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Compound-specific short-chain carboxylic acids identified in a peat dissolved organic matter using high-resolution liquid chromatography–mass spectrometry

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ABSTRACT

Dissolved natural organic matter (DNOM) is primarily composed of exuded or remnant biomolecules from plants and microorganisms. Labile amino acids and sugars have been well documented in the low molecular weight components of DNOM. However, little attention has been devoted to the isolation of labile short-chain carboxylic acids (SSCAs), which have demonstrated biogeochemical significance as sources of assimilable carbon, promoters of mineral dissolution, and ligands for metal complexation. Here we present an analytical method that identifies compound-specific SSCAs in a peat-derived DNOM isolate, Pahokee peat humic acid. Using high-resolution liquid chromatography (LC) coupled with high-accurate orbitrap mass spectrometry (MS), we targeted the identification of five SSCAs of different types: gluconate (a C_6 monocarboxylic acid), citrate (a branched C_6 tricarboxylic acid), 2-ketoglutarate (a C_5 dicarboxylic acid), and malate and fumarate (C_4 dicarboxylic acids). Following LC-separation and electrospray ionization, the compounds were annotated directly by orbitrap MS using their exact mass-over-charge (m/z) ions in the negative mode and their stoichiometric composition. Validated by LC–MS metabolite annotation in a bacterial matrix, we achieved identification of all five compounds in the peat DNOM isolate. Each targeted m/z channel also captured non-targeted compounds at different retention times, which represent isomers or different compounds. We found that the five targeted and the two non-targeted SSCAs identified collectively accounted for high parts-per-million to low parts-per-thousand of the total carbon, oxygen, or carboxyl content. Building on these findings, an important next step is to obtain a comprehensive profiling of SSCA structures in DNOM of different origins.

1. Introduction

Dissolved natural organic matter (DNOM) represents an important reservoir of organic carbon that is intimately involved in the fate and transport of inorganic and organic nutrients in a watershed. Characterization of molecular structures in DNOM has been a long-standing analytical challenge (Sutton and Sposito, 2005; Schmidt et al., 2011; Masoom et al., 2016). To characterize DNOM fractions including humic and fulvic isolates, various techniques have been applied: fractionation approaches using chromatography (Huber et al., 2011; Woods et al., 2012) and ultrafiltration (Li et al., 2004); chemical characterization using elemental analysis and acid-base titration (Ritchie and Perdue, 2003; Gondar et al., 2005; Lopez et al., 2012; Aristilde and Sposito, 2013; Klavins and Purmalis, 2013), spectroscopic examination using fluorescence (Provenzano et al., 2004), infrared (Niemeyer et al., 1992), nuclear magnetic resonance (Hertkorn et al., 2002; Woods et al., 2012), and mass spectroscopy (MS) (Remucal et al., 2012; DiDonato et al., 2016). Recent applications of electrospray ionization–Fourier transformed ion cyclotron resonance (ESI–FTICR) MS to characterize DNOM isolates have highlighted an abundance of low molecular weight molecules with m/z values < 800 (Remucal et al., 2012; Ikeya et al., 2015). Therefore, DNOM is increasingly portrayed as a supramolecular structure comprised of common biomolecules that are resistant to degradation due to multiple intermolecular interactions (Sutton and Sposito, 2005; Schmidt et al., 2011). The detection and quantitation of amino acids and sugars within DNOM have been well documented (Hedges et al., 1994; Thomas, 1997; Swenson et al., 2015; Abdelrahman et al., 2016), but little attention has been paid to short-chain carboxylic acids (SSCAs), which represent an important class of biomolecules. Here we combine high-resolution liquid chromatography (LC) with the high-accurate

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ESI-MS technique to elucidate SSCAs in a DNOM isolate of peat origin.

Peat humic acid (HA) was chosen to conduct our proof of concept experiment because it is a commonly extracted fraction of DNOM in characterization studies of peat environments (Gao et al., 1999; Zaccone et al., 2007; Klavins and Purmalis, 2013). Accumulation of metals, nutrients and pollutants in peat matrices has been attributed to oxygen-containing functional groups such as carboxyl, hydroxyl, and ketone (Provenzano et al., 2004). These functional groups are common in SSCAs present in biological metabolic pathways (Sasnow et al., 2016). Stemming from the fact that peat environments are comprised primarily (> 80%) of biologically derived organic matter from microbial and plant remnants as well as exudates (Gondar et al., 2005; Zaccone et al., 2007), we hypothesized that SSCAs of metabolic origin will be present in peat-derived DNOM. In this technical note, we evaluated this hypothesis by targeting the identification of five SSCA-type metabolites in different solvent-treated solutions of Pahokee peat HA. The targeted compounds are metabolic intermediates in sugar metabolism (gluconate, a C_6 carboxylic acid) or in the tricarboxylic acid cycle (citrate, a C_6 branched tricarboxylic acid; 2-ketoglutarate, a C_5 dicarboxylic acid; and malate and fumarate, C_4 dicarboxylic acids) (Fig. 1). Our findings confirmed the persistence of labile biologically derived SSCAs in DNOM isolates and our targeted compounds accounted for up to ppt fractions of total elemental composition.

2. Materials and methods

Pahokee peat HA was obtained from the International Humic Substances Society (1S103H) and dissolved completely (2 g/L) in ultrapure water (18.2 MΩ cm, Millipore) adjusted to pH 7 using concentrated KOH (Aristilde and Sposito, 2013). To extract the low molecular weight components from the peat HA, as previously done for an aquatic HA (Remucal et al., 2012), we subjected the water-dissolved peat HA (20 mL) to dialysis treatment wherein dialysis bags (6–8 kDa MWCO Spectra/Por cellulose ester membrane at 3.2 cm diameter from Spectrum Laboratories) were submerged (in 250 mL glass beakers) in four different solvents (100 mL): ultrapure water alone, methanol:acetone:water (40:40:20, v:v:v), methanol:water (50:50, v:v), or acetone:water (50:50, v:v). Sodium azide (1 mM final concentration) was added to prevent bacterial growth (Remucal et al., 2012). The dialysis bags, tied tightly on both ends, were suspended in the solutions using sterile plastic clamps. Experiments were done in two independent replicates; control experiments were also performed in the absence of peat HA.

After 24 h dialysis treatment, aliquots of the well mixed dialysates were dried under nitrogen gas and resuspended in ultrapure LC–MS water (concentrated 5×) and analyzed by LC–MS using an ultra-high performance LC (Thermo Scientific DionexUltimate 3000) with high-resolution/high-accurate mass spectrometer (Thermo Scientific Q Exactive quadrupole-Orbitrap hybrid mass spectrometer). We employed established LC–MS methods for metabolite identification in bacterial extracts based on accurate masses and their associated natural isotopic patterns (Lu et al., 2010). Briefly, at a total LC–MS run of 25 min, injected samples were subjected to reverse-phase LC separation at different gradients of two solvents, (i) 100% methanol and (ii) a 97:3 mixture of water:methanol with acetic acid (15 mM) and the ion-pairing agent tributylamine (10 mM); the MS was operated in full scan negative mode (m/z 70–800) whereby specific m/z ranges were targeted throughout different phases of the LC gradient (Lu et al., 2010). Metabolite quantification was conducted using standards purchased from Sigma–Aldrich. For continuous validation of metabolite identification, we compared the LC–MS analysis of the peat HA to cellular extracts of the soil bacterium *Pseudomonas putida* KT2440. The targeted metabolites were previously identified in this bacterium in our lab using a methanol:acetone:water solvent, which was shown to be optimal for isolating biological metabolites in bacteria cells (Sasnow et al., 2016; Aristilde, 2017; Aristilde et al., 2017).

3. Results and discussion

We compared both the total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of the peat HA solution treated with the four different solvents with those of the cellular extracts of *P. putida* KT2440 (Fig. 2A and B). Well-resolved peaks with high intensities were obtained in the TIC of the cellular extract with the methanol:acetone:water solvent (Fig. 2A). All four treatments resulted in the detection of multiple ions in the peat HA dialysate over the course of the LC–MS run; the highest peak intensities in the peat TIC were obtained with the acetone:water and water treatments (Fig. 2A). Using EICs of the water dialysate at the targeted m/z channels, we identified well-resolved peaks for all four SSCAs in the peat HA solution and validated them by EICs of the bacterial extract (Fig. 2B). The gluconate peak (m/z 195.0502) was isolated at 5.49 min (Fig. 2B and C). The other four compounds were detected at very close RTs, 12.60–12.85 min, but with distinct MS signatures (Fig. 2B and C). The m/z values for citrate, α-ketoglutarate, malate, and fumarate were, respectively, 191.0190, 145.0131, 133.0130, and 115.0036 (Fig. 2C).

Comparison with the bacterial EICs highlighted an additional peak both in the gluconate m/z channel (0.51 min downstream) and the fumarate m/z channel (0.87 min upstream) in the peat HA dialysate (Fig. 2B). These additional peaks were attributed to non-targeted molecules of similar stoichiometric composition as the targeted ones. Our metabolite detections in the peat HA solutions were obtained at high mass accuracy whereby the mass accuracy offset was calculated to be between 4.1 and 11.3 ppm (1 ppm = 0.0001% offset of the measured m/z from the theoretical m/z) (Fig. 2C). We note that the different solvents did not perform equally in extracting the SSCAs from the peat HA solution. In terms of carbon content, 2-ketoglutarate and malate were the most abundant in the water-treated dialysate whereas gluconate, citrate, and malate were the most abundant in the dialysate of methanol:acetone:water (Fig. 2D, left panel). In terms of the extraction of the carboxyl-enriched metabolites, water treatment performed better

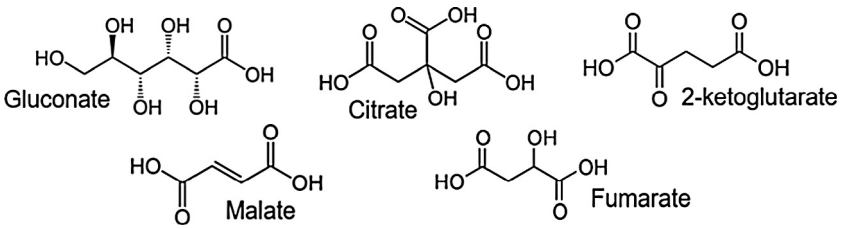

Fig. 1. Chemical Structures of the targeted short-chain carboxylic acids (SSCAs). Gluconate (C_6 carboxylic acid), citrate (C_6 branched tricarboxylic acid), 2-ketoglutarate (C_5 dicarboxylic acid), malate and fumarate (C_4 dicarboxylic acids).