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Separation of Soil Organic Phosphorus Compounds Using Reverse-Phase Ion-Pair Chromatography

April B. Leytem[1,*] and Robert L. Mikkelsen[2]

[1]USDA-ARS, NWISRL, Kimberly, Idaho, USA
[2]Potash & Phosphate Institute, Davis, California, USA

ABSTRACT

Methods were developed for the extraction and separation of soil organic phosphorus compounds using reverse-phase ion-pair chromatography (RP-ICP). Nucleotides (ATP, ADP, and AMP) were separated using a mobile phase of 15 mM TBAHS, 15 mM KH_2PO_4, and 7% acetonitrile. Inositol hexakisphosphate was separated using a mobile phase composition of 0.05 M formic acid:methanol (49:51 v/v) and 1.5 mL/100 mL of TBAOH. Extraction procedures were developed for the nucleotides which would be compatible with the RP-ICP system developed for their separation.

*Correspondence: April B. Leytem, USDA-ARS, NWISRL, Kimberly, ID 83341, USA; E-mail: leytem@nwisrl.ars.usda.gov.

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INTRODUCTION

The transport of phosphorus (P) from agricultural land to watercourses can contribute to the growth of algae and general water quality deterioration associated with eutrophication.[1] Areas with intensive livestock production can contribute P to runoff through land application of manure which can increase the organic P fraction of soils and therefore potentially lead to enhanced losses of P. Understanding how organic P compounds behave in soils requires the ability to accurately measure organic P compounds in soils, manures and other organic amendments.

Traditionally, soil organic P has been crudely determined by the difference between total P and inorganic P.[2–4] This method is commonly used because of the difficulties associated with direct measurement of organic P compounds. While the difference method may be useful for estimating the total soil organic P fraction, there may be substantial and unknown error associated with its measurement as well as the inability to identify specific organic P compounds. The development and implementation of improved detection methods are necessary to ensure accurate measurement of the concentration of organic P compounds in soil in order to study their behavior.[5]

The use of high performance liquid chromatography (HPLC) has been used to identify and measure the organic P fraction in plants, foods, and aqueous samples.[6–10] More recently the identification of organic P compounds in soils and soil extracts has been accomplished using phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy.[11–13]

Although these methods have been developed and used successfully, there are still some drawbacks to the utilization of these methods for studying the reactivity and transport of organic P compounds in soils. Many HPLC procedures utilize ion exchange columns for the separation of organic P. These columns tend to be expensive, not very durable, and impractical for analysis of large numbers of samples. Sample preparation can also be very time consuming and manipulation of samples prior to analysis can involve many steps that potentially alter or degrade organic P compounds. The use of NMR is limited by the requirement for sample pre-concentration and long run times, which can be both expensive and cause sample degradation.

Practical methods to identify organic P in soil extracts and aqueous samples are necessary when performing research requiring high throughput of samples. In addition, reliable extraction methods must also allow recovery of these compounds from the soil without modification and be compatible with the analytical procedure.

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Separation of Select Organic Phosphorus Compounds

Reverse phase ion pairing chromatography (RP-ICP) was used in this study for the separation and detection of Inositol Hexakisphosphate (IHP), Adenosine 5′ Triphosphate (ATP), Adenosine 5′ Diphosphate (ADP), and Adenosine 5′ Monophosphate (AMP). The RP-IPC system uses elements of both reverse phase and ion exchange chromatography. The reverse phase chromatography system is composed of a non-polar stationary phase, usually carbon chains of varying length, with a polar mobile phase. Unlike regular reverse phase chromatography, the RP-ICP system has a mobile phase containing an ion-pairing reagent, typically a charged molecule with a hydrophobic portion that will 'pair up' with ions in solution. The ion pairs are retained by the hydrophobic stationary phase. Ion pairs do not exist in the aqueous solution, but in the nonpolar stationary phase. Because the stationary phase retains ion pairs, the reverse-phase column effectively functions as an ion exchange column. This methodology offers several advantages. First, ionic compounds can be separated without having to use an ion exchange column, which eliminates complicated sample preparation. Second, reverse phase chromatography is relatively easy to use and the columns are durable and inexpensive. This allows for the analysis of large sample sets with minimal preparation time and without costly sample preparation techniques.

Extraction of Organic Phosphorus Compounds

Sequential acid and alkali extraction has been commonly utilized to determine soil organic P.[14–17] Initial soil extraction with strong acid followed by an alkali solution removes more organic P than when either extractant is used alone. In some procedures, elevated temperatures are also used, which can cause hydrolysis of labile organic P forms.[14,15,17] With this extraction procedure, a major concern is recovering all the organic P from the soil without hydrolyzing any to the inorganic form.

A method proposed by Bowman and Moir[18] to extract soil organic P in one step involves the use of Na_2EDTA with NaOH. The NaOH is used to solubilize organic P associated with soil organic matter, therefore removing the majority of the organic P fraction in the soil. The resistance of soil organic P to alkaline extraction is overcome by adding EDTA, which extracts organic P adsorbed via a metal cationic bridge. EDTA is a hexadentate ligand that binds strongly to Group 2 cations such as Mg^{2+} and Ca^{2+} and with other common soil metals such as Fe^{3+} and Al^{3+}. These metal-EDTA complexes have 1:1 formation constants ranging from 5.8×10^7 to 5.0×10^{10}.[19]

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With such high formation constants, EDTA is able to complex metal cations that are binding organic P molecules to soil particles and soil organic matter, thereby increasing organic P recovery from the soil. This method is a one-step procedure, which is an improvement upon the acid–base extraction.

MATERIALS AND METHODS

Reagents

Tetrabutylammonium hydrogen sulfate (BAHS) and KH_2PO_4 were obtained from Fisher Scientific. Tetrabutylammonium hydroxide (TBAOH; 40% in water) was obtained from Fluka. Standards of IHP, ATP, ADP, and AMP, were obtained from Sigma Chemical Company.

Mobile Phase

Preliminary experiments were conducted using different mixtures of the solvents and ion-pairing reagents described by Patthy et al.[7] to determine the optimal mixture for nucleotide separation. It was determined that the optimal mobile phase composition for separation of all three nucleotides consisted of 15 mM TBAHS, 15 mM KH_2PO_4, and 7% acetonitrile. The mobile phase was adjusted to pH 5.5 with concentrated H_2SO_4, filtered (0.45 μm) and degassed.

The mobile phase used for separation of IHP consisted of 0.05 M formic acid:methanol (49:51 v/v) and 1.5 mL TBAOH/100 mL.[6] The pH was adjusted to 4.3 with H_2SO_4, filtered (0.45 μm) and degassed.

HPLC Procedure

A reverse phase C-18 column (Waters Nova-Pak C_{18} 3.9 × 75 mm) 4 μm particle size was equilibrated with the mobile phase overnight. Analyses were conducted with a Waters HPLC system, and either a refractive index detector (Waters 410) for the IHP or a photo diode array (PDA) detector (Waters 991) for the nucleotides. Separation of ADP and AMP was optimal with a 1 mL min^{-1} flow rate, 10 μL injection volume, and detection at 260 nm (Fig. 1). Separation of ATP was optimal at a flow rate of 2.5 mL min^{-1}, 10 μL injection volume and detection at 260 nm (Fig. 2). Separation of IHP was optimal at a flow rate of 3 mL min^{-1} with a 30 μL injection volume (Fig. 3). Retention times and peak areas were calculated with the Millennium

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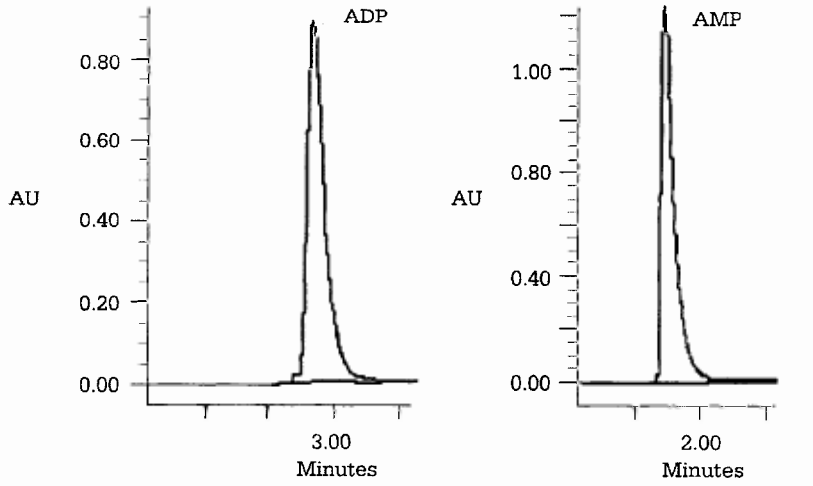

Figure 1. ADP (a) and AMP (b) detection in an isocratic RP-IPC system. Column: Waters Nova-Pak C18 3.9 × 75 mm, 4 μm particle size. The mobile phase consisted of 15 mM TBAHS, 15 mM KH_2PO_4, and 7% acetonitrile with mobile phase adjusted to pH 5.5. Flow rate was 1 mL min^{-1} with an injection volume of 10 μm and detection at 260 nm.

Chromatography Manager.[20] These methods gave reproducible results with linearity from 0 to above 1000 mg P L^{-1} ($r^2 > 0.99$).

Extraction Techniques

The surface horizon of three soils were used to determine the extractability and recovery of added organic P compounds (Table 1). Several combinations of the extracting solution composition, extraction time, amount of soil, and amount of added organic P were tested (Table 2). All extractions were conducted at 25°C, to avoid potential compound degradation from elevated temperatures. The initial extraction procedures were as follows: 4 or 8 mg of ATP was added to 4 or 8 g of soil (screened to <2 mm), 25 mL of extracting solution (NaOH-EDTA or H_2O) was added five minutes after addition of the ATP to the soil and shaken for 1 or 1.5 hr. Some samples were also extracted with water: these were shaken for an additional 0.5 hr and then combined with the original extracted solution (Table 2). All extracts were filtered through Whatman 42 filter paper, adjusted to pH 6 with H_2SO_4 and filtered (0.45 μm) prior to HPLC analysis.

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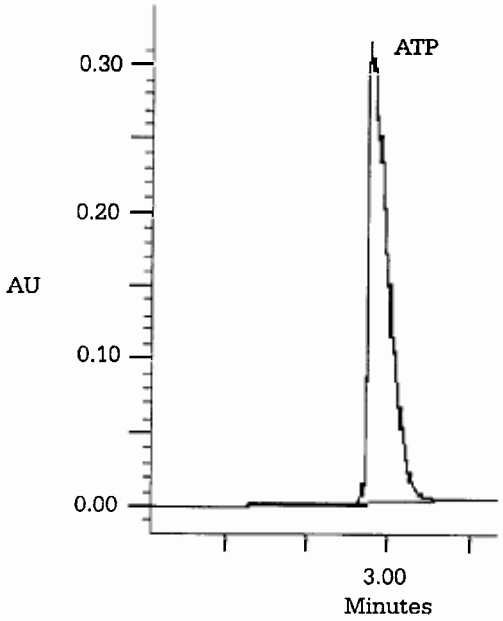

Figure 2. ATP detection in an isocratic RP-IPC system. Column: Waters Nova-Pak C18 3.9 × 75 mm, 4 μm particle size. The mobile phase consisted of 15 mM TBAHS, 15 mM KH_2PO_4, and 7% acetonitrile with mobile phase adjusted to pH 5.5. Flow rate was 2.5 mL min^{-1} with an injection volume of 10 μm and detection at 260 nm.

The extraction technique providing the highest recovery of ATP (99.5%) was as follows: 4 g of soil shaken with 25 mL 0.3 *M* NaOH + 0.05 *M* Na_2EDTA for 1.5 hours, filtered through Whatman 42 filter paper (or centrifuged for 15 min at 10,000 rpm), adjusted to a pH of 6 and filtered (0.45 μm) prior to HPLC analysis. Since this combination produced the highest percentage of recovery, it was used for all of the subsequent nucleotide extractions.

Extraction of IHP with the NaOH-EDTA extractant proved to be problematic. Alternative extraction solutions were investigated to determine compatibility with IHP and the analytical technique. Standards containing IHP were subsequently prepared using extracting solutions consisting of 0.5 *M* HCl,[6] 5% tri-chloroacetic acid,[22] and 5% acetic acid.

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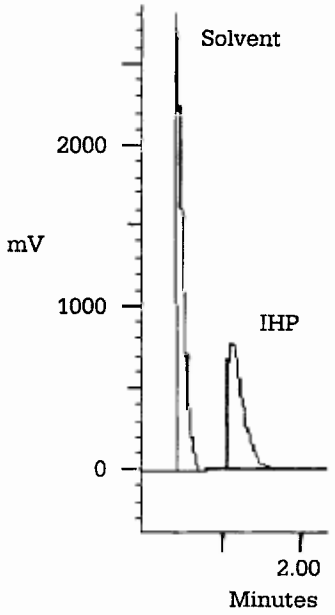

Figure 3. IHP detection in an isocratic RP-IPC system. Column: Waters Nova-Pak C18 3.9 × 75 mm, 4 μm particle size. The mobile phase consisted of 0.05 M formic acid:methanol (49:51 v/v) and 1.5 mL 100 mL^{-1} of TBAOH with mobile phase adjusted to pH 4.3. Flow rate was 3 mL min^{-1} with an injection volume of 30 μm.

Statistical Analysis

The extraction and recovery experiments were performed in triplicate. Statistical analysis was performed using mean separation by Duncan's Multiple Range Test at a 5% confidence level.

RESULTS

Separation of Organic Phosphorus Compounds

To achieve separation between ATP, ADP and AMP using the same mobile phase composition, the amount of acetonitrile was decreased from 17 to 7%, which increased the retention time of ATP at a flow rate of 1 mL min^{-1}, but also broadened the peak somewhat. This was overcome by increasing

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Table 1. Selected chemical and physical properties of the soils used in the extraction and recovery experiments.

	Soil physical and chemical properties						
Soil series	Sand (%)	Silt (%)	Clay (%)	OM ($g\,kg^{-1}$)	pH	Al_{ox} ($mmol\,kg^{-1}$)	Fe_{ox} ($mmol\,kg^{-1}$)
Blanton sand	90	4	6	13	5.4	22	6
Cecil sandy clay loam	62	14	24	48	6.4	44	25
Belhaven sandy loam	52	29	19	111	5.7	391	85

Al_{ox} and Fe_{ox} indicates oxalate-extractable Al and Fe (Jackson et al.[21]).

the flow rate to $2.5\,mL\,min^{-1}$. Using the mobile phase containing 7% acetonitrile, it was possible to detect all three nucleotides using the same analytical technique.

This HPLC method and mobile phase composition was reliable for analysis of samples tested from batch adsorption studies for the nucleotides (ATP, ADP, and AMP), but only separated ATP and ADP from soil extracts

Table 2. Recovery of ATP added to Blanton Sand as influenced by extracting solution composition and shaking duration.

Extracting solution	Soil (g)	ATP (g)	Shaking duration (hr)	Percent recovery (%)
Na_2EDTA-NaOH	4.0	0.004	1.5	99.5
Na_2EDTA-NaOH	4.0	0.004	1	91.7
Na_2EDTA-NaOH	8.0	0.008	1	74.4
Na_2EDTA-NaOH + H_2O	4.0	0.004	1 + 0.5	62.3
Na_2EDTA-NaOH + H_2O	8.0	0.008	1 + 0.5	70.6
H_2O	4.0	0.004	1	65.4
H_2O	8.0	0.008	1	51.7
H_2O + H_2O	4.0	0.004	1 + 0.5	72.5
H_2O + H_2O	8.0	0.008	1 + 0.5	67.3

Samples were either continuously shaken for 1 or 1.5 hr or shaken for 1 hr then washed with H_2O and shaken for an additional 0.5 hr. All Na_2EDTA concentrations were 0.05 M and all NaOH concentrations were 0.3 M.

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using the extraction method discussed above.[5] The NaOH-EDTA extracting solution had the same retention time as AMP, so it was difficult to quantitatively measure the concentration of AMP in these samples.

The HPLC method for IHP separation was more problematic, because the mobile phase was sensitive to changes in pH, which caused difficulties with peak separation. Mobile phase preparation required careful pH adjustment and re-equilibrating the column to obtain consistent and reproducible separation of IHP.

Extraction of Organic Phosphorus Compounds

There was a decline in ATP and ADP recovery from soil as soil reactivity increased (due to an increase in clay, organic matter, and Fe + Al content) following the trend Blanton = Cecil > Belhaven (Fig. 4). There was no significant difference in ADP recovery on the Blanton and Cecil soils, but there was a significant difference between these two soils and the Belhaven soil. The trend in percent recovery of ADP followed the order Blanton = Cecil > Belhaven.

When the NaOH-EDTA extraction method was tested for IHP, there was an observable decrease in the peak area of the standards prepared in the extraction matrix compared with those prepared in water. The peak area of the IHP in the alkaline extracting solution was approximately half of that resulting from the same IHP concentration dissolved in

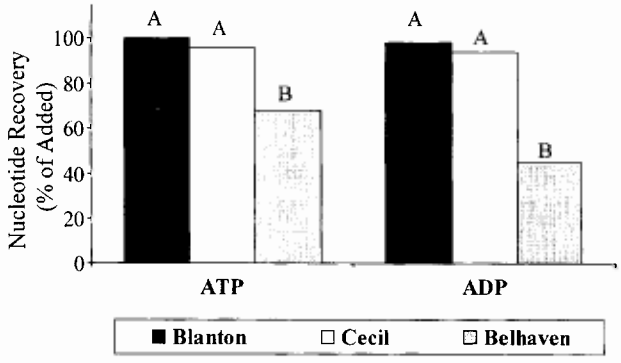

Figure 4. Recovery of ATP and ADP from Blanton, Cecil and Belhaven soils following addition of 1000 mg kg^{-1} of the compounds.

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water, and decreased below a detectable level within 24 hours when the IHP was left dissolved in the extracting solution overnight. To overcome this limitation, other extraction techniques were investigated (0.5 *M* HCl, 5% tri-chloroacetic acid, and 5% acetic acid). There was a decrease in peak area in excess of 50% with IHP standards prepared in all the extraction matrices compared to standards prepared in water. The extent of peak area change was determined in varying strengths of these extracting solutions, compared with IHP in water alone (Fig. 5). There was a significant decrease in peak area of IHP at low concentrations of either acidic or alkaline extracting solutions, with TCA resulting in the highest decrease.

The extractability of IHP from soil was measured with water, 0.3 M acetic acid, or 0.1 *M* NaOH + 0.025 *M* EDTA using the procedures previously described for extraction of ATP. The extractant giving the highest IHP recovery was the NaOH-EDTA solution (Fig. 6). The water extracted 40% of the added IHP from the Blanton soil, but the acetic acid did not extract IHP from any of the soils. The trend in extraction between the soils similar to that for ATP, the order of extraction was Blanton > Cecil > Belhaven.

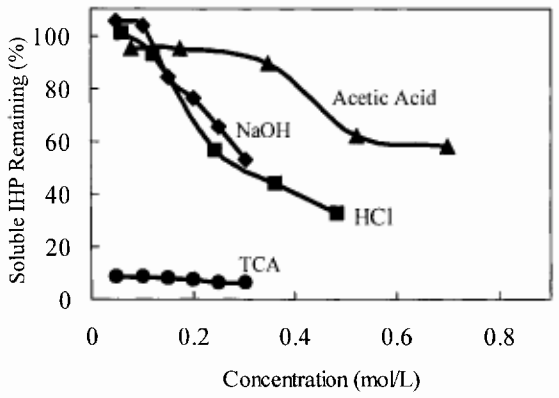

Figure 5. Changes in IHP detection with increasing strength of acid or base after a reaction time of 5 minutes (no soil added). Percent remaining was determined by comparing IHP in extracting solution to the concentration of IHP in water.

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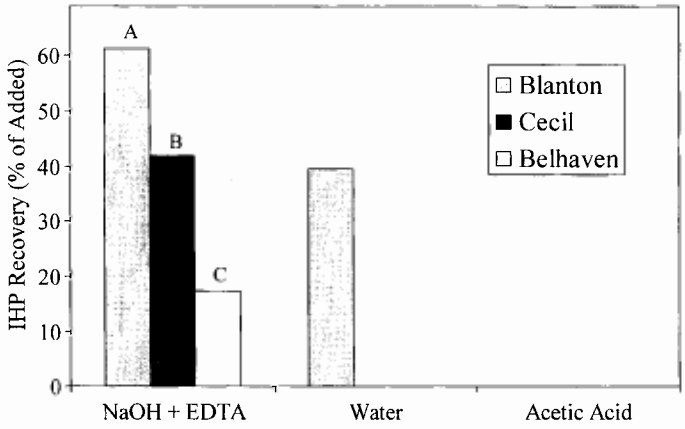

Figure 6. Recovery of IHP from Blanton, Cecil and Belhaven soils using 0.3 *M* acetic acid, 0.1 *M* NaOH + 0.025 *M* Na_2EDTA or H_2O as extractants.

DISCUSSION

Separation of Organic Phosphorus Compounds

Separation of four organic P compounds was successful using RP-IPC. This method required minimal sample preparation, which decreased the time and cost of analyzing large sample sets. The separation speed, efficiency, low buffer salt concentrations and back-pressures made this an attractive alternative to anion-exchange HPLC methods. In addition, there was no observable effect of the extracting solution matrix on compound separation and detection.

The separation of the nucleotides (ATP, ADP, and AMP) was accomplished with a mobile phase composition containing 7% acetonitrile. While this did allow separation of the compounds, the separation of ADP and AMP could possibly be improved by further decreasing the concentration of acetonitrile in the mobile phase. The use of a gradient pump may also improve the separation, by altering the acetonitrile concentration over time.[7]

Analysis of IHP was possible with the RP-ICP method investigated. Detection of the lower Inositol phosphate esters (such as tetra-, tri-, di- or mono phosphates) would not be possible with this method unless the pH of the mobile phase was increased. However, this pH change in the mobile phase would create problems with the peak shape of IHP. Sandberg and Ahderinne[6]

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successfully used this separation technique for the determination of tri-, tetra-, penta-, and hexaphosphates in foods and intestinal contents. The Sandberg and Ahderinne[6] technique required extensive sample preparation (pre-separation with ion-exchange) prior to analysis, which was not performed with the soil samples used in this study. Rounds and Nielsen[9] used a gradient anion-exchange HPLC technique to successfully separate IHP and its lower esters from plant materials with minimal sample preparation, which could provide an alternative to the RP-ICP technique. Clarkin et al.[23] also used a gradient anion-exchange technique to separate Inositol phosphate congeners in aqueous samples. While they obtained good compound separation with this technique, the time requirement was very long (160 min/sample) which is not practical for analyzing large numbers of samples.

CONCLUSIONS

Methods for using RP-IPC for the separation of soil organic P compounds in solutions and extracts were developed. These methods will allow more accurate determination of important P-containing compounds in soils, leading to improved characterization of their reactivity and mobility in soil. The ability to directly measure these compounds is important, due to increasing concern over the addition of organic P compounds to soils and how their behavior will affect overall P losses to the environment.

Extraction methods were developed that gave up to 95% recovery of nucleotides from soil while minimizing their degradation. These extraction procedures were developed to be compatible with the RP-ICP system developed for their separation. In contrast, extraction of IHP using strong acid or alkaline extracting solutions caused significant decrease in peak areas and was unsuitable for studies where accurate measurement of IHP was needed.

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