Title

Untargeted Soil Metabolomics Using Liquid Chromatography-Mass Spectrometry and Gas Chromatography-Mass Spectrometry.

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Untargeted Soil Metabolomics Methods

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Summary/ Abstract

The molecular composition of soil organic matter (SOM) sets the foundation for terrestrial microbial community structures and carbon cycling dynamics. However, the specific chemical constituents of SOM are underexplored. In this chapter we present a protocol for the extraction of small molecule metabolites from soil followed by compound analysis and identification using liquid chromatography/ mass spectrometry and gas chromatography/ mass spectrometry. There are options within the protocol to assess either the extracellular pool of metabolites or the total pool (including intracellular) and polar and non-polar metabolites, depending on the reader's research interests. These methods can be followed individually for a more targeted analysis or all methods can be combined to obtain a more comprehensive understanding of SOM metabolite composition (such as amino acids, nucleobases, organic acids, fatty acids, carbohydrates, secondary metabolites and antibiotics).

Key Words (5-10 words)

soil organic matter, liquid chromatography/ mass spectrometry, gas chromatography/ mass spectrometry, metabolomics

Introduction

Soils play an essential role in the global carbon cycle ***(1)***. Despite the importance of soils, the molecular composition of soil organic matter (SOM) is still largely unknown. This information can be used to understand and predict the roles of terrestrial ecosystems in climate change through, for example, the lability of SOM and its connection to microbial activities. Fortunately, many recent advances in analytical techniques have begun to shed light on SOM molecular structure and composition. One of the biggest insights has been through the use of imaging technology which completely shifted our understanding of SOM structure. Historically, SOM was thought to be composed of large polymeric humic substances ***(2)***, but now SOM is increasingly recognized to be largely composed of a variety of plant- and microbe-originating small molecules ***(3)***. Although bulk soils may contain thousands of compounds, what may be of the most interest for microbiologists are the water-soluble metabolites (<1000 Da) that are exchanged within soil microbial communities (*i.e.* the water-extractable exometabolite pool). Ultimately, it is the composition of this metabolite pool that shapes microbial communities and therefore the biological activity (carbon cycling) of soil ***(4)***. Other fractions that may also provide critical insights into soil ecosystems include the intracellular microbial metabolites (which may be released into the environment during cell lysis) and non-polar metabolites involved in microbial defenses (*e.g.* secondary metabolites and antibiotics).

A variety of techniques currently exist to characterize the small molecule composition of a soils including capillary electrophoresis/ mass spectrometry ***(5)***, gas chromatography /mass spectrometry (GC/MS) ***(6, 7)***, nuclear magnetic resonance ***(8)***, Fourier transform ion cyclotron resonance mass spectrometry ***(9)*** and high performance liquid chromatography ***(10)***. Of these, we focus on GC/MS and LC/MS approaches which are widely available. Of these GC/MS is most common and is relatively inexpensive method, but requires sample derivatization to increase

volatility of analytes and has some limitations in the range of detectable metabolites. Another frequently-used method that is becoming useful for soil metabolomics is liquid chromatography/ mass spectrometry (LC/MS) ***(11)***. This technique yields thousands of chemical features that can be identified by comparison with authentic chemical standards relative to retention time, *m/z* values and fragmentation spectra. LC/MS also provides a path to identification of novel compounds, typically in conjunction with isolation and NMR structural analysis ***(12)***. GC/MS and LC/MS approaches can either be used individually or samples can be fractionated and analyzed using a complementary set of methods. For example, for this protocol, GC/MS is used to identify carbohydrates and small organic acids while LC/MS is used for the analysis of larger and more diverse sets of polar and non-polar metabolites.

In this chapter, we present a protocol to extract and analyze the small molecule composition of soil, either extracellular or total (intracellular + extracellular) (Figure 1). Within the protocol, there are options to extract either polar metabolites (*e.g.* amino acids, nucleobases, organic acids) or non-polar metabolites (*e.g.* secondary metabolites, antibiotics) followed by analysis using either LC/MS or GC/MS, depending on the reader's instrument accessibility and scientific goals. There are many LC column options that are used to separate different classes of molecules, but here we present two common and broad chromatographies: normal-phase (to detect polar metabolites) and reverse-phase (to detect non-polar metabolites). The following details are specific to the instruments and therefore it is recommended that the reader consult instrument manuals for method optimization.

2. Materials

2.1. Soil drying and sieving

Chemicals:

1. 70% ethanol

Equipment:

1. Labconco FreeZone 2.5 lyophilizer
2. 50 mL polypropylene conical tubes
3. -80°C freezer
4. 18-gauge needles
5. AS 200 sieve shaker, sieve (2 mm mesh), collection pan (Retsch)
6. balance
7. weigh boats and spatulas

2.2. Soil extraction (polar metabolites)

Chemicals

1. LC/MS-grade water (JT Baker), kept at 4°C

Equipment

1. serological pipettes (10 mL) and pipettor
2. Q125 QSonica probe sonicator
3. Orbital-Genie orbital shaker (Scientific Industries), kept at 4°C
4. Eppendorf 5810R centrifuge, kept at 4°C
5. 10 mL syringes
6. 32 mm 0.45 µm syringe filters (Pall Acrodisc Supor membrane)
7. 15 mL polypropylene conical tubes

8. -80°C freezer
9. 18-gauge needles
10. Labconco FreeZone 2.5 lyophilizer

2.3. Normal-phase LC/MS

Chemicals

1. HILIC internal standards solution: 4-(3,3-dimethyl-ureido)benzoic acid (DUBA) (Sigma-Aldrich), d4-lysine (Sigma-Aldrich), d5-Benzoic acid (Sigma-Aldrich), 9-anthracene carboxylic acid (ACA) (Sigma-Aldrich), 13C-15N-L-phenylalanine (Sigma-Aldrich) all at 5 ug/mL in LC/MS grade methanol
2. (*optional*) GC/MS internal standard solution: d_{27}-myristic acid at 50 µg/mL in LC/MS grade methanol
3. Mobile phase A: 5 mM ammonium acetate in water
4. Mobile phase B: 95% acetonitrile, 5% 100 mM ammonium acetate in water

Equipment

1. vortexer
2. 1.5 mL Eppendorf tubes
3. Eppendorf 5430R centrifuge
4. 0.22 µm centrifugal membranes (Ultrafree-MC-GV PVDF, Millipore)
5. 2 mL screw cap glass vials (Agilent)
6. 250 µL glass vial inserts with polymer feet (Agilent)
7. vial screw caps with septa (Agilent)
8. ZIC-HILIC column (100 mm x 2.1 mm, 3.5 um, 200 Å) (Millipore)
9. Agilent 1290 UHPLC
10. Thermo QExactive MS

2.4. GC/MS

Chemicals

1. LC/MS grade methanol
2. GC/MS internal standard solution: d_{27}-myristic acid at 50 µg/mL in LC/MS grade methanol
3. O-methoxyamine hydrochloride (Supelco)
4. pyridine
5. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (Restek)
6. Fatty acid methyl ester (FAME) marker solution: methylcaprylate (20 mg), methylperlargonate (20 mg), methylcaprate (20 mg), methyllaurate (20 mg), methylmyristate (20 mg), methylpalmitate (20 mg), methylstearate (10 mg), methyleicosanoate (10 mg), methyldocosanoate (10 mg), methyllignocerate (10 mg), methylhexacosanoate (10 mg), methyloctacosanoate (10 mg), methyltriacontanoate (10 mg), all in 25 mL chloroform

Equipment

1. vortexer
2. 1.5 mL Eppendorf tubes
3. Eppendorf 5430R centrifuge
4. 0.22 µm centrifugal membranes (Ultrafree-MC-GV PVDF, Millipore)
5. Savant SpeedVac SPD111 (Thermo Scientific)
6. glass serological pipette (1 mL) and pipettor
7. Eppendorf thermomixer

8. 2 mL screw cap glass vials (Agilent)
9. 250 µL glass vial inserts with polymer feet (Agilent)
10. vial screw caps with septa (Agilent)
11. Agilent 7890 GC equipped with a 15 m length x 0.25 mm ID Rtx-5MS column with a 10 m Integra-Guard column (Restek)
12. Gerstl Autosampler**********
13. Agilent 5977 single quadrupole MS

2.5. Non-polar metabolite extraction and reverse-phase LC/MS

Chemicals

1. non-polar extractant: 50% ethyl acetate, 50% water
2. C18 internal standard solution: 2-Amino-3-bromo-5-methylbenzoic acid (ABMBA) (Sigma-Aldrich) at 5 µg/mL in LC/MS grade methanol
3. Mobile phase C: 0.1% formic acid in water
4. Mobile phase D: 0.1% formic acid in acetonitrile

Equipment

1. glass serological pipettes (10 mL) and pipettor
2. Orbital-Genie orbital shaker (Scientific Industries), kept at 4°C
3. Eppendorf 5810R centrifuge, kept at 4°C
4. glass Pasteur pipettes
5. 15 mL polypropylene conical tubes
6. Savant SpeedVac SPD111 (Thermo Scientific)
7. vortexer
8. 1.5 mL Eppendorf tubes
9. Eppendorf 5430R centrifuge
10. 0.22 µm centrifugal membranes (Ultrafree-MC-GV PVDF, Millipore)
11. 2 mL screw cap glass vials (Agilent)
12. 250 uL glass vial inserts with polymer feet (Agilent)
13. vial screw caps with septa (Agilent)
14. C18 column: Zorbax Eclipse Plus C18 (50 mm x 2.1 mm, 1.8 um) (Agilent)
15. Agilent 1290 UHPLC
16. Thermo QExactive MS

3. Methods

The following protocol was established using a sandy loam soil. Appropriate adjustments may need to be made depending on the soil type and mineral content. It is highly recommended that the protocol be followed initially with a small set of pilot samples for example a mixture of target compounds to determine recovery prior to full-scale analysis. Extraction controls should also be included throughout the protocol (samples without soil) to account for extraction background. *Note that it is critical that researchers follow best practices as far as lab safety, it is beyond the scope of this protocol to describe safety protocols and researchers.*

3.1. Soil drying. To ensure comparable extraction efficiency from sample-to-sample, soil should be dried prior to extraction. Lyophilization is a preferred method over heating (*i.e.* speedvac) to limit metabolite degradation. It is recommended to measure soil weight before and after drying to determine the percent water weight of original sample (see **Note 1**).

1. Place each soil sample in a container suitable for lyophilization such as a 50 mL polypropylene conical tube.
2. Freeze at -80°C.

3. Poke holes in lids using 18-gauge needle and place samples in pre-cooled lyophilizer. Turn on vacuum according to instrument protocol.
4. Allow soil to dry completely. This process may take anywhere from a few hours to a few days, depending on the amount of sample and its water content.

3.2. Soil sieving. These steps may not need to be done for certain fine-textured soils (such as sand-based desert soils).

1. Prepare a 2 mm mesh sieve and a collection pan by spraying with 70% ethanol and wiping clean. Be sure everything is dry prior to the addition of soil samples.
2. Place soil in sieve, place over collection pan and sieve for approximately 2 minutes at medium vibration or until all the sample has passed through.
3. For each sample, place 2 g sieved soil (dry weight) in appropriately labeled 50 mL conical tube for extraction (see **Note 2**).

3.3. Polar Metabolite Extraction. These steps are for the extraction of mostly polar metabolites typically found in dissolved organic matter (*e.g.* amino acids, nucleobases, sugars). If the focus is on non-polar metabolites, follow steps in section **3.6** instead. Alternatively, at this time, soil samples can be divided into two fractions; one for polar metabolite extraction and one for non-polar metabolites.

1. Add 8 mL LC/MS-grade water to each (2 g) sample (see **Note 3**).
2. If intracellular metabolites are to be analyzed, sonicate samples using a probe sonicator at 50% amplitude for 2 x 30 seconds.
3. Place samples on a refrigerated orbital shaker and shake at 200 rpm for 1 hr (angle the tube rack approximately 45 degrees to allow for adequate sample movement and extraction).
4. Centrifuge samples at 3220 x g for 15 min (at 4°C).
5. Decant supernatant into a 10 mL syringe fitted to a 0.45 µm (32 mm) filter disc and filter into 15 mL conical tubes (see **Note 4**).
6. Freeze extracts at -80°C.
7. Poke holes in lids using 18-gauge needle and lyophilize extracts for 6-8 hrs or until dry.
8. Once samples are dry, proceed with resuspension according to section **3.4** for LC/MS or **3.5** for GC/MS. To use both LC/MS and GC/MS, follow section **3.4** until noted.

3.4. Polar metabolite LC/MS sample preparation and analysis. An Agilent 1290 UHPLC and Thermo QExactive MS were used in this protocol. Parameters and methods will need to be adjusted and optimized according to the specific instruments. Here, polar metabolites are separated using normal-phase chromatography and detected by MS.

1. Resuspend dried water extracts in 200 µL of HILIC internal standards solution (see **Notes 5 and 6**), making sure to recover sample that may have adhered to the sides of the vial during lyophilization. Vortex samples to get extracts into solution (see **Note 7**).
2. Transfer samples to 1.5 mL tubes.
3. Centrifuge samples 5000 x g for 5 min to pellet insoluble salts.
4. Add samples to centrifugal membranes and filter by centrifuging at 5000 x g for 3 min.
5. At this point, **if performing both LC/MS and GC/MS**, remove an aliquot (*e.g.* 100 µL) for GC/MS and place into a new 1.5 mL tube. Add 100 µL of GC/MS internal standard solution. Proceed to section **3.5.5**.
6. Transfer samples to glass LC/MS vials with glass inserts, capping immediately.
7. For normal phase chromatography, use a ZIC-HILIC column with the following LC parameters: autosampler temperature: 4°C; sample injection volume: 2 µL; column compartment temperature: 40°C left / right.

8. Chromatographic separation of polar metabolites can be achieved by the gradient shown in Table 1 using mobile phases A and B.
9. For mass spectra, collect data in both negative and positive polarities with a mass range of 70-1050 *m/z* in centroid data mode. Acquire MS/MS fragmentation spectra using collision energies of 10-40 eV.
10. It is recommended that a known mixture of quality control standards be injected approximately every 10 samples to check for consistent instrument performance (*m/z* accuracy and retention time shifting) throughout the sequence. Methanol blanks should also be included frequently to check for metabolite carryover.
11. Data analysis can be done with the reader's software choice (*e.g.* MZmine) and may be targeted or untargeted.

3.5. Polar metabolite GC/MS sample preparation and analysis. For GC/MS, there are many derivatization procedures to detect different classes of metabolites, but here we report the protocol for a standard MSTFA derivatization to detect a broad range of metabolites.

1. Resuspend dried water extracts in 200 µL of methanol (see **Note 5**). Add 100 µL of GC/MS internal standard solution (see **Note 6**). Be sure to recover sample that may have adhered to the sides of the vial during lyophilization. Vortex samples to get extracts into solution (see **Note 7**).
2. Transfer samples to 1.5 mL tubes.
3. Centrifuge samples 5000 x g for 5 min to pellet insoluble salts.
4. Add samples to centrifugal membranes and filter by centrifuging at 5000 *x g* for 3 min.
5. Dry samples using a speedvac for about 30 min (see **Note 8)**.
6. Prepare a 40 mg/mL methoxamine solution in pyridine (using a glass pipette) and dissolve by shaking at 60°C for 15 on a thermomixer. Be sure to make an adequate amount (10 µL is needed per sample).
7. Add 10 µL methoxamine solution to each dried sample.
8. Shake samples in thermomixer at maximum speed at 30°C for 1.5 hours.
9. In the meantime, FAME markers can be prepared by adding 20 µL FAME marker solution to each bottle (1 mL) of MSTFA (see **Note 9**). FAMEs are included with samples to track GC column stability and for use as a retention time index.
10. Add 90 µl MSTFA/FAME standard mix to each sample, capping immediately.
11. Shake in thermomixer at maximum speed at 37°C for 30 minutes.
12. Centrifuge at 5000 x g for 1 min to pellet any undissolved material.
13. Transfer liquid contents to glass GC/MS vials with glass inserts, capping immediately.
14. For this protocol and specific GC/MS, the following GC gradient is used: 50°C initial oven temperature, ramp to 65°C at 5°C/min, held for 0.2 min, ramp to 80°C at 15°C/min, held for 0.2 min, ramp to 310°C at 15°C/min, held for 12 min.
15. MS parameters: transfer line: 250°C, electron ionization: 70 eV, ion source: 230°C, MS range from 50-700 *m/z* at an acquisition rate of 8 spectra/ sec.
16. As with LC/MS, it is recommended to include quality control standards throughout the run to ensure instrument consistency. Data analysis can be

done with the reader's software choice. An example of GC/MS-detected soil metabolites are shown in Figure 2.

3.6. Non-polar metabolite extraction and LC/MS sample preparation and analysis. These steps can be followed to extract non-polar metabolites such as sterols, fatty acids, lipids, secondary metabolites, *etc*. The same LC/MS system as indicated above is used for the detection of non-polar metabolites. However, reverse-phase LC is performed for the separation of non-polar metabolites.

1. Add 8 mL non-polar extractant to each (2 g) sample using glass serological pipette.
2. Place samples on a refrigerated orbital shaker and shake at 200 rpm for 1 hr (angle the tube rack approximately 45 degrees to allow for adequate sample movement and extraction).
3. Centrifuge samples at 3220 x g for 15 min (at 4°C).
4. Using a glass Pasteur pipette, remove the upper ethyl acetate layer and place into 15 mL conical tubes.
5. The ethyl acetate layer containing non-polar metabolites can be dried using a speedvac for approximately 1 hr (time will depend on the exact instrument).
6. Resuspend dried ethyl acetate extracts in 200 uL of C18 internal standard solution (see **Notes 5-7**).
7. Transfer samples to 1.5 mL tubes.
8. Centrifuge samples 5000 x g for 5 min to pellet insoluble salts.
9. Add samples to centrifugal membranes and filter by centrifuging at 5000 x g for 3 min.
10. Transfer samples to glass LC/MS vials with glass inserts, capping immediately.
11. For non-polar metabolites, use a C18 column with the same LC parameters as indicated in **3.4.7.**
12. Chromatographic separation of non-polar metabolites can be achieved by setting the LC to a flow rate of 0.4 mL/min and using the gradient shown in table 2 with mobile phases C and D.
13. For mass spectra, collect data in both negative and positive polarities with a mass range of 70-2050 m/z in centroid data mode. Acquire MS/MS fragmentation spectra using collision energies of 10-40 eV.

Notes

1. In order to determine the amount of soil to begin with, the reader will have to determine or approximate dry weight. This protocol uses 2 g of dry soil for each sample. For example, if the soil is expected to be 50% water weight, start with at least 4 g wet soil (although, if possible, it is recommended to start with more to account for sample loss during sieving and sample transfers).
2. If sample quantity is limited, the protocol can be scaled down. The extractant should be adjusted accordingly to maintain a 1:4 soil:extractant ratio. For example, for 0.5 g soil, use 2 mL extractant.
3. Throughout the extraction procedure, keep extractants and samples as cool as possible to limit continued microbial activity.
4. Filters can clog easily with some soils. In this case, the supernatant can be decanted into a 15 mL tube and re-centrifuged at a higher speed followed by filtration. Additionally, a pre-filter with a larger pore size (*e.g.* 3 µm) may be necessary**.**
5. Resuspension volume may vary depending on the type of soil (*i.e.* rich soils may require a larger volume) and instrument sensitivity. A good starting point is 200-500 µL. Instrument injection volumes can also be modified to adjust analyte signal intensity.

6. Internal standard composition can be modified depending on instrument type and sensitivity. Typically, stable isotopes and/or xenobiotic compounds are used.
7. Some soil extracts are difficult to get into solution and may require a bath sonicator (for 5 min) or extensive vortexing.
8. All moisture must be removed from samples. Any water present will interfere with derivatization reactions.
9. MSTFA is extremely volatile. The addition of FAMEs must be performed in a hood and done quickly to limit loss.

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Figure Captions
Figure 1. Workflow of soil metabolite extraction and analysis.
Figure 2. Soil metabolites detected GC/MS

Tables

Time (min)	A (%)	B (%)	flow (mL/min)
0.0	0	100	0.45
1.5	0	100	0.45
15.0	35	65	0.45
18.0	100	0	0.60
23.0	100	0	0.60
25.0	0	100	0.45
29.0	0	100	0.45
30.0	0	100	0.45

Table 1. Recommended LC gradient for normal-phase (HILIC) chromatography.

Time (min)	A (%)	B (%)
0.0	100	0
1.0	100	0
8.0	0	100
9.5	0	100
10.5	100	0
12.0	100	0
29.0	0	100
30.0	0	100

Table 2. Recommended LC gradient for reverse-phase (C18) chromatography.