RSCPublishing Faraday Discussions

Online supercritical fluid extraction mass spectrometry (SFE-LC-FTMS) for sensitive characterization of soil organic matter

Journal:	*Faraday Discussions*
Manuscript ID	FD-ART-01-2019-000011.R1
Article Type:	Paper
Date Submitted by the Author:	14-Mar-2019
Complete List of Authors:	Shen, Yufeng; Pacific Northwest National Laboratory, Richland, WA 99352, USA, Biological Sciences Division; CoAnn Technologies Zhao, Rui; Pacific Northwest National Laboratory, Environmental Molecular Sciences Laboratory Tolic, Nikola; Environmental Molecular Sciences Laboratory Tfaily, Malak; University of Arizona School of Plant Sciences; Environmental Molecular Sciences Laboratory Robinson, Errol; Pacific Northwest National Laboratory, Environmental Molecular Sciences Laboratory Boiteau, Rene; Oregon State University; Pacific Northwest National Laboratory Pasa-Tolic, ljiljana; Pacific Northwest National Laboratory, EMSL Hess, Nancy; Pacific Northwest National Laboratory, EMSL

Online supercritical fluid extraction mass spectrometry (SFE-LC-FTMS) for sensitive characterization of soil organic matter

Yufeng Shen, [1,3] Rui Zhao, [2] Nikola Tolić, [2] Malak M. Tfaily, [2,4] Errol W. Robinson, [1] Rene Boiteau, [2,5] Ljiljana Paša-Tolić, [2*] and Nancy J. Hess [2*]

[1]Biological Sciences Division, and [2]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99354, United States

[3]CoAnn Technologies, Richland, WA 99354, United States

[4]Department of Soil, Water and Environmental Science, University of Arizona, Tucson, AZ 85721-0038, United States

[5]College of Earth, Ocean and Atmospheric Sciences, Oregon State University, Corvallis, OR 97331, United States

KEYWORDS: supercritical fluid extraction (SFE), liquid chromatography (LC), electrospray ionization (ESI), Fourier transform mass spectrometry (FTMS), Fourier transform ion cyclotron resonance (FTICR), soil organic matter (SOM)

ABSTRACT: We report a novel technical approach for subcritical fluid extraction (SFE) for organic matter characterization in complex matrices such as soil. The custom platform combines on-line SFE with micro-solid phase extraction, nano liquid chromatography (LC), electrospray ionization and Fourier transform mass spectrometry (SFE-LC-FTMS). We demonstrated the utility of SFE-LC-FTMS, including results from both Orbitrap and FTICR MS, for analysis of complex mixtures of organic compounds in solid matrix by characterizing soil organic matter in peat, a high-carbon soil. For example, in a single experiment, >6,000 molecular formulas can be assigned based upon FTICR MS data from 1-50 µL of soil samples (roughly 1-50 mg of soil, dependent on soil density), nearly twice that typically obtained from direct infusion liquid solvent extraction (LSE) from an order of magnitude larger volume of the same soil. Detected species consisted predominately of lipid-like, lignin-like and protein-like compounds based on their O/C and H/C ratios with predominantly CHO and CHONP molecular composition. These results clearly demonstrate that SFE has the potential to effectively extract a variety of molecular species and could become an important member of a suite of extraction methods for studying SOM and other natural organic matter. This is especially true when comprehensive coverage, minimal sample volumes, and high sensitivity are required or when the presence of organic solvent residue in residual soil is problematic. SFE based extraction protocol could potentially enable spatially resolved characterization of organic matter in soil with resolution of ~1 mm^3 to facilitate studies probing spatial heterogeneity of soil.

Introduction

High resolution mass spectrometry (MS) is becoming a preferred method for characterizing organic molecules in complex matrices such as dissolved or soil organic matter (D/SOM) or petroleum. [1-6] Recent applications of this approach have shed new light on the molecular composition of natural organic matter (NOM) and closely coupled microbial community composition and function, which both change in response to a wide variety of environmental factors including surface adsorption, photochemisty, redox conditions, etc. [7-14] Such studies provide mechanistic insight into the source, reactivity, and degradation pathways of the carbon stocks on our planet and will ultimately inform and advance models of land-atmosphere interactions at larger scales.

One of the grand challenges in this field is improving comparisons of organic matter samples (e.g. across ecosystems). This requires development of robust analytical procedures that capture a comprehensive array of organic analytes while also minimizing effects of the sample matrix on analytical results. Characterizing SOM by mass spectrometry (MS) generally necessitates extraction of the organic molecules from the rock or soil matrix prior to analysis. This is typically accomplished using liquid solvents, such as water, inorganic acids/bases, organic solvents, and their combinations. [15] Recent work from our group and others demonstrated the effectiveness of the liquid solvent extraction (LSE) for isolating SOM from a variety of ecosystems. However, the polarity of the solvent has a strong bias towards extracting organic molecules with similar chemistry. [16,17] As a result, overall organic matter extraction efficiencies are highly dependent on matrix composition and typically low, which hampers comparisons between samples exhibiting different chemical compositions. Additionally, different ionization techniques –electrospray ionization (ESI), atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI), or laser desorption ionization (LDI)- will preferentially ionize a specific classes of molecules and further bias analytical results. It has been generally recognized and acknowledged that multiple approaches are required for robust assessment of natural organic matter composition. However, limited resources, including the amount of material available, usually result in partial characterization, most often using LSE followed by ESI coupled to ultra-high performance Fourier transform (FT) MS, such as Orbitrap or Fourier transform ion cyclotron resonance (FTICR) MS.

Expanding the arsenal of tools available for mass spectrometric natural organic matter characterization, herein we investigate the use of subcritical fluid extraction (SFE) system to further explore the chemical selectivity for specific classes of SOM molecules and improve the yield from small volumes of soil to allow for more sensitive measurement needed for e.g. characterization of soil spatial heterogeneity. SFE has been widely explored as an environmentally friendly and selective technique to obtain valuable analytes from many different matrices primarily in large-scale industrial applications. [18,19] More recently, SFE has been applied as an unconventional sample preparation technique for processing polymers, pharmaceuticals, and specialty chemicals with reduced consumption of organic solvents and increased yields. [20,21] Supercritical fluids (SCFs) have ten to a hundred times faster molecular diffusion rates than liquid solvents and lower surface tension. [22] While many different solvents have been employed as SCFs, CO_2 is the most commonly used because it is chemically stable, safe, cheap and easily available, as well as removable simply by relieving pressure. CO_2 also does not leave chemical residues in either the final extraction products or in the sample matrix. This results in a solvent-free, pure, concentrated, and intact extract allowing for improved analysis recovery and sensitivity compared to conventional LSE. One of major disadvantages though is limited ability to extract polar analytes. Previous work has demonstrated that altering SCF operation pressure and/or temperature or modifying SCF composition with the addition of polar solvents (e.g. ethanol, methanol, ethyl lactate, etc.) can modulate SFE selectivity to enable sequential SFE extraction of compounds with selected molecular properties. [18] [22]

Current SFE analytical practices are limited to extracting and analyzing simple, targeted (mixtures of) compounds. Due to its unique physical properties, SCFs exhibit enhanced penetration into porous soil matrices to extract contaminants and thus potentially achieve high extraction efficiencies (relative to LSE). SFE has been widely investigated to remediate environmental matrixes of organic contaminants such as polycyclic aromatic hydrocarbons. [23] However, the utility of SFE for comprehensive molecular characterization of intact organic compounds in soils has yet to be demonstrated, especially when coupled with advanced MS methods such as FTMS.

Since SFE provides clean extracts, it is often directly compatible with downstream analytics. For instance, coupling SFE with gas chromatography (GC) or supercritical fluid chromatography (SFC) can be rather simple due to the compatibility of the chromatographic mobile phases employed. On the contrary, mismatch between the SFE, which produces a large volume of gas, and gas-free liquid-phase ESI-FTMS represents a significant technical hurdle. This difficulty can be resolved using a liquid or solid phase trap for deposition of the extract following SC-CO_2 decompression. To couple SFE to ESI-FTMS for molecular level SOM characterization, we have implemented a solid phase extraction (SPE) trap column.[24] In this way, SFE extracts are trapped on the column and the SCFs are displaced with aqueous solvents for introduction into the mass spectrometer by ESI. [25]

We have further combined these techniques with online high-performance liquid chromatography (HPLC). An online coupling of SFE to LC-FTMS minimizes sample losses and maximizes coverage and sensitivity. This advancement enables the analysis of smaller sample volumes with fully automated operations compared with offline implementation. Automated online SFE-microSPE-nanoLC-FTMS platform enabled characterization of the many thousands of analytes present in soil. We observed an unprecedented diversity of analytes – more than 24,000 LCMS features (i.e., analytes)- from only a few milligrams of soil. This diversity of analytes indicates the wide compositional range of molecules that are observable by SFE-LCMS for the deep characterization of SOM. Further, the data showed that SFE has its own chemical selectivity for extracting specific SOM components that is complementary to a suite of LSE using a range of polar to non-polar solvents. [16,26] These results clearly demonstrate the potential of SFE to expand the chemical characterization of SOM and also assess the composition of much smaller soil samples that are attainable by conventional LSE approaches.

Materials and methods

Chemicals and soil sample manipulations.

All chemicals and H_2O used in the experiments were HPLC-grade materials from Fisher Scientific (Hanover Park, IL). The soil samples analyzed in this study consisted of peat containing 51% carbon by weight, collected from Northern Minnesota at a depth of 75cm. We also used low-C sandy-silt soil collected from a wetland at the Savannah River Site (SRS) in South Carolina. It contained 4% carbon by weight. In all cases, soils were dried and ground to a fine powder prior to analysis.

Design and operation of automated online SFE-LCMS system.

The system constructed for this study is illustrated in Figure 1. The SFE-grade CO_2 (Praxair, Danbury, CT) was placed into a 100 mL high-pressure syringe pump with a pressure limit of

10K psi (ISCO 100DX, Lincoln, NE). It was maintained at 4 ºC by using a recirculating cooler (Pharmacia Biotech, Manasquan, NJ). After filling, the system was closed with an on/off valve (VICI, Huston, TX) and detached from the CO_2 cylinder for convenient SFE coupling to different mass spectrometers. Each SFE consumed only about 0.3 mL liquid CO_2. Hence, a single filing of the syringe pump is enough for hundreds of SFE experiments. The CO_2 was purified using a 100 × 4.6 mm ID LC column packed with 3 µm porous graphitic carbon (Thermo Scientific, Waltham, MA) and a 200 × 4.6 mm ID LC column containing 5 µm C18-bonded silica particles (Phenomenex, Terrence, CA) prior to extraction.

SC-CO_2 flow was controlled with an automatic four-port switch valve (V1, VICI). We tested several different volumes of extraction vessels ranging from 1 to 50 µL. Unless otherwise indicated, the results presented here were derived using the 50 µL stainless steel extraction vessel specially manufactured by VICI. The dried and ground soils were transferred into the extraction vessel and placed in an oven (Quincy Lab, Chicago, IL) at 31 ºC for the duration of the extraction procedure (1 hour). The extraction products were then transferred the to an in-house manufactured microSPE fused silica capillary column (20 cm × 150 µm ID) containing 5 µm C18 particles (Phenomenex) through a 50 cm × 50 µm ID fused silica capillary tube with a volume of ~1 µL. The microSPE was connected to an in-house made nanoLC column (50 cm × 75 µm ID fused capillary column packed with 3 µm Phenomenex C18) with the "backflush" mode through a six-port valve (V2, VICI). [27] Unless otherwise indicated, SFE was performed at 3000 psi and 31 ºC for 1 hour, with a liquid CO2 flow of 4-6 µL/min as indicated by the ISCO pump controller.

After extraction, the microSPE was washed with LC mobile phase A at 5 µL/min for 10 min to remove residual SCF bubbles and then switched to the nanoLC column for gradient separation of the extracts (controlled by a 4-port valve V3, VICI). Acidic mobile phases (H_2O for A and ACN for B with 0.1% formic acid, v/v) and neutral mobile phases (H_2O for A and ACN for B with 0.1% ammonia acetate, v/v) were applied for negative mode ESI-FTMS. The LC platform previously reported [28] was used for nanoLC separation with gradient from A to 60% B in 120 min with a flow rate of 300 nL/min. The nanoLC column outlet was fitted with an emitter (3 cm × 20 µm ID fused silica tube with etched tip) for ESI to either of two different mass spectrometers. All packed capillary columns used for both microSPE and nanoLC were manufactured in-house using methods previously reported. [28]

Mass spectrometry experiments.

We used two MS platforms for this study: a Thermo Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA) and 12 Tesla Bruker solariX Fourier transform ion cyclotron resonance (FTICR) MS (Billerica, MA). The Exactive Orbitrap mass spectrometer was used for evaluating SFE performance under following operating conditions: -2.3 kV for ESI, $100<m/z<2000$ range at a resolution of 100,000 (at $m/z = 400$), an AGC of 3×10^6, and a single microscan for data acquisition. 12T FTICR operated with an ESI voltage of -4.4 kV and 150 low *m*/*z* cutoff for Q1. The calibration was performed as recommended by the vendor. Mass measurement accuracy <1 ppm was obtained for singly charged ions within $200<m/z<1200$ range.

Data analysis.

For FTICR datasets, we used an in-house developed Visual Basic for Applications (VBA) script utilizing the Bruker Data Analysis automation engine to average 5 minute non-overlapping LCMS segments and compile a list of peaks using “FTMS” peak picker with the signal-to-noise parameter set to 7. Peaks from 20 averaged spectra were internally calibrated using linear regression function. The list of calibration peaks included fatty acids and humic acid homologous series over $200<m/z<800$ range. We submitted peaks from spectra to the Compound Identification Algorithm (CIA) developed by Kujawinski and Behn [29]. Mass measurement accuracy of 1 ppm and a formula propagation with CH_2, H_2 and O building blocks was used for assigning the formula. In the case of ambiguous assignments, the formula with lower count of heteroatoms (N+S+P) was reported or in the case when heteroatom count was the same the formula with lower mass error.

For Exactive datasets, in-house developed software (Decon-Tools and VIPER [30]) were used to pick peaks and to average m/z measurements over chromatographic peaks. We internally calibrated the resulting list of m/z values using the same calibrantion peaks with initial mass tolerance of 5 ppm. We submitted the calibrated peaks to the CIA for formula assignment with peak mass tolerances of 2 ppm and a formula propagation with CH_2, H_2 and O building blocks.

Results

Performance of the online SFE-LC-FTMS for characterization of SOM from a low-C soil.

The performance of the SFE-LC-FTMS system was optimized on an Exactive Orbitrap mass spectrometer. The microSPE interface used to couple SFE and nanoLCMS produced stable ESI, as evidenced by the smooth chromatogram baselines shown in Figure 2. Additionally, we visually confirmed a stable ESI spray using the microscope on the spectrometer. The background intensity of the system blank was on a 10^5 level (Xcalibur Software). In comparison, the intensities of chromatographic peaks observed for 1 mg of the SRS soil was two orders of magnitude higher. Increasing sample size from 1 to 20 mg resulted in more chromatographic peaks, but further increases in sample size up to 35 mg did not lead to significant changes in the chromatogram. Both the number of LCMS features detected and the molecular formulas assigned followed the same sample trends as chromatograms (Figure S1, Supporting Information).

Molecular formulas were assigned to the analytes extracted by SFE using accurate masses (within 2 ppm mass error achievable by Exactive Orbitrap) as summarized in Figure 3. Similar composition distribution was obtained for the SRS soil samples ranging in size from 1 to 35 mg with maximum number of formula assignments (1,618) attained using 20 mg of soil. Approximately 50% of the LCMS features remained unassigned, regardless of the size of the sample. CHO compounds comprised ~ 20% of the total LCMS features detected; CHON and CHOS comprised ~10 each%; and CHOP, CHNOP and CHONS and CHOSP compounds constituting the majority of the remaining 15% of the LCMS features detected.

508 MS peaks were detected in the system blank and 266 of these peaks, or approximately 52%, could be assigned molecular formula. The compounds assigned were limited to several alkyl elongations, according to their O/C and H/C ratios. [31] In contrast, the smallest soil sample assessed (1 mg) yielded 820 formula assignments and only 157 were also found in the system blank, indicating that system background is relatively low.

The extraction recovery of the SCFE system was investigated for the 20 mg sample described above by repeated extractions of residual soil (each lasting one hour). The intensities of chromatographic peaks were reduced by 75% after the second extraction and by greater than 99% after the third extraction as shown in Supporting Information Figure S2. It is important to note that such recovery cannot be attributed to the total C compounds present due to the chemical selectivity of SFE-CO_2 (see results below). Increasing extraction time may improve the extraction recovery for SFE-CO_2 extractable species, but potentially results in some extracts washing off of the microSPE column during the longer collection time.

Performance of the online SFE-LC-FTMS for characterization of SOM from high-C soil.

Findings from the Exactive Orbitrap study (described above) guided FTICR experiments. A 12T FTICR mass spectrometer was used for detection of SOM extracted by SFE from peat soil. We have previously reported extensive characterization of the same peat soil using LSE with multiple solvents by direct infusion ESI on the same 12T FTICR mass spectrometer. [16] This study serves as a reference for evaluating novel analytical methodology introduced herein.

In a single SCFE-LC-FTICR MS analysis using 8 mg (50 µL) of peat soil we have detected 24,476 LCMS features (including ^{13}C and other minor isotopologues), of which 6,414 distinct formulas were assigned as shown in Figure 4 and Table S1, Supporting Information. This compares favorably to the 5,800 total formulas that were assigned for the same sample on the same mass spectrometer using LSE separately with four solvents MeOH, ACN, H_2O and hexane. [16]

Identifying more features by an LCMS-based method compared to direct infusion ESI method is expected because the separation reduces ion suppression effects. However, note that we obtained such high coverage of SOM using nearly an order of magnitude smaller soil sample (e.g., 50 µL or 8 mg for SFE versus 100 mg for LSE). This demonstrates the high sensitivity of the new SFE based extraction protocol potentially facilitating probing spatial heterogeneity of soil with spatial resolution of ~1 mm^3. Additionally, though more molecular formulas were assigned, a significant fraction (>60%) of LCMS features detected from the SFE extracts remained unassigned even with the high mass accuracy (<1 ppm) provided by the 12T FTICR MS. This indicates that perhaps compounds extracted by SFE are not included in existing chemical databases used for formula assignment or include elements not considered in common formula assignment algorithms.

We found that CHO compounds were the most represented category assigned for peat soil using SFE (66% of all assignments), followed by CHONP compounds (10% of all assignments), with 6,414 formulas assigned in total (Figure 4, Table S1). The van Krevelen diagram [32,33] shows that most formulas assigned have O/C <0.5 and H/C ratios between 0.7 and 2.2 (Figure 5). Note that 3,431 formulas (54% of all assignments) are lipid-like compounds according to their O/C and H/C ratios as defined by Sleighter and Hatcher. [34] (See the overlay in Figure 5.) This percentage of lipid-like compounds is similar to what was previously obtained with LSE using hexane [16]. Coverage for protein-like compounds (962 formulas, or 15% of all assignments) was higher than obtained by LSE with any solvent except hexane, which suggests enhanced SFE selectivity for this category of compounds. The representation of lignins (~15%) was significantly higher than for LSE-hexane, but lower then LSE-water, and similar to LSE-ACN or LSE-MeOH. However, condensed hydrocarbons, tannins and amino sugars were less represented in SFE extracts in terms of the number of formulas assigned, compared to LSE using ACN, H_2O and MeOH. [16] The peat datasets reported for LSE using ACN, H_2O and MeOH (total 5,800 formulas assignments) [16] were pooled for the complementary role of various extraction methods. In terms of assigned formulas, SFE had the largest overlap with LSE with hexane and the least overlap with LSE with water, suggesting that water might be the most effective modifier to SFE (SC-CO_2) to increase the diversity of SOM extractable from soil.

The normalized mass distribution of the molecules identified by the two techniques shows that while distribution of MS peaks is quite similar for the two techniques, more molecules are detected by the SFE in the $700<m/z<900$ range whereas LSE detects more species with $m/z <300$ (see Supporting Information, Figure S3). These observations support the chemical selectivity suggested by Figure 4, namely the SFE appears to be more selective for lipid-like, lignin-like and protein-like compounds than LSE (with results for all solvents combined) or that the chromatography improved the detection of less polar ions that were suppressed with direct infusion. Although not tested in this study, the higher proportion of lipid-like, lignin-like and protein-like compounds in SFE may also suggest that these compounds reside in pore spaces only accessible using SFE.

Discussion

The SFE-based MS characterization of natural organic matter demonstrates a significantly richer composition than attained previously using LSE-based approach, highlighting the utility of this method for obtaining improved coverage of soil organic matter compound classes from smaller sample sizes. The greater depth of coverage compared to conventional LSE can be attributed to (1) the greater extraction efficiency of organic matter using SFE and (2) reduction of ion suppression due to incorporation liquid chromatography separation step.

The high performance of the online SFE-microSPE-nanoLCMS was only achieved by considering several design and technical factors discussed here. First, the high purity of the SCF applied as extraction solvent was essential for achieving optimal performance. The contaminants in SFE-grade CO_2 can be enriched by the online microSPE, which can result in high MS background. Purifying SFE-grade CO_2 using graphitic carbon LC column prior to soil extraction considerably reduced the background (Figure 2). We also tested porous silica and silica-bonded C18 packed columns for purification of SFE-grade CO_2, but significant background was still observed (Figure S2). Second, the dimension of the microSPE affected the sample capacity for SFE and subsequent LC performance. We selected a 20 cm × 150 µm ID (i.e., 3.5 µL) column for microSPE component of the system as the most effective for handling small (≤ 20 mg) soil samples (in a 50 µL SFE vessel) (Figure 2, Figure S1). Increasing the microSPE column inner diameter was found to diminish coupled nanoLC efficiency, whereas lengthening the microSPE column could increase wash time needed to remove gas bubbles left by SFE, which is critical for effective ESI-MS, thus decreasing the overall throughput.

We incorporated the nanoLC in the system to minimize ion suppression during ESI and maximize sensitivity and coverage. Chromatography reduces ion suppression by separating species in time, such that less heterogeneous mixtures are delivered to the analyzer allowing for lower abundance compounds or compounds that are difficult to ionize are more easily detected thanks to the simplified matrix and less competition for charge. Furthermore, chromatography can separate species with very similar masses that may not be fully resolved even by the 12T FTICR MS. These benefits may explain why a greater proportion of analytes were detected by SFE based LCMS compared to LSE using several solvents with subsequent direct infusion MS. Increased resolving power mass measurement accuracy as well as faster data acquisition, such as those achievable at 21T, [1,35] would certainly further improve the detection of species within complex soil organic matter regardless of the extraction method used.

Our comparison of compound classes of SOM extracted by SFE and LSE methods on high C peat soil based on O/C and H/C ratios indicates that SFE-LCMS has the most similarity to the nonpolar hexane solvent showing selectivity for lipid-like and protein-like compounds (Table S1). The addition of H_2O or MeOH as co-solvent to the online SFE method could potentially allow tuning of the selectivity for other classes of SOM. The representation of lignin-like compounds appear to be significantly higher for the SFE than the LSE regardless of the solvent used. (Table S1) We also note that SFE is selective for CHNOP chemical compounds that are similarly represented in by the LSE-H_2O analyses but does not appear to extract CHOP or CHNOSP compounds. Since formula assignment algorithms should be independent of the extraction techniques we believe these results suggest a difference in extraction chemistry. It is likely that the observed increased coverage results in part from the known benefit of LC to decrease charge suppression compared to direct infusion ESI. However one can speculate that the ability of SC-CO_2 to penetrate into small pore spaces of the peat soil is likely to play a role in improved extraction efficiency. The resulting high sensitivity and comprehensive chemical characterization on small sample volumes could suggest new applications in spatially resolved characterization of organic molecules contained in complex matrices such as soil with ~1 mm^3 resolution. SOM exhibits tremendous heterogeneity not only in terms of its composition, but also in terms of its distribution within the soil. [36] Spatially resolved molecular characterization of organic matter in soil is critical for identifying biologically active sites, so-called hotspots. By enabling analysis of small volume of soil, SFE-LC-FTMS provides a means to get insights into localization and distribution of organic matter within the soil mineral matrix (e.g., within soil aggregates or on mineral surfaces). Recent report on direct analysis of small amounts (500 μg) of unprocessed soil sample using laser desorption ionization coupled with ultrahigh MS provides an attractive alternative approach for assessment of soil heterogeneity. [37]

Lastly, the results presented herein were obtained employing low temperature (31 ºC) and neat SC-CO_2 for extraction of intact SOM. However, the SFE-LCMS system described herein is designed for operation at higher temperatures (up to 150 ºC) and with modifiers such as water, methanol or isopropyl alcohol. These modifiers could extend SFE extraction coverage and modify chemical selectivity. Online coupling with HPLC enables additional experimental control through modification of LC mobile phases such as pH or solvent composition.

5. Conclusions

Online SFE-microSPE-nanoLC-FTMS is a sensitive approach that enables remarkable sensitivity in the molecular characterization of SOM and yields the assignment of >6,000 molecular formulas from as little as a few milligrams of soil. The sensitivity provided by the online SFE system enables analyses of limited samples and investigations into the spatial heterogeneity of soil on the cubic-millimeter scale. The selectivity of SFE with neat CO_2 for extraction of SOM is similar to LSE with hexane and provides rich information for lipid-like, lignin-like and protein-like compounds based on their O/C and H/C ratios. SFE of SOM tannins, cellulose and amino sugars potentially requires addition of polar solvents such as water as modifiers. These results suggest SFE could have an important role as an efficient extraction method for characterization of SOM potentially as a complementary method or re-extraction of previously LSE-extracted samples to enrich datasets of SOM currently acquired using LSE methods. To our knowledge, this is the first report of the use of SFE, a widely recognized green sample preparation technique, for organic matter characterization in complex matrices such as soil.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

ACKNOWLEDGMENTS

We acknowledge support by the U.S. Department of Energy (DOE) Office of Science and its Office of Biological and Environmental Research (BER). We thank Dr. Daniel Kaplan for providing soil samples from SRS, South Carolina. MMT thanks the BER Terrestrial Ecosystem Science program for support. This research was performed using EMSL, a DOE Office of Science User Facility sponsored by BER and located at Pacific Northwest National Laboratory (PNNL). PNNL is operated by Battelle for DOE.

FIGURE CAPTIONS

Figure 1. Schematic of the online SFE-micro-SPE-nanoLC-ESI-FTMS system implemented for MS characterization of a complex mixture of organic compounds from solid matrices. The soil samples were placed in the extraction vessel.

Figure 2. Optimization of the SFE-micro-SPE-nanoLC-ESI-FTMS system for characterization of SOM from low C soil employed a Thermo Exactive Orbitrap. NanoLC-ESI-FTMS base peak chromatograms of SOM extracted by SFE from 1-35 mg of low C soil from SRS.

Figure 3. Compositional distribution of the assigned formulas from SFE-micro-SPE-nanoLC-ESI-FTMS of SOM from SRS soil of different sample sizes.

Figure 4. Performance of the SFE-micro-SPE-nanoLC-ESI-FTMS system for characterization of SOM from high C peat soil. 8 mg of peat soil was used for SFE-CO_2 with ammonia acetate buffers as nanoLC mobile phases. a: Composition distribution; and b: compound classes of formulas assigned using FTICR mass spectra.

Figure 5. A van Krevelen diagram showing the distribution of CHO, CHON, CHOS and CHONP compounds detected by SFE-micro-SPE-nanoLC-ESI-FTMS system in high-C peat soil.

REFERENCES

(1) Shaw, J. B.; Lin, T. Y.; Leach, F. E., 3rd; Tolmachev, A. V.; Tolic, N.; Robinson, E. W.; Koppenaal, D. W.; Pasa-Tolic, L. 21 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer Greatly Expands Mass Spectrometry Toolbox. *Journal of the American Society for Mass Spectrometry* **2016,** *27* (12), 1929.

(2) Smith, D. F.; Podgorski, D. C.; Rodgers, R. P.; Blakney, G. T.; Hendrickson, C. L. 21 Tesla FT-ICR Mass Spectrometer for Ultrahigh-Resolution Analysis of Complex Organic Mixtures. *Analytical chemistry* **2018,** *90* (3), 2041.

(3) Hawkes, J. A.; Dittmar, T.; Patriarca, C.; Tranvik, L.; Bergquist, J. Evaluation of the Orbitrap Mass Spectrometer for the Molecular Fingerprinting Analysis of Natural Dissolved Organic Matter. *Analytical chemistry* **2016,** *88* (15), 7698.

(4) Kujawinski, E. B. Electrospray ionization fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS): characterization of complex environmental mixtures. *Environ. Forensics* **2002,** *3* (207-2016).

(5) Stenson, A. C.; Marshall, A. G.; Cooper, W. T. Exact masses and chemical formulas of individual Suwannee River fulvic acids from ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectra. *Analytical chemistry* **2003,** *75* (6), 1275.

(6) Hertkorn, N.; Frommberger, M.; Witt, M.; Koch, B. P.; Schmitt-Kopplin, P.; Perdue, E. M. Natural organic matter and the event horizon of mass spectrometry. *Analytical chemistry* **2008,** *80* (23), 8908.

(7) Bhattacharyya, A.; Campbell, A. N.; Tfaily, M. M.; Lin, Y.; Kukkadapu, R. K.; Silver, W. L.; Nico, P. S.; Pett-Ridge, J. Redox Fluctuations Control the Coupled Cycling of Iron and Carbon in Tropical Forest Soils. *Environ Sci Technol* **2018,** *52* (24), 14129.

(8) Bottos, E. M.; Kennedy, D. W.; Romero, E. B.; Fansler, S. J.; Brown, J. M.; Bramer, L. M.; Chu, R. K.; Tfaily, M. M.; Jansson, J. K.; Stegen, J. C. Dispersal limitation and thermodynamic constraints govern spatial structure of permafrost microbial communities. *FEMS Microbiol Ecol* **2018,** *94* (8).

(9) Stegen, J. C.; Johnson, T.; Fredrickson, J. K.; Wilkins, M. J.; Konopka, A. E.; Nelson, W. C.; Arntzen, E. V.; Chrisler, W. B.; Chu, R. K.; Fansler, S. J.et al. Influences of organic carbon speciation on hyporheic corridor biogeochemistry and microbial ecology. *Nat Commun* **2018,** *9* (1), 585.

(10) Yao, Q.; Li, Z.; Song, Y.; Wright, S. J.; Guo, X.; Tringe, S. G.; Tfaily, M. M.; Pasa-Tolic, L.; Hazen, T. C.; Turner, B. L.et al. Community proteogenomics reveals the systemic impact of phosphorus availability on microbial functions in tropical soil. *Nat Ecol Evol* **2018,** *2* (3), 499.

(11) Gueneli, N.; McKenna, A. M.; Ohkouchi, N.; Boreham, C. J.; Beghin, J.; Javaux, E. J.; Brocks, J. J. 1.1-billion-year-old porphyrins establish a marine ecosystem dominated by bacterial primary producers. *Proceedings of the National Academy of Sciences of the United States of America* **2018,** *115* (30), E6978.

(12) Kallenbach, C. M.; Frey, S. D.; Grandy, A. S. Direct evidence for microbial-derived soil organic matter formation and its ecophysiological controls. *Nat Commun* **2016,** *7*, 13630.

(13) Cory, R. M.; Ward, C. P.; Crump, B. C.; Kling, G. W. Carbon cycle. Sunlight controls water column processing of carbon in arctic fresh waters. *Science* **2014,** *345* (6199), 925.

(14) Ward, C. P.; Nalven, S. G.; Crump, B. C.; Kling, G. W.; Cory, R. M. Photochemical alteration of organic carbon draining permafrost soils shifts microbial metabolic pathways and stimulates respiration. *Nat Commun* **2017,** *8* (1), 772.

(15) Mopper, K.; Stubbins, A.; Ritchie, J. D.; Bialk, H. M.; Hatcher, P. G. Advanced instrumental approaches for characterization of marine dissolved organic matter: extraction techniques, mass spectrometry, and nuclear magnetic resonance spectroscopy. *Chem Rev* **2007,** *107* (2), 419.

(16) Tfaily, M. M.; Chu, R. K.; Tolic, N.; Roscioli, K. M.; Anderton, C. R.; Pasa-Tolic, L.; Robinson, E. W.; Hess, N. J. Advanced solvent based methods for molecular characterization of soil organic matter by high-resolution mass spectrometry. *Analytical chemistry* **2015,** *87* (10), 5206.

(17) Steelink, C. Investigating humic acids in soils. *Analytical chemistry* **2002,** *74* (11), 326A.

(18) Herrero, M.; Mendiola, J. A.; Cifuentes, A.; Ibanez, E. Supercritical fluid extraction: Recent advances and applications. *J Chromatogr A* **2010,** *1217* (16), 2495.

(19) King, J. W. Modern supercritical fluid technology for food applications. *Annu Rev Food Sci Technol* **2014,** *5*, 215.

(20) Yen, H. W.; Yang, S. C.; Chen, C. H.; Jesisca; Chang, J. S. Supercritical fluid extraction of valuable compounds from microalgal biomass. *Bioresource technology* **2015,** *184*, 291.

(21) Sanchez-Camargo, A. D. P.; Parada-Alonso, F.; Ibanez, E.; Cifuentes, A. Recent applications of on-line supercritical fluid extraction coupled to advanced analytical techniques for compounds extraction and identification. *J Sep Sci* **2018**, DOI:10.1002/jssc.201800729 10.1002/jssc.201800729.

(22) McHugh, M.; Krukonis, V. J. Supercritical fluid extraction: principle and practice. . *Supercritical fluid extraction: principle and practice.* **1994**.

(23) Wicker, A. P.; Carlton, D. D., Jr.; Tanaka, K.; Nishimura, M.; Chen, V.; Ogura, T.; Hedgepeth, W.; Schug, K. A. On-line supercritical fluid extraction-supercritical fluid chromatography-mass spectrometry of polycyclic aromatic hydrocarbons in soil. *J Chromatogr B Analyt Technol Biomed Life Sci* **2018,** *1086*, 82.

(24) Sanchez-Camargo, A. D.; Parada-Alfonso, F.; Ibanez, E.; Cifuentes, A. On-line coupling of supercritical fluid extraction and chromatographic techniques. *J Sep Sci* **2017,** *40* (1), 213.

(25) Batlle, R.; Carlsson, H.; Holmgren, E.; Colmsjo, A.; Crescenzi, C. On-line coupling of supercritical fluid extraction with high-performance liquid chromatography for the determination of explosives in vapour phases. *J Chromatogr A* **2002,** *963* (1-2), 73.

(26) Tfaily, M. M.; Chu, R. K.; Toyoda, J.; Tolic, N.; Robinson, E. W.; Pasa-Tolic, L.; Hess, N. J. Sequential extraction protocol for organic matter from soils and sediments using high resolution mass spectrometry. *Anal Chim Acta* **2017,** *972*, 54.

(27) Shen, Y.; Moore, R. J.; Zhao, R.; Blonder, J.; Auberry, D. L.; Masselon, C.; Pasa-Tolic, L.; Hixson, K. K.; Auberry, K. J.; Smith, R. D. High-efficiency on-line solid-phase extraction coupling to 15-150-microm-i.d. column liquid chromatography for proteomic analysis. *Analytical chemistry* **2003,** *75* (14), 3596.

(28) Shen, Y.; Zhao, R.; Berger, S. J.; Anderson, G. A.; Rodriguez, N.; Smith, R. D. High-efficiency nanoscale liquid chromatography coupled on-line with mass spectrometry using nanoelectrospray ionization for proteomics. *Analytical chemistry* **2002,** *74* (16), 4235.

(29) Kujawinski, E. B.; Behn, M. D. Automated analysis of electrospray ionization fourier transform ion cyclotron resonance mass spectra of natural organic matter. *Analytical chemistry* **2006,** *78* (13), 4363.

(30) Monroe, M. E.; Tolic, N.; Jaitly, N.; Shaw, J. L.; Adkins, J. N.; Smith, R. D. VIPER: an advanced software package to support high-throughput LC-MS peptide identification. *Bioinformatics* **2007,** *23* (15), 2021.

(31) Marshall, A. G.; Rodgers, R. P. Petroleomics: chemistry of the underworld. *Proceedings of the National Academy of Sciences of the United States of America* **2008,** *105* (47), 18090.

(32) Kim, S.; Kramer, R. W.; Hatcher, P. G. Graphical method for analysis of ultrahigh-resolution broadband mass spectra of natural organic matter, the van Krevelen diagram. *Analytical chemistry* **2003,** *75* (20), 5336.

(33) van Krevelen, D. *Fuel* **1950,** *29*, 269.

(34) Sleighter, R. L.; Hatcher, P. G. The application of electrospray ionization coupled to ultrahigh resolution mass spectrometry for the molecular characterization of natural organic matter. *J Mass Spectrom* **2007,** *42* (5), 559.

(35) Hendrickson, C. L.; Quinn, J. P.; Kaiser, N. K.; Smith, D. F.; Blakney, G. T.; Chen, T.; Marshall, A. G.; Weisbrod, C. R.; Beu, S. C. 21 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer: A National Resource for Ultrahigh Resolution Mass Analysis. *Journal of the American Society for Mass Spectrometry* **2015,** *26* (9), 1626.

(36) Smith, A. P.; Bond-Lamberty, B.; Benscoter, B. W.; Tfaily, M. M.; Hinkle, C. R.; Liu, C.; Bailey, V. L. Shifts in pore connectivity from precipitation versus groundwater rewetting increases soil carbon loss after drought. *Nat Commun* **2017,** *8* (1), 1335.

(37) Solihat, N. N.; Acter, T.; Kim, D.; Plante, A. F.; Kim, S. Analyzing Solid-Phase Natural Organic Matter using Laser Desorption Ionization Ultrahigh Resolution Mass Spectrometry. *Analytical chemistry* **2018**, DOI:10.1021/acs.analchem.8b04032 10.1021/acs.analchem.8b04032.

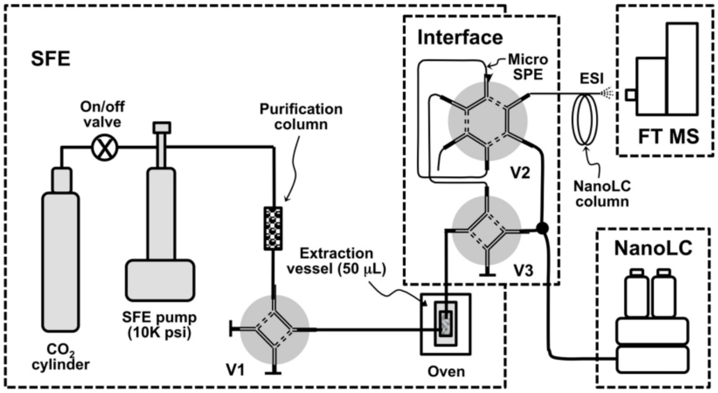
SFE
On/off valve
Purification column
CO$_2$ cylinder
SFE pump (10K psi)
V1
Extraction vessel (50 μL)
Oven
Interface
Micro SPE
V2
V3
ESI
NanoLC column
FT MS
NanoLC

Fig. 1

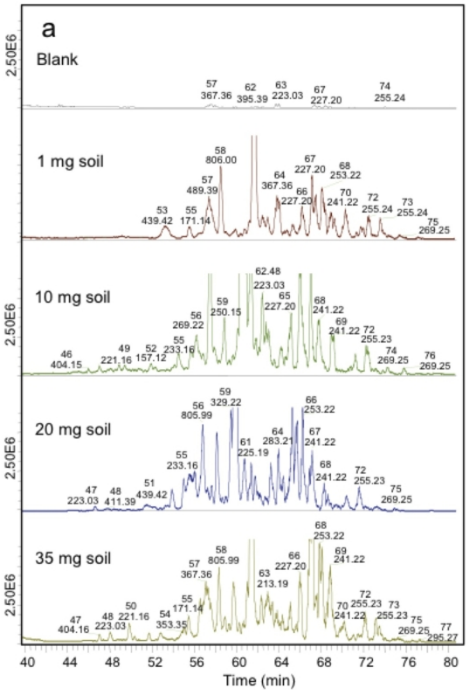
a
2.50E6
Blank
57
367.36
62
395.39
63
223.03
67
227.20
74
255.24
1 mg soil
53
439.42
55
171.14
57
489.39
58
806.00
64
367.36
66
227.20
67
227.20
68
253.22
70
241.22
72
255.24
73
255.24
75
269.25
2.50E6
10 mg soil
46
404.15
49
221.16
52
157.12
55
233.16
56
269.22
59
250.15
62.48
223.03
65
227.20
68
241.22
69
241.22
72
255.23
74
269.25
76
269.25
2.50E6
20 mg soil
47
223.03
48
411.39
51
439.42
55
233.16
56
805.99
59
329.22
61
225.19
64
283.21
66
253.22
67
241.22
68
241.22
72
255.23
75
269.25
2.50E6
35 mg soil
47
404.16
48
223.03
50
221.16
54
353.35
55
171.14
57
367.36
58
805.99
63
213.19
66
227.20
68
253.22
69
241.22
70
241.22
72
255.23
73
255.23
75
269.25
77
295.27
40
44
48
52
56
60
64
68
72
76
80
Time (min)

Fig. 2

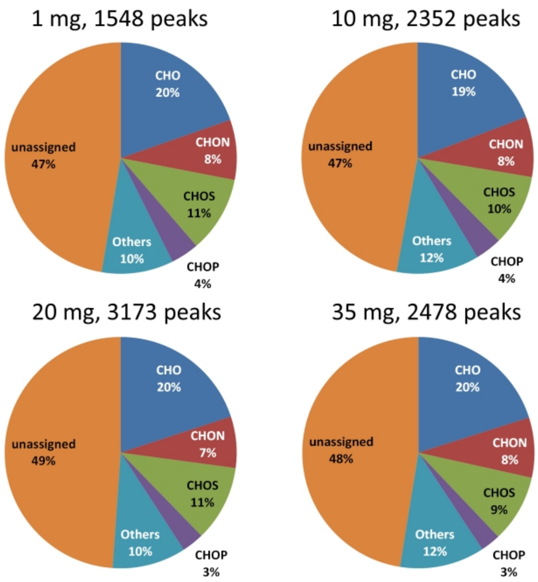
1 mg, 1548 peaks
CHO 20%
CHON 8%
CHOS 11%
CHOP 4%
Others 10%
unassigned 47%
10 mg, 2352 peaks
CHO 19%
CHON 8%
CHOS 10%
CHOP 4%
Others 12%
unassigned 47%
20 mg, 3173 peaks
CHO 20%
CHON 7%
CHOS 11%
CHOP 3%
Others 10%
unassigned 49%
35 mg, 2478 peaks
CHO 20%
CHON 8%
CHOS 9%
CHOP 3%
Others 12%
unassigned 48%

Fig. 3

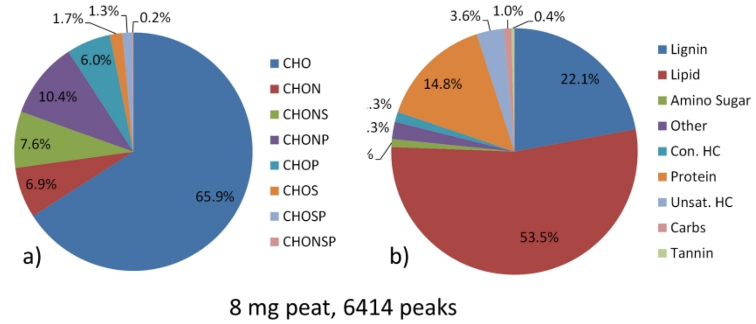
1.7%
1.3%
0.2%
6.0%
10.4%
7.6%
6.9%
65.9%
a)
CHO
CHON
CHONS
CHONP
CHOP
CHOS
CHOSP
CHONSP
3.6%
1.0%
0.4%
14.8%
22.1%
.3%
.3%
%
53.5%
b)
Lignin
Lipid
Amino Sugar
Other
Con. HC
Protein
Unsat. HC
Carbs
Tannin
8 mg peat, 6414 peaks

Fig. 4

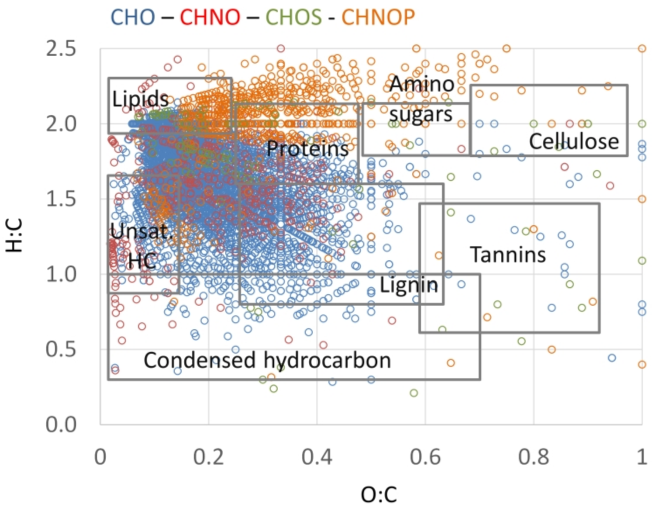
CHO – CHNO – CHOS - CHNOP
H:C
O:C
2.5
2.0
1.5
1.0
0.5
0.0
0
0.2
0.4
0.6
0.8
1
Lipids
Amino sugars
Proteins
Cellulose
Unsat. HC
Tannins
Lignin
Condensed hydrocarbon

Fig. 5