

5.1. Equip the fraction collector with 96 small scintillation vials (capacity of ~6 mL) and fill each vial with 2 mL of a suitable scintillation cocktail (e.g., Ultima-Flo AP liquid scintillation cocktail) compatible with buffers with low pH and high ammonium phosphate concentration (see **Table of Materials**).

NOTE: The number of vials and the size of the vials depend on the fraction collector and scintillation counter used. It is important to at least collect the **first 90 fractions**, if the gradient described here is used, to obtain a full inositol polyphosphate profile. Also make sure to **properly label** every vial and its respective lid, to prevent mix-up of fractions or samples.

5.2. Start the HPLC system/pumps and have it ready to run. Activate the piston seal wash and keep it activated during the whole run. Load the sample by manually injecting the complete supernatant from step 4.5 (approximately 750 µL) using a suitable syringe (see **Table of Materials**). If automatic injection is possible, transfer the sample to the corresponding sample vial. Turn the valve from “load” to “inject” position and start the gradient and the fraction collector.

NOTE: Depending on the HPLC system used, the starting procedure might differ, especially when comparing older systems (as described here) with a fully software-controlled newer model. It is very important to ensure that the gradient, the sample injection and fraction collection start simultaneously.

5.3. While the HPLC run is ongoing, check the pressure regularly. The starting pressure should be around 18–24 bar (1.8–2.4 MPa) and should slowly rise to 50–60 bar (5–6 MPa) once 100% buffer B is reached.

CAUTION: Decreased pressure might indicate a leak in the system while increased pressure indicates a blockage. Pressure fluctuations (≥ 3 bar in a few seconds) can indicate the presence of air in the system. Keep in mind that everything that leaves the column, as well as every leakage that occurs at the injector or afterwards is **radioactive**.

NOTE: The pressure also depends on the HPLC system and can be lower or higher than stated here. It will slowly increase after approximately 15–20 runs. However, this does not necessarily influence the quality of the obtained runs.

5.4. After the run, close the vials tightly and mix the fractions with the scintillation cocktail by vigorous shaking. Proceed directly with the measurement or keep the vials in an upright position, ideally in the dark.

NOTE: Fractions mixed with scintillation cocktail are stable for weeks and can be measured later. Since the half-life of tritium is 12.32 years, the signal loss is negligible.

5.5. Once the run of the last sample of the day is finished, stop both HPLC pumps.

5.6. (Optional) To increase the longevity of the system, especially when it is not used regularly, wash pump B and capillaries by placing the capillary from buffer B into a bottle with buffer A and let the pump run for 10–15 min. Before the next use, **remember** to replace the capillary into buffer B and to uncouple pump B from the mixer to flush it with buffer B. Once the pump and capillaries are filled again with buffer B, reconnect it with the mixer and the system is ready to use.

6. Measuring the fractions

6.1. Insert the vials into scintillation counter racks and measure each vial for 5 min in a liquid scintillation counter.

6.2. Ideally, use racks that directly fit small vials and avoid hanging in the vials in bigger (e.g., 20 mL) vials to reduce counting errors. The software settings used in this protocol are shown in **Supplemental Figure 1**.

NOTE: Regularly perform an SNC (self-normalization and calibration) protocol using unquenched ^{3}H standards. Shorter counting times (1–5 min) are possible to reduce the waiting time. However, to ensure a high counting reproducibility and accuracy, 5 min are recommended.

7. Data analysis

7.1. Export the measurements from the scintillation counter as a spreadsheet file or a compatible/convertible file format. Evaluate the data with a computer equipped with Excel or similar software, and a suitable analysis software like Origin.

7.2. Prepare a 2-D line chart where the measured counts per minutes (cpm) are plotted against the retention time (see **Figure 1**, **Figure 2**).

7.3. To compare samples with each other, **normalize the data** by summing up the cpm from each eluted fraction from minute 25 to 96 for each individual sample.

NOTE: Minute 25 is used as cut-off to exclude unincorporated [^{3}H]-*myo*-inositol, $InsP_1$ and $InsP_2$ from the analysis, as those tend to fluctuate strongly and cannot be well separated (at least with

the gradient proposed in this protocol) and thus strongly change the normalization factor due to their high activity.

7.4. Normalize all data to the sample with the lowest total cpm (in fractions 25–96) by dividing the total cpm from the sample with the lowest cpm (in fractions 25–96) by the total cpm (in fractions 25–96) of the other samples. The resulting factor can then be used to normalize the cpm from each fraction by multiplying the cpm of each fraction with the factor.

NOTE: In the end, the sum of the cpm values from minute 25 to the end should be equal for all samples compared with each other. Only normalized runs should be presented in the same graph/figure (when presented as actual profiles). **Supplemental Figure 2** shows an example of how these calculation steps are made (using only fractions 25–35 of two samples for simplification). However, in some cases it is not necessary to normalize data. For instance, when peaks are quantified according to step 7.4 and presented as percentages of total InsPs (as shown in **Figure 3D**). As stated before, when presenting multiple analyses side by side as profiles, or when the actual measured activity is used for conclusions (e.g., treatment xy increases $InsP_7$ by xy% compared to control, referring to the cpm values of $InsP_7$ of both samples and not to their percentage of total InsPs) normalization is needed. To analyze the effect of genotype or treatment differences on labeling efficiency, it is important **not** to **normalize**, as this would invalidate these differences. However, absolute quantification with this method is challenging because the extraction efficiency with this protocol can be variable for various reasons and are sometimes even observed when replica of same genotype and treatment are analyzed. Keep in mind that depending on the HPLC system, column and gradient used for the analyses, the cut-off might need to be changed.

7.5. To **perform relative quantifications** of certain inositol polyphosphate peaks and to subsequently create **bar graphs** that contain data of replications for statistical analyses, continue the analysis with a specialized software that can calculate peak areas of chromatograms (e.g., Origin). See **Supplemental Figure 3**.

NOTE: Most HPLC systems that are software-controlled are supplied with a respective software capable of this task. Peaks are determined as the fractions with cpm values above background (that varies to a certain degree between runs) and retention times that are similar to previously published data. The retention time of a specific peak is determined in spreasheet software (e.g., Excel) and used to assign peaks for calculation of definite integrals (e.g., in Origin). **Supplemental Figure 3** illustrates this process of peak determination, background subtraction and integration of peaks.

REPRESENTATIVE RESULTS:

The results shown here aim to illustrate possible outcomes obtained according to variations at technical and biological levels. The first is exemplified by analyses using new versus aged columns (**Figure 1**) and fresh versus stored samples (**Figure 3**), and the second by evaluating extracts from two different plant systems, *A. thaliana* (**Figures 1**, **Figure 3**) and *L. japonicus* (**Figure 2**).

An optimal SAX-HPLC run is depicted on **Figures 1A–C**, which shows a complete inositol polyphosphate spectrum obtained from *A. thaliana* extracts after scintillation counting. Note that peaks are nicely separated and can be assigned to different isomers (or enantiomer-pairs) based on chromatographic mobilities described earlier[5,7].

Figure 2 shows the representative result of a SAX-HPLC analysis of *L. japonicus* seedlings that were grown and labeled under the same conditions as the *Arabidopsis* seedlings. While presumably all InsP species and peaks that are known from *Arabidopsis* can be seen, there are substantial differences regarding the relative (e.g., ratios between isomers) amount of specific InsP isomers, when comparing the profiles of both species. For instance, the Lotus extracts showed increased $InsP_{3c}$, $InsP_{4b}$, $InsP_{5b}$ and reduced $InsP_{3a}$, $InsP_{4a}$, $InsP_{5a}$ and $InsP_{5c}$ compared to *Arabidopsis* which leaves room for further investigations. **Figure 2D** illustrates the different ratios between InsP isomers between *Arabidopsis* and *Lotus*.

Figure 3 shows two InsP profiles of a sample that was split after the extraction. The first half was immediately analyzed and the second half one day later, after storage at -80 °C. Despite minor differences observed between the different samples (i.e., black and red lines on **Figure 3A–C**), those are not significant (**Figure 3D**). This illustrates that one freeze-thaw cycle does not harm the sample and that the method itself generates reproducible results.

FIGURE AND TABLE LEGENDS:

Figure 1: Typical InsP profile of a successful and of an unsuccessful SAX-HPLC analysis performed with this protocol. (**A–C**) SAX-HPLC profile of 17-day-old wild-type (Col-*0*) *Arabidopsis* seedlings radiolabeled with [^{3}H]-*myo*-inositol. Global InsP extraction and SAX-HPLC run were performed on the same day. (**A**) Full spectra; (**B**, **C**) Zoom-ins of the profile shown in A. All visible peaks are highlighted and assigned to the corresponding InsP species. Based on published chromatographic mobilities[5,7], $InsP_{4a}$ likely represents Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4, $InsP_{5a}$ represents $InsP_5$ [2-OH], $InsP_{5b}$ represents $InsP_5$ [4-OH] or its enantiomeric form $InsP_5$ [6-OH], and $InsP_{5c}$ represents $InsP_5$ [1-OH] or its enantiomeric form $InsP_5$ [3-OH]. The isomeric natures of $InsP_{3a-c}$, $InsP_{4b}$, $InsP_7$, and $InsP_8$ are still unknown. Panel (**D**) shows a SAX-HPLC profile of identically grown plants but using an aged column (>40 runs). A clear reduction of $InsP_6$ compared to other InsP species and the absence of PP-InsPs is visible.

Figure 2: Representative InsP profile of *L. japonicus* plants. SAX-HPLC profile (**A–C**) of 17-day-old wild-type (Gifu) *L. japonicus* seedlings radiolabeled with [^{3}H]-*myo*-inositol. (**A**) Full spectra; (**B**, **C**) Zoom-ins of the profile shown in A. All visible peaks are highlighted and assigned to the corresponding InsP species. Based on published chromatographic mobilities[5,7], $InsP_{4a}$ likely represents Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4, $InsP_{5b}$ likely represents $InsP_5$ [4-OH] or its enantiomeric form $InsP_5$ [6-OH], and $InsP_{5c}$ likely represents $InsP_5$ [1-OH] or its enantiomeric form $InsP_5$ [3-OH]. The isomeric natures of $InsP_{3a-c}$, $InsP_{4b}$, $InsP_7$, and $InsP_8$ are unknown. (**D**) Comparison between the individual InsP species (in % of total activity from elution 25–96) of *A. thaliana* (data from **Figure 1A–C**) and *L. japonicus* (data from **Figure 2A–C**).

Figure 3: InsP profiles of a split sample illustrating the reproducibility of SAX-HPLC analyses. (**A**–**C**) SAX-HPLC profiles of 17-day-old wild-type (Col-*0*) *Arabidopsis* seedlings radiolabeled with [^{3}H]-*myo*-inositol. Prior the run, the sample was split and one half run immediately and the other half one day later after storage at -80 °C. (**A**) Full spectra; (**B**, **C**) Zoom-ins of the profile shown in A. All visible peaks are highlighted and assigned to the corresponding InsP species. Based on published chromatographic mobilities[5,7], $InsP_{4a}$ likely represents Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4, $InsP_{5a}$ represents $InsP_5$ [2-OH], $InsP_{5a}$ represents $InsP_5$ [2-OH], $InsP_{5b}$ represents $InsP_5$ [4-OH] or its enantiomeric form $InsP_5$ [6-OH], and $InsP_{5c}$ represents $InsP_5$ [1-OH] or its enantiomeric form $InsP_5$ [3-OH]. The isomeric natures of $InsP_{3a-c}$, $InsP_{4b}$, $InsP_7$, and $InsP_8$ are still unknown. Panel **D** shows the quantification of $InsP_6$ and the PP-InsPs $InsP_7$ and $InsP_8$ of both runs. The values represent the amount (in %) of the respective InsP species relative to all InsP (total activity from elution 25–96).

Supplemental Figure 1: Software settings for liquid scintillation counting using a light scintillation counter. Screenshots showing the software version, as well as settings used for scintillation counting of [^{3}H] samples performed with this protocol are depicted.

Supplemental Figure 2: Representative example of data normalization. A screenshot of a worksheet shows all steps and formulas used to normalize SAX-HPLC runs to each other. For simplification, only fractions 25–35 of samples are shown.

Supplemental Figure 3: Peak determination, background subtraction and integration using analysis software. (**A**) Data from SAX-HPLC analysis is loaded into the software (minutes 28–96) and the peak analyzer tool is selected. (**B**–**E**) The baseline is defined manually by setting points between individual peaks and the background is subtracted. (**F**) Peaks are determined manually based on appearance and published chromatographic mobilities[5,7]. (**G**) Peak ranges are defined manually by cpm values. (**H**) Peaks are integrated and calculated as % of all peaks.

DISCUSSION:

Here we present a versatile and sensitive method to quantify InsPs including PP-InsPs in plant extracts and provide practical tips on how to get this method established. Even though the protocol is generally robust, suboptimal runs and analyses can occur. In most cases, those runs can be identified by a strong reduction or even complete loss of highly phosphorylated InsPs, especially the PP-InsP species $InsP_7$ and $InsP_8$. Possible reasons can be microbial contaminations of the plant material and insufficient deactivation of endogenous plant PP-InsP hydrolases during extraction due to insufficient grinding and thawing of plant material that will not be in immediate contact with extraction buffer. Further reasons include inaccurate pH adjustment by insufficient or excess addition of neutralization buffer, or simply insufficient sample material. The latter can make it difficult to detect PP-InsPs, since those are often present in very low amounts in the cells. An excess of sample material or inefficient drying during step 3.5 may cause dilution of the perchloric acid, therefore also leading to insufficient enzyme deactivation and a specific loss of $InsP_6$ and PP-InsPs. The amount of plant material, as well as radiolabel used in this protocol were optimized based on costs and performance, and is therefore close to the lowest amount that is still sufficient for providing optimal results.

In addition, the column resin will gradually loose its binding capacity. The first sign of this process is (for reasons not entirely clear to the authors) a specific loss of higher phosphorylated InsP species like the PP-InsPs. With further aging, even $InsP_6$ will bind less efficiently to the column (**Figure 1D**). Therefore, the use of an adequate column, as well as meticulous handling of the sample and proper maintenance of the HPLC components is crucial for ensuring accurate results.

When comparing samples and runs, especially when generated with different equipment (e.g., HPLC systems and columns) or on different days, it is crucial to normalize the samples to each other (as described in step 7.3) and to analyze them in the same way. Only through normalization it is possible and accurate to show multiple samples in the same graph (**Figure 3**). For quantification of individual InsPs relative to total InsPs, or to another specific InsP species, it is not necessary to normalize, as long as only relative values and not absolute values are shown. Ideally, both the InsP profiles and the quantifications are shown. However, in some cases it is not possible to adequately show two or more runs in the same graph. Different retention times or different levels of background activity can make it difficult to compare unquantified SAX-HPLC profiles alone. The same is the true when many samples need to be compared. In such cases, a further evaluation using an additional software (e.g., Origin) for individual peak quantification is necessary.

The authors are aware that the protocol described here can be optimized and needs to be adapted to each individual research question. Although being optimized for *Arabidopsis* extracts[7,17] in this protocol, this method is versatile and can help determining InsP profiles of other plant species as well. Here we exemplify this possibility by presenting for the first time a InsP profile for *L. japonicus*, which required no modifications of the labeling conditions, InsP extraction or SAX-HPLC run (**Figure 2**). Notably, while overall similar, differences are observed between *L. japonicus* and *Arabidopsis* InsP profiles. For instance, in *L. japonicus* $InsP_5$ [4-OH] or its enantiomeric form $InsP_5$ [6-OH] are more abundant than $InsP_5$ [1-OH] or its enantiomeric form $InsP_5$ [3-OH] in comparison to *Arabidopsis*, where $InsP_5$ [1-OH] or its enantiomeric form $InsP_5$ [3-OH] are the dominant $InsP_5$ species. Likewise, we anticipate that alterations in the media composition, [^{3}H]-*myo*-inositol concentration, plant age, environmental conditions (e.g., light and temperature), addition of chemical compounds or analyses of plant-microbial interactions among other factors, might need to be tested and adapted.

One important drawback of this method that needs to be considered is that the labeling is done in a (sterile) liquid culture, which does not represent a physiological environment for most land plants. In addition, due to the high costs of [^{3}H]-*myo*-inositol, the volume of the labeling solution and the size of the culture vessel is generally limited, which also restricts the size of the plants that can be used. Cultivation in liquid culture can be avoided by directly infiltrating for instance leaves of soil-grown plants with [^{3}H]-*myo*-inositol and subsequently following the protocol described here, as previously reported[10].

There are several drawbacks of this protocol in comparison to alternative methods, such as TiO_2 pull-down followed by PAGE or mass spectrometry based techniques. Due to the [^{3}H]-*myo*-inositol labeling, only InsP species that directly originate from radiolabeled *myo*-inositol will be

detected in the end. The method described here is blind to other Ins isomers such as *scyllo*-inositol and other isomers some of which have been identified in certain plants[44]. Furthermore, *myo*-InsPs derived from other pathways will be excluded, including those synthesized by *de novo* synthesis of *myo*-inositol and *myo*-inositol-3-phosphate via isomerization of glucose-6-phosphate, catalyzed by *myo*-inositol-3-phosphate synthase (MIPS) proteins[45]. Although [^{32}P] or [^{33}P]-*ortho*-phosphate can be used as alternative labels, their use poses a major disadvantage, since every phosphate-containing molecule, including the abundant nucleotides and its derivatives, will be labeled. Those molecules can also be extracted with this protocol and bind to the SAX column, which will result in a high level of background activity that will interfere with the identification of individual InsP peaks[5]. In addition, quantification of [^{32}P]- or [^{33}P] -labeled InsPs and PP-InsPs can be strongly influenced by phosphate and pyrophosphate moiety turnover and might not report a mass readout for inositol species.

On the other hand, [^{3}H]-*myo*-inositol specifically labels *myo*-inositol-containing molecules. InsPs, inositol-containing lipids, such as phosphoinositides, and galactinol are in this case labeled. However, only InsPs will be analyzed with this protocol, since lipids are insoluble in the extraction buffer and galactinol does not bind to the partisphere column.

So far, the differences from a plant InsP profile generated by [^{3}H]-*myo*-inositol labeling compared with one determined by TiO_2 pulldown/PAGE remains unknown, since such comparisons have not been performed in plants. A recent study in animal cells addressed this question[46]. In that work, a pool of $InsP_6$ that is invisible by [^{3}H]-*myo*-inositol labeling, which should thereby be directly derived from glucose-6-phosphate, was identified by comparing SAX-HPLC profiles with PAGE gels of mammalian cell lines. 24 h of phosphate starvation resulted in a 150% increase of $InsP_6$ when quantifying PAGE gels of InsPs purified using TiO_2 pulldown. SAX-HPLC analyses of [^{3}H]-*myo*-inositol-labeled cells that were treated identically only showed an increase by 15% of [^{3}H]-$InsP_6$. As previously mentioned, InsPs lower than $InsP_5$ are undetectable with PAGE analysis in most cases. Radiolabeling followed by SAX-HPLC appears to be the method of choice, as long as mass spectrometric protocols are not optimized to detect this group of highly negatively charged molecules.

Another remaining challenge is to distinguish enantiomers in SAX-HPLC analyses (or in any other method for InsP analysis)[10,17]. This challenge can be tackled by the addition of chiral selectors, i.e., enantiopure compounds like L-arginine amide that interact with the respective enantiomeric molecules to form diastereomeric complexes that can be separated[10]. To our knowledge, this approach has only been implemented to discriminate the enantiomeric $InsP_5$ isomers $InsP_5$[1-OH] and $InsP_5$ [3-OH] by NMR analyses. Discrimination of other enantiomeric pairs or successful discrimination of enantiomers by chiral SAX-HPLC analysis or chiral PAGE-based methods have not yet been reported and should be further developed. Considering the conserved synthesis and the conserved regulation of PP-InsPs by phosphorous availability, we envision that especially non-radioactive methods such as PAGE or MS-based methods, together with nutrient analyses, will help ground truthing efforts to calibrate remote sensing data designed to diagnose nutrient deficiencies in crops[17,18,24,25]. However, the method presented here can currently still be

considered the gold standard for InsP analyses and will be instrumental to discover new functions of these intriguing messengers in plants.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Berridge, M. J., Irvine, R. F. Inositol Phosphates and Cell Signaling. *Nature.* **341** (6239), 197-205, (1989).

2 Streb, H., Irvine, R. F., Berridge, M. J., Schulz, I. Release of Ca-2+ from a Nonmitochondrial Intracellular Store in Pancreatic Acinar-Cells by Inositol-1,4,5-Trisphosphate. *Nature.* **306** (5938), 67-69, (1983).

3 Krinke, O., Novotna, Z., Valentova, O., Martinec, J. Inositol trisphosphate receptor in higher plants: is it real? *Journal of Experimental Botany.* **58** (3), 361-376, (2007).

4 Kuo, H. F. *et al.* Arabidopsis inositol pentakisphosphate 2-kinase, AtIPK1, is required for growth and modulates phosphate homeostasis at the transcriptional level. *Plant Journal.* **80** (3), 503-515, (2014).

5 Kuo, H. F. *et al.* Arabidopsis inositol phosphate kinases IPK1 and ITPK1 constitute a metabolic pathway in maintaining phosphate homeostasis. *Plant Journal.* **95** (4), 613-630, (2018).

6 Gillaspy, G. E. in *Lipid-mediated Protein Signaling* 10.1007/978-94-007-6331-9_8 (ed Daniel G. S. Capelluto) 141-157 (Springer Netherlands, 2013).

7 Stevenson-Paulik, J., Bastidas, R. J., Chiou, S. T., Frye, R. A., York, J. D. Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. *Proceedings of the National Academy of Sciences of the United States of America.* **102** (35), 12612-12617, (2005).

8 Lemtiri-Chlieh, F., MacRobbie, E. A. C., Brearley, C. A. Inositol hexakisphosphate is a physiological signal regulating the K^+-inward rectifying conductance in guard cells. *Proceedings of the National Academy of Sciences.* **97** (15), 8687-8692, (2000).

9 Lee, H. S. *et al.* InsP6-sensitive variants of the Gle1 mRNA export factor rescue growth and fertility defects of the ipk1 low-phytic-acid mutation in Arabidopsis. *Plant Cell.* **27** (2), 417-431, (2015).

10 Blüher, D. *et al.* A 1-phytase type III effector interferes with plant hormone signaling. *Nature Communications.* **8**, (2017).

11 Murphy, A. M., Otto, B., Brearley, C. A., Carr, J. P., Hanke, D. E. A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. *Plant Journal.* **56** (4), 638-652, (2008).

12 Poon, J. S. Y., Le Fevre, R. E., Carr, J. P., Hanke, D. E., Murphy, A. M. Inositol hexakisphosphate biosynthesis underpins PAMP-triggered immunity to Pseudomonas syringae

pv. tomato in Arabidopsis thaliana but is dispensable for establishment of systemic acquired resistance. *Molecular Plant Pathology*. **21** (3), 376-387, (2020).
13 Wild, R. *et al.* Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. *Science.* **352** (6288), 986-990, (2016).
14 Shears, S. B. Inositol pyrophosphates: Why so many phosphates? *Advances in Biological Regulation.* **57** 203-216, (2015).
15 Shears, S. B. Intimate connections: Inositol pyrophosphates at the interface of metabolic regulation and cell signaling. *Journal of Cellular Physiology.* **233** (3), 1897-1912, (2018).
16 Desai, M. *et al.* Two inositol hexakisphosphate kinases drive inositol pyrophosphate synthesis in plants. *Plant Journal.* **80** (4), 642-653, (2014).
17 Laha, D. *et al.* VIH2 Regulates the Synthesis of Inositol Pyrophosphate InsP8 and Jasmonate-Dependent Defenses in Arabidopsis. *Plant Cell.* **27** (4), 1082-1097, (2015).
18 Laha, D. *et al.* Arabidopsis ITPK1 and ITPK2 Have an Evolutionarily Conserved Phytic Acid Kinase Activity. *Acs Chemical Biology.* **14** (10), 2127-2133, (2019).
19 Dorsch, J. A. *et al.* Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. *Phytochemistry.* **62** (5), 691-706, (2003).
20 Flores, S., Smart, C. C. Abscisic acid-induced changes in inositol metabolism in Spirodela polyrrhiza. *Planta.* **211** (6), 823-832, (2000).
21 Brearley, C. A., Hanke, D. E. Inositol phosphates in barley (Hordeum vulgare L) aleurone tissue are stereochemically similar to the products of breakdown of InsP(6) in vitro by wheat-bran phytase. *Biochemical Journal.* **318** 279-286, (1996).
22 Laha, N. P. *et al.* ITPK1-Dependent Inositol Polyphosphates Regulate Auxin Responses in *Arabidopsis thaliana*. *bioRxiv.* 10.1101/2020.04.23.058487 2020.2004.2023.058487, (2020).
23 Laha, D. *et al.* Inositol Polyphosphate Binding Specificity of the Jasmonate Receptor Complex. *Plant Physiology.* **171** (4), 2364-2370, (2016).
24 Dong, J. S. *et al.* Inositol Pyrophosphate InsP(8) Acts as an Intracellular Phosphate Signal in Arabidopsis. *Molecular Plant.* **12** (11), 1463-1473, (2019).
25 Zhu, J. *et al.* Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis. *Elife.* **8**, (2019).
26 Couso, I. *et al.* Synergism between Inositol Polyphosphates and TOR Kinase Signaling in Nutrient Sensing, Growth Control, and Lipid Metabolism in Chlamydomonas. *Plant Cell.* **28** (9), 2026-2042, (2016).
27 Ito, M. *et al.* Hydrophilic interaction liquid chromatography-tandem mass spectrometry for the quantitative analysis of mammalian-derived inositol poly/pyrophosphates. *Journal of Chromatography A.* **1573** 87-97, (2018).
28 Wilson, M. S. C., Saiardi, A. Inositol Phosphates Purification Using Titanium Dioxide Beads. *Bio-Protocol.* **8** (15), (2018).
29 Loss, O., Azevedo, C., Szijgyarto, Z., Bosch, D., Saiardi, A. Preparation of Quality Inositol Pyrophosphates. *Jove-Journal of Visualized Experiments.* UNSP e3027
10.3791/3027 (55), (2011).
30 Harmel, R. K. *et al.* Harnessing C-13-labeled myo-inositol to interrogate inositol phosphate messengers by NMR Electronic supplementary information (ESI) available. See DOI: 10.1039/c9sc00151d. *Chemical Science.* **10** (20), 5267-5274, (2019).

31 Azevedo, C., Saiardi, A. Extraction and analysis of soluble inositol polyphosphates from yeast. *Nature Protocols.* **1** (5), 2416-2422, (2006).
32 Shears, S. B. in *Inositol Phosphates: Methods and Protocols* 10.1007/978-1-0716-0167-9_1 (ed Gregory J. Miller) 1-28 (Springer US, 2020).
33 Wilson, M. S. C., Saiardi, A. Importance of Radioactive Labelling to Elucidate Inositol Polyphosphate Signalling. *Topics in Current Chemistry.* **375** (1), (2017).
34 Stevenson-Paulik, J. *et al.* Inositol phosphate metabolomics: Merging genetic perturbation with modernized radiolabeling methods. *Methods.* **39** (2), 112-121, (2006).
35 Liu, C., Riley, A. M., Yang, X., Shears, S. B., Potter, B. V. L. Synthesis and Biological Activity of d- and l-chiro-Inositol 2,3,4,5-Tetrakisphosphate: Design of a Novel and Potent Inhibitor of Ins(3,4,5,6)P4 1-Kinase/Ins(1,3,4)P3 5/6-Kinase. *Journal of Medicinal Chemistry.* **44** (18), 2984-2989, (2001).
36 Hughes, P. J., Hughes, A. R., Putney, J. W., Shears, S. B. The regulation of the phosphorylation of inositol 1,3,4-trisphosphate in cell-free preparations and its relevance to the formation of inositol 1,3,4,6-tetrakisphosphate in agonist-stimulated rat parotid acinar cells. *Journal of Biological Chemistry.* **264** (33), 19871-19878, (1989).
37 Shears, S. B., Kirk, C. J., Michell, R. H. The pathway of myo-inositol 1,3,4-trisphosphate dephosphorylation in liver. *The Biochemical journal.* **248** (3), 977-980, (1987).
38 Stevenson-Paulik, J., Odom, A. R., York, J. D. Molecular and Biochemical Characterization of Two Plant Inositol Polyphosphate 6-/3-/5-Kinases. *Journal of Biological Chemistry.* **277** (45), 42711-42718, (2002).
39 Stephens, L. R., Hawkins, P. T., Downes, C. P. An analysis of myo-[3H]inositol trisphosphates found in myo-[3H]inositol prelabelled avian erythrocytes. *The Biochemical journal.* **262** (3), 727-737, (1989).
40 Saiardi, A. *et al.* Mammalian inositol polyphosphate multikinase synthesizes inositol 1,4,5-trisphosphate and an inositol pyrophosphate. *Proceedings of the National Academy of Sciences.* **98** (5), 2306, (2001).
41 Azevedo, C., Burton, A., Bennett, M., Onnebo, S. M. N., Saiardi, A. in *Inositol Phosphates and Lipids: Methods and Protocols* 10.1007/978-1-60327-175-2_5 (ed Christopher J. Barker) 73-85 (Humana Press, 2010).
42 Saiardi, A., Caffrey, J. J., Snyder, S. H., Shears, S. B. The Inositol Hexakisphosphate Kinase Family: CATALYTIC FLEXIBILITY AND FUNCTION IN YEAST VACUOLE BIOGENESIS. *Journal of Biological Chemistry.* **275** (32), 24686-24692, (2000).
43 Brearley, C. A., Hanke, D. E. Inositol phosphates in the duckweed Spirodela polyrhiza L. *Biochemical Journal.* **314** 215-225, (1996).
44 Pollard, J. K., Steward, F. C., Shantz, E. M. Hexitols in Coconut Milk - Their Role in Nurture of Dividing Cells. *Plant Physiology.* **36** (4), 492-&, (1961).
45 Donahue, J. L. *et al.* The Arabidopsis thaliana Myo-inositol 1-phosphate synthase1 gene is required for Myo-inositol synthesis and suppression of cell death. *Plant Cell.* **22** (3), 888-903, (2010).
46 Desfougeres, Y., Wilson, M. S. C., Laha, D., Miller, G. J., Saiardi, A. ITPK1 mediates the lipid-independent synthesis of inositol phosphates controlled by metabolism. *Proceedings of the National Academy of Sciences of the United States of America.* **116** (49), 24551-24561, (2019).

Figure 1

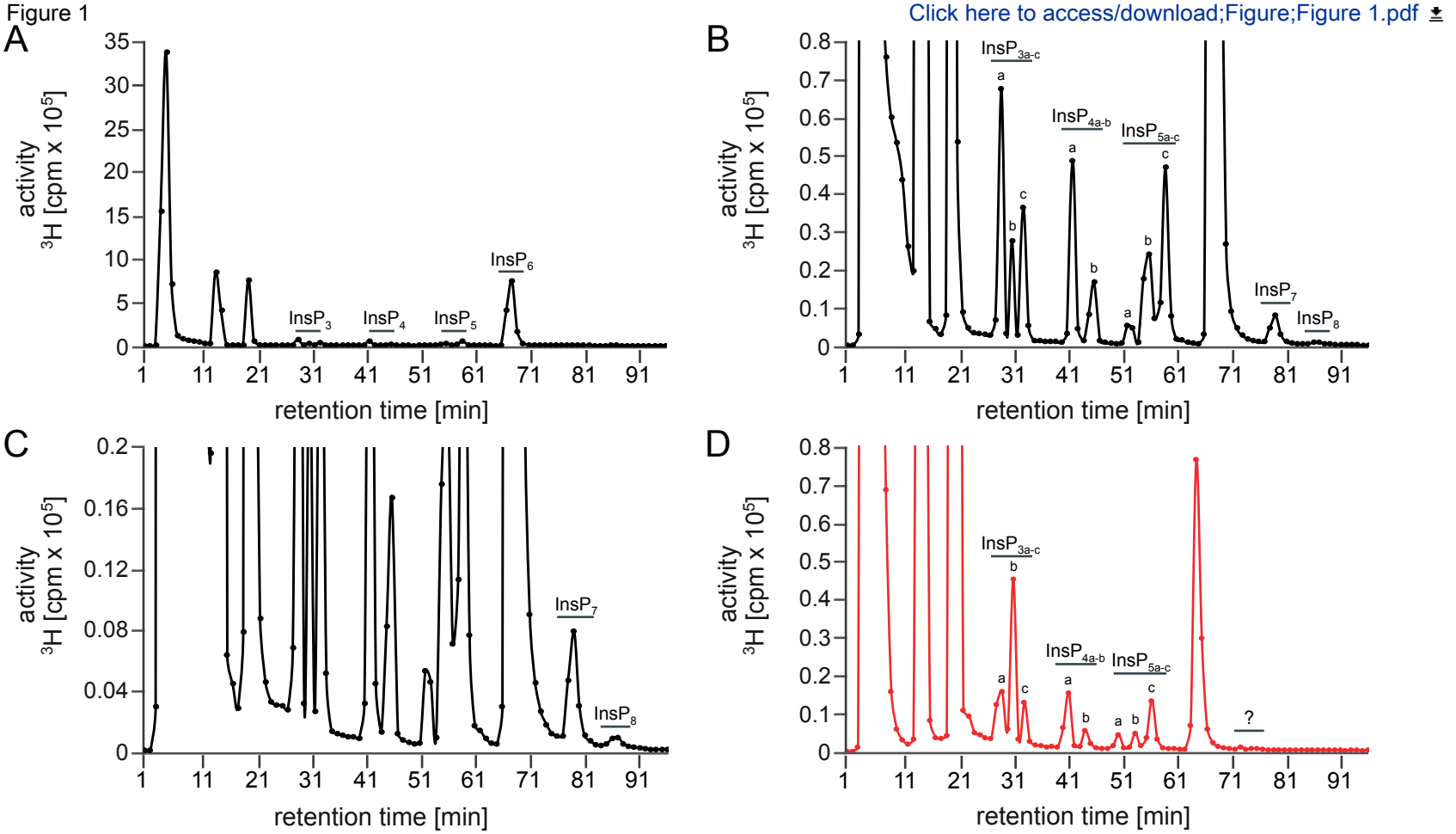


Figure 2

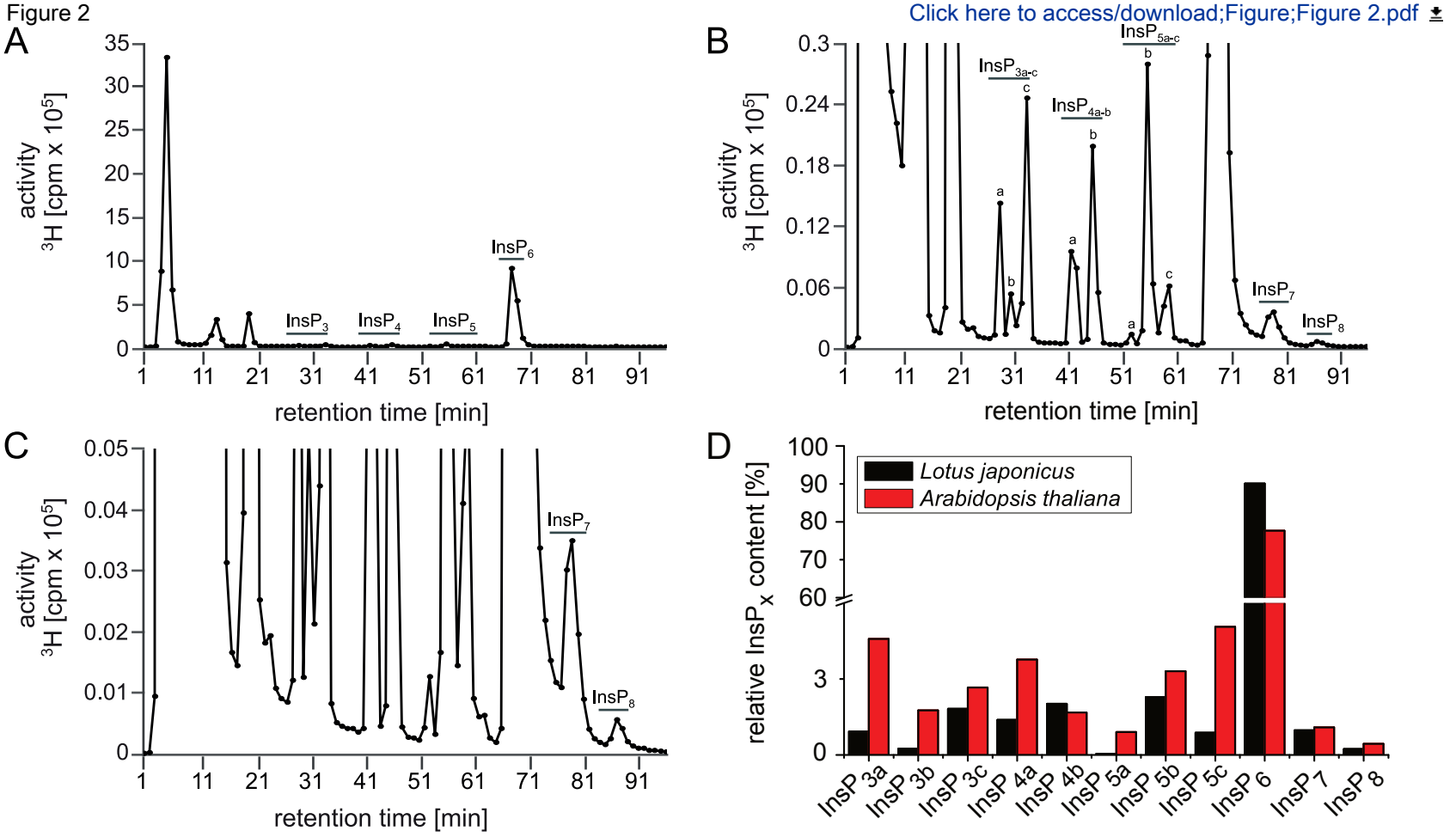
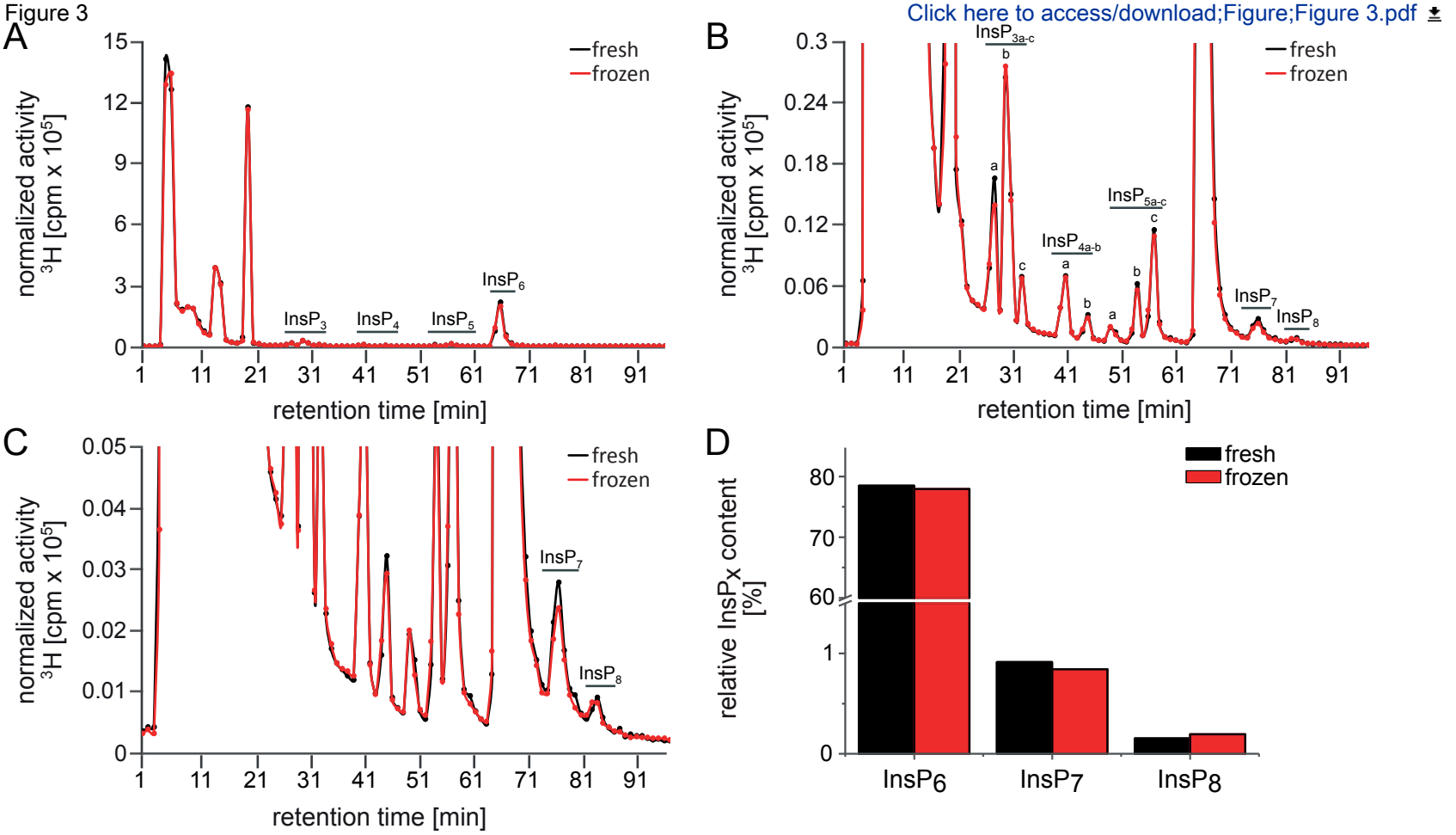


Figure 3



Supplemental Figure 1

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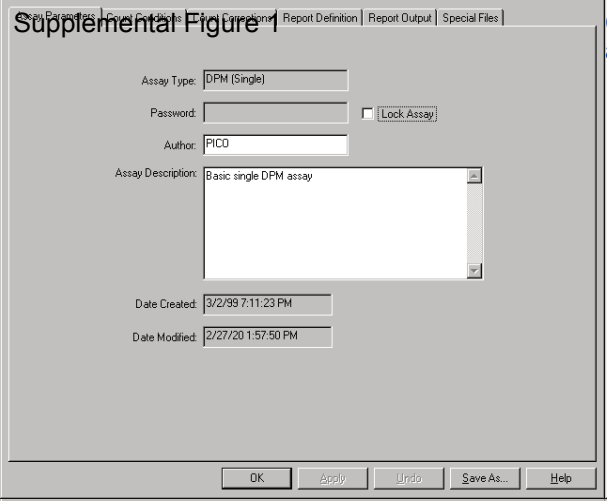

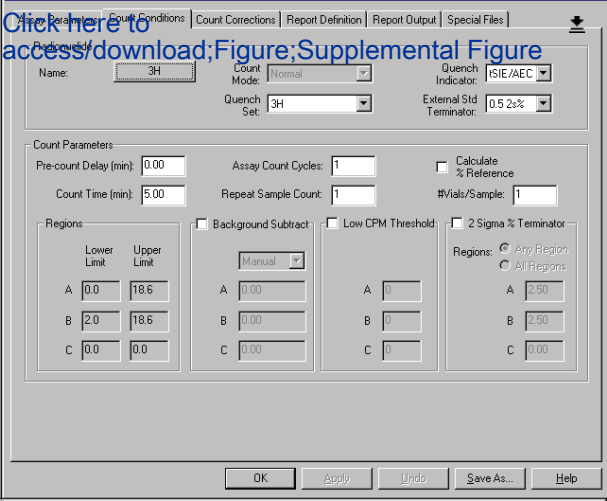

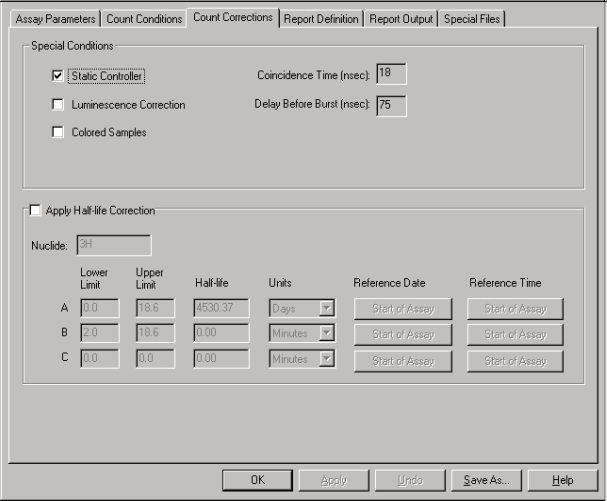

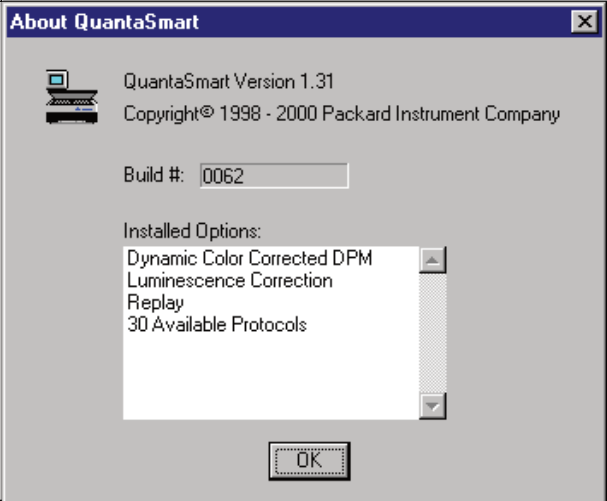

Supplemental Figure 2

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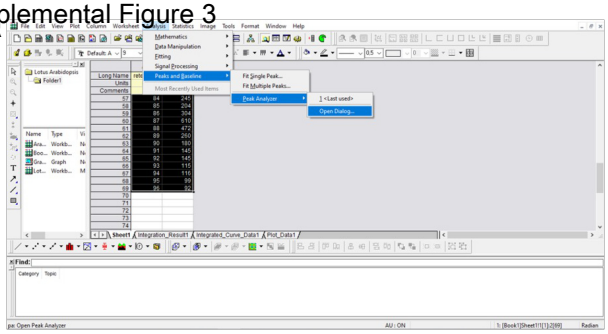
	A	B	C	D	E	F	G	H	I
1									
2	fractions	Sample 1		normalized values of Sample 1		Sample 2			normalization factor
3	25	4149	B3*I5	4063		4250			
4	26	3871	B4*I5	3790		3740		F15 < B15	
5	27	7858	B5*I5	7694		8214		F15/B15	0,979167349
6	28	16723	B6*I5	16375		14061			
7	29	3705	B7*I5	3628		3646			
8	30	26619	B8*I5	26064		27697			
9	31	15093	B9*I5	14779		14519			
10	32	2629	B10*I5	2574		2665			
11	33	6990	B11*I5	6844		6793			
12	34	2290	B12*I5	2242		2357			
13	35	1708	B13*I5	1672		1784			
14									
15	SUM(B3:B13)	91635	SUM(D3:D13)	89726	SUM(F3:F13)	89726			
16									
17									

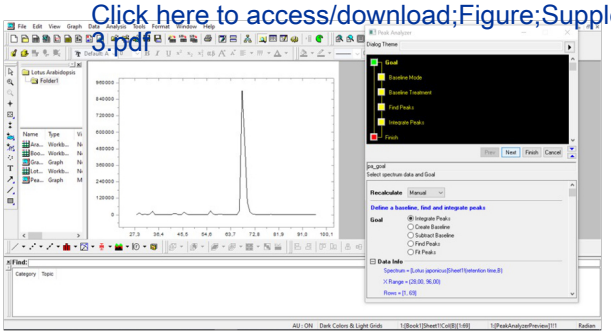
Supplemental Figure 3

Click here to access/download;Figure;Supplemental Figure 3.pdf

A



B



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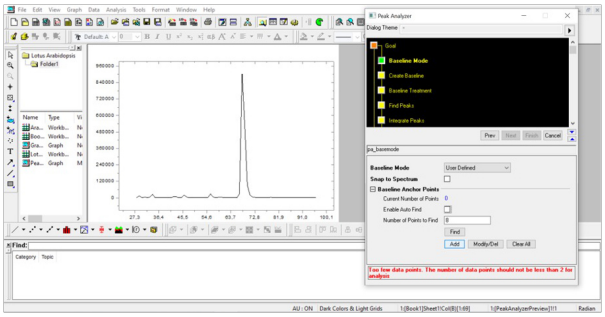

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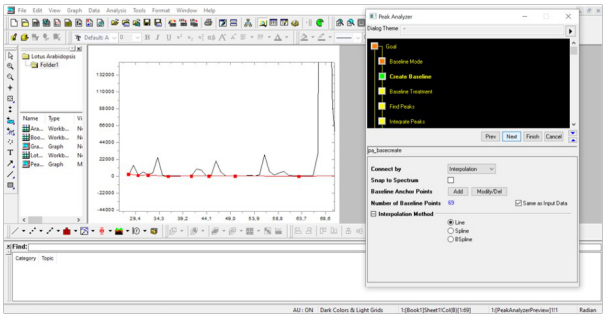

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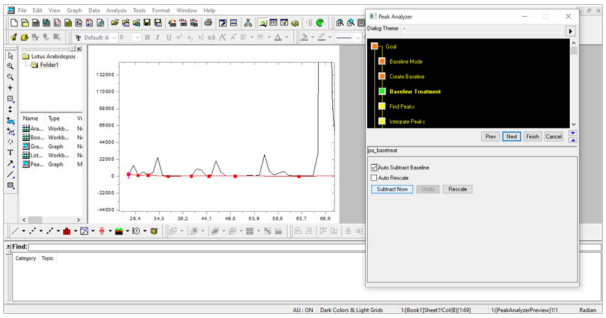

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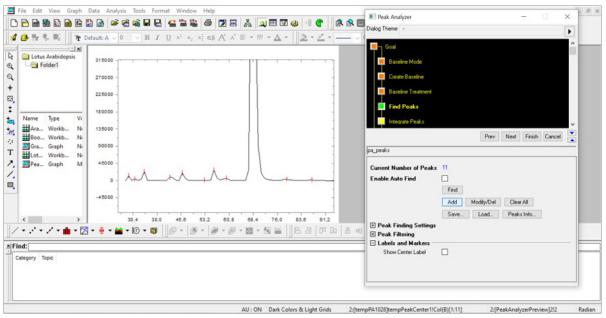
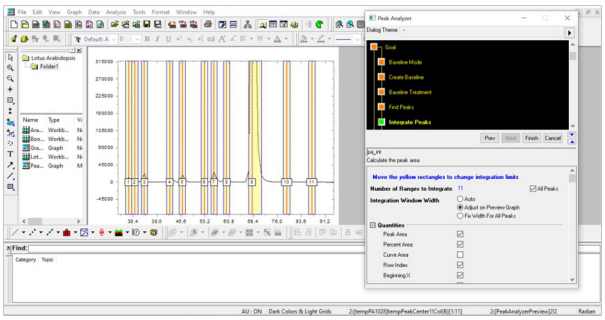
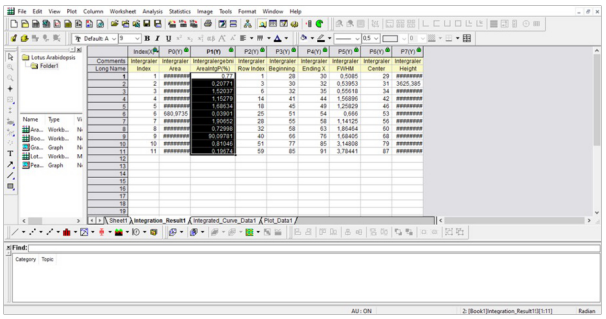


G



H



Name of Material/Equipment	Company	Catalog Number
Centrifuge	Eppendorf AG	model: 5430 R
Diammonium hydrogen phosphate, ≥97 %	Carl Roth GmbH + Co. KG	0268.1
Ethylenediamine tetraacetic acid disodium salt dihydrate,	Carl Roth GmbH + Co. KG	8043.2
Falcon 12-well clear flat bottom TC-treated multiwell cell	Corning Inc.	353043
Fraction collector	LAMBDA Instruments GmbH	model: OMNICOLL single channel
Growth incubator	poly klima GmbH	model: PK 520-LED
HPLC pumps	Kontron Instruments	model: 420
HPLC syringe for Rheodyne valves, 1 mL	Hamilton Company	81365
Injector for HPLC	Supelco	model: Rheodyne 9725
Inositol, myo-[1,2-3H(N)]	American Radiolabeled Chemicals Inc.	ART 0261
Liquid nitrogen	University, Chemistry Department	
Liquid scintillation counter	PerkinElmer Inc	model: TRI-CARB 2900TR
Micro pestle	Carl Roth GmbH + Co. KG	CXH7.1
Mixed cellulose Eester filter, ME range (ME 24), plain, 0.2 µm pore size, 47 mm circle	GE Healthcare Life Sciences	10401770
Mixer for HPLC	Kontron Instruments	model: M 800
Murashige & Skoog medium, salt mixture	Duchefa Biochemie	M0221
OriginPro software	OriginLab Corp.	
Orthophosphoric acid, ≥85 %, p.a., ISO	Carl Roth GmbH + Co. KG	6366.1
Partisphere 5 µm SAX cartridge column, 125 x 4.6 mm	Hichrom Limited	4621-0505
Perchloric acid, 70 %, 99.999 % trace metals basis	Sigma-Aldrich	311421
Petri dish, square, PS, clear, 120/120/17 mm, sterile	Greiner Bio One International GmbH	688161
pH-indicator paper pH 5.5 - 9.0, Neutralit	Merck KGaA	109564
Phytagel	Sigma-Aldrich	P8169
Potassium carbonate	Carl Roth GmbH + Co. KG	P743.2
Safe-Lock tubes, 1.5 mL	Eppendorf AG	30120086
Sample loop for 9725 injectors, volume 2 mL, PEEK	Supelco	57648
SNAPTWIST scintillation vial, 6.5 mL	Simport Scientific Inc.	S207-5
Sterile bench	LaboGene	model: ScanLaf MARS 900
Sucrose, ≥99,5 %, p.a.	Carl Roth GmbH + Co. KG	4621.1
Ultima-Flo AP liquid scintillation cocktail	PerkinElmer Inc	6013599
Ultra-pure deionized water	Milli-Q	

Wrenchless WVS End Fitting Kit	Hichrom Limited	4631-1001

Comments/Description

Dear Dr. DSouza,

Thank you very much for handling our manuscript and for providing us with valuable advice.

We are grateful that all reviewers found our protocol useful and that it will help plant scientists working with inositol phosphate research interested in employing this method.

We are also grateful for the reviewers' concerns and believe that they helped to substantially improve the quality of our manuscript.

Please find a point-by-point response to the reviewers' comments below. Corresponding changes in the manuscript are highlighted in red.

We hope that with the additional data and information, our revised manuscript now meets the high standards for publication in JoVE.

With best regards,

Philipp Gaugler

Dear Dr. Gaugler,

Your manuscript, JoVE61495 "Extraction and quantification of soluble, radiolabeled inositol polyphosphates from different plant species using SAX-HPLC," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **May 01, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888
Follow us: Facebook | Twitter | LinkedIn

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

• **Protocol Highlight:** The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We believe that our protocol has all the specific details included that are necessary to replicate the methodology with a different technical setup. We decided not to give precise instructions on how exactly the HPLC components are started (e.g. button clicks), since most likely other scientists using our protocol will have their own setup, which will be controlled differently. We recommend to get acquainted with the respective HPLC setup available and how it is controlled before the first try with our protocol and with this method in general. Furthermore, we did not highlight steps in the protocol that we judge to be easily understandable or that are routinely performed in a lab working with plants (e.g. seed sterilization). We believe this will keep the video concise and with a focus on the complex steps that profit the most from being shown in a video, while still having a cohesive and logical narrative. Similarly, in the discussion we mainly focussed on the described method, not only mentioning the critical steps and potential difficulties that can be encountered, but also providing additional information about limitations and advantages of our protocol, especially compared to other existing methods. Furthermore, we suggested future applications and indicated where and how further improvements could be applied to this protocol.

• **References:** Please spell out journal names.

Response: We thank the editor for making us aware of this and changed the names accordingly.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This is a very useful protocol for scientists who work with inositol phosphates. Analysis of IPs can be a barrier to many labs embarking on IP research, so this protocol would prove to be very useful.

Major Concerns:
None.

Minor Concerns:
I have a list of questions/suggestions that struck me as I read through the protocol.

Line 118: 1.1. "Set up a system consisting of two independent HPLC pumps (binary pump)" Authors should state if the pumps have to be binary (i.e. could quaternary pumps be used or is there a particular reason why these could not be used)

Response: Indeed, we did not specify that the use of quaternary pumps is also possible without compromising the results (at least in our hands). We routinely use this method with a binary pump (Kontron) and a quaternary pump (Waters Alliance), but the latter still with the indicated binary gradient and see no apparent differences. This information is now included.

Line 164: 'Leucopore tape', is a brand name, maybe try microporous surgical tape (such as Leucopore or Micropore tape)

Response: Changed accordingly.

Line 166: "through bare skin. Always wear gloves when handling radioactive material or equipment that has direct or indirect contact to radioactive material"

Authors could add something here about training in safe use of radiochemicals or following the local rules for safe handling (for example, I would swab down surfaces for scintillation counting to check for spillage etc.)

Response: We agree that additional information about safe handling is useful – it is now included in the revised version.

Line 160: How important is it to obtain tritiated inositol with a specific activity of 30 - 80 Ci/mmol? Lower specific activities are available, but would these limit the detection of less abundant IPs?

Response: Despite not having tested this ourselves, we expect that the detection of less abundant InsPs (especially of $InsP_8$) will be negatively affected by using a lower activity. Even using the activity that we are recommending in the current version of our protocol, we are often at the limit of reliably detecting $InsP_8$. One might overcome a lower specific activity by simply using a larger volume of the activity, but this might result in ethanol toxicity if inositol solved in 90 % ethanol is used (as described in the Table of Materials). This toxicity effect is not present if it is solved in water, but we chose to use ethanol-solved inositol to minimize the risk of bacterial and fungal contaminations. Considering that there is a minimal price difference between *myo*-inositol with lower and higher specific activity, we do recommend the use of the one mentioned in our protocol.

Line 171: "Do not overfill the tube, and place no more than 100 mg FW/tube, in case too many seedlings were labeled."
I am not sure what the authors mean by 'too many seedlings were labelled'. Wouldn't they all be labelled? Or do some individual plants take up more label than others and you don't want too many of these individuals in any one extraction? The following sentence about the potential to dilute the acid extraction solution is much clearer.

Response: We rephrased the sentence and hope that it is now more understandable.

Line 190: Change to something like this 'Immediately prior to extraction, add EDTA to both solutions to a final concentration of 3 mM (eg from a filtered 250 mM EDTA stock solution).'

Both are made using ultra-pure deionized water followed
195 by vacuum filtration through 0.2 uM pore-sized membrane filters.

Response: Changed accordingly.

Line 215: flow-rate should not exceed 2 mL/min. After washing, the column is ready for the analysis and, when properly handled, can be used for 20 - 40 runs.
What needs to be done after 20-40 samples have been analysed on the column? Washing with water? (Or is this all the analysis that can be done on one column?)

Response: We included more information on how to handle the column once it shows signs of decay and that it ultimately needs to be replaced if those measures don't help. However, in our experience, columns cannot be used more than 40 times. We believe this is caused by the harsh conditions of the eluent (i.e. low pH and high salt).

Line 237: gloves
The authors should mention eye protection too.

Response: Changed accordingly.

Line 239: 'disposed of accordingly.'
The authors should mention that the research should follow their Local Rules for safe disposal of radioactive material. (This should remind naïve researchers to check with their Radiation Officer before embarking on a tritium labelling experiment).

Response: We agree that no one should perform radioactive experiments without being aware of the local rules and included the recommendation as suggested.

241 4.1. 'Prepare the working solutions by adding EDTA to the extraction buffer and the neutralization buffer to a final concentration of 3 mM. Prepare at least 600 µL of extraction buffer and 400uL neutralization buffer per sample and keep them on ice.'
The authors probably don't need to mention addition of the EDTA as this is already covered in section 3.1
Perhaps try: Prepare the working solutions for the extraction and neutralization buffer. 600ul of extraction buffer and 400ul neutralization buffer will be needed for each sample.

Response: Changed accordingly.

Line 260: Remind the reader that any tubes used for extractions or containing pellets, should be treated as solid (low-level) radioactive waste, and that they should follow their local rules for safe disposal.

Response: Inserted a reminder as suggested.

Line 282: What volume of sample is injected?

Response: We included the information that the whole sample/supernatant is injected (approx. 750 µL).

Line 293: The authors should use a better term than 'older' for the column after '15-20' runs (as this is not many runs).

Response: We agree with the reviewer. ‘Old’ referred to number of runs not actually time after purchase. We removed the term to avoid confusion.

Line 316 6. Measuring the fractions
Mention the programme/settings used on the TRI-CARB 2900TR Scintillation Counter. What should be used as a Blank for the Counter?

Response: We agree that this information is important, especially for scientists that never used an LSC or measured ^{3}H samples, and included as Supplemental Figure 1 screenshots of the settings used. We hope that this will be helpful to program other available scintillation counters.

Line 334: " 7.3. To compare samples with each other, normalize the data by summing up the values from minute 25 to the end of the run (minute 96) for each individual sample. Then divide the lower sum by the higher sum and multiply all individual values of the run with the higher some with this factor. In the end, the sum of the cpm values from minute 25 to the end should be equal for all samples compared with each other. Only normalized runs should be presented in the same graph/figure."

This description of normalization was not clear. 'Summing the values', be more specific, eg For every HPLC analysis/sample, sum the CPM from each eluted fraction from minute 25 to 96. The rest of the instructions are also a bit vague. The authors should be a bit more detailed. eg (if I have understood this correctly) Normalise all data to the sample with the highest total cpm (in fractions 25-96) by dividing the total cpm from a sample by the total cpm from the sample with the highest cpm, the resulting factor can be used to normalize the cpm from each fraction.
This raises a few questions: How different can the labelling be between samples in the same treatment?
Also, if comparing mutants or different growth conditions, should these be normalized to the WT/control treated plants, or should samples from different treatments be normalized within the treatment group? Presumably, some treatments or mutations might affect the destination of the labelled inositol (eg more inositol channeled into the cell wall), so the total cpm in the IP pool may be genuinely lower or higher than in the control treated or WT plants. In this case, wouldn't it be a bad idea to normalize to the most highly labelled control plants? I may be misunderstanding the data normalization procedure, so I think a few more details on how to carry this out (maybe a worked example, here or in the video) are necessary.

Response: We rephrased the paragraph and included additional information to make the normalization procedure clearer. This included Supplemental Figure 2, which shows the formulas used for the calculations in Excel, and Supplemental Figure 3, which shows the process of data analysis in Origin.
Absolute quantification with this method is quite challenging because the extraction efficiency with our protocol can be variable for reasons not clear to the authors, e.g. sometimes observed when replica of the same genotypes and treatments are analyzed. Therefore, we recommend to normalize samples. We agree with the reviewer that important genotype or treatment differences with respect to the overall labeling efficiency will get lost with our normalization procedure. In the revised version of our manuscript, we point this limitation out. We also included a sentence that normalization should be omitted in cases were labeling efficiency is to be analyzed.

It might be worth suggesting that researchers carry out a QC run after every 5 or 6 samples. The QC could be tritiated IP standards, made up to a particular concentration, aliquoted and frozen. This would give an indication of loss of sensitivity or resolution (although not in real time). However, if it is only possible to use the column for 30-40 samples before it needs replacing, this may be a waste of column. If the column can only be used for a relatively small number of samples, it would be good if the authors could explicitly state this early on (as most HPLC users wouldn't consider a column to be even close to 'old' after 30-40 runs).

Response: We agree that QC runs improve the confidence of results. However, we want to point out that only very few ^{3}H InsP standards, such as Ins(1,4,5)P_3 and InsP_6, are commercially available (the quality of InsP_6 that we purchased was in fact insufficient, as in our hands these standards eluted in several peaks) and that the PP-InsPs, which are generally the first InsPs that become difficult to detect when the column gets older, are not commercially available. In our hands, runs of WT Arabidopsis or WT yeast grown under the standard conditions can serve as QC runs, as those provide the full array of InsPs in one sample, with a high degree of reproducibility if following the recommendations stated in the protocol. We now reinforce the information that the column will most likely deteriorate after

30 – 40 runs and then needs to be replaced. We have not been able to find a method to rejuvenate older columns. While these columns are expensive, they still make up less than 20 % of the overall costs for consumables of one run (assuming that they last 40 runs).

This said, we point out in the revised version of our manuscript that standards are very helpful to provide information about loss of sensitivity and resolution and also help to assign peaks to possible isomers (despite the shortcoming that enantiomers and probably also not all non-enantiomeric isomers cannot be separated by HPLC). There is a great body of work particularly from the lab of Charles Brearley at the University of East Anglia that established methods to generate various standards that his lab has successfully used in his SAX-HPLC protocols. This work (and also that of some other labs) is now referred to in the revised version of our manuscript.

Line 347 7.4. To quantify certain inositol polyphosphate peaks…
Can the peaks be quantified? Peak areas of the same IP can be compared between samples, or the relative proportion of different IPs within a sample, so would this be better described as relative quantification?
Could some of the peaks be quantified if compared to tritiated IP standards? I noticed that it is possible to buy tritiated IP3 and IP6, but at lower specific activities than the inositol used for labelling here (which was why I was wondering if inositol with a lower specific activity could be used for labelling), or does the variability in labelling IPs in vivo make quantification impossible.
Also, if a researcher is comparing the relative amount of particular IPs between different treatments, should they use raw or normalized data?

Response: We agree that the term quantification alone can be misleading, as in most cases only relative quantification is presented. Regarding the use of standards, please see our response above.
To compare the relative amount of particular InsPs between different treatments, normalized data are necessary, unless they are calculated as percentage of total InsPs of each individual run.

FIGURES
Line 388: In the figure legend the authors refer to an 'old' column. It would be useful to know what exactly they mean by 'old'. How many samples can be analysed on the column before the chromatography deteriorates? As columns are expensive, it would be good to know how much analysis can be achieved per column.

Response: We specified the term ‘old’ in this case and state now earlier in the manuscript the average number of times these Partisphere HPLCcolumns can be used.

Line 380 Figure 1:
Are B and C zoom-ins of A? It is not clear from the wording.
It is not immediately obvious how the different scales compare in this set of panels, and there is a temptation to just look at the number scale (at least I did this for a while). It might be clearer to re-label the y axis to be all at 105.

Response: We rephrased the sentence to make it more clear that B and C are zoom-ins of A. We agree that the different scales of the y-axis can be confusing and therefore changed them all to 10^5.

Figure 2. Are A,B and C the same chromatogram?
It might be useful to add one of the chromatograms from figure 1 to make comparison with the Arabidopsis profile a bit easier.

Response: A, B and C show indeed the same HPLC run as B and C are zoom-ins to allow visualization of the individual $InsP_{3-5}$ isomers and the more highly phosphorylated PP-InsPs. As suggested by Reviewer 3, we included panel D that shows bar graphs of the quantification of $InsP_{3-8}$ from Arabidopsis and Lotus side by side to make the comparison easier.

Figure 3. How was IP6 relatively quantified? In the bar graph it has a relative level of 80%. Is this 80% of the total IP profile? It would be worth describing how this was calculated.

Response: Yes, it was 80 % of all InsP species eluting after minute 25. We agree that this information was missing and described this now in more detail.

Reviewer #2:
Manuscript Summary:
This protocol is very clearly written, with sufficient introduction and a wealth of experimental details. I want to thank the authors for providing such a detailed method to the community.

Major Concerns:
none

Minor Concerns:
Line 433, the authors mention the need to normalize to compare different experiments. Could they quickly comment on the methods that can be used for normalization.

Response: We reworked the whole normalization and data analysis paragraph to provide more details on how normalization can be done, what the options are and when normalization might not be needed or should be avoided. Please see also answer to Reviewer 1 above.

Reviewer #3:
Manuscript Summary:
The use of in vivo [3H]-myo-inositol labeling and SAX-HPLC to analyze InsP profile in plant tissues have been previously reported in several studies. This manuscript is intended to provide an accessible workflow for InsP analysis in plant tissues, from system set-up, in planta radiolabeling, InsP extraction, and SAX-HPLC operation to data processing. The results from studying two plant species were presented in this manuscript and the post-extraction stability of InsP was assessed. The authors provided a thoughtful discussion on the pros and cons of InsP profiling by SAX-HPLC and its limitation in detecting PP-InsP (InsP7 and InsP8) in comparison with PAGE analysis followed by TiO2 enrichment.

Major Concerns:

(1) Because [3H]-myo-inositol labeling and SAX-HPLC analysis have been used to study InsP in plants since two decades ago (e.g., Brearley & Hanke, 1996, Biochem. J.; Stevenson-Paulik et al., 2006, Methods), the authors should compare or comment on the differences among methods and the modifications and/or improvements in their current protocol.
(2) InsP standards should be used for the assignment of InsP isomers on the chromatograms presented in this manuscript.

Response: We agree that it is important to compare the described method not only with available alternatives like MS, NMR or PAGE, but also to compare it with previously published protocols using [^{3}H]-*myo*-inositol and SAX-HPLC. In the revised version of the manuscript we point out that one of the first SAX-HPLC protocols for the analysis of InsPs from plant samples was developed by the lab of Charles Brearley. Different labs have contributed to the improvement and adaptability of the method, such as the labs of John York and Adolfo Saiardi. Our protocol describes improvements to more reliably detect PP-InsPs due to modified labeling conditions (e.g. more plant material and the use of [^{3}H]-*myo*-inositol with higher specific activity, 30 – 80 Ci/mmol), modified extraction and neutralization buffer (1 M $HClO_4$ and 1 M K_2CO_3) and a modified gradient to better separate higher anionic species (i.e. $InsP_5$ isomers, $InsP_6$ and PP-InsPs). In addition, we provide a guideline for relative quantification of these molecules.

We agree that the use of standards is very helpful in such chromatography-based methods. As mentioned before in the response to Reviewer 1, we point that out in the revised version of our manuscript.

Minor Concerns:
To demonstrate how data analysis is performed according to step 7.3 and 7.4 of the protocol, an additional figure is highly recommended that presents quantification bar graphs based on the results of A. thaliana and L. japonicas to support the conclusion drawn in the discussion (line 443-454).

Response: We agree that a figure like this will be helpful and included it as Figure 2 D.

Reviewer #4:
This article, "Extraction and quantification of soluble, radiolabeled inositol polyphosphates from different plant species using SAX-HPLC" describes an advanced technique that is critical for studying inositol pyrophosphates (PP-InsPs) and their precursors. Understanding PP-InsP synthesis and signaling is becoming exceedingly important and while there are other methods to study PP-InsPs, this technique is the most sensitive and quantitative to date. Visualization of this technique will help laboratories who are unfamiliar techniques perform them efficiently and accurately. Overall, this article is well written and very meticulous, though there are some minor revisions are necessary.

line 41: add comma after "PP-InsPs"

Response: Changed accordingly.

line 61: Although minor, the authors should remove the word "extensively" and replace it with "rigorously" or another synonymous term. As PP-InsPs in plants have been studied by a handful of groups and their roles in signaling are still being explored, an alternative word to extensively would be more appropriate.

Response: We agree and rephrased the sentence accordingly.

lines 62-65: The authors state that InsP8 regulates defenses against herbivores/pathogens and then state that this was proposed to be mediated by the complex. While the data in Laha et al 2015 is compelling in suggesting InsP8 regulates these defenses, there is still work to be done before this can be concluded. Please rephrase this statement accordingly.

Response: We agree that still more work needs to be done on this topic to completely elucidate the mechanism and rephrased the sentence accordingly.

lines 118-123: It would be helpful for readers, especially those who are less familiar with this technique, to include the buffer name, molarity, and pH when introducing line A and B as well as a brief rationale. Perhaps: "Designate one pump for buffer A (termed pump A, consisting of 1mM EDTA) and one for buffer B (termed pump B, consisting of 1.3 M (NH4)2HPO4; pH 3.8 with H3PO4). Utilizing these specific buffers results in elution of highly charged PP-InsPs from the column." Alternatively, the authors could switch the order of section 2 (plant cultivation and labeling with [3H]-myo-inositol) and section 3 (preparation of buffers, column, and HPLC system) to provide a more logical workflow so that the buffers are discussed prior to sample preparation.

Response: We agree and switched the order of sections 2 and 3.

lines 273-275: The authors redirected the reader to the materials and methods section for the scintillation cocktail however from this table, it was not straightforward as to which solution is the cocktail. Please reword this sentence to include the abbreviated scintillation cocktail name stated from the materials and methods section in the sentence.

Response: We included the name of the scintillation cocktail.

lines 334-339 (section 7.3). Please rewrite this section and provide full details on this very important process, data normalization. I recommend starting with an overall rationale for why only incorporated counts are used. In describing the normalization process, the authors need to be clear on each step such as why a lower number is divided by a higher value. A formula perhaps might be useful to describe these steps. The software program "Origin" was mentioned, however, there are details that must be included: determining peak areas (how are the peaks themselves determined for each InsP/PP-InsP species?), are overlapping fractions considered for adjacent species?, cut-off values for the analysis (area under the curve), and other key details for analyzing this data. From my experience, there are many cohesive ways this data can be analyzed. This section needs to be better explained to help those who are unfamiliar with the technique to understand how to process their data. Change "some" to "sum" in line 336.

Response: We rephrased the paragraph and included additional information to make the normalization procedure clearer. This included Supplemental Figure 2, which shows the formulas used for the calculations in Excel, and Supplemental Figure 3, which shows the process of data analysis, including peak determination and background subtraction in Origin.