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Identification and quantification of *myo*-inositol hexakisphosphate in complex environmental matrices using ion chromatography and high-resolution mass spectrometry in comparison to ^{31}P NMR spectroscopy

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Highlights:

- Ion chromatography (IC) with high-resolution mass spectrometry (HRMS) was used to identify and quantify *myo*-IP6 in soil extracts.
- Ion suppression in IC facilitated the electrospray ionisation of the analyte for negative ion HRMS identification.

- IC provided faster quantification of *myo*-IP6 in extracts of complex matrices than ^{31}P NMR

Abstract

Myo-inositol hexakisphosphate, or phytic acid, (*myo*-IP6) is a key organic phosphorus (P) compound in soils and manures. Determinations of *myo*-IP6 in soils and manure extracts are frequently performed by ^{31}P NMR spectroscopy. This approach is time-consuming in terms of both sample preparation and instrument time, with uncertainties existing in relation to accuracy of identification and quantification due to potentially interfering resonances from co-extracted P species. In contrast, ion chromatography (IC) in combination with high-resolution mass spectrometry (HRMS) negative ion, electrospray ionisation (ESI) has been shown to enable highly specific identifications of *myo*-IP6 isolated from complex mixtures. In this paper, IC and ESI-HRMS were applied to the identification and the quantification of *myo*-IP6 isolated from soils and manures using NaOH-EDTA extraction, and quantifications based on IC. ESI-HRMS analysis of eluate trapped from IC unequivocally confirmed identification of *myo*-IP6 from a soil extract. The ion suppression cell of the IC instrument provides isolates of the analyte free of ionic components that would interfere with ESI. The *myo*-IP6 was identified in the NMR by comparing spectra of extracts of soils with and without authentic *myo*-IP6 "spiked" prior to extraction. Comparison of quantification *via* standard addition in IC and NMR analysis gave good correlation (r = 0.955). IC with ESI-HRMS was found to be more sensitive, rapid and reliable for the identification and quantification of *myo*-IP6 with a limit of detection (LOD) of 0.7 mg kg^{-1} and limit of quantification (LOQ) of 2.1 mg kg^{-1} using IC versus > 10 mg kg^{-1} LOD using ^{31}P NMR.

Keywords: Phytic acid, soil, ion chromatography, high-resolution mass spectrometry, NMR

Introduction

myo-IP6 constitutes up to 50 % of organic P in soils [1,2] and is thought to provide a significant source of P to biota in the absence of readily available inorganic phosphate [3], thereby playing an important role in the soil P biogeochemical cycle. Understanding the nature and behaviour of *myo*-IP6, and indeed other organic P compounds, in P biogeochemical cycling requires accurate detection and quantification of the compound isolated from the soil matrix. Determination of individual P compounds in soils is a major challenge due to the complexity of the soil matrix [4]. *myo*-IP6 possesses unusual properties for a naturally occurring organic compound in that it is highly polar, with six charge-dense phosphate moieties. Extraction of *myo*-IP6 from soil requires a strongly basic extractant and inevitably high concentrations of organic and inorganic ions, metals, and natural polymeric substances are coextracted. Analytical methods must overcome interferences from these materials and contend with the relatively low concentrations of P in soil extracts.

The use of ^{31}P NMR spectroscopy for identification and quantification of organic P in soil extracts has the advantage of being P-specific, enabling identification of P compound classes (and individual compounds when available in high concentration) as well as quantification. Its application to soil organic P characterisation was first demonstrated in 1980 [5]. In the following decades, NMR was applied to the identification and quantification of *myo*-IP6 in a range of soils, manures, and sediments [6–8]. Identification of the compound is based on the presence of four peaks, theoretically appearing in a 1:2:2:1 ratio (see Figure 1), in the monoester region of the spectrum. Quantification of *myo*-IP6 is achieved either by reference to an appropriate internal standard (usually methylene diphosphonic acid (MDPA)), or by

determination of the total P of the extract and calculation of the proportion of *myo*-IP6 in the total spectrum [3].

While ^{31}P NMR is advantageous for the characterisation of organic P compounds in soil extracts to the compound class level (e.g. phosphate monoester, phosphate diester, phosphonate) identification of individual compounds, particularly those other than *myo*-IP6, is not straightforward and often uncertain due to low signal-to-noise ratios and overlapping signals in the spectrum. ^{31}P NMR analyses of soil extracts require long acquisition times (usually overnight) at considerable cost to achieve acceptable signal-to-noise ratios. Difficulties caused by overlapping signals are particularly evident in the case of the phosphate monoesters as these are typically the most abundant soil P compounds observed in the NMR spectrum [9] and their signals co-occur in a narrow chemical shift range, typically ~ 4 – 6 ppm. In the absence of spiking with standard compounds, assignment of individual peaks to specific compounds is questionable. Literature values have been reported for the chemical shifts of individual compounds in comparable solutions and soil extracts [10,11] and have been referred to in identifications of compounds [12,13]. However, the chemical shift of a signal can be altered by the ionic strength and pH of the solution, hence, ppm values for extracts can differ substantially from those reported in the literature [14,15].

McLaren *et al.* [16] identified a broad ^{31}P NMR peak underlying the monoester region in the >10 kDa fraction of soil extracts and attributed the feature to “humic” polymeric P. Quantification of individual compounds in NMR spectra, once identified, can be challenging due to this underlying P signal. This difficulty was addressed by Doolette & Smernik [17] where three spectral analysis methodologies were proposed for the deconvolution of NMR

spectra. It was suggested that where peaks are fitted and integrated to the baseline [3] that the 1:2:2:1 stoichiometry of the signals should be observed. If the ratio deviates from 1:2:2:1, then underlying signal may result in overestimation of *myo*-IP6. Individual peaks may be modelled by the spectral analysis software to fit the region and integrated to simplify the signal. The deconvolution of the spectrum to take account of polymeric-P may be achieved by fitting of a "broad feature" to the region, which is then subtracted prior to integration of peaks. However, none of these approaches to spectral deconvolution has been adopted as standard practice.

Ideally, NMR spectroscopy experiments utilise relaxation delay several times greater than the T_1 of all the nuclei being quantified to attain full signal. For quantification against total P, the relaxation delay must be increased relative to the longest T_1 of the sample, requiring lengthy experiments to determine the T_1's present. Where an internal standard is used, the chemical environment of the standard's P nucleus must be sufficiently similar to that of the analyte P nucleus for meaningful quantification. Differences in the T_1 of the nuclei will result in disproportionate signal from one of the compounds leading to inaccurate quantification. T_1 relaxation delays are also influenced by the chemical environment of the analyte nucleus. For example, paramagnetic ions bound to the analyte reduce the T_1 delay. Soils are an abundant source of paramagnetic ions such as Al, Ca, Mg, Mn, and these inevitably occur in extract solutions and influence T_1 delays of the P nuclei, thereby affecting the quantification of *myo*-IP6 using NMR. Relaxation delays of longer than 25 s are now more commonly used for quantification of P compound classes in reference to the total P of the extract [18].

As alternatives to NMR, chromatographic methods with colorimetric and mass spectrometric detection have been applied to the identification of organic P compounds, particularly inositol

phosphates (IPxs) including the lower inositol phosphates and stereoisomers of IP6, in soil and manure extracts, although there is little consensus regarding the optimal approach. Leytem *et al.* [19] extracted P compounds from broiler ileal digesta, litter and manure with HCl, rather than the more frequently used NaOH-EDTA extraction [20] and determined IPx concentrations *via* HPLC with post-column derivatisation with $FeCl_3$. Quantification of IPx and monoester P was compared to NaOH-EDTA extracts analysed by ^{31}P NMR and found to correlate well despite the different extraction procedures used. El-Rifai *et al.* [21] used size exclusion chromatography (SEC) with selected ion monitoring (SIM) in ESI-MS to identify *myo*-IP6 in extracts, however, it was not possible to quantify *myo*-IP6 under these conditions. Quantification using ESI-MS as sole detection method is dependent on the ionisation efficiency of the target compound and requires calibration of the detection conditions using a standard reference. Sjoberg *et al.* [22] and Paraskova *et al.*[23] combined HPLC with multiple reaction monitoring (MRM) mass spectrometry for identification of IPxs in lake sediments. Solid phase extraction (SPE) using a C_{18} cartridge was required to remove matrix interferences from "coloured material", which caused loss of peak resolution between analyses and required regeneration of the column with offline flushing with base and acid solutions to eliminate this effect.

Herein, we demonstrate a new protocol for the qualitative and quantitative analysis of *myo*-IP6 in complex biological and environmental matrices based on IC and ESI-HRMS. The chromatography aspect was informed by the IC approach employed by Waithasong *et al.* [24] who extracted soils separately with aqueous acid and base. NaOH extracts were acidified with 6 M HCl to precipitate "humic acids" prior to IC analysis. An SPE clean-up step was then introduced to remove chloride. Identification and quantification were achieved by retention time and standard addition. Our new protocol is more streamlined, still using the NaOH-EDTA

extraction, however, sample processing is kept to a minimum, requiring only filtration and dilution of the extracts prior to ion suppression IC to provide salt-free *myo*-IP6 isolates for ESI-MS analysis. The quantification and identification results obtained show a number of advantages in the use of IC-HRMS in the identification and quantification of *myo*-IP6 compared to NMR. The developed protocol provides a sensitive, specific and efficient method of analysis with potentially wide utility.

Materials and methods

Extraction chemicals EDTA ($Na_2EDTA.2H_2O$) and sodium hydroxide were purchased from Sigma Aldrich (UK), and Thermo Fisher Scientific (Hemel Hempstead, UK), respectively. Reference standard *myo*-IP6 (D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate sodium salt, $Na_{12}C_6H_{12}O_6(HPO_3)_6$) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Methylene disphosphonic acid (MDPA) was purchased from Sigma Aldrich (UK). Glassware was washed with Decon 90, acid (5 % HNO_3) washed, rinsed with double-distilled water (DDW) and furnaced at 450 °C for 4 h. IC vials were acid washed and rinsed x 6 with DDW.

Soil and manure sampling strategy

Three clay soils under different land uses (arable, grassland and woodland) were collected from within 500 m of each other in the Sem river catchment around Prior's Farm in Salisbury, a site where extensive characterisation of P and organic matter fluxes from intensive cattle farming in the catchment has been studied in previous publications [25,26]. Soils were collected from the upper 10 cm after removal of grass and litter. The manures were collected fresh from farms within the Hampshire Avon catchment near Shaftesbury and Salisbury, and the Chew Valley catchment near Bristol, UK. Soil properties are described in Table 1. The soils were air dried

at ambient temperature and sieved to 2 mm prior to extraction, and the manures freeze dried to minimise any risk of infection and crushed.

Extraction

Extraction of soil and manures was based on Turner's 2008 study [20]. Extraction was performed in triplicate for each soil or manure. Air-dried soil (or manure) samples were added to 50 mL centrifuge tubes in 1.5 g aliquots. A solution (30 mL) of 0.25 M NaOH/0.05 M Na_2EDTA was then added. Tubes were shaken at 240 rpm at room temperature for 4 h, before centrifugation at 3300 rpm for 45 min. The supernatant was decanted (20 mL) to a 28 mL vial for NMR analysis. Internal standard (1.0 mL 50 µg $P.mL^{-1}$) methylene diphosphonic acid (MDPA) was added to the 20 mL aliquot for NMR which was then freeze-dried.

A 1 mL aliquot of the supernatant was filtered through a 0.2 µm PTFE syringe filter to an IC vial for IC analysis. The remaining supernatant was retained for total NaOH-EDTA extracted P analysis. IC and TP aliquots were stored at - 20 ° C until analysis. Soils were spiked prior to extraction using the method described below.

Soil spiking with *myo*-IP6

For extraction efficiency experiments, triplicate portions (1.5 g air-dried) of each soil were mixed with either 1 mL DDW or 616 mg L^{-1} *myo*-IP6 stock solution. Soils were stirred to completely homogenise the mixture and incubated for 2 h at room temperature before extraction.

Extraction efficiency calculations

Extraction efficiency was calculated by combination of the concentration of *myo*-IP6 added to the soil ([A]) to each control soil *myo*-IP6 concentration ([C]) to obtain a value for the theoretical *myo*-IP6 concentration ([A] + [C] = [T]) for that soil. This value ([T]) would represent the soil *myo*-IP6 concentration, assuming 100 % extraction efficiency. The calculated extraction efficiency (Eq. 1) is then the spiked soil *myo*-IP6 concentration ([S]) expressed as a percentage of the theoretical *myo*-IP6 concentration.

$$\frac{[S]}{[T]} x\ 100 = EEIP6 \quad (1)$$

Instrumental analysis

IC was performed on a Dionex ICS-5000 (Thermo Scientific, Hemel Hempstead, UK) equipped with a KOH eluent generator, ion suppressor and conductivity detector. Compounds were separated on an Ionpac AS11 column (2 x 250 mm; Thermo Scientific) preceded by an AS11G guard column (2 x 50 mm). The flow rate was 0.250 mL min^{-1}, and column oven temperature was 30 °C. The elution gradient was based on the work of Waithasong *et al.* [24] and comprised an initial 10 min equilibration at 4 mM KOH, followed by 0 to 19 min: 4 mM KOH, 19 to 24 min: ramp to 70 mM, 24 to 29 min: 70 mM KOH, 29 to 30 mM: ramp to 4 mM KOH.

A cleaning gradient was set up as follows: 0 min: 4 mM KOH, 1 to 41 min: 95 mM, 42 to 60 mM: 4 mM KOH. The cleaning gradient was run between all samples to prevent carryover of *myo*-IP6 in the system. The parameters for the cleaning gradient were determined by injecting an *myo*-IP6 standard (1 µM), followed by the cleaning gradient and a Milli Q blank afterward,

then increasing the duration of the high concentration KOH until there was no *myo*-IP6 detectable in the Milli Q blank. Chromatograms were analysed in Chromeleon (Thermo Scientific).

Preparative IC

Eluate fractions were collected in furnaced amber glass vials post-detection at intervals corresponding with the *myo*-IP6 peak in the ion chromatogram (see Figure 2B) for HRMS analysis.

Calibration curve

A calibration curve for *myo*-IP6 was prepared using stock solutions at 0.005, 0.01, 0.05, 0.1, 0.5 and 1 µM concentrations of the standard compound. The lowest concentration standard (0.05 µM) was run ten times and the LOD and LOQ were calculated (Eq. 2, 3) using the standard deviation (σ) of the area of the *myo*-IP6 peak from the ten injections, and the slope (m) of the calibration according to the formulae:

$$LOD = \frac{3.3\ x\ \sigma}{m} \quad (2)$$

$$LOQ = \frac{10\ x\ \sigma}{m} \quad (3)$$

Standard addition

myo-IP6 in soil and manure extracts was quantified *via* standard addition of reference *myo*-IP6. Soil extracts, cattle manure and sheep manure extracts were diluted 20-fold with Milli Q water.

Pig manure and chicken manure extracts were diluted 50-fold as these have higher concentrations of *myo*-IP6. The concentration of *myo*-IP6 standard to be added was calculated after initial screening of the diluted extracts and quantification using the calibration curve. Three standard additions were made, in increments of 0.06, 0.08, 0.4 and 0.8 mM *myo*-IP6, to soil, cow and sheep, chicken, and pig manure extracts respectively. The concentration of *myo*-IP6 was determined with respect to the purity of the compound as established in McIntyre *et al*. [27]. The concentration of *myo*-IP6 in the untreated extract was calculated by preparation of a standard addition plot, according to Eq. 4:

$$[IP6] = c/m \quad (4)$$

Where c is the intercept, and m is the slope of the fitted line: y = mx + c.

ESI-HRMS

Reference *myo*-IP6 solution was prepared at 20 ppm in DDW. Preparatory IC eluate was analysed directly. HRMS analysis was performed on an Orbitrap Elite MS (Thermo Scientific) with an ESI source. The Orbitrap was operated in negative ion mode, calibrated using negative ion calibration solution (Thermo Scientific), and tuned automatically on the *m/z* 328.9 (*myo*-IP6 $[M\text{-}2H]^{2-}$). Solutions were directly infused at 10 µL min^{-1} for acquisition of full mass spectra. Source voltage was -1.8 kV, sheath gas (nitrogen) flow rate was 30 arbitrary units (arb), auxiliary gas (nitrogen) flow rate was 0 arb and the sweep gas (nitrogen) flow rate was 1 arb. The capillary temperature was set to 275 ° C. Full mass spectra were recorded at 120,000 resolution and 50 scans were averaged to increase the signal-to-noise ratio. Mass spectra were analysed using Xcalibur (Thermo Scientific). *Myo*-IP6 was identified by comparison of the negative ion mass spectrum of the eluate to that of reference *myo*-IP6 as described in McIntyre

et al. [27] and observation of characteristic ions with *m/z* values < 5 ppm of those reported for the standard compound.

^{31}P NMR

Freeze-dried soil extracts were prepared for NMR by dissolving 100 or 200 mg homogenised freeze-dried powder in 0.9 mL 1 M NaOH/ 0.1 M Na_2EDTA solution and 0.1 mL D_2O [20].The arable soil freeze dried extracts were analysed in 200 mg mL^{-1} solutions, while the remaining clay soil extracts were analysed at 100 mg mL^{-1}. Re-dissolved extracts were centrifuged at 6000 rpm for 20 min and the supernatant decanted to a 5 mm NMR tube for immediate analysis.

An equimolar MDPA:*myo*-IP6 solution was prepared and run under the same conditions as the soil extracts. A relative response factor was calculated versus MDPA for the peak area of each of the four *myo*-IP6 resonances after integration and these were used to quantify the compound in the soil extracts. NMR spectra were acquired on a 500 MHz Varian VNMR S500 equipped with an Agilent OneNMR probe. Parameters were 45 ° pulse, 0.66 s acquisition time, 3.0 s relaxation delay, 16000 scans at 25 ° C with proton decoupling.

NMR spectra were analysed using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). The PO_4^{3-} peak was set to 6 ppm. Control and spiked spectra for the same soil extract types were overlaid and *myo*-IP6 peaks were identified by the increase in peak height of the four peaks (*myo*-IP6a, *myo*-IP6b, *myo*-IP6c, *myo*-IP6d). The baseline was corrected using a Whittaker Smoother function in MestreNova. Internal standard MDPA, and the four peaks were integrated, and the integral values were corrected using the predetermined response factor for MDPA versus *myo*-IP6 under experiment conditions. Quantification of *myo*-IP6 was based

on the lowest corrected peak area as this was deemed to be least influenced by underlying signal from other ^{31}P nuclei in the extract.

Pearson's product moment correlation between quantifications of *myo*-IP6 by NMR and IC was determined in the statistical package 'R'.

Results and Discussion

Development of this new IC and ESI-HRMS approach to the determination of *myo*-IP6 proceeded with: (i) implementation of IC with ion suppression for the analysis of soil and manure extracts, (ii) identification of *myo*-IP6 using co-chromatography with the authentic compound and ESI-HRMS, and (iii) quantification by IC using standard addition compared with ^{31}P-NMR quantification using an internal standard.

Ion chromatography of soil and manure extracts

Figure 2 depicts the ion chromatograms of extracts of each type of soil (A - C) and the four manures (D - G). The *myo*-IP6 peak is highlighted in the enlarged section of each chromatogram. In each analysis, the majority of the matrix material elutes in the first 10 min, followed by a large EDTA peak (13 – 15 min), with the *myo*-IP6 peak appearing at ~ 20 min, shortly after the maximum concentration of KOH (70 mM) is reached in the eluent gradient. Peaks eluting immediately after the *myo*-IP6 peak have been identified as isomers of IP5 in the McIntyre study [27], and the tailing of *myo*-IP6 into these has been characterised. Due to the higher concentration of *myo*-IP6 in pig and chicken manure, these extracts were diluted 50-fold, whereas the soil extracts were diluted 20-fold.

Identification of *myo*-IP6 in IC chromatograms

The ion chromatograms (Figure 2) of the soil and manure extracts show good separation of *myo*-IP6 from the matrix material, which was identified by comparison of retention time with the reference compound, as well as by observation of the enhanced peak after standard addition. The *myo*-IP6 elutes sufficiently late in the chromatogram to prevent interference from the tailing EDTA peak. Its retention behaviour is in accordance with that found by Waithasong *et al.* [24] although variations in retention time are common in ion chromatography. The pig, sheep, and to a lesser extent cattle, manure extract chromatograms display peaks (as yet unidentified) in the same region as the *myo*-IP6 peak. A comparison of the 20 to 22 min region of a control versus spiked grassland soil extract is shown in Figure 3 (A). The enhanced *myo*-IP6 peak is clearly visible in the overlaid chromatogram aiding the identification of the compound. Figure 3 (B) depicts the overlaid ion chromatograms of a control soil extract, and the extract following addition of 0.062 µM commercial *myo*-IP6 reference standard (0.4 ng *myo*-IP6 per injection). Again, the putative *myo*-IP6 peak is visibly enhanced in area due to co-chromatography with the authentic compound, confirming the identity of the eluting compound.

ESI-HRMS identification of *myo*-IP6 in IC eluate

The eluate corresponding to the *myo*-IP6 peak (20.6 – 21.6 min) in the ion chromatogram of the grassland soil (Figure 2 B) was collected post-detection and directly infused into the ESI-Orbitrap HRMS. The negative ion mass spectrum of the eluate is presented in Figure 4 (A) together with the reference mass spectrum of standard *myo*-IP6 (B) for comparison and confirmation of the identification. A list of the ions and their identities are given in Table 1. The eluate mass spectrum shows the same pattern of multiple charge acquisition, fragmentation and adduct formation as seen for the reference *myo*-IP6. The characteristic behaviour of *myo*-IP6 in ESI-HRMS, and the ions generated in the mass spectrum, have been discussed

comprehensively in McIntyre *et al.* [27]. The ESI-HRMS spectra provide unequivocal confirmation of the identification of *myo*-IP6 isolated from the soil matrix. In preliminary experiments *myo*-IP6 was not identified in a soil extract mass spectrum even when spiked directly into the extract. This was likely due to the high ionic strength of the extract, caused by the high concentrations of hydroxide ion, metals and salts in the extractant and vast range of other charged species occurring in soils. Competition between these ions in the ESI source can cause clustering [28] and ion suppression of analytes of interest [29].

The advantages of using IC to isolate *myo*-IP6 are twofold. First, the chromatographic separation of compounds from the matrix material, provides a purified sample with fewer interfering compounds for ESI-HRMS. Second, the ion suppressor in the IC instrument removes K^+ ions from the eluate *via* cation exchange for H^+ ions across a membrane. Coupled with the reduction, or indeed elimination, of matrix compounds *via* chromatography, the ion suppression results in a predominantly aqueous solution containing the *myo*-IP6 analyte. Unhindered by competition from salts in solution in the ESI, ionisation of the analyte is achieved, enabling analysis of the compound and unambiguous identification.

Standard addition quantifications

myo-IP6 was quantified in IC *via* addition of *myo*-IP6 reference standard to the extract solution. Standard addition concentrations were chosen after an initial approximate quantification against the external calibration graph. The form of the ion chromatograms did not appear to be affected by the addition and the range of R^2 values for the standard addition plots was 0.9269 – 0.9996. It was concluded that the standard addition did not cause any deleterious effects and confirmed that SPE clean-up was not required.

NMR spectra

Figure 5 shows the overlaid full ^{31}P NMR spectra of the soil extracts of both control and spiked soils of each land use type. The internal standard (MDPA), the monoester-P, the diester-P, and pyrophosphate regions are indicated. The detail around the 4 – 6 ppm region is enlarged. The form of the spectra of the control and spiked extracts are almost identical for each soil type, with the exception of the *myo*-IP6 peaks. The identification of the four *myo*-IP6 resonances (chemical shifts: *myo*-IP6a 5.75, *myo*-IP6b 4.81, *myo*-IP6c 4.44 and *myo*-IP6d 4.33 ppm) is verified by the enhanced peak height in the spiked samples. *myo*-IP6 was not detected in one of the woodland control soil extracts and none of the arable control extracts, even at 200 mg mL^{-1} concentration. The *myo*-IP6 chemical shifts in the NMR spectra of the 200 mg mL^{-1} spiked arable soil extracts were altered by up to - 0.06 ppm compared to 100 mg mL^{-1} extracts. The chemical shifts were within a 0.02 ppm window for the 100 mg mL^{-1} spectra but shifted by up to 0.06 ppm in the 200 mg mL^{-1} spectra. This was likely due to the difference in ionic strength of the higher concentration solutions and illustrates the difficulty in relying on literature chemical shift values for identification of individual compounds in soil using ^{31}P NMR.

The low concentration of organic P in the arable extracts is evident by the lack of signal in the monoester- and diester-P regions of the NMR spectrum. The spectrum is dominated by the PO_4^{3-} peak. A low abundance of pyrophosphate is observed around − 4.5 ppm. The grassland soil extracts contained more monoester P, and the highest concentration of pyrophosphate of the three soil types. Monoester P was most abundant in the woodland soil extracts, as well as some pyrophosphate signal at − 4.5 ppm.

Identification and quantification of *myo*-IP6 based on NMR spectra

myo-IP6 was quantified from the NMR spectra by integration of the MDPA and the four *myo*-IP6 peaks. The value for each of the *myo*-IP6 peaks was adjusted using the response factor calculated from the equimolar solution of MDPA and *myo*-IP6 reference compounds. However, the peak areas of the four *myo*-IP6 resonances were not consistently observed in the expected ratio of 1:2:2:1, indicating underlying signals in this critical region. Therefore, the lowest peak area of the four was chosen as the basis of the quantification, as it was assumed that this peak was the least influenced by underlying signals. This was one of the methods proposed by Doolette & Smernik [17]. Almost no monoester P was detected in the control arable soil extract, and the *myo*-IP6 peak ratios in the spiked extract NMR spectrum are closest to 1:2:2:1 ratio of the three soil types.

Quantification, extraction efficiency, and comparison of methods

The concentrations of *myo*-IP6 in each of the soil extracts, as determined by IC and NMR, the extraction efficiency of NaOH-EDTA for *myo*-IP6, and the IC LOD and LOQ are presented in Table 2. The *myo*-IP6 concentration was greatest in the pig and chicken manures, reflecting the high grain content of their diets, particularly for the chicken. Poultry and pigs are less capable than ruminants of digesting the *myo*-IP6 in their feed and subsequently have higher concentrations of the compound in their manure [30,31]. The sheep and cattle were grass fed and as expected had lower concentrations of *myo*-IP6 in their manures than the grain fed animals. Determined *myo*-IP6 concentrations were in line with literature reports of *myo*-IP6 concentrations in soils and manures [6,32,33]. The woodland soil was found to have the highest concentration of *myo*-IP6 by IC. Extraction efficiency of NaOH-EDTA for *myo*-IP6 from the

soils, as determined by IC, ranged from 62.1 – 69.4 %. The woodland soil had the lowest extraction efficiency, and the arable soil the highest. The extraction efficiencies of NaOH-EDTA for *myo*-IP6 determined by spiking the soil prior to extraction given in Table 2 are complete for the IC data, but not for NMR quantification data. They are in the range of 62 – 69 %, however, the reasons for the different extraction efficiencies between soils is not clear from these data.

Figure 6 depicts the correlation of the quantification of *myo*-IP6 by both IC and NMR where *myo*-IP6 was detected by both methods for each sample. The slope of the fitted line is 0.9456, indicating an almost 1:1 relationship. The Pearson correlation coefficient was found to be 0.955 with $P < 0.001$, suggesting that both methods are comparable.

myo-IP6 was not detected in the arable soil control NMR spectrum, despite doubling the concentration of the extracts to 200 mg mL^{-1}. The arable soil extract NMR spectra contained very little organic P but did have a high concentration of PO_4^{3-} as evidenced by the intense peak in the NMR spectra. The low organic P content resulted in an almost 1:2:2:1 ratio for the *myo*-IP6 peaks in the spiked samples. The integration of these peaks was therefore determined to be least affected by the underlying broad P signal in the spectrum. This supports the conclusion that the use of lowest peak area in this experiment was the best quantitative approach and that variation in peak ratio is caused by underlying interferences, such as the broad feature identified by McLaren *et al.* [16]. The LOD of the NMR experiments was not directly determined, but can be considered >10 mg kg^{-1} using the approach applied here as this was the highest concentration determined by IC, but not by NMR.

Determination of the response factor for quantification of *myo*-IP6 relative to MDPA was a faster and more practical alternative to measurement of T_1 values for the *myo*-IP6 peaks which requires multiple lengthy experiments and calculations [34]. The response factor method appears to be reasonably accurate given the agreement in quantification with that determined by IC. The T_1 relaxation of a nucleus may be influenced by paramagnetic ions in the sample solution if they are in sufficiently high concentration and bound to the analyte of interest. We are confident there is no interference in the quantification from paramagnetic ions for two reasons. Firstly, while the paramagnetic ion concentration of a soil extract solution will be high due to co-extracted metal ions, the EDTA in the re-dissolved extract solution will prevent binding to the *myo*-IP6. Secondly, the effect of the paramagnetic ions would be to shorten the T_1 relaxation of the *myo*-IP6 nuclei, and the use of the response factor in this situation would cause overestimation of the *myo*-IP6 concentration. Since the quantification performed using the response factor correlated very well with that calculated *via* standard addition in IC, it was concluded that the response factor method of quantification using the lowest peak area was a reliable approach.

Comparison of IC v NMR

Quantification of *myo*-IP6 using standard addition in IC was found to be more rapid than quantification using NMR, even including the time taken for the cleaning step between sample injections to remove carry over. The total instrument time required per extract analysis in IC is 6 h, while each NMR analysis takes 16 h, making IC analysis 2.5 times faster than NMR. Additionally, sample preparation is much quicker and more straightforward for IC. Extracts are syringe filtered and diluted for standard addition in IC (ca. 30 min), while extracts for NMR must be frozen (12 h), and freeze-dried (~ 36 – 48 h), before being homogenised, weighed, re-dissolved and centrifuged (total = 1 h). Sample requirements for IC are also far lower than for

NMR. Each NMR analysis required at least 100 mg of freeze-dried powder, which equates to about 3.5 mL of extract solution. Each IC analysis required 200 µL (50 µL x 4) of extract solution for full quantitative results. The sensitivity of IC (LOD = 0.7 mg kg^{-1}, LOQ = 2.1 mg kg^{-1}) exceeds that of NMR (LOD > 10 mg kg^{-1}) under the applied conditions.

Quantification of *myo*-IP6 in individual extracts and standard deviations of the quantifications (Table 2) are similar between methods. The variances seen in the quantification are therefore an artefact of the extraction procedure and the variance between 1.5 g replicates using this method.

Conclusions

This work demonstrates the development of a novel and robust method for the identification and quantification of *myo*-IP6 extracted from soils. The main findings confirm:

1. The use of IC-HRMS for identification and quantification of *myo*-IP6 in soil and manure extracts is shown to be advantageous for these complex matrices. The *myo*-IP6 isolated by IC is free from significant interferences allowing the use of ESI-HRMS to make unequivocal identifications of the target compound. This contrasts to the difficulties encountered in NMR spectroscopy, particularly in the absence of spiking, where *myo*-IP6 identification and quantification may be confounded by variation in chemical shifts, overlapping peaks and/or underlying signals.

2. The ESI of the preparatively-isolated *myo*-IP6 is facilitated by the ion suppression component of the ion chromatograph. The removal of salt contaminants eliminates signal dispersion in the ESI caused by ion suppression and competition between analyte and matrix ions.

3. The standard addition method of quantification was found to be necessary as quantification with reference to an external calibration was impaired by matrix effects, which were unpredictable. Standard addition plots gave good R^2 values. The LOD and LOQ of *myo*-IP6 in IC were found to be 0.7 and 2.1 mg kg^{-1}, respectively whereas the LOD of *myo*-IP6 in ^{31}P NMR was > 10 mg kg^{-1}. A cleaning program was required between samples to remove residual *myo*-IP6 from the system. Nevertheless, the standard addition in IC method of quantification was much quicker than NMR in terms of sample preparation and analysis time. A much smaller amount of extract was required for each IC (200 µL) than for NMR (5 mL) analysis.

4. Quantification of soil *myo*-IP6 using both NMR and IC correlated well (r = 0.955) indicating that the methods were comparable. Standard deviations for replicate analyses of extracts of different sample type were similar between IC and NMR.

5. The extraction efficiency of the NaOH-EDTA extraction method for *myo*-IP6 from soils was briefly investigated. As expected, extraction efficiency results from NMR and IC were broadly similar where *myo*-IP6 was detected in both control and spiked samples. The reasons for the difference in extraction efficiency between soil types was not immediately apparent and this disparity will be investigated in a future study applying IC analysis of *myo*-IP6.

Together, these results show that the IC quantification method is more rapid and more sensitive than NMR, with a lower LOD, and just as accurate. Standard addition in IC can be used in place of NMR for quantification of *myo*-IP6, and offline ESI-HRMS can be used to unequivocally confirm the identification of *myo*-IP6 extracted from complex matrices. The sensitivity, rapidity and low sample requirements of the IC quantification may be exploited,

and multiple replicate analyses performed for different extracts, enabling more complex and ambitious experiments to be undertaken leading to more rigorous or comprehensive assessments being achieved.

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Tables and Figures

Table 1. Soil properties. (*Denotes average of 3 replicates.)

Soil	Moisture content	pH	Organic content LOI	Total P	IP6	NaOH-EDTA extracted P	Al	Ca	Fe	Mg	Mn	Total Metals
	(%)		(%)	(mg kg^{-1})								
Arable	38*	7.37*	10.7*	2006	7.2*	385.9*	2456	1087	2781	288	22	6633
Grassland	24*	6.08*	11.9*	1332	52.8*	293.8*	2586	354	2806	227	33	6006
Woodland	36*	4.60*	20.2*	1162	61.2*	344.5*	2371	212	1766	218	14	4580

Table 2. Ions identified by ESI-HRMS in Figure 4 including formulae and mass measurement errors.

	Ion	z	*myo*-IP6 soil *m/z*	*myo*-IP6 ref *m/z*	Formula	Soil *myo*-IP6 Δ ppm	*myo*-IP6 ref Δ ppm
A	$[M\text{-}HPO_3\text{-}2H]^{2-}$	2	288.9405	288.9408	$C_6H_{15}O_{21}P_5$	-1.4	-1.9
B	$[M\text{-}HPO_3\text{-}3H + Na]^{2-}$	2	299.9316	299.9317	$C_6H_{14}O_{21}P_5Na$	-1.6	-1.7
C	$[M\text{-}2H]^{2-}$	2	328.9235	328.9239	$C_6H_{16}O_{24}P_6$	-1.0	-1.6
D	$[M\text{-}3H+Na]^{2-}$	2	339.9145	339.9148	$C_6H_{15}O_{24}P_6Na$	-1.0	-1.4
E	$[M\text{-}4H+2Na]^{2-}$	2	350.9057	350.9057	$C_6H_{14}O_{24}P_6Na_2$	-1.3	-1.3
F	$[M\text{-}2HPO_3\text{-}H_2O\text{-}H]^{-}$	1	480.9115	480.9116	$C_6H_{13}O_{17}P_4$	-2.5	-2.7
G	$[M\text{-}2HPO_3\text{-}H]^{-}$	1	498.9222	498.9223	$C_6H_{15}O_{18}P_4$	-2.6	-2.8
H	$[M\text{-}2HPO_3\text{-}2H+Na]^{-}$	1	520.9042	520.9043	$C_6H_{14}O_{18}P_4Na$	-2.6	-2.8
I	$[M\text{-}HPO_3\text{-}H]^{-}$	1	578.8887	578.8888	$C_6H_{16}O_{21}P_5$	-2.6	-2.7

Table 3. *myo*-IP6 concentrations as determined by IC and NMR and limit of detection (LOD) and limit of quantification (LOQ) of IC. *C = control, S = spiked.*

Soil Extract	*myo*-IP6 by IC	Average *myo*-IP6	Extraction efficiency	*myo*-IP6 by NMR	Average *myo*-IP6	Extraction efficiency
	mg kg^{-1}		%	mg kg^{-1}	mg kg^{-1}	%
Arable C1	10.09			ND		
Arable C2	4.32	7.2 ± 2.9		ND		
Arable C3	7.12			ND		
Arable S1	199.54			182.75		
Arable S2	128.19	164.9 ± 35.7	69.4	142.59	159.5 ± 20.8	
Arable S3	167.01			153.01		
Grassland C1	79.42			74.42		
Grassland C2	32.92	52.8 ± 24.0		33.74	55.9 ± 20.6	
Grassland C3	45.99			59.47		

Grassland S1	124.54			171.49		
Grassland S2	233.9	188.9 ± 57.2	66.7	230.41	221.6 ± 46.3	77.4
Grassland S3	208.22			262.74		
Woodland C1	51.55			ND		
Woodland C2	68.55	61.2 ± 8.7		49.73	52.3 ± 3.7	
Woodland C3	63.48			54.95		
Woodland S1	248.8			239.79		
Woodland S2	138.24	181.0 ± 59.4	62.1	156.67	186.2 ± 46.5	65.8
Woodland S3	156.04			162.24		
LOD	0.7					
LOQ	2.1					

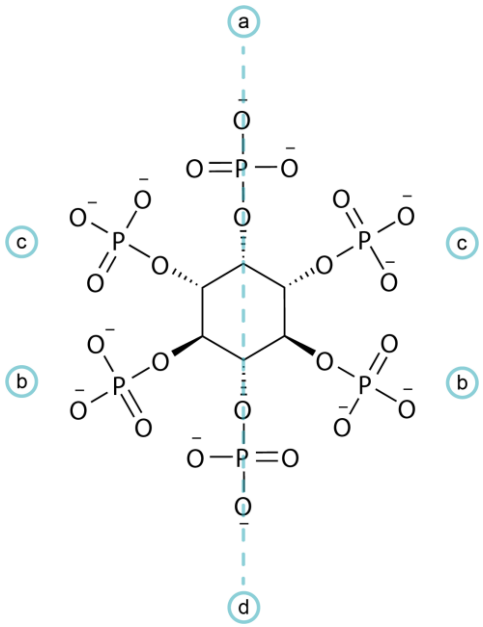

Figure 1. *myo*-IP6 at pH 10 – 13 with plane of symmetry indicated. Two P nuclei contribute to the b and c signals, leading to peak integrals in a ratio of 1:2:2:1[35].

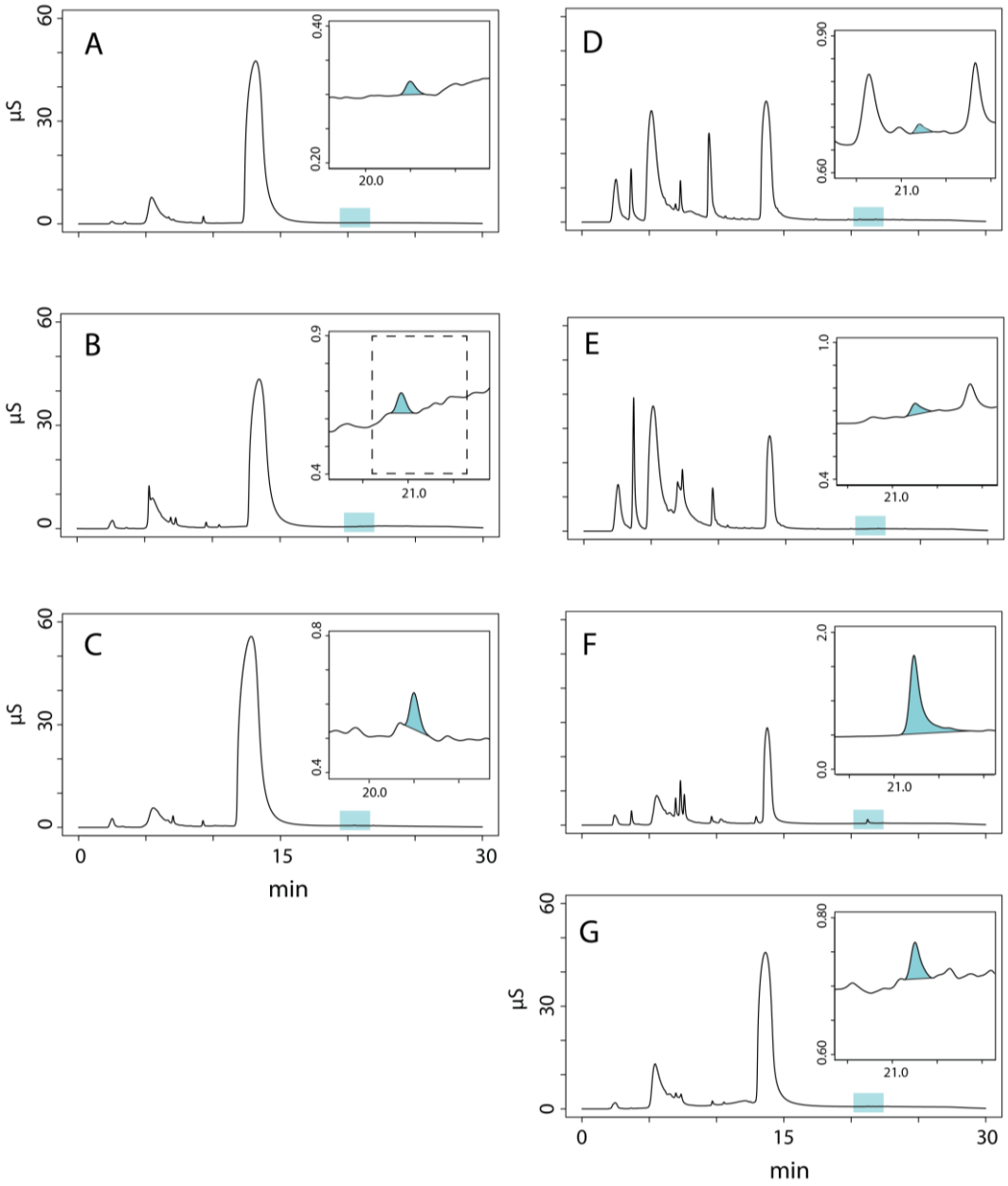

Figure 2. Ion chromatograms of soil (A: Arable, B: Grassland, C: Woodland) and manure (D: Sheep, E: Cattle, F: Chicken, G: Pig) extracts. The *myo*-IP6 peak is highlighted in the enlarged segment. Dotted line indicates fraction collected for ESI-HRMS.

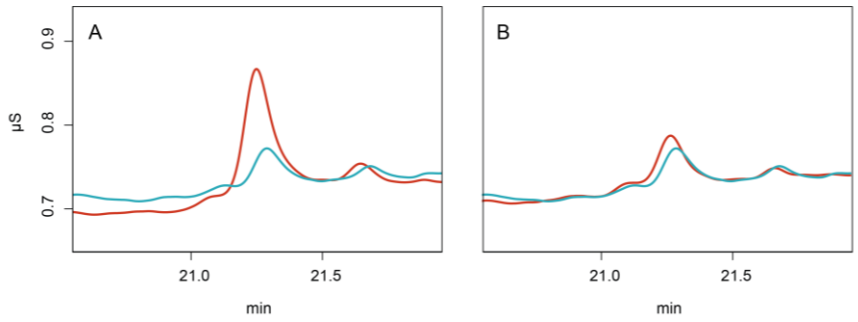

Figure 3. Overlaid ion chromatograms of grassland extract. (A) The chromatogram of the control soil extract (blue) overlays the chromatogram of the extract of the soil spiked prior to extraction (red). (B) The chromatogram of the control soil extract overlays the chromatogram of the extract including addition of commercial *myo*-IP6 reference compound (0.4 ng).

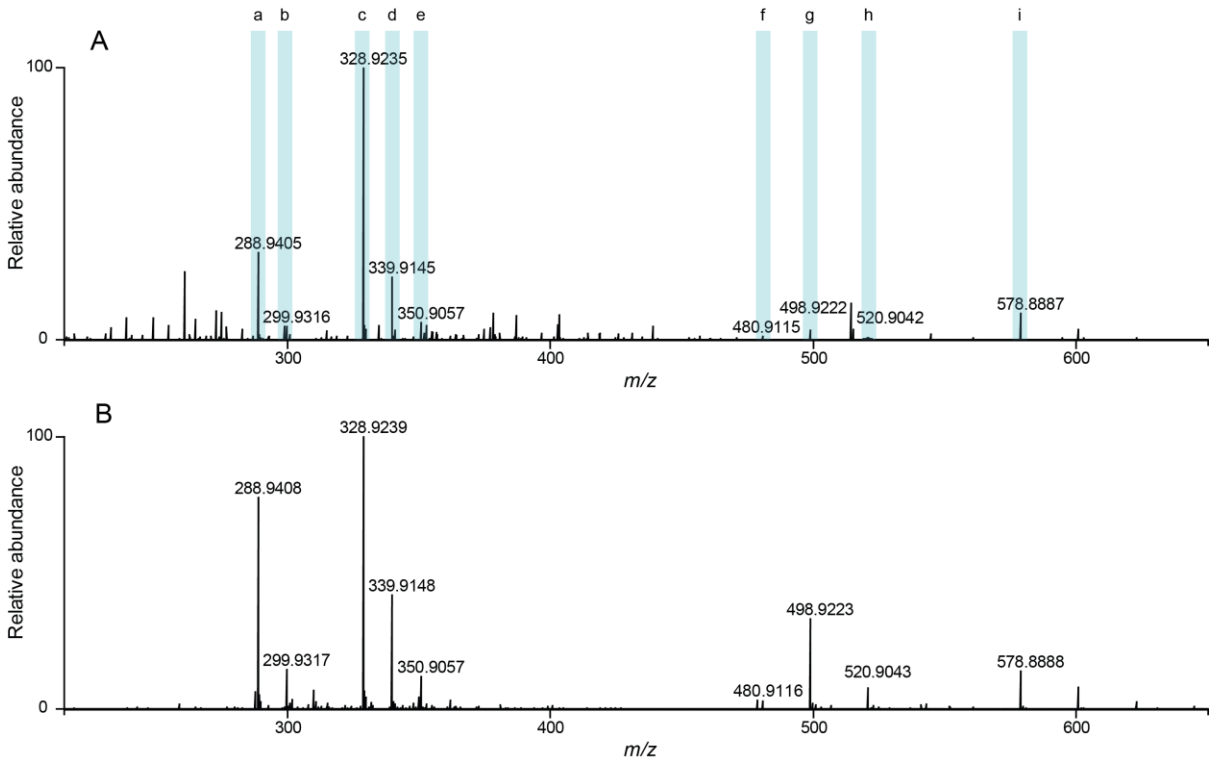

Figure 4. (A) ESI-HRMS negative ion mass spectra of isolated soil extract eluate (chromatogram B, Figure 2) from IC and (B) reference *myo*-IP6 standard. Ions highlighted are identified in Table 1.

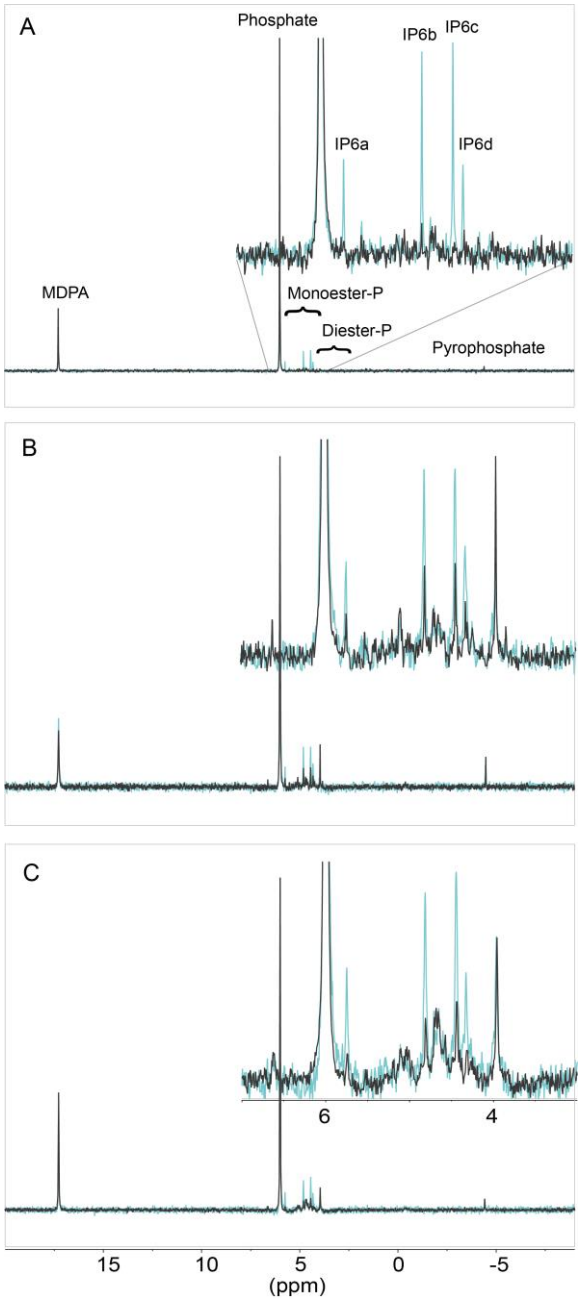

Figure 5. Overlaid NMR spectra of soil extracts (A: Arable, B: Grassland, C: Woodland) with control soil extracts in black and spiked extracts in blue. Internal standard (MDPA), orthosphosphate, monoester-P, diester-P and pyrophosphate regions are indicated. The monoester region (4-6 ppm) is enlarged in each. *myo*-IP6 resonances are identified by the four enhanced peaks in the enlarged region as labelled in A.

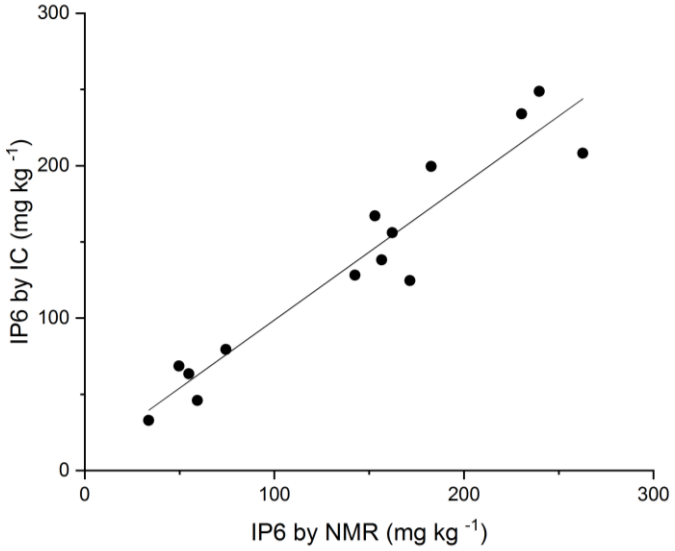

Figure 6. Plot of *myo*-IP6 concentrations determined by IC and NMR and Pearson's product moment correlation ($r = 0.9554$, $p \leq 0.001$).