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Assessing crop residue phosphorus speciation using chemical fractionation and solution ^{31}P nuclear magnetic resonance spectroscopy

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ABSTRACT

At physiological maturity, nutrients in crop residues can be released to the soil where they are incorporated into different labile and non-labile pools while the remainder is retained within the residue itself. The chemical speciation of phosphorus (P) in crop residues is an important determinant of the fate of this P. In this study, we used chemical fractionation and ^{31}P nuclear magnetic resonance (NMR) spectroscopy, first separately and then together, to evaluate the P speciation of mature oat (*Avena sativa*) residue. Two water extracts (one employing shaking and the other sonication) and two acid extracts (0.2 N perchloric acid and 10% trichloroacetic acid) of these residues contained similar concentrations of orthophosphate (molybdate-reactive P determined by colorimetry) as NaOH–EDTA extracts of whole plant material subsequently analysed by solution ^{31}P NMR spectroscopy. However, solution ^{31}P NMR analysis of the extracts and residues isolated during the water/acid extractions indicated that this similarity resulted from a fortuitous coincidence as the orthophosphate concentration in the water/acid extracts was increased by the hydrolysis of pyrophosphate and organic P forms while at the same time there was incomplete extraction of orthophosphate. Confirmation of this was the absence of pyrophosphate in both water and acid fractions (it was detected in the whole plant material) and the finding that speciation of organic P in the fractions differed from that in the whole plant material. Evidence for incomplete extraction of orthophosphate was the finding that most of the residual P in the crop residues following water/acid extractions was detected as orthophosphate using ^{31}P NMR. Two methods for isolating and quantifying phospholipid P were also tested, based on solubility in ethanol: ether and ethanol:ether:chloroform. While these methods were selective and appeared to extract only phospholipid P, they did not extract all phospholipid P, as some was detected by NMR in the crop residue after extraction. These results highlight the need for careful interpretation of results from chemical fractionation, as separation can be compromised by incomplete recovery and side reactions. This study also highlights the benefits of employing a technique that can simultaneously detect multiple P species (solution ^{31}P NMR) in combination with chemical fractionation.

1. Introduction

Phosphorus (P) is a highly mobile element in plants, moving readily between parts of the plant. The seed becomes the ultimate sink for most P in annual species, while the remaining P is distributed between roots, stem, leaves and chaff/pod material. In cropping systems, these latter plant parts (crop residues) remain in the field after grain harvest and are a potential source of nutrients such as P, for subsequent crops. The speciation of P in these residues plays an important role in determining its fate, i.e. whether it is released to soil as soluble P, assimilated by micro-organisms, or whether it adds to more chemically stable P pools in

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soil. Several methods have been used to characterise P in crop residues, the two most popular being chemical fractionation [1–5] and ^{31}P nuclear magnetic resonance (NMR) spectroscopy [6–9].

Chemical fractionation has long been used to characterise P in plant material. Consequently, an extensive body of data exists in the literature [1–4,10–12]. Sequential chemical fractionation characterises P species based on their assumed solubility in, or reactivity toward, a series of extractants. Generally, crop residue P is separated into (i) P soluble in weak acid or water (soluble P), (ii) P soluble in non-polar organic solvents (phospholipid P), (iii) P released by reaction with potassium hydroxide or strong acid (nucleic acid P), and (iv) residual P.

Solution ^{31}P NMR spectroscopy following sodium hydroxide ethylenediaminetetraacetic acid (NaOH–EDTA) extraction offers an alternative approach to determining P speciation in environmental and agricultural samples [13,14]. This method provides more detailed P speciation than is possible with sequential chemical extraction because several organic and inorganic P species can be identified simultaneously. Sample preparation for solution ^{31}P NMR involves alkaline extraction, usually with 0.25 mol L^{-1} NaOH and 0.05 mol L^{-1} Na_2EDTA. The solubility of both organic and inorganic P species is maximised at high pH, and the additon of EDTA complexes paramagnetic ions which increases the efficiency and diversity of P species extracted [15]. As with chemical fractionation, there is a portion of P that was not extractable (residual P). There is also the potential for some organic P compunds to be hydrolysed under the alkaline extraction conditions [16].

In plant studies, solution ^{31}P NMR spectroscopy has primarily been used to speciate P in immature material [6,17] or in material used in incubation/decomposition studies [7,8,18]. Recently, we used this technique to speciate P in field-collected mature cereal and legume residues [9] and found that crop residues (stem and leaf material) contained primarily orthophosphate, along with smaller quantities of phospholipids, RNA, phytate and pyrophosphate.

Few studies have compared results from these two different approaches [19–22] and to our knowledge, no study has correlated the P species obtained from ^{31}P NMR to that from chemical fractionation for plant material. There is a small body of work in which the two methods have been combined by using NMR to characterise P species isolated in chemical extracts [23,24]. Application of ^{31}P NMR analysis to the fractionated materials can be used to test basic assumptions that underpin the chemical fractionation approach. Chemical fractionation assumes that each extractant is selective for the targeted P species and that all of the targeted P species are released to solution during the treatment. Chemical fractionation methods also assume that other P species present are not extracted or transformed prior to the step designed to release that particular P species. Solution ^{31}P NMR provides a means to test these assumptions through determination of the P speciation of both the extracts and the treated crop residues following each fractionation step.

The first aim of this study was to compare P speciation of a single plant residue as determined by chemical fractionation with that determined by solution ^{31}P NMR spectoscopic analysis. The second aim of this study was to combine chemical fractionation with solution ^{31}P NMR spectoscopic analysis by analysing the extract and residue fractions of key steps in chemical fractionation methods (water/acid extraction and lipid extraction) using ^{31}P NMR spectroscopy. Better identification of P species in crop residues can lead to improved estimation of the turnover of these P species in soil, leading to a better assessment of the amount of P that may be provided for subsequent crops.

2. Methods

2.1. Residue properties

The stem and leaf material (hereafter referred to as the residue) of oat (*Avena sativa*) was collected from a farm near Truro (139°7′46″E, 34°24′42″S), South Australia, by cutting mature plants 1 cm above the soil surface at harvest and removing the chaff and seed by cutting off the heads. The residue was then oven-dried at 60 °C. Total P concentration in the residue (539 mg kg^{-1}) was measured using inductively coupled plasma optical emission spectroscopy (ICP–OES) (Perkin-Elmer, Optima 7000DV at 214.97 nm) following digestion of triplicate ground samples using boiling concentrated HNO_3 at 140 °C [25].

2.2. Chemical fractionation

Chemical fractionation was used to quantify (i) soluble P (both orthophosphate (molybdate-reactive P) and total P) and (ii) phospholipid P using two methods previously used for the identification of P species in tobacco [2] and tomato plants [5]. Although these steps would usually be carried out sequentially, in this study the water/weak acid and phospholipid extractions were all carried out on a fresh residue sample. Fig. 1 illustrates how chemical fractionation was combined with ^{31}P NMR spectroscopy for sample analysis.

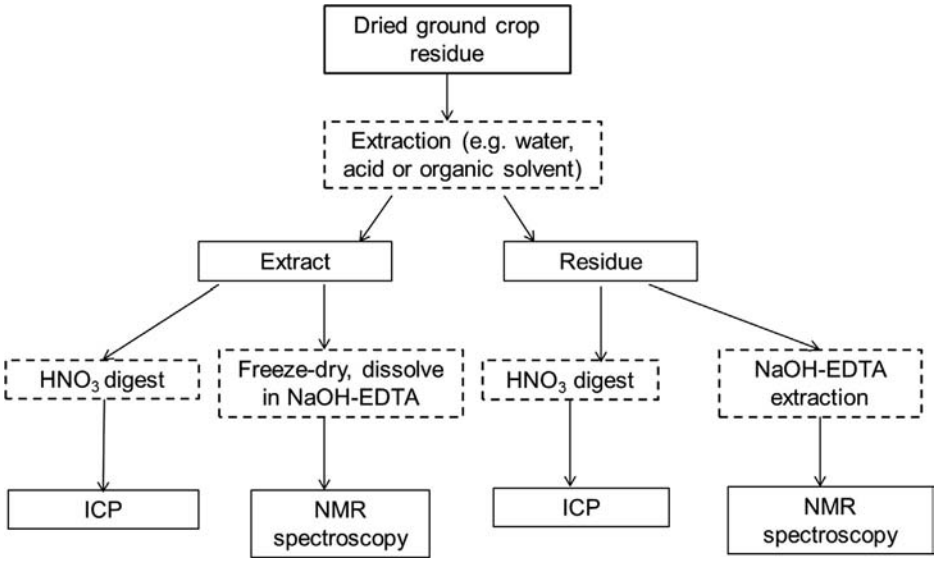

Fig. 1. Flow diagram of the extraction and analysis procedure for chemical fractionation and solution ^{31}P NMR spectroscopy.

Four methods that have previously been used to measure water/acid soluble residue P were tested: (i) water extraction using an end-over-end shaker (WSH); (ii) water extraction with sonication (WSO) [9]; (iii) extraction with 10% trichloroacetic acid (TCA) [2]; and (iv) extraction with 0.2 N perchloric acid (PA) [5]. In each case, four aliquots of 2 g of ground residue were weighed into 50 mL centrifuge tubes and 40 mL of the given extractant was added. All samples except WSO were placed on an end-over-end shaker for 1 h. The WSO sample was sonicated (Virtis Virsonic Sonicator®, USA) for 10 min at 90 W.

All samples were centrifuged ($1400 \times g$) and filtered through Whatman No. 42 filter paper. After extraction, the remaining residue was rinsed once with the given extractant and the filtered rinse combined with the extract. An aliquot of the supernatant was taken from each replicate tube, digested in HNO_3 and analysed for P using ICP–OES as described above [25]. Orthophosphate P (molybdate reactive P-MRP) in the soluble extracts was also measured colorimetrically [26]. The remaining supernatant (10–20 mL) was frozen and subsequently freeze-dried prior to NMR analysis. The residues of the water and acid extracts ($\sim$2 g) were oven-dried at 40 °C, weighed and extracted with 40 mL NaOH–EDTA based on the method of Cade-Menun and Preston [27] outlined below.

Two methods that have been previously used to measure the phospholipid concentration in plant residues were assessed: (i) extraction with ethanol:ether (E:E) [2]; and (ii) extraction with ethanol:ether:chloroform (E:E:C) [5]. For both extractions, four aliquots of 1 g of residue were weighed into 50 mL centrifuge tubes and to each tube 25 mL of the selected extractant was added. The E:E extractions were carried out on an end-over-end shaker for 1 h; the E:E:C extractions were carried out in a water bath heated to 50 °C for 1 h. The E:E:C extraction mixtures were shaken manually every 10 min.

Once the extraction was complete, all tubes were centrifuged ($1400 \times g$) for 10 min and extracts were filtered using Whatman No. 42 filter paper. The remaining residue in each tube was rinsed twice with an additional 5 mL of the given extractant (E:E or E:E:C) and these washings were combined with the extract. The residues of the E:E and E:E:C extractions were weighed and extracted with 20 mL of NaOH–EDTA based on the method of Cade-Menun and Preston [27] outlined below.

The supernatants of the E:E and E:E:C extractions were transferred to Teflon™ beakers and boiled to dryness, leaving a small pellet. The remaining pellet was digested using concentrated HNO_3 at 140 °C [25] and the total P concentration in the digest was determined using ICP–OES.

2.3. NaOH–EDTA extraction

The whole oat residue and the residues following treatment with various extractants were extracted with NaOH–EDTA (in triplicate) using the method of Cade-Menun and Preston [27], originally developed for soil extraction. This involved shaking approximately 2.0 g of dried residue with 40 mL of 0.25 mol L^{-1} NaOH and 0.05 mol L^{-1} Na_2EDTA for 16 h. The extracts were centrifuged ($1400 \times g$) for 10 min and filtered using Whatman No. 42 filter paper. An aliquot (20 mL) from each of the triplicate extracts was immediately frozen using liquid nitrogen and freeze-dried for NMR analysis.

2.4. NMR analysis of NaOH–EDTA extracts

Freeze-dried NaOH–EDTA extracts were combined for NMR analysis from which a 300–500 mg subsample was ground, re-dissolved in 5 mL of deionised water, and centrifuged at $1400 \times g$ for 20 min. Since the PA and TCA extracts did not attain dryness on freeze-drying, they were directly dissolved in 0.25 mol L^{-1} NaOH and 0.05 mol L^{-1} Na_2EDTA to make a final volume of 5 mL. In each case, the supernatant solution (3.5 mL), methylenediphosphonic acid (MDP) (0.1 mL at a concentration of 6 g L^{-1}), added as an internal standard, and deuterium oxide (D_2O) (0.3 mL) were added to a 10 mm NMR tube and mixed. The pH of these solutions was tested and where the pH was <13, 100–500 μL of 10 M NaOH was added to raise the pH to >13.

Solution ^{31}P NMR spectra were acquired at 24 °C on a Varian INOVA 400NMR spectrometer (Varian, Palo Alto, CA) at a ^{31}P frequency of 161.9 MHz. Recovery delays ranged from 8 s to 50 s and were set to at least five times the T_1 (spin lattice relaxation time) values of the orthophosphate resonance determined in preliminary inversion-recovery experiments (data not presented). We used a 90° pulse of 30–45 μs, an acquisition time of 1.0 s, and gated broadband ^1H decoupling. Between 1600 and 10,000 scans were acquired for each sample, depending on the P concentration. The spectra presented have a line broadening of 2 Hz.

2.5. Quantification of P species from ^{31}P NMR spectra

The relative concentrations of P species in the NaOH–EDTA extracts were determined from ^{31}P NMR spectra using integration and, in some cases, deconvolution. The absolute concentration of each P species (including those determined using integration alone and those determined using integration and deconvolution combined) was calculated by integration against a known concentration of the MDP that was added to each NMR tube. Pyrophosphate concentrations were determined using integration alone (integral region -4.5 ppm to -5.5 ppm). For the whole residue samples only, the relative concentrations of P species giving rise to the numerous individual peaks were quantified by spectral deconvolution, using a method similar to Bünemann et al. [28]. Each spectrum was fitted with up to twelve peaks as per Noack et al. [9]. These were identified as orthophosphate, α-and β-glycerophosphate, phytate (four peaks), and five peaks in the monoester region that can be assigned to ribonucleotides that result from alkaline hydrolysis of RNA [29]. Each peak was defined by three parameters: the chemical shift (frequency), intensity, and the line width, which were allowed to vary in the fit.

2.6. Statistical analyses

Treatment differences were evaluated by least significant difference (LSD, $p \leq 0.05$) derived from analysis of variance (ANOVA) using the GENSTAT version 13 statistical package (VSN International, Rothamsted, UK). Assumptions of constant error variance (homogeneity), normality of data distribution and additivity of treatment and replicate effects were tested for each analysis.

3. Results and discussion

3.1. Residue P speciation as determined by ^{31}P NMR spectroscopy

The speciation of P in the whole residue was determined by triplicate ^{31}P NMR analysis of NaOH–EDTA extracts (Fig. 2). Just over half (54%, 294 mg kg^{-1}) of total P in this whole oat residue was detected as orthophosphate (5.7 ppm). Phospholipids, detected as their alkaline degradation products α-and β-glycerophosphate [30] at 5.0 ppm and 4.6 ppm, respectively, represented the next largest P class, accounting for 11% (58 mg kg^{-1}) of total residue P (Fig. 2). Ribonucleic acid, again detected as its alkaline degradation products: a set of five peaks [9,29] between 4.0 ppm and 4.5 ppm, and pyrophosphate, identified as a peak at -5.0 ppm, each comprised a further 6% (33 mg kg^{-1} and 31 mg kg^{-1}, respectively) of the total residue P. Phytate, detected as four characteristic signals at 5.7 ppm, 4.7 ppm,

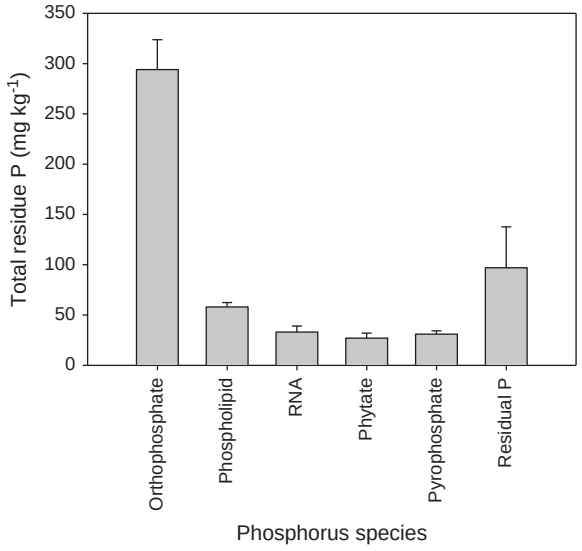

Fig. 2. Concentration (mg kg^{-1}) and standard deviation of P species detected in NaOH–EDTA extracts of the whole oat residues ($n=3$).

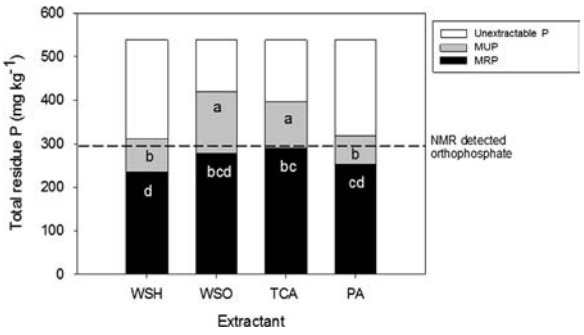

Fig. 3. Comparison of the concentration of molybdate reactive P (MRP) and molybdate unreactive P (MUP) determined by water/acid extraction and colorimetry with orthophosphate determined by NaOH–EDTA extraction and NMR detection. Within a P class, measurements appended by a different letter are significantly different ($p \leq 0.05$). WHS=water/shake, WSO=water/sonnicated, TCA=trichloroacetic acid and PA=perchloric acid.

4.4 ppm and 4.3 ppm in the ratio 1:2:2:1 [29], comprised 5% (27 mg kg^{-1}) of the total residue P (Fig. 2). Consequently 18% (97 mg kg^{-1}) of the total P was not detected by NMR, (designated as residual P in Fig. 2). There was little variation in either the chemical shift of peaks (<0.1 ppm) or P species concentrations detected by NMR between analytical replicates, with coefficients of variation in concentration (standard deviation as percentage of mean concentrations) ranging from 8 to 18% for most species; the exception was residual P (42%).

The presence of these P species is consistent with previous studies of P forms in plant material [8,9,18]. In a study where eight cereal and legume residues were collected from the field, Noack et al. [9] identified orthophosphate, phospholipids, RNA, phytate and pyrophosphate in stem and leaf material. Orthophosphate was the dominant P form, representing 25–75% of total residue P, with smaller quantities of lipid P (10–49%), RNA (5–30%), pyrophosphate (7–14%) and phytate (<1%) detected.

3.2. Water and acid extractable P

The extraction of P with either water or acid is the first step in all plant chemical fractionation methods and is reported to represent the soluble P fraction. The extracted P is commonly further classified as molybdate reactive P (MRP) and molybdate unreactive P (MUP). The former is determined by colorimetry following reaction with molybdate reagent and can be interpreted as soluble orthophosphate, while the latter is calculated as the difference between total P (determined by nitric acid digestion followed by ICP–OES analysis) and includes soluble organic P as well as condensed inorganic P forms such as pyrophosphate and polyphosphate [2,5,31]. It should be noted that in some studies only MRP is measured on this fraction [1]. Here, four different extraction conditions were tested and MRP and MUP were determined (Fig. 3).

For all extraction conditions tested, the MRP concentration ranged from 235 mg kg^{-1} to 290 mg kg^{-1} and was similar to the orthophosphate concentration determined by NMR analysis (Fig. 3). While the four extraction methods resulted in similar values for MRP, two extraction methods (TCA and WSO) extracted higher amounts of P from the residues, apparently due to greater extraction of MUP.

3.3. Speciation of P in water and acid extracts as determined by ^{31}P NMR spectroscopy

Solution ^{31}P NMR spectroscopy was used to further characterise the P species in the water and acid extracts. This was achieved by freeze-drying the extracts and re-dissolving them in NaOH–EDTA, to facilitate comparison of the composition of water/acid extracts with that of the NaOH–EDTA extract of the whole residue; the resulting ^{31}P NMR spectra are shown in Fig. 4. Integration of the ^{31}P NMR spectra of the water and acid extracts confirmed that the majority of signal detected (75–87%) could be ascribed to orthophosphate, with a slightly higher range of values for the weak acid extracts (85–87%) than the water extracts (75–80%). These values represent a higher percentage of orthophosphate than obtained by comparing colorimetry values to total P in the extracts (61–64% and 56–63% for weak acid and water extracts, respectively).

The overall composition of P in the water extracts is in broad agreement with a ^{31}P NMR study by He et al. [32] on P speciation in water extracts from seven crop residues. Four of these water extracts (clover, vetch, wheat and lupin) contained P only in the orthophosphate form [32]. For the remaining three residues (corn, alfalfa and soybean), orthophosphate was the dominant P form, while monoester P comprised 8–32% of the total soluble P [32].

Despite its water solubility, pyrophosphate was not detected in any of the water or acid extracts. The most likely explanation is that pyrophosphate was enzymatically hydrolysed during the water extractions or hydrolysed during acid extraction [33]. The plant residues would contain enzymes that would be released from the ground residues into water extracts. Numerous enzymes have been shown to catalyse the release of orthophosphate from a wide range of organic P compounds [34–36]. While these enzymes would likely remain active in the near-neutral pH of water extracts, they would be inactivated at the high pH of the NaOH–EDTA solutions, which would explain why pyrophosphate is detected in the NaOH–EDTA extract of the whole residue. Acidic extracts are unlikely to contain pyrophosphate as this molecule is easily hydrolysed to orthophosphate under acidic conditions [37].

A close inspection of the monoester region of the ^{31}P NMR spectra of the water and acid extracts (right side of Fig. 4) reveals some key differences in organic P speciation between the water and acid extracts and the NaOH–EDTA extract of the whole residue. For the two water extracts, a large peak at 5.0 ppm and a much smaller peak at 4.6 ppm are coincident with peaks we assigned as α-and β-glycerophosphate in the ^{31}P NMR spectrum of the whole residue. Spiking subsequently confirmed this assignment for the water extracts. The presence of glycerophosphate in the ^{31}P NMR spectrum of the whole residue is attributed

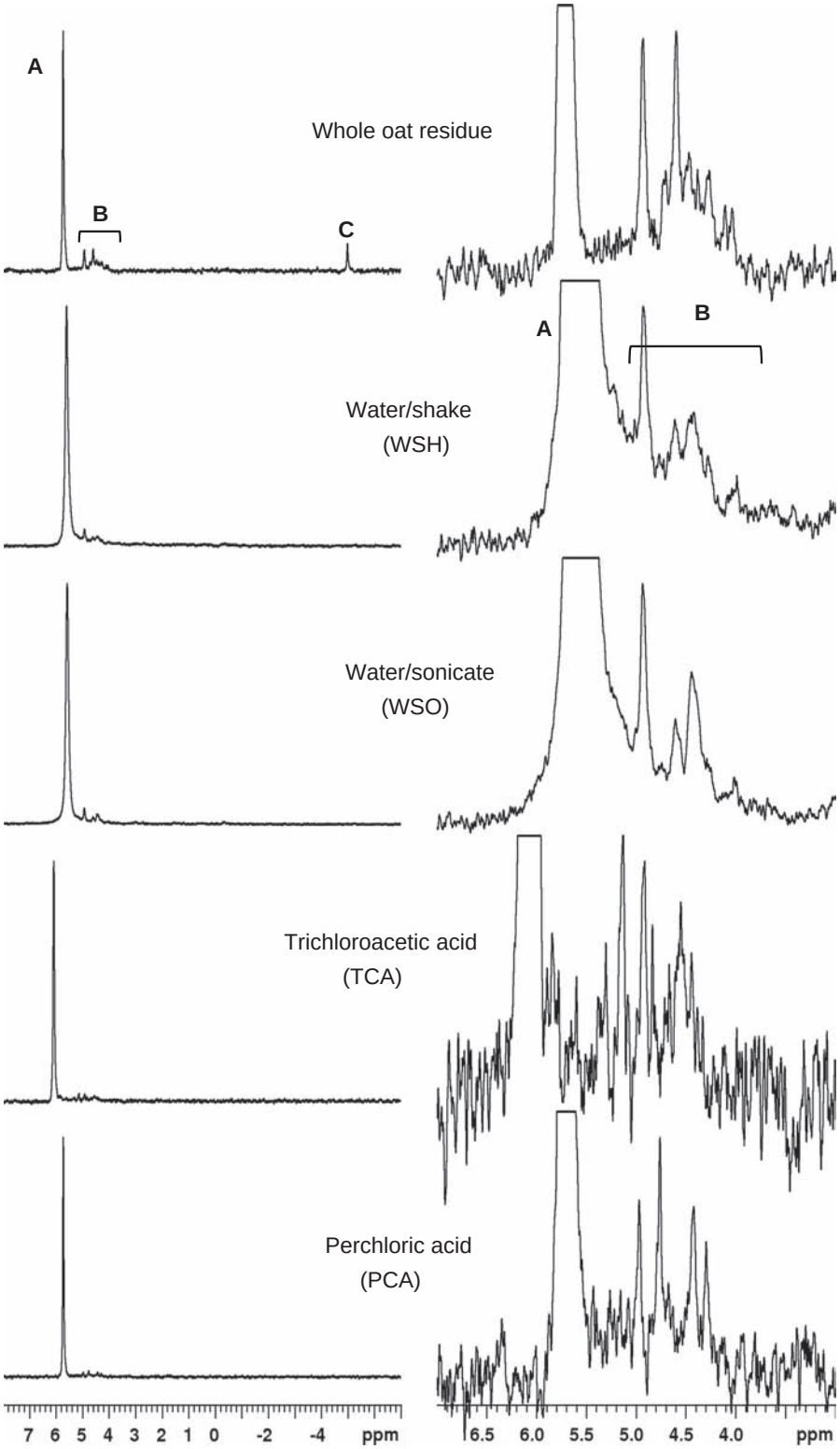

Fig. 4. Solution ^{31}P NMR spectra of NaOH–EDTA whole crop residue and extracts after soluble P fractionation steps. Left wide spectra view and right narrow spectra view. Spectra on the left have been vertically scaled to the maximum intensity of the orthophosphate peak (5.75 ppm). Spectra on the right have been vertically scaled to the maximum intensity of the most intense monoester peak. Peaks assigned as A=orthophosphate, B=monoester P and C=pyrophosphate.

to alkaline hydrolysis of phospholipids. Phospholipid, being hydrophobic, should not be water-extractable, which suggests that some phospholipid present in the whole residues was hydrolysed during the water and weak acid extraction treatments, releasing glycerophosphate (which is soluble in water and weak acid) to solution. Similar to pyrophosphate, this hydrolysis was most likely mediated by enzymes released from the ground residues into the extract solution [33–36]. This explanation is supported by the very different proportions of the two glycerophosphate isomers between the whole residue NaOH–EDTA extracts (approximately equal amounts of α-and β-glycerophosphate) and the water extracts (predominantly α-glycerophosphate).

The signal-to-noise (S/N) ratio of ^{31}P NMR spectra of the acid extracts is poorer than for the water extracts (Fig. 4). This is mainly a consequence of an inability to remove the acids from these extracts by freeze-drying (in contrast to the ease in removing water only from the water extracts). Thus, the acid extract spectra represent a much smaller total amount of extract and the samples analysed had proportionally lower P concentrations. Despite the poor S/N ratios, at least four monoester peaks are apparent. The presence of the conjugate bases of trichloroacetic acid and perchloric acid in the samples also appears to have affected the positions of peaks for both orthophosphate and monoesters, such that they do not align with the peak positions in other samples (Fig. 4). A similar effect of salts on peak positions has been noted previously [38]. Nonetheless, it is likely that the left-most monoester peaks are due to α-and β-glycerophosphate; the identity of the other two monoester peaks is unknown.

3.4. Speciation of P in the residue following water and acid extractions as determined by ^{31}P NMR spectroscopy

Solution ^{31}P NMR spectroscopy was also used to characterise P species in the residues of the plant material after the water and acid extractions (Fig. 5). An implicit assumption of chemical fractionation is that these residues remaining after an extraction step do not contain the same chemical species as are present in the corresponding extract. Furthermore, the P species in the extract and residue fractions when combined should be those P forms present in the whole residue.

Crucially, for all water and acid extractions, the majority (64–78%) of P detected in the residues was orthophosphate (Fig. 4). When orthophosphate detected in the water/acid extracts and remaining residues is combined, the total is substantially greater than the concentration of orthophosphate detected by NMR in the NaOH–EDTA extract of the whole residue. This can be partly attributed to hydrolysis of pyrophosphate during water and acid extraction; pyrophosphate comprised 6% of P in the whole residue but was virtually absent from all of the water and acid extracts and residues (Figs. 4 and 5). However, it appears likely that some organic P species were also hydrolysed to release orthophosphate during water and acid extraction. Again, for the water-based extractions, this is likely to have occurred through enzymatic hydrolysis, as there would be enzymes released into solution that are efficient at hydrolysing monoester organic P compounds to orthophosphate [36]. For the acid extracts there is the potential for weak acid to chemically hydrolyse some organic P, thereby releasing orthophosphate to solution [39].

The presence of orthophosphate in the crop residues following water and acid extraction is consistent with the findings of Dou et al. [40]. In a study on the water extractability of orthophosphate in poultry and dairy manure they found a single extraction did not release all orthophosphate, with a further 25–30% released in 3–4 subsequent 1 h extractions [40].

The main organic P species detected by NMR in the residues following water extraction were the phospholipid degradation products α-and β-glycerophosphate at 5.0 ppm and 4.6 ppm, respectively (right side of Fig. 5). Interestingly, the ratio of these two peaks (approximately 1:1) was similar to that seen for the whole residue, indicating that phospholipid in this fraction remained intact through the water extraction (neutral pH) and was subsequently converted to glycerophosphate under the alkaline conditions of NaOH–EDTA extraction [41]. Several organic P species were detected by NMR in the residues following acid extraction (right side of Fig. 5). We were able to identify the strongest monoester peaks in the ^{31}P NMR spectrum of the TCA residue as α-and β-glycerophosphate by spiking. We note that the chemical shift of these species was approximately 0.2 ppm higher than in the residues following water extraction, probably reflecting differences in pH and/or ionic strength. The close similarity of the monoester region of the ^{31}P NMR spectrum of the PCA residue to that of the TCA residue suggests that α-and β-glycerophosphate were also the major organic P species present. Interestingly, the α-isomer of glycerophosphate was dominant in the residues following acid extraction, in common with the water extracts, but in contrast to the acid extracts and residue following water extraction. This indicates there was some hydrolysis of phospholipids in these residues prior to extraction with NaOH–EDTA. A short review by Folch [41] reports under alkaline conditions there is a predominance of β-glycerophosphate and acid treatment results in predominance of α-glycerophosphate. Other organic P species in the ^{31}P NMR spectra of the residues following acid extraction were not identified. Differences in pH and/or ionic strength make it impossible to tell whether or not these minor organic P compounds are the same as those detected in the NaOH–EDTA extract of the whole residue.

3.5. Implications from NMR analysis of extract and residue fractions of water and acid extractions

The implications of these findings are far-reaching. Extraction of P with water or acid is the first step in all chemical fractionation methods [1,2] and these results demonstrate that this step does not achieve what it sets out to do, i.e. selectively separate intrinsically water soluble species (recovered in the extract) from those not soluble in water (recovered in the remaining residue). On face-value, the orthophosphate concentrations in the water/acid extracts aligned well with the orthophosphate concentrations determined by NMR analysis of the NaOH–EDTA extract of the whole residue. However, detailed analysis of the extracts and residues following water and acid extraction subsequently showed this to be a coincidence borne of two flaws in the water and acid fractionation procedures that cancelled out for this particular material. The incomplete extraction of orthophosphate meant that the orthophosphate concentrations in the water and acid extracts were underestimated. This problem was evidenced by the dominance of orthophosphate in the NaOH–EDTA extracts of residues following water/acid extraction. On the other hand, enzymatic (in the case of water extracts) or acid (in the case of acid extracts) hydrolysis of organic P and pyrophosphate meant that some P present as organic or condensed P in the plant material was detected as orthophosphate in the extracts, erroneously increasing the orthophosphate concentration. This problem was evidenced by the almost complete absence of pyrophosphate in any of the water/acid extracts or residues and also the finding that the organic P composition in the extracts and residues did not align with that of the whole crop residue.

Obviously, the problems identified here for water and acid extractions would compromise the remaining steps of sequential fractionation schemes. The incomplete recovery of orthophosphate would result in an overestimation of P species in subsequent fractionation steps (e.g. nucleic acid or residual P) and the transformation of organic P species invalidates the implicit assumption that unextracted P species are unaffected by preceding steps.

3.6. Phosphorus species detected in organic solvent treatments

For both organic solvent treatments, extraction with ethanol:ether (E:E) and extraction with ethanol:ether:chloroform (E:E:C), only a small proportion (1.2% and 1.4%, respectively) of total P (as determined by acid digestion of plant residue) was detected by ICP–AES in the extract (after acid digestion). It is possible that these values may be an underestimation of P in these extracts as the lipid material isolated following removal of the organic solvents was very hydrophobic and may have resisted acid digestion. These apparent phospholipid

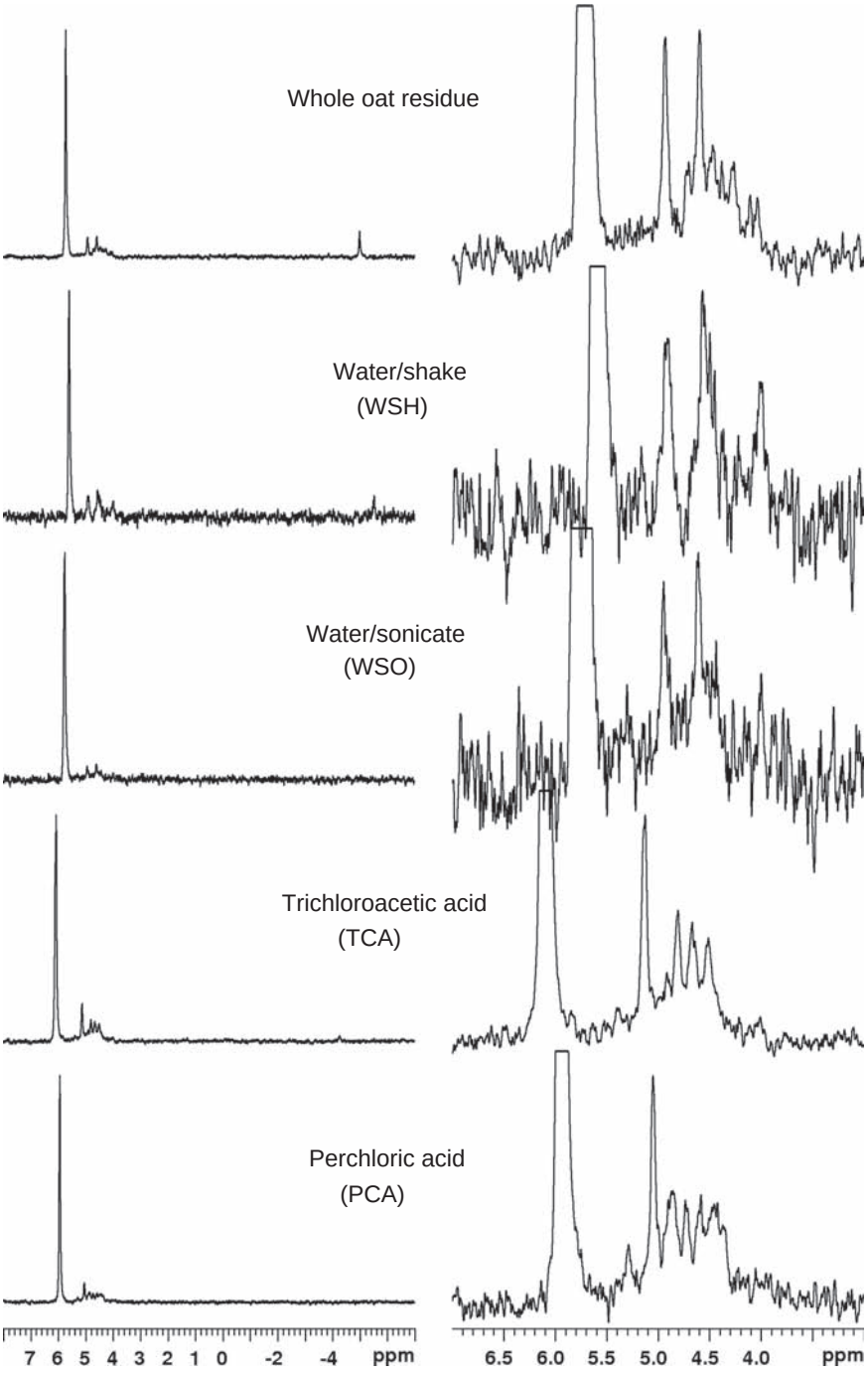

Fig. 5. Solution ^{31}P NMR spectra of NaOH–EDTA whole crop residue remaining after soluble P fractionation steps. Left wide spectra view and right narrow spectra view. Spectra on the left have been vertically scaled to the maximum intensity of the orthophosphate peak (5.75 ppm). Spectra on the right have been vertically scaled to the maximum intensity of the most intense monoester peak.

contents were certainly much lower than the 11% of total P determined to be phospholipid by NMR analysis of the whole residue (Fig. 2). It was not possible to obtain a comparable NMR analysis of the organic solvent extracts because they could not be dissolved in NaOH–EDTA, again due to their waxy, hydrophobic nature. However, we were able to analyse residue of organic solvent extraction by ^{31}P NMR after extracting the residues with NaOH–EDTA (Fig. 6). The spectra obtained are similar in appearance to the ^{31}P NMR spectrum of the whole residue NaOH–EDTA extract, except that the relative size of peaks for α-and β-glycerophosphate (the alkaline degradation products of phospholipids) was diminished for the E:E and E:E:C residues.

These results indicate that while both E:E and E:E:C treatments were selective for extracting phospholipid P, they were not exhaustive in that they did not extract all phospholipids originally present. In contrast to the water and acid extractions, the organic solvent extractions did not appear to transform organic P in the remaining residue, nor would they be expected to, as any released enzymes would be inactivated in such solvents and these treatments do not involve a major shift in pH that would result in chemical hydrolysis of organic P species. The apparent incomplete extraction of phospholipids under the treatment conditions reported here would result in an underestimation of this P form by chemical fractionation and, when

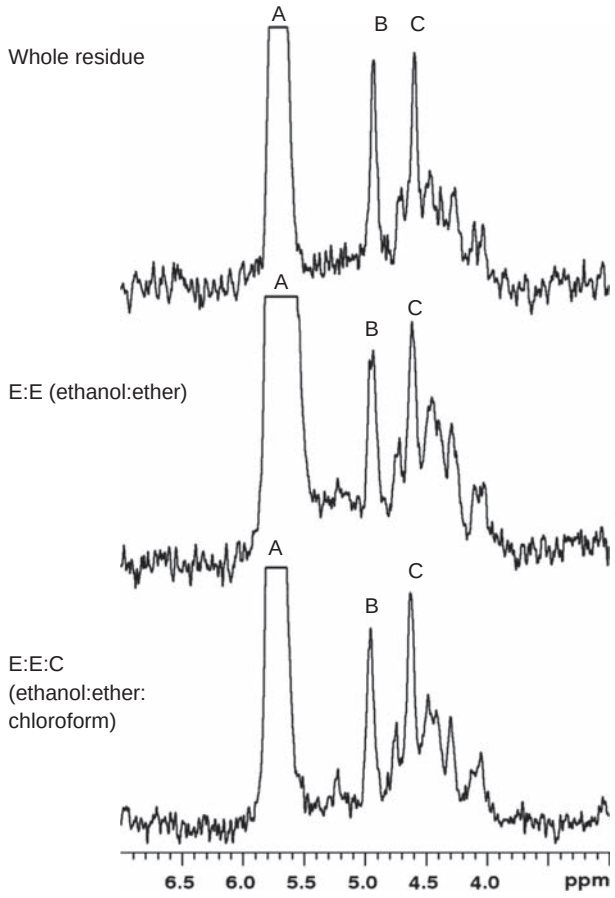

Fig. 6. Solution ^{31}P NMR spectra of NaOH–EDTA whole crop residue extracts and remaining residue after extraction with different phospholipid fraction steps. Peaks assigned as A=orthophosphate, B=α-glycerophosphate, C=and β-glycerophosphate.

used as a step in sequential fractionation, an overestimation of one or more P species identified in subsequent steps (i.e. nucleic acid or residual P).

4. Conclusions

A simple comparison of two alternative approaches for measuring the orthophosphate concentration in mature oat residue – ^{31}P NMR spectroscopy following NaOH–EDTA extraction and colorimetry following water or acid extraction – showed they produced similar values. However, this apparent consistency is illusory because in the latter approach an overestimation of orthophosphate due to hydrolysis of organic P and pyrophosphate was balanced by underestimation of orthophosphate due to incomplete extraction. This fortuitous coincidence cannot be relied on, and a re-think of chemical fractionation approaches, including sequential fractionation, to determine P speciation of plant material is required. The same is likely to be true for soil analysis. There is a need to further investigate the impact that hydrolysis of organic and condensed (e.g. pyrophosphate) P forms during fractionation procedures may have on the interpretation of fractionation results, as well as the degree to which incomplete extraction modifies results. In both instances, the use of ^{31}P NMR spectroscopy in combination with chemical fractionation appears to offer a way forward. These results can aid in the characterisation of P species in sequential chemical extracts of plant material and provide a better understanding of the fate of crop residue P post-harvest.

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