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Liquid extraction of low molecular mass organic acids and hydroxamate siderophores from boreal forest soil

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ABSTRACT

Low molecular mass organic acids (LMMOAs) and hydroxamate siderophores (HS) are molecules secreted by microbes and have previously been found in soil solution and in cultures. Mycorrhizal fungi are suggested to be involved in the nutrient uptake processes of trees and weathering of minerals. In this study soil samples taken from the O and E horizons of a podzol were extracted with 10 mM potassium phosphate buffer at pH 7.2. Variable parameters included addition of methanol to the extraction buffer and the use of ultrasonication or rotary shaking during extraction. LMMOAs and HS content of the soil extracts were determined. Analysis of soil extracts were carried out by liquid chromatography mass spectrometry (LC–MS) and the extraction results compared to results for soil solution samples obtained by centrifugation of the soils sampled. The extraction yields were significantly increased by addition of methanol to the extraction buffer, especially for the O horizon samples. Rotary shaking of the samples for 90 min gave slightly higher yields than ultrasonication for 15 min but the reduction in extraction time makes ultrasonication an attractive option. Of the HSs determined, ferricrocin was found in all samples. Optimal extraction conditions showed citric acid and isocitric acid to be the most abundant organic acids in the O and E horizons, respectively.

1. Introduction

Swedish boreal forests are dominated by coniferous trees growing on podzol. The podzol are characterized by an organic topsoil layer (O) followed by a weathered eluvial horizon (E), an enriched illuvial horizon (B), and a more or less unaffected mineral soil horizon (C) above the bedrock. The mineral nutrition status of the trees are influenced by the weathering processes taking place in the soil, which in turn are affected by several parameters, including the presence of mycorrhizal fungi living in symbiosis with the trees. From the mycorrhiza, chemical compounds such as low molecular mass organic acids (LMMOAs) (Ahonen-Jonnarth et al., 2000; van Scholl et al., 2006) and hydroxamate siderophores (HS) (Neilands, 1995; Watteau and Berthelin, 1994) can be exuded by fungi as reviewed by Rosling et al. (2004) and Winkelmann (2007). Both these compound classes have been shown to enhance the weathering rates of different minerals, and there have also been reports on synergistic effects when HS and LMMOAs have been combined in weathering experiments (Cheah et al., 2003; Courty et al., 2010; Drever and Stillings, 1997; Eckhardt, 1985; Jones and Edwards, 1998; Reichard et al., 2007; van Hees et al., 2003). Traditionally, analytical determination of siderophores has been performed by spectroscopic methods (Schwyn and Neilands, 1987) or biological assays (Bossier and Verstraete, 1986; Powell et al., 1980). More specific results can be achieved by the use of liquid chromatography (LC) (Haselwandter and Winkelmann, 2002; Jalal et al., 1984; Konetschny-Rapp et al., 1988) or electrospray ionization mass spectrometry (MS) (Gledhill, 2001; Mawji et al., 2008). These two techniques have also been combined into highly selective and sensitive LC–MS methods (McCormack et al., 2003; Moberg et al., 2003).

The levels of LMMOAs in soil solutions from podzols have been rather extensively studied as reviewed by Strobel (2001). Depending on vegetation, season and mineral affinity, the levels reported for different acids varies between nM to mM, but is typically within the μM range (Jones and Edwards, 1998; Strobel, 2001; van Hees et al., 2000). Much less is known on the natural abundance of HS within podzols, but soil solution concentrations within the low nM range for HS belonging to the ferrichrome family have been reported (Essén et al., 2006; Holmstrom et al., 2004).

The exact mechanisms by which these substances affect the weathering are not fully understood, but both LMMOAs and HS adsorb readily to minerals and humic material (Jones et al., 2003; van Hees et al., 2003). For the LMMOAs, this adsorption is

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believed to buffer the soil solution concentrations as the decomposition rates are much slower for the adsorbed molecules (van Hees et al., 2003). In order to get a more complete picture on the activities of these compounds, it would be of interest to measure the adsorbed fraction.

The adsorption of phosphate to forest mineral soil is strong, which make phosphate a suitable agent to extract other anionic components as organic acids (van Hees et al., 2003). The adsorption coefficients (adsorbed to soil/total content in soil) for phosphate are higher than for oxalic and citric acid at pH 4.5 for mineral soil. Jones et al. (2003) found that adsorbed citric acid was fully extracted by KH_2PO_4 in O horizon soil, but not in mineral soil. To further enhance the extraction yield addition of methanol has been tested (Chen et al., 2001).

In this work, ultrasonic extraction has been compared with ordinary rotary shaking. As an extraction medium, phosphate buffer at neutral pH has been used, either alone or together with methanol. The resulting soil extracts has been analyzed by LC–MS for the content of HS and LMMOAs, and the values compared with the levels found in soil solution.

2. Material and methods

2.1. Site, soils and sampling

The sampling site was located at Bispgården (63°07′N, 16°70′E), NW of Sundsvall in central Sweden. The soil selected was podzolized and forested mainly with Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). The chemical composition of the soil is described in Bylund et al. (2008a,b). Samples from the organic (O) and the eluvial (E) horizons were taken in the summer from the walls of two pits, each of the size 1 × 1 × 1 m. Green plant material was removed and the sampled soil was stored in plastic bags at 4 °C and used within 48 h. The moisture content was estimated for both organic and eluvial horizon.

2.2. Soil solution obtained by drainage centrifugation

The soil was packed in a plastic tube (id 45 mm, h 70 mm) and set in a double-bottomed centrifugation cup (Giesler and Lundström, 1993). The tube was centrifugated at 16,500 rpm for 40 min at 4 °C using Avanti J–20XP centrifuge with a JA 14 rotor (Beckman coulter, California, USA). The obtained soil solution was membrane filtered (Millex HV 0.45 μm, Millipore, Massachusetts, USA) and then stored frozen for further analysis.

2.3. Soil extraction of LMMOAs and HS

Soils were extracted with 10 mM K_2HPO_4 buffer at pH 7.2 (A) or a mixture of 10 mM K_2HPO_4 buffer with methanol in a ratio of 1:1 with final pH of 7.5 (B). The extraction was performed at ambient temperature, either in an ultrasonic bath for 15 min or on a rotary shaker for 90 min. For extraction of LMMOAs 20 g soil was extracted with 200 mL of buffer (A) or buffer–methanol mixture (B) and for HS 2 g of soil was extracted with 10 mL of buffer (A) or buffer–methanol mixture (B). Soil extraction was replicated three times. The extraction mixture for determination of LMMOAs was upon completion centrifugated at 16,250 rpm for 30 min at 4 °C and the supernatant was membrane filtered (Millex HV 0.45 μm, Millipore) and then stored frozen for further analysis. The extraction mixture for determination of HS was membrane filtered (Millex HV 0.45 μm, Millipore) and the filtrate frozen for further analysis.

2.4. The LC–MS system

A LC system consisting of a Shimadzu LC-10AD pump (Kyoto, Japan) and two Perkin Elmer 200 Micro Series LC-pumps and an Agilent 1100 autoinjector (California, USA) was coupled to an API3000 mass spectrometer (Applied Biosystems, Ontario, Canada). For column switching a 10-port switching valve (Valco Instruments, Texas, USA) was used.

2.5. LC–MS/MS analysis of LMMOAs

The LMMOAs included in the study were *cis*-aconitic, *trans*-aconitic, citraconic, citric, fumaric, glutaric, isocitric, α-ketoglutaric, lactic, maleic, malic, malonic, oxalic, pyruvic, shikimic, succinic and tartaric acid. Standards were of pure analysis quality from various suppliers. Stock solutions of 20 mM were prepared in ultrapure water and stored frozen until further dilution to working standards. Linearity and accuracy were determined for standard solutions within the range 0.01–50 μM.

Analytes were separated on a Supelcogel C610-H column (300 mm × 7.8 mm) from Supelco (Pennsylvania, USA) with an isocratic system, methanol–0.01% formic acid (10:90) at a flow rate of 0.400 mL/min. The injected volume was 100 μL. After the column the flow was split in a Valco tee union directing approximately

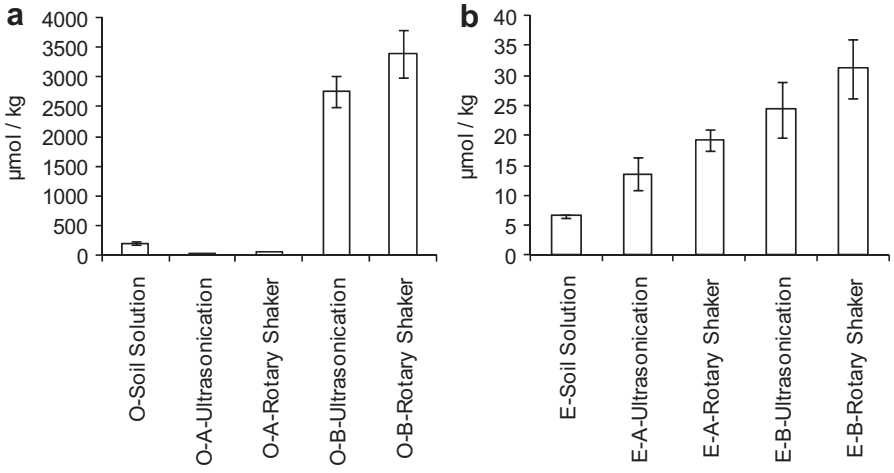

Fig. 1. Diagram of the total amounts of LMMOA for a) the O horizon and b) the E horizon. Abbreviations; Buffer: A and B.