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Application of a Stir Bar Sorptive Extraction sample preparation method with HPLC for soil fungal biomass determination in soils from a detrital manipulation study

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ABSTRACT

Ergosterol is a sterol found ubiquitously in cell membranes of filamentous fungi. Although concentrations in different fungal species span the range of 2.6 to 42 μg/mL of dry mass, many studies have shown a strong correlation between soil ergosterol content and fungal biomass. The analysis of ergosterol in soil therefore could be an effective tool for monitoring changes in fungal biomass under different environmental conditions. Stir Bar Sorptive Extraction (SBSE) is a new sample preparation method to extract and concentrate organic analytes from liquid samples. SBSE was here demonstrated to be a simple, fast, and cost effective method for the quantitative analysis of ergosterol from field-collected soils. Using this method we observed that soil ergosterol as a measure of fungal biomass proved to be a sensitive indicator of soil microbial dynamics that were altered by changes in plant detrital inputs to soils in a long-term field experiment.

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1. Introduction

Large amounts of litter fall to the forest floor in deciduous forests every year. The decomposition of this detritus may represent about 70% of total forest soil carbon (C) efflux (Raich and Schlesinger, 1992). Soil microbes including bacteria, fungi, and archaea ultimately process all detrital inputs and thus can be considered the "gatekeepers" of soil C pools and biochemical composition (Voříšková et al., 2014). While the quality of plant detritus, plant root exudation, microbial decomposers, and soil minerals all interact to form soil organic matter (SOM), recent research has highlighted the role of fungi, and specifically mycorrhizal fungi, in both forming and maintaining stable SOM pools (Averill, 2016; Averill et al., 2015; Brzostek et al., 2015; Clemmensen et al., 2013; Fernandez and Kennedy, 2015; Lindahl and Tunlid, 2015). Although soil microbial biomass carbon generally makes up only 1–5% of total soil organic carbon (SOC) (Jenkinson and Ladd, 1981), it is the most active factor in litter and SOC turnover and responds more rapidly to soil disturbance than does SOC (Xiaojun et al., 2013). Thus, accurate and simple methods to measure soil microbial biomass, particularly that of fungi (Pascoal and Cássio, 2004) are critical to all studies of ecosystem function.

There are several ways to measure the C content of soil fungal biomass. However, none are considered uniformly accurate under all environmental conditions (Beni et al., 2014; Wallander et al., 2013). Several studies have suggested that ergosterol, considered the most important sterol of fungi, could be a sensitive indicators of soil fungal biomass (Axelsson et al., 1995; Lau et al., 2006; Olsson et al., 2003; Teste et al., 2016; Weete and Gandhi, 1996). Ergosterol is widespread in the more developed fungi, although some primitive fungal taxa include other sterol compounds. Although concentrations in different fungal species span the range of 2.6 to 42 μg/mL of dry mass, many studies have shown a strong correlation between soil ergosterol content and fungal biomass (Pasanen et al., 1999). Many classic analyses of fungal C involve a large number of steps and have expensive reagents, and thus are time consuming and difficult to perform. The saponification process is an important part of the analytical process (Tardieu et al., 2007), which is followed by an extraction step (Bailly et al., 1999) before chromatographic analysis. Most ecological studies have used high pressure liquid chromatography (HPLC) followed by UV detection (Chiocchio and Matković, 2011; de Ridder-Duine et al., 2006) at 282 nm, which needs sample pre-concentration and clean up before the analysis. For this latter part of the procedure, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are widely used, but these techniques are time-consuming (Boonzaaijer et al., 2005; Mollahosseini et al., 2016; Valle-Algarra et al., 2009) and the LLE produces high volumes of toxic waste.

The development of a rapid and accurate extraction technique that is simpler, quicker and cost effective would be of use to a large number of researchers. The advantages of a micro-extraction technique are

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reduced extraction solvent volumes and extraction times as well as higher enrichment factors and recoveries (Aguirre et al., 2014; Bidari et al., 2007; Cacho et al., 2013; Chaichi et al., 2013; Kamankesh et al., 2015; Kawaguchi et al., 2013; Liang et al., 2005). Stir Bar Sorptive Extraction (SBSE) is a micro-extraction method that was introduced in 1999 (Baltussen et al., 1999). SBSE is for the extraction of non-polar and medium-polarity organic compounds, and it has high reproducibility (Nogueira, 2015). SBSE is a solid-liquid extraction method with a polydimethylsiloxane (PDMS) sorbent on the outer layer of glass-coated magnetic stir bar that is placed in the liquid sample to sorb organic compounds (Benedé et al., 2016; David and Sandra, 2007; Pavlović et al., 2007; Zhao et al., 2013). There are two options for the desorption of the analyte. The first is solvent desorption, where the stir bar is placed in a solvent solution to extract the analyte constituents, yielding a concentrated liquid solution which is necessary, for example, for liquid chromatography. The second is thermal desorption, which is commonly used with gas chromatography. While there are many advantages of SBSE including simplicity of operation, high pre-concentration factors, and low sample volume requirements (Kende et al., 2006; Kolahgar et al., 2002; Sampedro et al., 2009; Sánchez et al., 2013), there are several caveats to the technique. For example, only polydimethylsiloxane (PDMS) sorbent is currently commercially available, and thus polar analytes do not adsorb to the stir bar (Camino-Sánchez et al., 2014; Gilart et al., 2014). Other coatings have been made for specific polar analytes using the sol-gel (Fan et al., 2013; Gilart et al., 2014; Lei et al., 2016) process. For example, stir bars with a coating of ZnS nanoparticles loaded on activated carbon were made to preconcentrate trace levels of carbamate insecticides prior to analysis (Talebianpoor et al., 2017). These in-house coatings can extend the application potential of the SBSE technique (Gilart et al., 2014).

In this study, the SBSE technique was tested for the analysis of ergosterol in soils by HPLC to determine its usefulness for ecological studies of soil fungal biomass. Results from this analysis were compared to results from our formerly published method (Beni et al., 2014) that used a standard SPE column for sample preparation. The SBSE-HPLC technique was applied to soils from experimentally manipulated plots in the Síkfőkút DIRT (Detrital Input and Removal Treatment) project. Because the Síkfőkút soils have undergone detrital input reduction and additions for over a decade (Fekete et al., 2011a, 2011b, Kotroczó et al., 2014, Tóth et al., 2011), they were expected to have different soil organic carbon and fungal biomass levels, making the experiment an ideal place to test this new method of analysis.

2. Material and methods

2.1. Reagents

All aqueous solutions were prepared using water deionized through a Millipore Milli-Q system. Ergosterol standards (Sigma-Aldrich) were obtained that were >95% pure. Technical grade methanol (99.95%) and reagent grade potassium-hydroxide were used for soil extractions and saponification. Sodium chloride used for the extraction was >99.5% pure (Fluka). HPLC grade acetonitrile was used for ergosterol desorption from stir bar. HPLC grade methanol (99.99%) was used for HPLC analysis.

2.2. Apparatus

Twister Stir Bar Sorptive Extraction (SBSE) bars with Polydimethylsiloxane (PDMS), film thickness 1.0 mm, length 20 mm were obtained from Gerstel (http://www.gerstel.com).

The ergosterol content of samples was determined with a Gilson 506C HPLC system with auto-sampler and UV detection. The measurements were done at 282 nm and room temperature. The chromatography column was RP-C18 (150 × 4.6 mm; 3 μm), obtained from Phenomenex. The chromatograms were processed with Gilson 712 software. All other parameters were similar to those of Beni et al. (2014).

2.3. Experimental site and soil sampling

The Síkfőkút DIRT (Detrital Input and Removal Treatment) experimental site is located in the south part of the Bükk Mountains in North Hungary (47°55′N, 20°26′E), 320–340 m above sea level (Fekete et al., 2016). According to the FAO World Reference Base, the soils were Luvisols (Świtoniak et al., 2014). The forest is a semi-natural stand (*Quercetum petraeae-cerris* community) without forest management. Soil samples of two of the most extreme DIRT treatments were compared for this study: Double Litter (DL, where inputs of aboveground litter were doubled every year) and No Input (NI, where roots were excluded and aboveground litter was removed every year). Every treatment was replicated three times (Fekete et al., 2012, Fekete et al., 2011a, 2011b).

2.4. Statistical methods

The Mann-Whitney *U* test was used to determine if there were significant differences between the two methods for the determination of ergosterol in two litter manipulation (DL, NI) treatments. When $p \leq 0.05$ values were considered to be significantly different. Every treatment had 3 independent data points, because the field manipulations were replicated 3 times. We took soil samples three times in Síkfőkút, so the analysis of the ergosterol content of the soil samples was replicated 3 times as well.

2.5. Method development: ergosterol extraction and analysis

The principal steps of the new method were: collecting and preparing the soil sample, ultrasonic assisted extraction and saponification of lipids to release ergosterol, Stir Bar Sorptive Extraction (SBSE) with PDMS sorbent to enrich and purify the ergosterol, and finally HPLC measurement to determinate ergosterol concentration. Optimal parameters of sample preparation, such as time, reagent concentration and organic solvent were explored. All method development experiment was repeated at three times.

2.5.1. Optimisation of soil extraction and saponification for SBSE

Because the capacity of the PDMS sorbent on the stir bar is limited, the amount of soil that could be used without saturating the sorbent was determined. Analyses were performed using 0.5, 1.0, 5.0 and 10.0 g of sand as an artificial soil that initially contained no fungal biomass, with 0.500 mg/g ergosterol added to each soil and an extract solution of 0.14 mol/L potassium hydroxide in methanol. The highest yield of ergosterol was with 1 g of artificial soil and 10 mL of solution. After that the natural soil samples were tested, and the highest yields were also observed using 1 g of soil. Thus these parameters were used for all further analyses.

The extraction efficiencies of ultrasonic shaking were tested for 5, 10, 20 or 30 min, and optimum yield was observed at 20 min. It was critical to use ice water to cool the solution and maintain the temperature at 25 °C, as at ~40 °C ergosterol recovery significant decreases (Beni et al., 2014).

2.5.2. Optimisation of the SBS extraction

Ergosterol is hydrophobic and thus is easily adsorbed onto a PDMS sorbent. Different volumes of soil extract were tested, as were pH and extraction time. First, different volumes (1, 2, 5 and 10 mL) of the NaOH-methanol solution were tested, and the highest signal was observed with 10 mL of extract solution. The next step was the optimization of the adsorption time. Soils were extracted for 1, 2, 3, 4, 5 and 10 min, and results demonstrated that 4 min was sufficient. All extractions used a stir bar rotation speed of 130–135 rotations/min.

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