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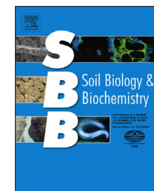

Development of liquid chromatography mass spectrometry method for analysis of organic N monomers in soil

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ABSTRACT

The aim of this study was to develop an analytical procedure based on liquid chromatography-mass spectrometry (LC–MS) for analysis of monomeric organic N compounds in soil extracts. To benchmark the developed LC–MS method it was compared with a capillary electrophoresis–mass spectrometry (CE–MS) method recently used for analysis of small organic N monomers in soil. The separation was optimized and analytical performance assessed with 69 purified standards, then the LC–MS method was used to analyse soil extracts. Sixty-two out of 69 standards were analysable by LC–MS with separation on a hydrophilic interaction liquid chromatography column. The seven compounds that could not be analysed were strongly cationic polyamines. Limits of detection for a 5 μL injection ranged between 0.002 and 0.38 μmol L^{-1}, with the majority (49 out of 62) having limits of detection better than 0.05 μmol L^{-1}. The overall profile and concentration of small organic N monomers in soil extracts was broadly similar between LC–MS and CE–MS, with the notable exception of four ureides that were detected by LC–MS only. In soil extracts that had been concentrated ten-fold the detection and quantification of (some) organic N compounds was compromised by the presence of large amounts of inorganic salts. The developed LC–MS method offered advantages and disadvantages relative to CE–MS, and a combination of the two methods would achieve the broadest possible coverage of organic N in soil extracts.

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1. Introduction

The profile and concentration of organic N molecules in soil solution and soil microbes are information-rich signals indicative of soil function. This is because organic N compounds in soil solution reflect biological processes such as uptake and efflux of organic molecules by plant roots and free-living and symbiotic microbes, activity of extracellular enzymes, and leaching of organic molecules from litter. In recent times the profile of organic N molecules in the soil solution has become of particular interest with the demonstration that plants can directly take up various small organic forms of N (Chapin et al., 1993; Jones and Darrah, 1993; Näsholm et al., 2009; Warren, 2013b) and recognition that knowledge of the identity and concentration of compounds in soil solution is key to designing and interpreting experiments on organic N uptake (Warren, 2014a). Knowledge of organic N in soil microbes holds great promise as an indicator of function of the microbial community. For example, stresses such as water deficits and freeze–thaw cycles that have strong effects on the physiology and composition of the microbial community (Schimel et al., 2007) ought to also affect the composition of organic compounds in soil microbes (e.g. concentrations of osmolytes: Warren, 2014c).

The most common approach for exploring organic N monomers in soil has involved use of methods for identification and quantification of amino acids. Various chromatographic and electrophoretic methods have been used successfully for separation and quantification of primary (and in some cases secondary) amino acids in soil extracts (e.g. Kielland, 1995; Yu et al., 2002; Warren, 2008; Farrell et al., 2011). These methods are generally based upon highly selective derivatization and/or detection schemes. The downside of this selectivity is that the methods are effectively blind to many of the other compound classes of organic N present in soil (Warren, 2013a). To address questions such as what organic N compounds are used for osmotic adjustment or what organic N compounds are taken up by plants requires a broader exploration of organic N than is possible with methods that target amino acids only.

Few methods have been developed for the comprehensive analysis of organic N monomers in soil-based samples, at least in part because the properties of organic N in soil limit the application

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of common analytical platforms such as nuclear magnetic resonance (NMR) and gas chromatography (GC). NMR spectroscopy shows inherent limitations in terms of detection sensitivity (Gika et al., 2012) and thus when applied to soil will be able to detect only the most abundant compounds. Gas chromatography is unsuitable for broad-based exploration of organic N in soil because many organic N molecules (e.g. quaternary ammonium compounds, oligomers and polymers) are not volatile and cannot be derivatized using common reagents, or are degraded during passage through the hot injection port and column (Kaspar et al., 2009).

Capillary electrophoresis in combination with electrospray ionization and mass spectrometry (hereafter referred to as CE–MS) has recently been demonstrated as a useful tool for profiling small organic N compounds in soil extracts (Warren, 2014a). The major advantages of CE–MS are that compounds do not need to be volatile or derivatized, and it can separate molecules of widely varying polarity while also resolving isomeric and isobaric ions (Kuehnbaum and Britz-McKibbin, 2013). However, the widespread adoption of CE–MS may be prevented by some limitations. First, the described CE–MS separation (Warren, 2014a) was limited to those compounds that were cationic at the electrolyte's pH (~2). Organic N compounds that are weakly cationic (e.g. ergothioneine) yield broad peaks and poor detection limits, while compounds that are neutral (e.g. N-acetylglucosamine) or anionic (e.g. ureides) cannot be analysed. The second factor that may limit the more widespread adoption of CE–MS is the comparatively poor reproducibility of absolute migration time (Ramautar et al., 2009; Sugimoto et al., 2010), which complicates data analysis because the same peak can appear at (slightly) different migration times in replicate analytical runs. The final factor limiting use of CE–MS is that the installed base of CE–MS instruments remains small, and thus there is limited access to instrumentation and expertise.

Liquid chromatography in combination with electrospray ionization and mass spectrometry (hereafter referred to as LC–MS) may offer an alternative to CE–MS for analysis of small organic N compounds. One of the biggest assets of LC–MS is that the installed base of LC–MS instruments is large, and thus instrumentation and expertise are widely accessible. Unfortunately, LC–MS of hydrophilic compounds such as small organic N compounds is far from straightforward. The challenge arises because organic N compounds exhibit negligible retention or separation on the reversed phase columns commonly used for LC–MS. Retention and separation can be improved by adding ion pairing reagents to the mobile phase (Hakkinen et al., 2007; Lu et al., 2008; Sanchez-Lopez et al., 2009), but methods utilizing ion pairing reagents are generally unsuitable because ion pairing reagents suppress mass spectrometry signals and lead to persistent contamination of the mass spectrometer's ion source (Rutters et al., 2000; Holcapek et al., 2004). Another alternative is to derivatize compounds so as to improve their chromatography (and in many cases also detection limits) (Inagaki et al., 2010; Murphy et al., 2014), but not all compounds can be derivatized and thus derivatization cannot achieve a sufficiently large breadth of coverage. Two separation modes capable of LC–MS of hydrophilic compounds without ion pairing or derivatization are hydrophilic interaction liquid chromatography (HILIC) (Alpert et al., 1994) and aqueous normal phase liquid chromatography (Pesek and Matyska, 2007). In recent times LC–MS with HILIC separations have been used by numerous groups for analysis of complex mixtures of hydrophilic compounds (Lu et al., 2008; Kato et al., 2009; Schiesel et al., 2010; Creek et al., 2011; Iwasaki et al., 2011; Rappold and Grant, 2011; Boudra et al., 2012; Buszewski and Noga, 2012; Chen et al., 2012; Fraser et al., 2012; Zhang et al., 2012) but it is unclear if methods developed for other biological samples (e.g. animals, plants, microbial cultures) can be directly applied to soil. Soil-based samples are particularly challenging because concentrations of organic N compounds are typically several orders of magnitude smaller than concentrations of (potentially interfering) inorganic ions (Oikawa et al., 2011; Warren, 2014a).

The aim of this study was to develop an analytical procedure based on LC–MS with HILIC separation for analysis of monomeric organic N compounds in H_2O extracts and 2.5% $CHCl_3$ extracts of soil. To benchmark the developed LC–MS approach it was compared with a CE–MS approach recently demonstrated as useful for broad-based analysis of small organic N monomers in soil (Warren, 2013a). To provide a fair benchmarking of performance, common samples and standards were analysed on both analytical platforms and the same mass spectrometer was used for LC–MS and CE–MS.

2. Methods

2.1. Experimental design

To characterize analytical performance the same standards and samples were analysed by LC–MS and CE–MS. The first test of performance involved injecting 69 purified standards (see Section 2.2) so as to determine which organic N monomers could be analysed, their detection limits, and the shape and width of peaks. Special attention was paid to the separation of five pairs of structural isomers (Fig. 1).

To examine applicability of LC–MS and CE–MS to soil-based samples, two types of soil extract (see Section 2.3) were analysed. Soil extracted with ultra-pure water was used to provide a sample indicative of compounds in free solution, while soil extracted with aqueous 2.5% (v/v) $CHCl_3$ was used to provide a sample indicative of compounds present in microbial biomass. These two types of soil extract were then analysed a) after preparation in appropriate injection buffer/solvent but with no pre-concentration and, and b) after 10-fold pre-concentration.

The final tests of analytical performance involved determining the repeatability of LC–MS and CE–MS analyses of a soil extract. Five replicate injections of the same 2.5% $CHCl_3$ extract of soil were used to calculate for each peak the precision of concentration estimates, and precision of retention time (LC–MS) or migration time (CE–MS).

2.2. Chemicals and standards

Methanol, acetonitrile and formic acid were LC/MS grade; while ammonium formate, ammonium hydroxide (28–30% NH_3), and chloroform were analytical grade. All electrolytes, rinsing solutions, standards and extracts were prepared with 18.2 MΩ cm ultra-pure water (Arium 611UV, Sartorius, Goettingen, Germany). The majority of method development was carried out with 69 purified standards of monomeric organic N compounds. Standards were prepared from their free acids or salts purchased from Sigma. All standards of chiral amino acids were L enantiomers. Stock solutions of 1 mg mL^{-1} were made in ultra-pure water or 0.1 M HCl. To determine which compounds were analysable, the individual stock solutions were combined into five different mixtures at concentrations of 20 μg mL^{-1} (Table S1). Mixtures were prepared ensuring that there were no compounds with the same nominal mass within each mixture, and thus assignment of peaks was generally unambiguous. In cases where peaks could not be assigned unambiguously, individual standards were analysed separately. Finally, in a few instances where compounds (apparently) present in a mixture did not appear in a chromatogram or electropherogram, the mixture was subsequently analysed by direct infusion-mass

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