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Separation of inositol phosphate isomers in environmental samples by ion-exchange chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

A method for isomeric separation of inositol phosphates ($InsP_n$) in environmental samples originating from different sources such as soil, manure/compost, and aquatic sediments has been developed. The method includes a single NaOH-EDTA extraction step, centrifugation and direct injection of a particle free solution into an ion chromatographic column. Isomeric liquid chromatographic separation was achieved with an ammonium carbonate gradient compatible with electrospray ionization tandem mass spectrometric detection (LC-ESI-MS/MS). The detection limits of the LC-ESI-MS/MS method were between 0.03–0.16 μM for the different $InsP_n$, corresponding to 37–99 ng P/g sample DW. The method has shown to be able to analyze more than 200 samples from soil, manure and sediment without any severe matrix effects. This will allow future studies of the fate of inositol phosphates in the environment.

1. Introduction

Knowledge of sources, fluxes, turn over, and inactivation/burial patterns of different phosphorus (P) forms in the environment is necessary to target the P that is ecologically relevant, i.e. the P that will be available for primary production in general, such as in agriculture production, and more specifically, for effective measures to counteract eutrophication. P bound in organic matter represents per see ecologically relevant P since it is easily transported by surface water and has the potential to be mineralized and support future growth.

A key process is the mineralization of organic matter in aquatic sediments, where some of the organic P is labile enough to get mineralized and the dissolved P recycled back to the water column where it might support future growth. However, some organic-P is apparently refractory enough to resist degradation and remains stored in the sediment.

So called P fractionation methods divide inorganic-P forms depending on their solubility due to ion exchange, low redox potential, and high and low pH [1]. In these procedures, organic-P forms are extracted using NaOH, and are detected after digestion/oxidation to mobilize phosphate to be quantified as a sum [2]. Alternatively, the different organic-P forms can be determined using ^{31}P-NMR, yielding speciation due to the different binding characteristics of the P atom [3]. However, to gain specific identification, we have developed methods to extract and identify organic-P forms such as DNA, phospholipids [4,5] and inositol phosphates ($InsP_n$) [6].

$InsP_n$ is suggested to represent a dominant group of organic phosphorus compounds in many soils and sediments [7,8]. Despite their relative abundance compared to other organic-P compounds they are rarely considered contributors to eutrophication due to their stable character [9]. $InsP_n$ are nevertheless transported and accumulated in aquatic systems, and their degradation and the subsequent mobilization of phosphate may contribute to eutrophication effects such as the growth of cyanobacteria [8].

Different chromatographic techniques, such as size exclusion chromatography [10], ion-exchange chromatography [11–17], and reversed-phase chromatography [18] have been used for studying $InsP_n$. We have in a previous study reported a method based on ion-chromatography connected to mass spectrometry with the capability to separate ($InsP_n$), after oxalate extraction, into different groups based on the number of phosphate groups attached to the inositol ring. The method has the possibility to provide more information about $InsP_n$ flux and turn-over in the environment. However, the method has some limitations, such as the relative short lifetime of the separation column due to mobile phase

Abbreviations: $InsP_n$, inositol phosphates; IC, ion chromatography; ESI, electrospray; MS, mass spectrometry; P, phosphorus

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composition.

In this study a liquid chromatographic method (LC) is presented that shows improved long-term stability (higher pH tolerance) as well as better performance (improved selectivity and efficiency). Furthermore, the method is compatible with both electrospray ionization tandem mass spectrometry (ESI-MS/MS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES).

The aim of this study was to develop a method with the capability to analyze and retrieve reliable quantitative data about inositol phosphates from a larger number of environmental samples without losing analytical system performance.

2. Experimental

2.1. Samples

Samples representing various environmental matrices such as soil, manure and aquatic sediments were analyzed. The samples were air-dried, sieved through a 2 mm sieve and homogenized with a mortar and pestle before analysis.

2.2. Chemicals

All chemicals were of analytical grade (Sigma-Aldrich, Germany), unless otherwise specified. Dilution was performed with Milli-Q (MQ) water (Millipore, Bedford, MA). Standard solutions were prepared in MQ water from myo-inositol hexakis(dihydrogenphosphate) solution (~40% in H_2O, 80180 – Sigma-Aldrich) and the dipotassium salt of myo-inositol hexakis(dihydrogenphosphate) (P5681 – Sigma-Aldrich). Further chemicals used were sodium hydroxide (NaOH, p.a. EKA Bohus-Sweden), ethylenediaminetetraacetic acid (EDTA, disodium salt) and ammonium oxalate (Merck, Switzerland).

2.3. Extraction of inositol phosphates from sediment

Samples (approx. 100 mg) were placed in a 2.0 mL micro centrifuge tube (WWR International LLC, Randor, PA) and extracted, either with 1 mL 0.25 M NaOH containing 0.050 M EDTA or 1 mL 0.2 M oxalate/oxalic acid (pH3.0), by shaking for 4 h using a Multi Reax (Heidolph Instrument, Schwabach, Germany). The samples were centrifuged (Spectrafuge 7 M, Labnet International Inc. Edison, NJ) to remove particles at 10,000 rpm for 15 min. A clear solution (0.5 mL) was transferred to a 1.5 mL glass vial and placed in the LC autosampler for analysis.

2.4. Preparation and characterization of in-house standard

In-house standard solution was prepared in the same way as previously [6] described by hydrolyzing the dipotassium salt of myo-inositol hexakis(dihydrogenphosphate) to produce a mixture of myo-inositol phosphate isomers: mono- ($InsP_1$), bis- ($InsP_2$), tris- ($InsP_3$), tetrakis- ($InsP_4$), pentakis- ($InsP_5$) and hexakisphosphate ($InsP_6$). The dipotassium salt of myo-inositol hexakis(dihydrogenphosphate) was dissolved in 2.5 mL 3.2 M acetic acid, heated to 120 °C for 1.5 h, dried under a stream of nitrogen and redissolved in 5 mL MQ-water. Characterization of the in house standard was done in the same way as previously [6] described using ICP-AES detection. Briefly, the ICP-AES was calibrated using a set of inorganic-P standards before the total concentration of P in the in-house standard was determined. Subsequently, the distribution of the different $InsP_n$ was determined by LC-ICP-AES. A series of standard solutions were then prepared from the in-house stock solution in the range between 0.1 and 55 μM for calibration of the ESI-MS system.

Table 1
Gradient used for the elution and separation of inositol phosphates and the injection program. B-phase – 600 mM $(NH_4)_2CO_3$, Injection program NaOH/EDTA −0.25/0.05 M, FA −0.1% formic acid.

Step	Time [min]	Mobile phase B [%]	Inj prog. parameter
1	0.0	3	Draw 5 μL (sample) - inject
2	1.0	3	
3	2.0	20	
4	4.0	28	
5	6.5	43	
6	8.0	55	
7	12.5	95	
8	15.0		Valve bypass
9	16.0		Valve mainpass
10	17.0		Draw 10 μL MQ - inject
11	20.0	95	Valve bypass
12	20.1	1	
13	21.0		Draw 50 μL NaOH/EDTA – inject
14	24.0		Draw 50 μL FA – Inject
15	30.0	1	
16	30.1	95	
17	35.0	95	
18	35.1	3	
19	49.0		Valve bypass
20	50.0		Valve mainpass
21	60.0	3	

2.5. Liquid chromatography

Screening experiments were done with three different ion exchange columns, PRP-X100 (Hamilton, Reno, NV), Asahipak NH2P-40 2D (Shodex, Showa Denko Europe Munich, Germany) and IEC PIKESS DEAE-2B (Shodex). Most promising results were achieved with the Asahipak column and further method development was done with this column. The chromatographic system used was 1260 Infinity (Agilent Technologies, Waldbronn, Germany) with binary pumps, an autosampler and a column oven. The dwell volume in the system was reduced by disconnecting the mixer, pulse damper and heat exchanger. The main work was done with a Shodex Asahipak NH2P-40 2B column, (50 mm × 2.1 mm, particle size 4 μm). A 15 cm column with the same stationary phase was also tested. The mobile phases for the chromatographic separation were A: MQ-water and B: 600 mM aqueous $(NH_4)_2CO_3$. Gradient elution was used (see Table 1) with the flow rate of 150 μL min^{-1}. The column temperature was set to 50 °C. The injected sample size was 5 μL and an injection program (see Table 1) was used to inject 50 μL NaOH-EDTA (0.25/0.05 M) and 50 μL formic acid (0.1%) solutions after the last $InsP_6$ peak has eluted for cleaning the system. Total run time was 60 min, where 15 min were used for the separation and 45 min for washing and equilibration of the column between the runs.

2.6. Electrospray mass spectrometry

The chromatographic system was coupled with a 3200 Q Trap LC/MS/MS system (AB Sciex, Concord, ON, Canada). The flow from the column (150 μL min^{-1}) was mixed in a T-junction with a flow of 2-propanol (50 μL min^{-1}) resulting in a total flow of 200 μL min^{-1} prior to entering the ESI-MS interface. The ESI source was operated in negative ion mode with ion spray voltage set to −4.0 kV, curtain gas flow to 15 psi, nebulizer gas flow to 60 psi, turbo gas flow to 60 psi and temperature to 700 °C. Optimal conditions for the detection were screened through direct infusion experiments. The selected flow for infusion experiments was 10 μL min^{-1} (ramping of collision energy (CE), collision cell exit (CXP) potential, etc.). Each $InsP_n$ was detected in multiple reaction monitoring mode (MRM) by monitoring suitable precursor and product ion transitions at optimal CE and CXP (see Table 2) with a