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Comparison of sample preparation techniques for the (-)ESI-FT-ICR-MS analysis of humic and fulvic acids

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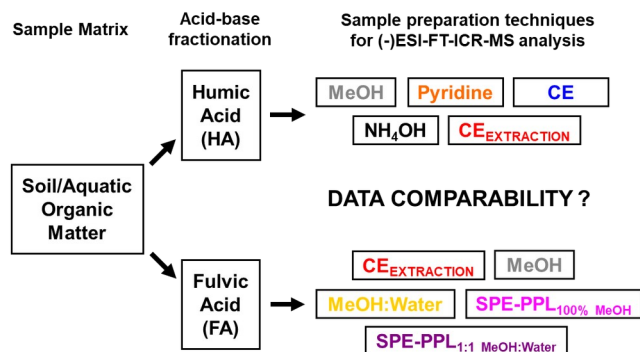
ABSTRACT

Soil organic matter (SOM) plays a key role in the global carbon and nitrogen cycles. Soil biogeochemistry is commonly studied by extracting the base-soluble fractions of SOM: the acid-insoluble humic acid (HA) and acid-soluble fulvic acid (FA). Electrospray ionization – Fourier transform – ion cyclotron resonance – mass spectrometry (ESI-FT-ICR-MS) is commonly utilized for molecularly characterizing these fractions. Different sample preparation techniques exist for the analysis of HA and FA though questions remain regarding data comparability following different preparations. Comparisons of different sample preparation techniques revealed that the negative-mode ESI-FT-ICR-MS analytical window can be skewed to detect different groups of molecules, with primary differences in oxygenation, aromaticity, and molecular weight. It was also observed that HA and FA from soils versus an aquatic matrix behaved very differently and thus, we concluded that sample preparation techniques determined to be “most optimal” in our study are in no way universal, and we recommend that future studies of HA and FA involve such comparative studies for determining the most suitable sample preparation technique for their particular type of HA or FA matrices. This will enhance data comparability among different studies and environmental systems and ultimately, allow us to better understand the complex composition of environmental matrices.

Subjects: Soil organic matter, electrospray ionization (ESI), FT-ICR-MS, data comparability.

Synopsis: Minimal research exists on the comparability of mass spectral data of humic and fulvic acid extracts. This study compares different sample preparation techniques and discusses potential analytical biases to characterized molecular composition.

Graphical Abstract:



1. INTRODUCTION

Humic substances (HS) are the biogeochemical products of the natural decomposition of plant litter by microorganisms^{1, 2} and abiotic processes³ contributing to the production of soil organic matter (SOM). HS account for 60-75 % of SOM⁴ and have a unique structure dissimilar to other compound

groups present in SOM⁵⁻⁹. HS play an essential role in soil fertility, agricultural productivity, stabilization of aggregates, carbon sequestration, and as a whole are fundamental players in the biogeochemical cycling of SOM^{1, 2, 4, 10-16}. HS have been a significant focus of soil science research and are still objects of intense discussion, research, and even controversy^{4, 11, 17-27}. Therefore, understanding the molecular composition of HS is important for deciphering their enigmatic cycling as well as their involvement in carbon sequestration processes and mitigating climate change.

Ultrahigh resolution mass spectrometry, namely Fourier transform - ion cyclotron resonance - mass spectrometry (FT-ICR-MS), is a powerful technique providing a detailed view of the molecular composition of natural organic matter²⁸⁻³², including HS^{3, 32-42}. Nowadays, electrospray ionization (ESI), particularly in negative ionization mode (-), has become the norm in the environmental sciences^{43, 44}. As SOM cannot be directly solubilized (a requirement for ESI analysis), HS are extracted using an alkali extraction (0.1 M NaOH). The unextracted remainder of SOM, the humin, is insoluble in most solvents making it difficult to analyze and thus, the humin fraction is rarely employed in SOM research. The NaOH-extracted HS are further fractionated into a fulvic acid (FA) fraction and a humic acid (HA) fraction based on the adjusting the solution to pH = 1 by the addition of HCl: the FA fraction remains fully solubilized while the HA fraction precipitates out. These two HS are then usually freeze-dried into a powder for sequential analyses⁴⁵.

HA and FA fractions have been studied for decades and it has been found that they are analytically challenging samples for ESI methods: FA samples are difficult to purify and thus, are often rich in inorganic species – a severe issue, which can bias, and even prevent the ionization of molecules in the ESI source. The HA fraction, in contrast, has high aromatic content and tends to aggregate⁴⁶, and sometimes it can also contain inorganic (e.g., Ca, Fe) constituents^{47, 48} additionally skewing its solubility and ionizability. Once acid-precipitated and dried, the HA fraction is also difficult to re-solubilize back into solution.

Different sample preparation techniques have been developed to overcome the challenging matrices of HA and FA. HA fractions have been previously dissolved in neutral MeOH:Water mixtures^{49, 50}, basified MeOH: Water mixtures with NaOH⁵¹, basified MeOH: Water mixtures with NH₄OH^{35-37, 51}, acidified MeOH: Water mixtures with acetic acid⁵¹, basified MeOH: Water mixtures with NaOH followed with cation-exchange⁵², neutral MeOH⁴⁴, basified water with NH₄OH^{44, 53-55}, as well as in basified water with NaOH followed by cation-exchange and solvent extraction of the resin³. Commonly, NH₄OH is added after HA had been already solubilized and thus, NH₄OH only serves as an ionization enhancer^{35-37, 49, 50}. The addition of NH₄OH is necessary as HA fractions are often rich in poorly ionizable aromatic and condensed aromatic molecules^{56, 57}. In other studies, however, NH₄OH was simultaneously added when the powdered HA was mixed with the solubilization solution and thus, NH₄OH served both as a basification agent and an ionization enhancer^{44, 51, 53-55}. Recent work has shown that the use of NH₄OH can induce nitrogen-incorporation and possibly bias the mass spectrometric analytical window⁵⁸, but this has not been assessed in HA matrices.

FA fractions have been previously solubilized in neutral MeOH: Water mixtures^{43, 51}, basified isopropanol:water mixtures with NH₄OH^{51, 59}, basified MeOH: Water mixtures with NH₄OH³⁸, acidified MeOH: Water mixtures with acetic acid⁵⁹, basified water with NH₄OH⁵⁵. Solid-phase extraction using Bond Elut Priority Pollutant cartridges (SPE-PPL), a popular approach for obtaining high purity organic matter extracts from aquatic samples⁶⁰, has also been employed⁶¹.

Clearly, there is a large diversity in the possible sample preparation techniques to solubilize HA and FA fractions in solution and prepare samples suitable for ESI-FT-ICR-MS analysis. Different sample preparation techniques have been previously discussed in the literature⁶² though questions remain about the comparability of data from these different sample preparation techniques. The composition of solvent (e.g., 100% MeOH, MeOH:Water or isopropanol:water mixtures) and addition of modifiers (NH₄OH, acetic acid, etc.) are controlling factors in the ionizability of molecules in the ESI source^{63, 64}. It has been shown that when HA is analyzed in MeOH: Water mixtures of different ratios (e.g., 25:75, 50:50, 70:30), the highest quality spectra are obtained when MeOH is more than 50%^{53, 59, 65}. Solvents and modifiers affect the molecular conformation, charge distribution, the stability of the formed ions, and consistency of ionization currents⁶⁶⁻⁶⁹. Additional factors include the pH of the solution, ratio of organic solvent:water, as well the quantity of ions (i.e., the ionic strength)^{59, 70, 71}. Thus, variability in the sample preparation will affect the ionizability of molecules and thus likely affect the observed molecular composition of HA and FA using ESI-FT-ICR-MS.

Previous studies have shown that different solvents fractionate organic matter and further bias the analytical window of ESI-FT-ICR-MS in the context of sediments⁷², aerosols⁷³, soils⁷⁴, and petroleum and its fractions^{67, 69}. However, in these studies the solvent was used both for obtaining solvent-extractable organic matter and for a carrier solvent in the ESI analysis. By contrast in HA and FA analysis, the humic substances are already extracted and only need to be solubilized.

In this manuscript, we assess how data for freeze-dried HA and FA compares when different sample preparation techniques are used. This is the first comprehensive study of sample preparation techniques for HA and FA fractions. Though these fractions are key SOM constituents and are commonly employed in soil science studies, this humic fractionation technique has started to appear in aquatic dissolved organic matter (DOM) studies too. This is likely because DOM partly originates from SOM leaching into aquatic environments establishing the connection between terrestrial (land) and aquatic interfaces. Different sample preparation techniques were tested on HA and FA fractions from several soils and one river. We investigate compositional differences using (-)ESI-FT-ICR-MS and assess how sample preparation and/or solvent composition affect the molecular results. Results from this study will enhance our understanding of HA and FA and create a framework for researchers to perform similar sample preparation comparative studies in order to obtain more representative molecular information about HA, FA, and other environmental samples.

2. EXPERIMENTAL SECTION

2.1 Soil samples and extraction of humic substances

Two haplorthox (red-yellow latosol) soil samples, representing agricultural and forest soils, were collected in the Embrapa Southeast Livestock Research Center (Embrapa Pecuária Sudeste, São Carlos, São Paulo State, Brazil). A spodosol soil profile, characteristic of Amazonian soils, was obtained from nearby Barcelos city, Amazon State, Brazil. Obtained were samples of its Horizon A (0 – 15 cm, sample labeled Amazonian-A) and its Horizon C (350 cm, sample labeled Amazonian-C).

Additional information about the four soils can be found in Section 1 of the supporting information (SI). Powdered HA and FA samples were prepared from the four soils following the procedure recommended by the International Humic Substances Society (IHSS)⁴⁵. The full protocol is described in Section 2 of the SI. Additional standardized and internationally recognized HA and FA samples were purchased from the IHSS (<https://humic-substances.org/>): Elliott Soil HA (sample label ESHA, cat. no. 5S102H, lot V) and FA (sample label ESFA, cat. no. 5S102F, lot V) as well as Suwannee River HA (sample label SRHA, cat. no. 2S101H, lot II) and FA (sample label SRFA, cat. no. 2S101F, lot II).

2.2 Sample preparation techniques

Calculated sample amounts (0.5 – 0.7 mg), depending on carbon content, were weighed into glass vials to result in 0.2 mg-carbon equivalents. The solid HA or FA powders were then solubilized in 2000 μ L solvent (as described below) to yield a HA or FA solution at a concentration of 100 mg sample-carbon per L solution ($\text{mgC}\cdot\text{L}^{-1}$). This solution was then diluted 2-fold to yield a final sample concentration of 50 $\text{mgC}\cdot\text{L}^{-1}$ that was to be infused in the ESI-FT-ICR-MS per the standard operating procedure for the instrument. For all sample preparations, MilliQ laboratory-grade water (18.1 M Ω) and methanol (MeOH, Fisher Scientific, Optima LC-MS grade) were used, and all glassware was acid-cleaned and combusted (8 hours at 450 °C) prior to usage.

Five different solubilization procedures were employed to solubilize HA fractions. Procedural blanks corresponding to the five sample preparation techniques were also prepared.

- **Solubilization in methanol (MeOH approach)** – tested only on the agricultural HA sample. The powdered HA sample was mixed with 2000 μ L MeOH and then sonicated for 10 minutes. Due to the incomplete visual dissolution of the sample, the suspension was filtered using a 0.45 μ m acid-washed filter (ThermoFisher Scientific Target2 F2513-3, PTFE membrane). The sample was then diluted with additional 2000 μ L MeOH. Solubilized HA carbon was not quantified. A 30 min sonication was also employed as a separate test but complete HA solubilization was not achieved. A (-)ESI-FT-ICR mass spectrum was not acquired for the 30 min sonication test.
- **Solubilization in pyridine (Pyridine approach)** – tested only on the agricultural HA sample. The powdered HA sample was mixed with 2000 μ L pyridine (Sigma-Aldrich, \geq 99.9%, HPLC grade) and then sonicated for 10 minutes. Due to the incomplete visual dissolution of the sample, the suspension was filtered using a 0.45 μ m acid-washed filter (Fisher Target2 F2513-3, PTFE membrane). The sample was then diluted with additional 2000 μ L pyridine. Solubilized HA carbon was not quantified. A 30 min sonication was also employed as a separate test but complete HA solubilization was not achieved. A (-)ESI-FT-ICR mass spectrum was not acquired for the 30 min sonication test.
- **Solubilization with ammonium hydroxide (NH₄OH approach)** – The powdered HA sample was mixed with 2000 μ L basified MilliQ water (pH 9, NH₄OH, Fluka, \geq 25 wt.%, LC-MS grade) and then sonicated for 10 minutes. Complete dissolution was achieved. A 500 μ L aliquot of the aqueous

supernatant was mixed with 500 μL MeOH to result in a 1:1 MeOH: Water solution at a final sample-carbon concentration of 50 $\text{mgC}\cdot\text{L}^{-1}$.

- **Base-solubilization with cation-exchange (CE approach)** – The powdered HA sample was mixed with 1800 μL basified MilliQ water (pH 9, NaOH, Fisher Chemical SS254-4, 50% w/w Certified) and then sonicated for 10 minutes. Complete dissolution was achieved. To this solution, 200 μL of a suspension of cation-exchange resin (Dowex 50WX8 100-200 mesh, Acros Organics) in water was added yielding a total water volume of 2000 μL . The resin suspension was vortexed for 30 seconds, and let sit for 10 minutes. A 500 μL aliquot of the aqueous supernatant was mixed with 500 μL MeOH to result in a 1:1 MeOH: Water solution at a final sample-carbon concentration of 50 $\text{mgC}\cdot\text{L}^{-1}$.
- **Base-solubilization with cation-exchange and solvent-extraction ($\text{CE}_{\text{EXTRACTION}}$ approach)** – The powdered HA sample was mixed with 1800 μL basified MilliQ water (pH 9, NaOH, Fisher Chemical SS254-4, 50% w/w Certified) and then sonicated for 10 minutes. Complete dissolution was achieved. To this solution, 200 μL of a suspension of cation-exchange resin (Dowex 50WX8 100-200 mesh, Acros Organics) in water was added yielding a total water volume of 2000 μL . The resin suspension was vortexed for 30 seconds, and let sit for 10 minutes. Then, the supernatant was aliquoted out of the resin, and 2000 μL of MeOH were added to the resin to solvent-extract any organic molecules that were retained onto it. The vial with the resin solvent-extraction was vortexed for 30 seconds and let sit for 10 min. Exactly 500 μL of the methanolic supernatant were mixed with 500 μL of the cation-exchanged aqueous solution to result in a 1:1 MeOH: Water solution at a final sample-carbon concentration of 50 $\text{mgC}\cdot\text{L}^{-1}$. The $\text{CE}_{\text{EXTRACTION}}$ approach is graphically described on Figure S2.

Four different solubilization procedures were employed to solubilize FA fractions. Procedural blanks corresponding to the four approaches were also prepared.

- **Base-solubilization with cation-exchange and solvent-extraction ($\text{CE}_{\text{EXTRACTION}}$ approach)** – see above.
- **Solubilization in methanol (MeOH approach)** – The powdered FA sample was mixed with 2000 μL MeOH and then sonicated for 10 minutes. Complete dissolution was achieved. The sample was then diluted with additional 2000 μL MeOH.
- **Solubilization in methanol-water mixture (MeOH:Water approach)** – The powdered FA sample was mixed with 2000 μL MeOH and then sonicated for 10 minutes. Complete dissolution was achieved. Then, 500 μL of the methanolic solution was mixed with 500 μL of MQ water resulting in a 1:1 MeOH: Water solution.
- **Solid-phase extraction using PPL cartridges (SPE-PPL approach)** – The powdered FA sample was solubilized in 5000 μL of neutral (pH = 7) MilliQ water and then sonicated for 10 minutes. Complete dissolution was achieved. Then, the solution was acidified to pH = 2 using dropwise addition of HCl (Fisher, Certified ACS Plus). Solid-phase extraction was performed using PPL cartridges (Agilent Technologies, Bond Elut Priority Pollutant (PPL), 100 mg styrene divinyl copolymer). The extraction was performed following previously published procedures^{60, 75} with slight modifications including more extensive cartridge washing, as described below, to minimize the number of contaminant species introduced into the samples from the PPL resin. The PPL cartridge is firstly conditioned with three cartridge volumes of MeOH and then washed using six cartridge volumes of acidified (pH = 2) MilliQ water. Then, the acidified sample solution is passed through the column. Then, the cartridge is washed with six cartridge volumes of acidified (pH = 2) MilliQ water. Volumes passing through the PPL cartridges were allowed to flow through by gravity, i.e., the flow rate was not enhanced using a peristaltic pump setup. The cartridges are then dried under a flow of ultrapure N_2 gas (AirGas, NI UHP300). Cartridges are eluted with 2000 μL of MeOH to result in a methanolic extract at a final sample-carbon concentration of 50 $\text{mgC}\cdot\text{L}^{-1}$ (assuming 50% recovery).

A complete summary of samples and sample preparation techniques is presented in Table SX.

2.3 Ultrahigh resolution mass spectrometry: (-)ESI-FT-ICR-MS

Prepared HA and FA samples with different sample preparation techniques were directly injected into an Apollo II ESI source of a Bruker Daltonics 10-Tesla Apex Qe FT-ICR-MS at the College of Sciences Major Instrumentation Cluster (COSMIC) at Old Dominion University (Norfolk, VA). Samples were analyzed in (-)ESI mode with the same tuning parameters instrumental variability from influencing the spectral comparability. The samples of this project were analyzed in three separate days and data comparability was ensured by tune validation using SRFA and the recommendations by Hawkes and D'Andrilli⁴³. All spectra are internally calibrated using naturally present fatty acids, dicarboxylic acids, and compounds belonging to CH_2 -homologous series⁷⁶. Peaks with signal-to-noise ratio ≥ 3 were used for further

processing. Using an in-house MATLAB script, peaks found in the procedural blank, peaks of inorganic origin ("salt" peaks), and isotopologue (^{13}C , ^{32}S) peaks are removed. Salt peaks are defined as following:

Table S1. Definition of salt peaks

m/z range	Mass defects of salt peaks
< 300	0.4 – 0.97
300-500	0.5 – 0.96
500-800	0.6 – 0.95
> 800	0.7 – 0.94

Mass spectra are assessed based on total number of formulas and number of salt peaks. Number of assignable peaks is also calculated as total peaks – salt peaks – blank peaks – isotopologue peaks. Salt and assignable peaks are assessed based on number-based percentages (i.e., salt % = number of salt peaks/number of total peaks x 100).

To assign molecular formulas and assess molecular composition, refined mass lists of assignable peaks were combined in a dataset of 81 samples in total. This dataset included SRFA quality control spectra as well as mass spectra of various agricultural and Amazonian samples from previous studies of Tadini^{77, 78}. The mass spectra of all samples have been published in the *Mendelay Data* repository⁷⁹⁻⁸¹. Spectra were then aligned to minimize formula assignment errors⁸² using a previously published MATLAB script⁸³. Aligned spectra were then assigned molecular formulas using a previously published MATLAB script⁸⁴. Assignment was limited to $^{12}\text{C}_{5-100}$, $^1\text{H}_{5-100}$, $^{16}\text{O}_{1-50}$, $^{14}\text{N}_{0-5}$, $^{32}\text{S}_{0-4}$, and $^{31}\text{P}_{0-2}$ with maximum assignment error of 1 ppm. Formulas that did not adhere to previously established molecular rules for natural organic matter⁸⁵⁻⁸⁷ were eliminated. Mass peaks with any residual ambiguous assignments (multiple possible molecular formulas per mass peak) were further refined using inclusion within homologous series (CH_2 , H_2 , COO , CH_2O , $\text{C}_2\text{H}_4\text{O}$, O , H_2O , NH_3)^{85, 86}. Any further ambiguous assignments were refined based on assignment error and the molecule with lower assignment error was selected. In the final list of molecular formulas, there was only one molecular formula per mass peak. For all formulas, the modified aromaticity index^{88, 89} is calculated as shown below:

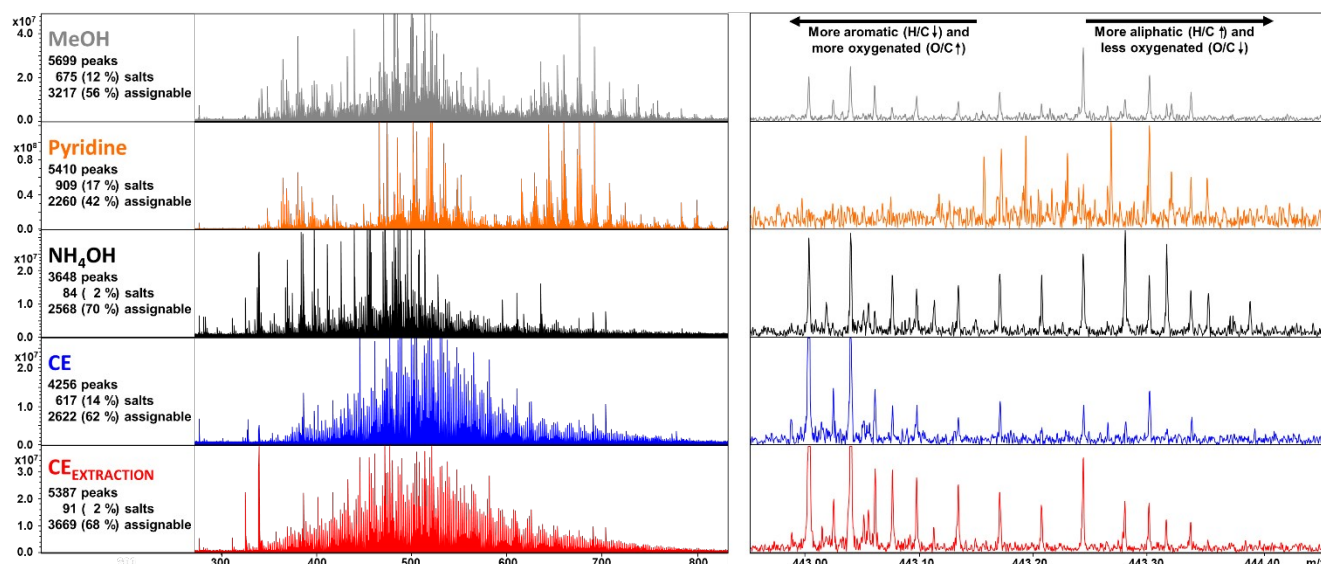
$$AI_{MOD} = \frac{1 + C - \frac{1}{2}O - S - \frac{1}{2}(N + P + H + Cl)}{C - \frac{1}{2}O - N - S - P}$$

Formulas were classified as following: Condensed aromatic ($AI_{MOD} \geq 0.67$), aromatic ($0.67 < AI_{MOD} \leq 0.50$), olefinic/alicyclic ($0 < AI_{MOD} < 0.50$), and aliphatic ($AI_{MOD} = 0$).

3. RESULTS AND DISCUSSION

3.1 Sample preparation techniques for humic acids (HA)

The agricultural soil HA was prepared using five solubilization approaches and was analyzed on the same day with identical tuning parameters (Figure 1). Corresponding procedural blank samples are shown in Figure S4.



270

271 **Figure 1:** Whole (-)ESI-FT-ICR mass spectra (left) and expanded windows at nominal mass of 443 (right)
 272 for an agricultural soil humic acid prepared using the MeOH, Pyridine, NH₄OH, CE, and CE_{EXTRACTION}
 273 sample preparation techniques.

274

275 The mass spectra had variable shapes indicative of different ionization distribution when different
 276 preparation techniques were employed (Figure 1). The MeOH and Pyridine approaches yielded trimodal
 277 spectra whereas the NH₄OH, CE, and CE_{EXTRACTION} approaches yielded unimodal Gaussian-like spectra.
 278 Unimodal spectra are commonly observed for natural organic matter, including for HA and FA fractions³⁰.
 279 ^{31, 35-37, 51, 74, 90, 91}. The observed spectral multimodality following the MeOH and Pyridine approaches was
 280 likely caused by aggregation, selective ionization of different molecular classes, or preferential extraction
 281 of different molecular classes because the HA samples were not entirely solubilized in MeOH or pyridine.
 282 A detailed discussion of the observed spectral multimodality in the MeOH and Pyridine treatments can be
 283 found in Section 7 of the SI.

284 The peak distributions at single nominal mass ranges are inspected to evaluate differences among
 285 the detected ions in each sample (right panels of Figure 1). As spectral shape and magnitude are highly
 286 liable to bias by instrumental parameters^{92, 93}, per-m/z distributions are more reliable for evaluating how the
 287 sample preparation techniques affected the observed HA molecular composition. These expanded ranges
 288 reflect the chemical stability, prevalence, and ionizability of molecules of different compositions present in
 289 the analyzed sample⁹³. The distribution of ionized molecules with the MeOH approach appeared to contain
 290 a variety of different molecules covering mass defects of 0.00 – 0.35. The distribution of ionized molecules
 291 with the Pyridine approach appears to be missing ions with mass defects 0.95 – 0.15 indicative of
 292 discrimination against more oxygenated aromatic molecules and preferential ionization of poorly
 293 oxygenated aliphatic molecules. Additionally, molecular formulas were assessed based on the abundance
 294 non-oxygen heteroelements (i.e., abundance of CHO, CHON, CHOS, and CHOP formulas). NSP
 295 heteroelements are actively involved in the global biogeochemical cycles and in soil systems are
 296 responsible for modulating plant growth as well as carbon decomposition and humification reactions⁹⁴⁻⁹⁶.
 297 Highly elevated contents of CHON formulas were observed following the MeOH and Pyridine approaches
 298 and these are discussed in detail in Section 8 of the SI. Because these two approaches (MeOH and
 299 Pyridine) did not fully solubilize the samples and appeared to significantly bias the molecular composition,
 300 they were not further considered for testing on other HA samples.

301 To further assess this, characteristic H/C and O/C clustering patterns of molecular formulas were
 302 assessed using van Krevelen diagrams^{90, 97} (Figure S6). The modified aromaticity index (AI_{MOD}) metric
 303 allowed for distinguishing aliphatic, olefinic, aromatic, and condensed aromatic molecules^{88, 89}. The MeOH
 304 approach enhanced the ionization of aliphatic/olefinic molecules (AI_{MOD} < 0.50) but still allowed for the
 305 observation of aromatic (0.67 < AI_{MOD} ≤ 0.50) and condensed aromatic compounds (AI_{MOD} ≥ 0.67). When
 306 the agricultural HA was solubilized in pyridine (Figure 1), the ionization of aliphatic and olefinic molecules
 307 (AI_{MOD} < 0.50) was enhanced, and aromatic molecules (AI_{MOD} > 0.50) were almost entirely undetected.
 308 Aromatic molecules could have been suppressed in the ESI source (outcompeted by the aliphatic
 309 molecules), but aromatic molecules could also have been not solubilized by the pyridine at all. Previous
 310 studies have shown that pyridine extractions of organic matter are selective towards molecules of less
 311 polar aliphatic character⁷³ agreeing with our findings.

312 Using basified aqueous solutions (either with NaOH or NH₄OH) achieved complete solubilization of
313 HA samples. Therefore, all sample molecules could be introduced into the ESI source. Using NH₄OH is
314 very practical and is commonly employed in HA analysis: In addition to serving as a basification agent,
315 NH₄OH is an ionization enhancer in (-)ESI⁹⁸. Upon nebulization, NH₄OH transforms into gaseous species,
316 which are easily evacuated while promoting molecular ionization:
317



318 In contrast, when powdered HA is solubilized by basified solutions with NaOH, sodium cations
319 negatively affect the ionization and also cause the formation of peaks of inorganic origin, such as solvent-
320 salt or HA-salt adducts^{51, 59} also known as “salt” peaks. Thus, sodium cations must be removed using
321 cation-exchange (CE) resin prior to analysis. When the spectra following NH₄OH and CE approaches are
322 compared (Figure 1), it is evident that peaks with mass defect > 0.20 are lost after the CE approach. Van
323 Krevelen diagrams of the assigned formulas (Figure S6) indicate that higher molecular weight and poorly
324 oxygenated molecules of both aromatic and aliphatic character sorbed onto the resin³. To recover any
325 possible CE-entrapped molecules, the resin was further solvent-extracted with MeOH. This MeOH fraction
326 was mixed with the CE-exchanged aqueous solution (CE_{EXTRACTION} approach, Figure S2). Thus, all
327 molecules were combined to provide a more representative solution of the original HA sample. The peaks
328 that were not observed when only CE was employed have now been detected following the CE_{EXTRACTION}
329 approach (Figure 1). These ions may also have been lost due to unoptimized instrument parameters, but
330 this is highly unlikely as all agricultural HA samples were analyzed on the same day with the same
331 parameters and all samples exhibited stable ionization currents.

332 The spectra following the NH₄OH and CE_{EXTRACTION} appeared most to contain the highest
333 diversity of HA ions (Figure 1), but it is evident that the per-m/z magnitude distributions were different.
334 When NH₄OH was used, ions of higher mass defect were detected indicative that less oxygenated
335 molecules (primarily aliphatic but some aromatic too) are preferentially ionized at the expense of more
336 oxygenated of various aromatic character. Not all aliphatic molecules that were observed following the
337 NH₄OH approach were detected when the CE_{EXTRACTION} approach was employed. This indicates that while
338 there is a significant overlap, the NH₄OH and CE_{EXTRACTION} approaches both yield a bias towards different
339 molecular classes. Notably, the CE_{EXTRACTION} approach allowed for detecting 48% more assignable peaks
340 (5387) than the NH₄OH approach (3648) making the CE_{EXTRACTION} approach superior for this particular
341 sample matrix. We hypothesize that NH₄OH sucks up all of the charge which would be otherwise
342 available for more molecules to be ionized (i.e., ionization poorly transfers to molecules and they do not
343 become ions). Alternatively, NH₄OH may be overionizing the oxygen-rich molecules cheating
344 supercharged ions. Such ions would likely have too many charged groups (e.g., COO⁻) in close proximity
345 that would cause the ions to collapse and degrade by the time they reach the ICR cell. These two
346 hypotheses explain why NH₄OH reduced the number of total and assignable peaks, as well as why less
347 oxygenated compounds are preferentially detected with the NH₄OH approach.

348 Only the three solubilization approaches which allowed for full solubilization (NH₄OH, CE,
349 CE_{EXTRACTION}) were employed on the HA from the forest soil and similar trends were observed (Figure SX),
350 as described in Section X of the SI. The NH₄OH and CE_{EXTRACTION} approaches were additionally tested on
351 HA from the Elliott Soil (ESHA) and Suwannee River (SRHA). The spectral trends for ESHA (Figure SX)
352 were similar to those observed for the agricultural and forest HA: CE_{EXTRACTION} allowed for the detection of
353 higher number of peaks over a large mass defect range. By contrast, SRHA performed very differently:
354 the CE_{EXTRACTION} spectrum had a bimodal distribution and there was an evident loss of ions with higher
355 mass defect (> 0.23). The NH₄OH preparation allowed for detecting a higher number of total and
356 assignable peaks, and the per per-m/z distributions appeared to contain most diverse types of ions.

357 To better understand how the NH₄OH and CE_{EXTRACTION} approaches affect the molecular
358 composition of HA fractions, molecular formulas of the CE_{EXTRACTION} and NH₄OH samples of the four HA
359 samples were assessed as using presence/absence analysis⁹⁹ (Figure 2). The formulas of soil HA
360 samples preferentially observed for following the NH₄OH approach were less oxygenated than those
361 observed following the CE_{EXTRACTION} approach. The common formulas of these samples (Figures X) had
362 H/C and O/C clustering patterns characteristic of soil HA fractions indicating that both NH₄OH and
363 CE_{EXTRACTION} approaches manage to representatively characterize soil HA fractions³. Furthermore, the
364 CE_{EXTRACTION} also enhanced the detection of CHON and CHOS species for the soil HA matrices (Figures
365 SX) but suppressed the detection of CHON species in SRHA (Figure SX).

366 In summary: 1) the unique formulas observed following the CE_{EXTRACTION} approach generally had
367 the same H/C and O/C clustering pattern as the common formulas (Figures SX and SX); and 2):
368 CE_{EXTRACTION} increased the number of total and assignable peaks; and 3) CE_{EXTRACTION} allowed for observing
369 the most diverse types of molecules (Figure S6) including more CHON species as well as condensed
370 aromatic compounds of different degrees of oxygenation characteristic for the heterogenous nature of HA

fractions³. For these reasons we conclude that $CE_{EXTRACTION}$ is the most optimal approach for the analysis of the soil HA matrices assessed here. Thus, the $CE_{EXTRACTION}$ sample preparation technique should be considered more often for the analysis of HA samples. There was a notable difference in how the aquatic HA sample behaved relative to the HA samples. The $CE_{EXTRACTION}$ approach enhanced the detection of less oxygenated compounds of uniform aromaticity and higher molecular weight (> 600 Da) whereas the NH_4OH approach enhanced the detection of more aromatic and more oxygenated compounds across the entire MW range (300-800 Da). The NH_4OH approach also allows for the detection of more total and assignable peaks. For these reasons, and because the uniquely detected peaks following the NH_4OH approach have a similar H/C and O/C clustering pattern to the common peaks among the NH_4OH and $CE_{EXTRACTION}$ approaches, we conclude that the NH_4OH sample preparation technique is more optimal for aquatic HA samples.

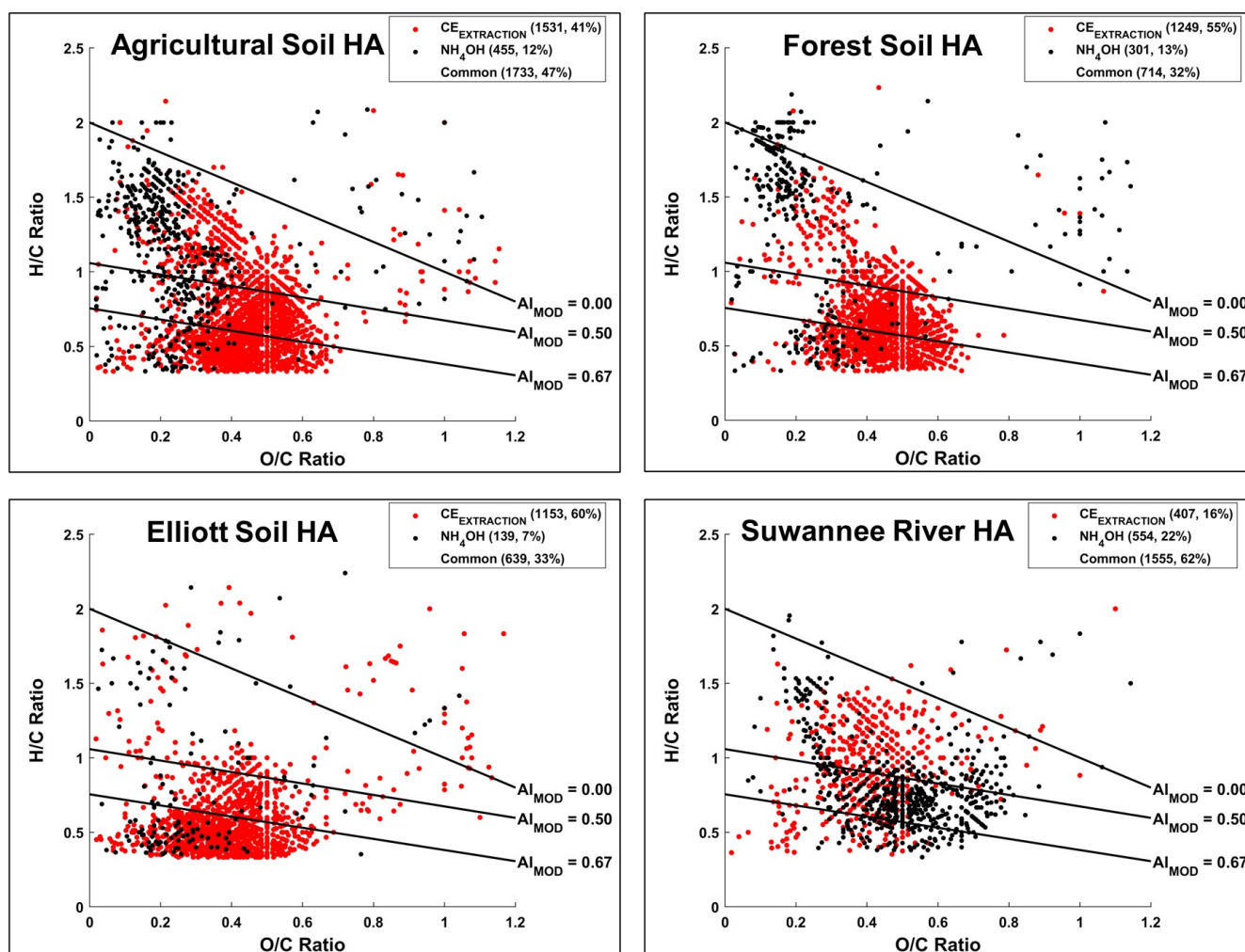


Figure 2. Van Krevelen diagrams (H/C versus O/C molar ratios) of assigned molecular formulas to humic acids prepared using the NH_4OH and $CE_{EXTRACTION}$ sample preparation techniques. Formulas are assessed using presence/absence analysis⁹⁹ and only unique formulas to each sample are shown. The number of formulas found in each of these pools is listed in the legends along with corresponding percentages (relative to total number of formulas in the two samples being compared). The black lines indicate modified aromaticity index cutoffs (AI_{MOD})^{88, 89}. Common formulas and assessments relative to molecular weight are shown in Figures S7 and S8.

3.2 Sample preparation techniques for fulvic acids (FA)

The FA fraction is generally less problematic than the HA one due to the solubility of FA in both acid and base. However, FA can have high mineral content even after clean-up with resins. To analyze the FA samples, Amazon-C FA was first prepared using the $CE_{EXTRACTION}$ approach. This approach was chosen as we assumed that the sample is white due to its high content of Al complexes or minerals¹⁰⁰ that had survived cation-exchange and DAX-8 resin purifications. Employing the same solubilization approach (i.e., $CE_{EXTRACTION}$) for both HA and FA fractions would have been ideal for achieving fully comparable data if HA and FA from the same soil are compared. The resultant (-)ESI-FT-ICR mass spectrum was of poor quality

and only 1156 peaks were detected, 792 (58%) of which were salts (Figure 3). The sample also exhibited unstable ionization currents which provided early evidence that inorganic species which were not successfully removed by the CE resin. The molecular formulas corresponding to the 287 assignable peaks did not exhibit characteristic H/C and O/C clustering patterns for FA (Figure SX) but formulas within homologous series (CH₂, H₂O, CO, etc.) were found validating their existence in the Amazon-C sample. Thus, solid-phase extraction using Bond Elut Priority Pollutant (PPL) cartridges⁶⁰ was employed (SPE-PPL approach) yielding a spectrum of very high quality. The observed stable ionization currents during the analysis and the detected 6694 peaks indicated that the SPE-PPL preparation was successful in removing the inorganic impurities.

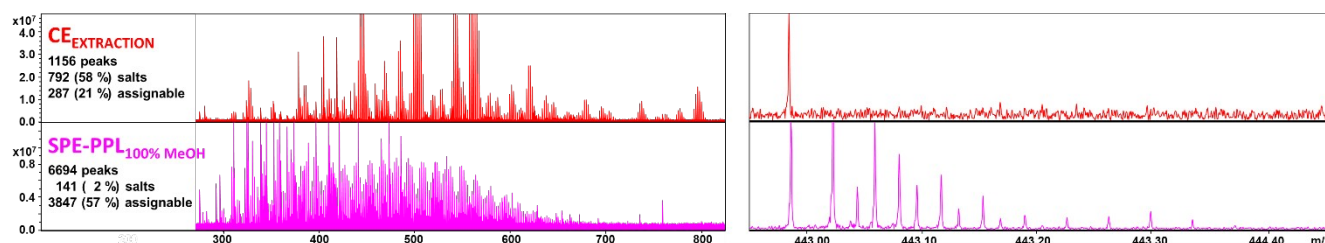


Figure 3: Whole (-)ESI-FT-ICR mass spectra (left) and expanded windows at nominal mass of 443 (right) for Amazon-C fulvic acid prepared using the **CE EXTRACTION** and **SPE-PPL_{100%} MeOH** sample preparation techniques.

Though SPE-PPL is widely used in the environmental sciences, it is well known that it only recovers 40-60% of the sample-carbon which fractionates the sample and biases the composition towards. Thus, we compared SPE-PPL with two more sample preparation techniques: MeOH and MeOH:Water approaches (Figure 4). For these additional tests we used Amazonian-A FA as the Amazonian-C FA was in limited amounts.

We first solubilized the powdered Amazonian-A FA in MeOH (MeOH approach). We assumed that MeOH will only solubilize the organic matter and avoid most inorganic substances due to the poor solubility of inorganic salts in MeOH¹⁰¹. The resultant spectrum was again populated with salt peaks. Then, we mixed the MeOH solution with water, which we have seen sometimes to improve the spectral quality (MeOH:Water approach)⁵⁷. Unfortunately, this approach did not produce a usable spectrum for the Amazonian-A FA either. By contrast, the resultant mass spectrum of following SPE-PPL contained 7553 peaks, which included 2% salt peaks and 4747 assignable peaks. The assigned molecular formulas to Amazonian-A and Amazonian-C spectra after SPE-PPL showed H/C and O/C clustering patterns typical for fulvic acids providing additional support that the SPE-PPL approach was most appropriate for the soil FA samples of this study.

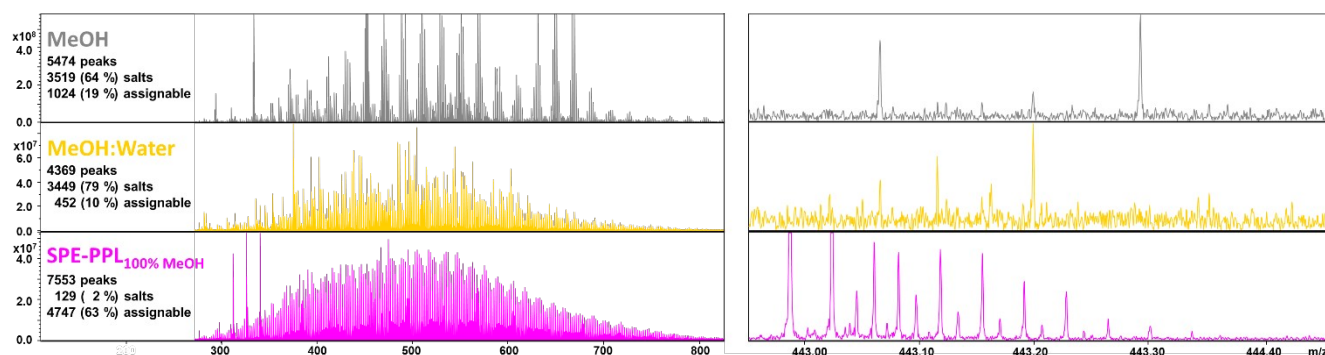
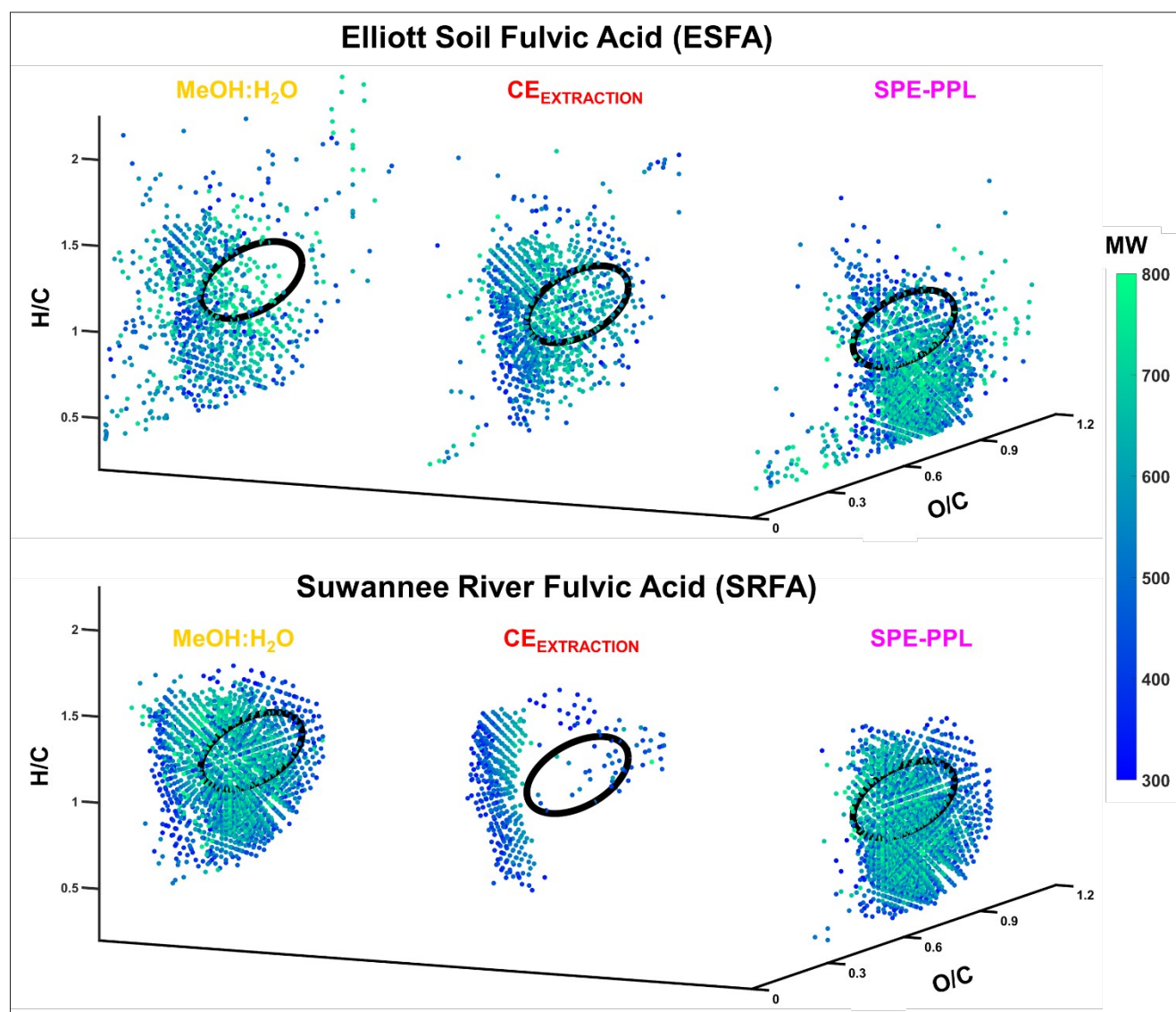


Figure 4: Whole (-)ESI-FT-ICR mass spectra (left) and expanded windows at nominal mass of 443 (right) for Amazon-A fulvic acid prepared using the **MeOH**, **MeOH:Water**, and **SPE-PPL_{100%} MeOH** sample preparation techniques.

However, because the MeOH: Water approach was ineffective for our Amazonian FA samples, and this is the recommended sample preparation technique for standardized FA samples from IHSS⁴³, we suspected that the “most optimal” sample preparation technique will differ for FA of different levels of purity or types of environments (e.g., soil versus aquatic) or matrices (e.g., peat versus loam soil). To investigate this, the Elliott Soil FA (ESFA) and Suwannee River FA (SRFA) samples from IHSS were solubilized using three different approaches: MeOH:Water (recommended for IHSS FA standards⁴³), CE EXTRACTION (most optimal for the soil HA of our study), and SPE-PPL (most optimal for the Amazon soil FA samples).

445 For both ESHA and SRFA, the MeOH:Water approach yielded high number of peaks (4723 and
 446 5681 peaks, respectively) with spectra (Figures SX) comparable to those published by the interlaboratory
 447 comparison study⁴³. Assigned molecular formulas also had typical H/C and O/C clustering patterns for
 448 these two samples (Figure 5)⁴³. For ESFA, the molecular composition following ESFA and CE_{EXTRACTION}
 449 approaches was of high similarity. This was probably because the molecules lost during the SPE-PPL
 450 extraction were likely small and undetectable by (-)ESI-FT-ICR-MS. Similar comparisons of CE and SPE-
 451 PPL preparations on ESFA were published previously paralleling our findings⁶¹. By contrast, the
 452 CE_{EXTRACTION} approach caused a significant loss of lignin and tannin-like compounds of O/C > 0.4 in the
 453 SRFA sample indicating that CE_{EXTRACTION} is not suitable for this matrix. The SPE-PPL approach produced
 454 similar spectra to those after the MeOH:Water approach but a closer inspection revealed that SPE-PPL
 455 skewed the molecular compositions towards a more oxygenated composition causing a loss of molecules
 456 with O/C > 0.5. Thus, for both Elliott soil and Suwannee River FA, the most appropriate sample
 457 preparation technique appears to be MeOH:Water concluding that the “most optimal” sample preparation
 458 technique will vary with the type of FA sample (e.g., aquatic versus soil) and its matrix constituents (e.g.,
 459 inorganic contents). These findings validated that the MeOH:Water sample preparation technique is the
 460 most suitable approach for working with SRFA and ESFA, which are key reference materials used for
 461 mass spectrometric tuning, tune validation and general quality control, and for ensuring inter- and intra-
 462 laboratory data comparability⁴³. It must be noted that SPE-PPL greatly enhanced the detection of CHON
 463 species which might make the SPE-PPL technique more optimal for cases where soil nitrogen dynamics
 464 are researched.
 465



466
 467 **Figure 5.** Presence/absence analysis⁹⁹ on Elliott soil fulvic acid (ESFA, top) and Suwannee River fulvic
 468 acid (SRFA, bottom) molecular compositions obtained following MeOH: Water, CE_{EXTRACTION}, and SPE-PPL
 469 solubilization approaches. Shown are van Krevelen diagrams (H/C versus O/C molar ratio plots) of unique

formulas to two or three samples. Formulas are color-coded based on molecular weight (MW). The black ovals serve as visual references for comparisons. Common formulas are shown in Figures SX and SX.

The SPE-PPL technique is highly utilized in the community of aquatic sciences, but little knowledge exists on how data compares if the methanolic eluent is analyzed with 2-fold dilution with MeOH (i.e., as 100% MeOH) or with Water (i.e., as 1:1 MeOH:Water). Different research groups analyze SPE-PPL extracts differently without published rationale for the chosen solvent system. We have found that the addition of water can sometimes improve the spectral quality, but in other cases can deteriorate it. We have seen that this can vary even within the same type of sample matrix. For example, biochar extracts analyzed for the study by Wozniak⁵⁶ gave the highest quality spectrum following SPE-PPL_{100% MeOH} whereas biochar extracts analyzed for the studies by Goranov^{57, 102} gave the highest quality spectrum following SPE-PPL_{1:1 MeOH:Water}. Samples were analyzed by the same analyst and sample preparation technique comparisons were made for each dataset. To test this for a soil FA, we analyzed ESFA using both SPE-PPL_{100% MeOH} and SPE-PPL_{1:1 MeOH:Water}. The addition of water reduced the total number of peaks from 6209 to 5006 and caused molecules with O/C > 0.5 to be suppressed whereas molecules with O/C < 0.5 to be preferentially ionized (Figure SX).

3.3 Implications and recommendations for future studies

The employment of sample preparation techniques for HA and FA fractions can significantly alter the analytical window of the (-)ESI-FT-ICR-MS technique. The utilization of MeOH or pyridine as solvents did not provide representative spectra of the samples of this study likely due to the incomplete HA solubilization and the enhanced ionization of aliphatic compounds at the expense of aromatic ones (Figure 1). However, it must be noted that the observed trends in this study are possibly specific to our sample matrices: the agricultural and forest HA samples used here were not fully soluble in MeOH but MeOH had been previously successful in solubilizing the aquatic SRHA without the addition of NH₄OH or NaOH⁴⁴. Thus, MeOH and Pyridine approaches should not be disregarded and be tested in future studies for HA matrices other than the ones employed here. The two approaches that were shown most prominent for the HA of our study, NH₄OH and CE_{EXTRACTION}, produced high-quality spectra but introduced biases in the observed molecular composition caused from different solvent composition affecting the (-)ESI. The CE_{EXTRACTION} approach has been rarely employed³ while using NH₄OH has been the most common approach in HA analysis^{35-37, 44, 51, 53-55}. Furthermore, while we did not observe this in our study (see section X of the SI), NH₄OH has been shown to react with molecules and produce N-adducts⁵⁸. Thus, it is likely that previously published HA data has been biased towards a more aliphatic (H-rich), N-rich, and less oxygenated (O-poor) composition, which may lead to false interpretations that the refractory HA fraction of SOM is biologically reactive and possibly labile towards microbial consumption.

The Amazonian FA samples of our study had challenging salt-rich matrices and the organic matter could not be purified using CE or DAX-8 resins. The SPE-PPL approach is rarely used for analyzing FA samples⁶¹ but here we show that it has a significant merit for preparing FA samples of challenging composition (e.g., with high inorganic content). This agrees broad application of SPE-PPL in desalinating marine and other inorganic-rich aquatic matrices^{60, 75} but questions remain regarding the molecular fractionation following SPE-PPL. Our comparisons reveal that SPE-PPL fractionates the sample causing a loss of less oxygenated molecules, but this may be unavoidable for salty FA matrices. For cleaner FA matrices, such as ESFA, employing SPE-PPL is not necessary as both MeOH:Water and CE_{EXTRACTION} produced comparable data. Similar comparisons by Han⁶¹ agree with our results for ESFA and disagree with those for SRFA, likely because of the significant differences among soil and aquatic molecules and how their chemistries.

While we make conclusions for the “most optimal” approach in our study, we must emphasize that these approaches are in no way universally optimal. Our work shows that the “most optimal” sample preparation technique is dependent on the type of sample and its matrix. Thus, we recommend that the most effective sample preparation technique is determined on a per-dataset basis. For future studies researchers should identify 1-3 key samples that are representative of the particular sample matrix of a dataset and test several different sample preparation techniques to determine the most optimal sample preparation technique. Performing such methodical comparisons will enhance (-)ESI-FT-ICR-MS data quality allowing us to better understand the molecular-level role of HS in the global biogeochemical cycles.

This study fills a key research gap in our knowledge on the (-)ESI-FT-ICR-MS analytical window as well as on data comparability. Ideally, all samples should be prepared using the same technique, but this is incredibly challenging for HS as the HA and FA fractions are incredibly different in their composition. We showed the utility of CE_{EXTRACTION} for HA analysis and our comparisons on ESFA revealed that CE_{EXTRACTION} is useful for FA analysis too. Thus, depending on the sample matrix, it may be possible that both HA and FA fractions of certain datasets are prepared using the same techniques. The large differences in trends

531 of aquatic versus soil HS indicate that comparing terrestrial and aquatic data is extremely challenging and
532 more universal sample preparation techniques should be developed for such comparisons.

533 Any future studies combining datasets should carefully assess what sample preparation techniques
534 were used and consider possible ways to trim formulas that are not reproducible among methods. For
535 example, one possible way for this would be to align all formulas throughout the dataset and trim formulas
536 found in only one sample while keeping formulas found in at least two (or more) samples. Another
537 possible option would be to optimize for higher signal-to-noise peak picking thresholds removing
538 irreproducible peaks of poor signal-to-noise from the spectra⁹⁹. Such refinements are incredibly important
539 for combining datasets and assessing them using multivariate statistical methods¹⁰³. The usage of multiple
540 sample preparation techniques, on the other hand, can be useful in expanding the ESI-FT-ICR-MS
541 analytical window and by combining the data of the multiple preparations one can obtain a more
542 comprehensive catalog of molecular formulas for a particular sample. Though only (-)ESI was employed
543 here, it is well known that other ionization techniques can provide additional aspects to the molecular
544 composition of environmental matrices. Thus, future studies should consider other ionization methods,
545 such as positive-mode ESI or atmospheric pressure photoionization (APPI) techniques, and perform
546 similar sample preparation technique comparisons to assess data comparability. This would advance the
547 knowledge of the complex composition and heterogeneity of HS and more broadly enhance our
548 understanding of the global biogeochemical cycles.

549

550 SUPPORTING INFORMATION

551

552 Graphical description of sampling sites; Sample preparation flowchart for the CE_{EXTRACTION} approach;
553 Description of ESI-FT-ICR-MS tuning and quality control; Mass spectra of procedural blanks; Assigned
554 molecular formulas and van Krevelen diagrams; Discussion of mass spectral multimodality; Discussion of
555 potential nitrogen-incorporation.

556

557 Mass spectral data (formula assignments) has been published in the *Mendeley Data* repository⁷⁹ (doi:
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559

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561

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