On-line nano-solid phase extraction Fourier-transform ion cyclotron resonance mass spectrometry workflow to analyze small scale gradients of soil solution organic matter in the rhizosphere

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Abstract

A new method combining on-line nano-solid phase extraction coupled with Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) was developed to extract and analyze organic matter (OM) from microliter volumes of salt containing soil solution samples. The system allows the reproducible analysis of only minute amounts of organic carbon (down to 10 ng C) without the need of further sample preparation. The new method was applied to unravel developing small-scale patterns of dissolved organic matter (DOM) in soil solution of a soil column experiment in which *Zea mays* plants were grown for three weeks. Soil solution was sampled by micro suction cups from the undisturbed soil-root system once a week. Growth of the root system and, hence, position of individual roots relative to the suction cups was followed by X-ray computed tomography (X-ray CT). Our method allowed resolving the chemical complexity of soil solution OM (up to 4300 molecular formulas). This makes it possible to observe chemical gradients in the rhizosphere on a molecular level over time. The increasing influence of roots on soil solution OM is visible from higher molecular masses, an increasing degree of oxygenation and a higher fraction of formulas containing heteroatoms. The on-line nano-solid phase extraction-FT-ICR-MS method

provides novel insight into the processes affecting DOM in the rhizosphere, such as root exudation, microbial processes, and soil organic matter stabilization.

Soil organic matter (SOM) formation and composition are highly influenced by the interaction with plants via the root system. Comparison of biomarkers for roots and shoots indicates that root-derived carbon dominates organic matter (OM) formation in agricultural soils compared to above-ground litter.[1] The presence of bioavailable carbon sources, such as root exudates, for soil microorganisms can influence the microbial growth strategy, suggesting a strong effect of root exudation on microbial communities.[2]

The temporal and spatial heterogeneity in the rhizosphere (i.e. the soil influenced by roots) has already been studied in terms of chemical composition,[3] nutrient level,[4] physical parameters like hydraulic properties,[5] oxygen,[6] and pH,[7] as well as in the organization of microbial communities.[8] It is apparent that analyzing root mediated processes requires a time resolved, non-destructive sampling operating on the small spatial scale of the rhizosphere (0.5 – 4 mm).[9]

Soil solution is a highly dynamic component of the rhizosphere, enabling mass flux of carbon and nutrients. Direct sampling of soil solution (e.g. via micro suction cups (MSC)) yields *in situ* information about the rhizosphere state and processes[10,11] like nutrient dynamics[12,4] and organic acids turnover.[13,10] As an alternative to MSC, micro-dialysis has also been applied to sample the dynamic pools of enzymes,[14] nutrients[15], and amino acids.[16] A non-targeted view into the vast number of organic components present in the rhizosphere would help to reveal the complex interplay of root mediated carbon input, microbial degradation, and carbon sequestration beyond the individual compound level.

Previous non-target studies of soil solution OM used sample collection by suction cups, subsequent solid phase extraction (SPE) and analysis by FT-ICR-MS.[17] SPE by packed sorbents is a standard method to desalt and enrich aqueous OM samples.[18] Hyphenation of SPE and MS measurements has been demonstrated for OM utilizing a liquid chromatography (LC)-system.[19] An automated on-line micro-extraction by packed sorbents was used for the direct analysis of salt rich marine samples at the scale of 2 mL sample volume.[20] However, a further downscaling of packed column SPE workflows for enrichment and desalting of OM-samples does not seem feasible

due to the high risk of sample contamination and a lack of commercially available solid phases with less than one mg of sorbent mass.

Bailey et al. presented results for the direct infusion (DI) analysis of soil solution utilizing FT-ICR-MS.[21] However, high and varying salt concentrations usually limits the application of direct infusion of samples into the mass spectrometer due to ionization suppression.[22,23] It is also possible to study the organic carbon distributions in soil samples using destructive extraction based or imaging methods.[24–26] A workflow for an on-line extraction method for small quantities of solid soil samples was recently presented by Shen et al. who utilized on-line supercritical fluid extraction mass spectrometry (SFE-LC-FT-MS) for the analysis down to 1 mg of soil.[27]

All approaches to analyze soil solution or soil OM mentioned above either require several milliliters of sample volume or use destructive methods for sample acquisition and thus lack the required spatial and temporal resolution needed to reveal the processes caused by the interplay of root structure and OM in the rhizosphere.

The aim of this study was to develop a sample preparation and measurement workflow for the extraction of OM from a few microliters of soil solution. Analysis via FT-ICR-MS allows for a non-targeted molecular insight into small scale rhizosphere processes despite the high salt concentrations in the soil solution samples. To account for the limited (microliter) sample volume and its high ratio of salt to organic carbon (approx. 100:1 m/m) a robust and sensitive on-line nano-SPE-FT-ICR-MS based workflow for the on-line extraction and direct analysis of soil solution OM was developed and validated.

The combination of molecular information from FT-ICR-MS with the structural data of the root system as provided by X-ray computed tomography (X-ray CT) will allow for the first time a non-destructive, time resolved analysis of complex rhizosphere biogeochemical processes at high spatial resolution (Figure 1).

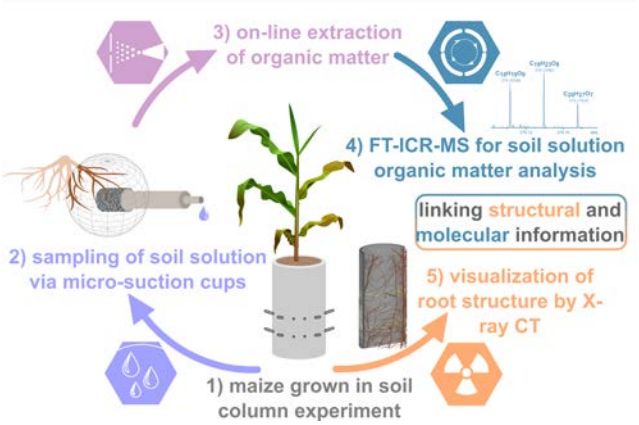

Figure 1: Workflow to link information about root structure to changes in the molecular composition of soil solution OM in the rhizosphere. It consists of: 1) growing maize in soil columns, 2) sampling of soil solutions via micro-suction cups and 3) apply on-line extraction of OM (on-line nano-SPE) with 4) subsequent analysis of soil solution OM by ultra-high resolution FT-ICR-MS and 5) X-ray CT to visualize the root structure.

Experimental section

Samples

Soil solution samples were collected three times (7 d, 14 d, 21 d) at 16 cm below the soil surface during a three week growth of *Zea mays* in a soil column experiment. Experimental details on the preparation of the soil column experiment, the soil solution sampling, X-ray CT[12,28], and anion chromatography can be found in the supporting information (SI) to this article.

Suwannee River Fulvic Acid-standard (SRFA II, International Humic Substances Society, 20 mg L^{-1}, approx. 10 mg L^{-1} carbon) was used for the on-line nano-SPE method development. For the evaluation of eluent composition, formic acid concentration, and comparison to DI-ESI measurements a SRFA concentration of 10 mg L^{-1} was used. Although not a soil solution OM, SRFA represents a well characterized complex OM mixture.[29]

Effective separation of salts from OM was tested by adding sodium chloride (NaCl, 1 g L^{-1}) to SRFA, similar to OM-to-salt ratios (1:100, min. 25 mg L^{-1} dissolved organic carbon (DOC)) determined in soil solutions. Total salt concentration was approximated based on the anion concentration assuming that all anions are present as the respective sodium salt (max: 2.5 g L^{-1} salts). SRFA and soil solution samples were diluted with the aqueous eluent (1:2, water with 0.005 vol-% formic acid) immediately prior to analysis.

Chemicals

Details regarding all the used chemicals can be found in the SI (Table S1).

On-line nano-solid phase extraction

The nano-LC system (Ultimate 3000 nanoRSLC, Thermo Fischer Scientific, Waltham, MA, U.S.A.) consisted of a pump and autosampler with 5 µL sample loop. The sample was retained on a C-18 precolumn (Acclaim™ PepMap™ 100, 2 cm x 75 µm, 3 µm, Thermo Fischer Scientific, Waltham, MA, U.S.A.) and eluted with 90% MeOH, 10% water (both with 0.005 vol-% formic acid). A 10-way valve was used to direct the salt containing matrix to the waste while allowing the OM-fraction to pass to the nano-ESI source and FT-ICR-MS.

The nano-LC gradient was modified to allow separation of salts from OM in a low overall run time (29 min) while ensuring stable and reproducible spray conditions. For the washing and equilibration steps, the flow rate was set to 1500 nL min^{-1}. During the elution of OM the flow rate was lowered to 200 nL min^{-1} in order to allow sufficient time for the acquisition of scans in the FT-ICR-MS. After the OM was eluted the flow rate was increased again to flush the system. HPLC-grade water and methanol with 0.005 vol-% formic acid (pH of aqueous eluent 3.4 at 22 °C) were used as eluents. Eluents with pH 8 (2 mM ammonium acetate adjusted with NH_4OH), pH 4 (2 mM ammonium formate adjusted with formic acid), and pH 6.22 (no additives to eluent) were compared to test the effect of the eluent pH on the extraction reproducibility. A scheme of the nano-LC setup is shown in Figure S1 and the gradient conditions are provided in Table S2.

FT-ICR-MS-measurements

All MS measurements were performed with an FT-ICR mass spectrometer with a dynamically harmonized analyzer cell (solariX XR, Bruker Daltonics, Billerica, MA, U.S.A.) and a 12 T refrigerated actively shielded superconducting magnet (Bruker Biospin, Wissembourg, France). The mass spectrometer was controlled with ftmsControl 2.2.0 (Bruker Daltonics, MA, U.S.A.). Mass spectra were recorded in the mass range setting 147 – 1000 m/z in magnitude mode (four megaword time domain, 1.677 s transient length) and reduced profile mode (97% data reduction). External mass calibration was done with SRFA. A nano-electrospray ionization (nano-ESI) source for the nano-LC-coupling, CaptiveSpray-Source (Bruker Daltonics, Billerica, MA, U.S.A.), was used in negative mode. Parameters for the Captive Spray nano-ESI

source were as follows: dry gas temperature: 150 °C, dry gas flow rate: 3.0 L min^{-1}, capillary voltage: 1300 V. The same conditions were applied for the nano-solid phase extraction and DI-nano-ESI measurements. For DI-nano-ESI measurements the C-18 precolumn was removed from the nano-LC system.

DI-ESI-FT-ICR-MS measurements

A standard ESI source (Apollo II, Bruker Daltonics, Billerica, MA, U.S.A.) in negative ionization mode (capillary voltage: 4.3 kV, flow rate: 240 µL h^{-1}, dry gas temperature: 200 °C, dry gas flow rate: 3.0 L min^{-1}, nebulizer gas flow rate: 1.0 bar) was used for direct infusion measurements. For one mass spectrum 256 scans were co-added in the mass range 147–1000 m/z.

Data processing

Mass spectra from LC acquisition runs were averaged from 7 to 15 min (approx. 247 single scans) to generate the mass spectrum of the OM-containing fraction.

Internal re-calibration of averaged spectra was done with a list of masses commonly present in natural organic matter (m/z 250–600, n = 188, linear calibration function). The root mean square error (RMSE) of the calibration masses was below 0.2 ppm. Peaks were considered detected if the signal-to-noise (*S/N*) ratio was greater than four. Raw spectra were processed with Compass DataAnalysis 5.0 (Bruker Daltonics, MA, U.S.A.).

Molecular formulas (MF) were assigned to peaks in the range 150–750 m/z allowing for elemental compositions $C_{1–60}$ $H_{0–122}$ $O_{0–40}$ $N_{0–2}$ $S_{0–1}$ with an error range of ±0.5 ppm according to Lechtenfeld et al.[30] and Koch et al.[31] Briefly, the following rules were applied: 0.3 ≤ H/C ≤ 3.0, 0 ≤ O/C ≤ 1.2, 0 ≤ N/C ≤ 1.5, 0 ≤ DBE ≤ 25 (double bound equivalent, DBE = 1 + 1/2 (2C − H + N), Koch et al.),[32] −10 ≤ DBE-O ≤ 10 (Herzsprung et al.[33]), and element probability rules proposed by Kind and Fiehn.[34] Isotope formulas were removed from the data set as they represent duplicate chemical information. The mass error range in the final data set was limited to the 5^{th}–95^{th} percentile of errors of CHO formulas in the initial data set (here approx. ± 0.45 ppm). *S/N* for the soil solution analysis was set to 8 as explained in the results and discussion section. All MF present in the MSC or eluent blank samples were removed from the final data set.

Results and Discussion

Optimization of the on-line nano-solid phase extraction method

Most studies focusing on the characterization of OM apply direct infusion-(DI)-ESI FT-ICR-MS after removal of salt and OM enrichment via SPE.[35] Enrichment and desalting are crucial as ionization of OM can be largely suppressed by salts.[23] However, extraction of OM from soil solution samples from column experiments of only 50 – 150 µL could not be achieved with a conventional off-line micro-SPE and subsequent DI-nano-ESI measurement (using 10 mg sorbent, Figure S2). Although OM-signals were detected, contaminant peaks and salt clusters dominated the mass spectrum resulting in low sensitivity and incomplete coverage of OM complexity. Combining the benefits of an automated miniaturized SPE for desalting with the low risk of contamination was possible using a nano-LC-nano-ESI system which can be directly hyphenated with a mass spectrometer. The main advantage of a nano-LC-system is the low flow rate which allows injection and analysis of small sample volumes with limited dilution of the analytes prior to injection. In addition, nano-ESI offers a higher tolerance for buffers and salt as well as increased sensitivity compared to ESI.[36] For FT-ICR-MS hyphenation, the low flow rate allows for more scans and a higher intensity per sample volume since the duty cycle of the MS is mostly limited by the ion detection speed and not their accumulation time.

Figure 2A shows an example chromatogram and the averaged mass spectrum of 20 mg L^{-1} SRFA with 1 g L^{-1} of NaCl of the optimized method using eluents with pH 3.4. As discussed, the most important factor is the ability of the method to remove salts from the soil solutions. It was possible to generate a mass spectrum with a typical OM pattern (Figure 2B-D). A 100-fold excess of salt over organic carbon was present while injecting only 5 µL of sample. The total amount of organic carbon needed to generate the mass spectrum was just 25 ng. This is more than three orders of magnitude less material than required by the recently published on-line SFE-LC-FT-MS-workflow.[27]

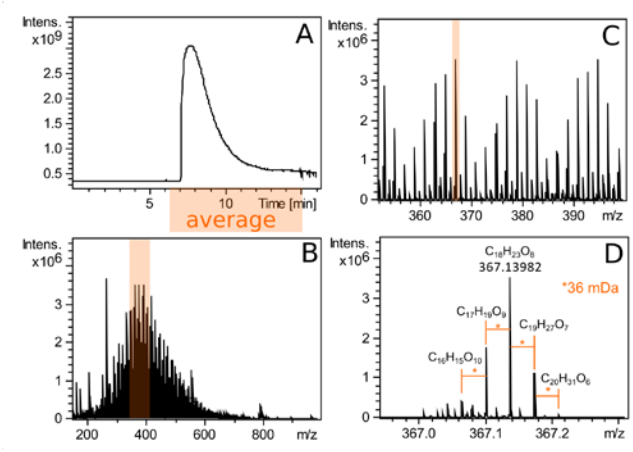

Figure 2: Extraction and measurement of OM samples with the on-line nano-SPE method at pH 3.4. (A) Total ion count (TIC). (B) Averaged full scan FT-ICR mass spectrum (7 – 15 min, 250 scans). (C) Zoom into the mass spectrum (m/z 340 – 400) with regular patterns of OM. (D) Zoom into nominal mass 367 (most intensive OM peak). Mass spacing of 36 mDa (indicated by an asterix) represents the mass difference of an exchange of "O" vs "CH_4" as indicated by the ion formulas of the CHO class. 20 mg L^{-1} SRFA with 1 g L^{-1} NaCl, 5 µL injection volume, diluted 1:2 with aqueous eluent (water with 0.005 vol-% formic acid).

Even from 10 ng of organic carbon with a 600-fold excess of salt (6 g L^{-1}) a spectrum with the typical OM pattern could be obtained (Figure S3) showing the feasibility of our on-line nano-SPE method for even smaller amounts of carbon and matrices with higher salt content. Further increasing salt concentration in the sample led to a loss in the number of assigned MF (Figure S4) so that sample dilution was required (see below).

Optimization of the solvent composition

DI-ESI experiments using SRFA (10 mg L^{-1}) were performed to determine the optimal solvent composition for OM analysis. The highest number and highest reproducibility of MF were obtained using 90% MeOH (Table S3). This agrees with previous findings that a high organic solvent fraction is advantageous for ESI analysis of OM.[37] Adding a buffer to the eluent (e.g. ammonium formate, pH 4) decreased the number of assigned MF significantly for negative ionization mode due to signal suppression.[38] However, diluted formic acid (0.005 vol-%, pH ~ 3.4) is already sufficient to keep the pH of OM solutions constant despite the acidic functional groups of the components in SRFA (Figure S5), while minimizing ion suppression (Figure S6).

Optimization of the eluent pH

The effect of pH of the eluent was tested for a range of pH values (4, 6, and 8, Figure S7). Although a similar number of MF (5300) could be assigned after on-line extraction

of OM (20 mg L^{-1} SRFA with 1 g L^{-1} NaCl) for all three pH values tested, the fraction of shared formulas between triplicate measurements at each pH varied between 1728 (33% of all unique formulas), 1018 (19%) and 1380 (26%) for pH 8, 6 and 4 respectively. A low pH generally favors the retention of humic and fulvic acids.[39] However, the number of nitrogen-containing formulas was slightly higher at pH 8 (7.9% of MF) as compared to pH 4 (5.4%) indicating an increase of retention- and/or ionization efficiency for basic compounds at higher pH (Figure S7).

To allow for a complete protonation of acidic components and an overall better retention and reproducibility on a C-18 phase a formic acid buffer at pH 3.4 was used for further analysis. The applied one-step-"elution" of OM with 90% methanol has two major advantages i) it allows for fast run times as most OM fractions elute together and ii) it provides constant eluent composition for ESI and hence less discrimination due to varying ionization conditions.[40,37] The elution of OM in one step for an on-line extraction is thus different from an actual chromatography of OM as described in literature for off-line,[41] offline 2D-,[42] and on-line LC separations.[43,44]

FT-ICR-MS parameter optimization for on-line extraction

Mass resolution and mass accuracy in FT-ICR-MS are strongly depended on the number of ions in the ICR cell, which can be controlled via the ion accumulation time (IAT).[45]

Optimization of the ion accumulation time

The high sensitivity of the FT-ICR-MS allows that soil solutions can even be diluted before the on-line extraction to lower the salt concentration. The corresponding decrease in organic carbon concentration may be compensated for by increasing the IAT. To find the optimal ratio between dilution and IAT, a mixture of 20 mg L^{-1} SRFA with 1 g L^{-1} NaCl was diluted with the aqueous eluent and processed with the on-line nano-SPE and FT-ICR-MS measurements at different IATs. As expected, increasing the IAT led to a larger number of assigned MF (Table 1). The results indicate that the reduction of ion suppression via sample dilution has a larger effect than the loss of sensitivity on the number of detected MF. Since all the samples were diluted with the aqueous eluent, an undiluted sample has a higher pH (~ 5.2, Figure S5), the OM is less retained and as a result, a lower number of formulas could be assigned.

Table 1: Effect of dilution of a 20 mg L^{-1} SRFA sample with 1 g L^{-1} NaCl and variation in ion accumulation time (IAT) on the molecular formula (MF) assignment and the intensity weighted averaged (WA) molecular parameters. The aqueous eluent (water with 0.005 vol-% formic acid) was used for dilution.

Dilution factor	**none**	**2**	**2**	**5**	**10**
IAT (ms)	10	5	10	25	35
number of MF	1902	2182	3003	3731	3976
RMSE of assigned formulas (ppb)	200	190	174	179	172
Intensity of highest OM Peak *m/z* 363.1449 (10^5)	7.3	3.4	7.9	16	18
TIC (10^8)	7.1	5.4	7.3	11.6	12.8
WA *m/z*	440.8	462.7	467.6	457.7	451
WA O/C	0.36	0.41	0.42	0.44	0.45
WA H/C	1.24	1.18	1.14	1.12	1.13

RMSE: root mean squared error, TIC: total ion count

Expectedly, higher IAT resulted in increased peak intensities (e.g. as indicated from the total ion count (TIC)) while the RMSE of formula assignments remained at sub-ppm level (Table 1). However, the mass error distribution at high IAT (above 25 ms) reveals a bimodal pattern, likely due to a too high number of ions of the ICR cell (8). All the spectra were dominated by formulas of the CHO class. With increasing IAT a higher number of heteroatom containing MF could be observed (Figure S9).

Optimization of the sample dilution

To account for possible variation in the organic carbon concentration of soil solutions, three replicates of SRFA and a soil solution were analyzed at 2- and 4-fold dilution using the on-line nano-SPE method. While the number of assigned MF in the soil solutions seems to be independent of the dilution (dilution 1:2 and 1:4 tested), the reproducibility of the number of shared formulas between three measurement replicates was at a maximum after a 1:2 dilution of the samples (Table S4). Decreasing carbon concentration due to dilution did not affect the quality of OM mass spectra. The two soil solution samples and the SRFA sample grouped acceptably according to their aggregated molecular parameters (Table S4). We conclude that our method is robust against DOC concentration variability among different soil solution samples.

The major difference between the replicate measurement of a soil solution and SRFA was the higher fraction of MF unique for a single measurement (Table S4). Increasing the *S/N* threshold of MF in the final data set reduced the number of unique assignments (Figure S10). As a compromise between the number of assigned MF and non-reproducible peaks, the *S/N* threshold for the analysis of soil solutions was set to eight resulting in 50% MF shared between triplicate measurements. For SRFA the increase of the *S/N* threshold led to 61 % shared formulas between triplicates, while for the higher dilution level of the soil solution the reproducibly remained lower at 38% (Table S4).

This value is lower than reported for DI-ESI FT-ICR-MS measurement.[37] In contrast to published values of mass spectral reproducibility, we cannot distinguish extraction and MS effects on reproducibility, and a small influence of the sample matrix cannot be excluded for our on-line method. Another explanation for the lower spectral reproducibility is the data reduction during MS acquisition as differences in baseline noise between replicates affects the number of detected signals irrespective of a post-measurement *S/N* filtering.

For further analysis of soil solution samples with the on-line workflow all samples were diluted 2-fold, the IAT set to 10 ms, and *S/N* threshold set to 8.

Comparison between on-line nano-solid phase extraction and direct infusion measurements

To assess systematic differences between our on-line nano-SPE and the conventional DI-ESI method six replicates of SRFA were analyzed with both methods.

A similar number of MF could be assigned in SRFA for the on-line nano-SPE as compared to a DI measurement (Table 2). Mass accuracy and mass-resolving power was slightly lower during the on-line extraction which can be explained by the averaging of a transient signals with variable ion numbers causing small shifts of the ion cyclotron frequencies.[46]

The on-line nano-SPE method generally resulted in a higher intensity weighted average (WA) H/C ratio and a lower O/C ratio compared to the DI-ESI (Table 2). This effect can mostly attributed to the different ion sources (nano-ESI vs conventional ESI), source parameters, and solvent composition used.[37,47] An additional, however smaller, bias of the nano-LC pre-column on the OM composition was also observed (Figure S11). In addition, the average m/z of assigned MF also increased by 20% with the new

on-line nano-SPE workflow as compared to DI-ESI measurements, indicating a better coverage of the OM mass distribution. Since standard DI-ESI is also inevitably selective on the determined OM composition, the difference caused by the application of different ionization sources was expected.[47]

Table 2: Comparison of the new on-line nano-solid phase extraction workflow and DI-ESI regarding spectral quality, number of molecular formulas (MF), Intensity weighted average (WA) molecular composition (mean ± standard derivation, *n* = 6). For the on-line nano-SPE 20 mg L^{-1} SRFA was prepared in water (10 ms IAT). For the DI-ESI measurements 10 mg L^{-1} SRFA was prepared in 50% MeOH / 50% water (15 ms IAT).

Sample introduction to FT-ICR-MS	On-line nano-SPE	Direct infusion ESI
Number of MF	3045 ± 153	2951 ± 177
RMSE formula assignment (ppb)	148.7 ± 13.2	108.4 ± 1.93
Mass-Resolving power at *m/z* 400 ± 1	432783 ± 27102	480881 ± 38919
WA *m/z*	503.7 ± 2.2	391.38 ± 10.2
WA H/C	1.196 ± 0.009	1.112 ± 0.006
WA O/C	0.388 ± 0.007	0.469 ± 0.014
Volume of Sample consumed per run (µL)	2.5	31
Mass of carbon used for spectrum generation (ng) per run	25	155

For one SRFA-standard without salt addition, we tested if a fractionation of OM on the C-18 phase may contribute to the observed differences in molecular composition (Table 2, Figure S11) between the two methods. The applied gradient program for the on-line extraction resulted in a hydrophilic fraction eluting at high water content (99%) whereas the later eluting hydrophobic fraction (90% MeOH) contributed to the majority of the total intensity (Figure S12). The majority of MF (98%) was detected in the hydrophobic fraction indicating no major loss of molecular information from highly polar OM compounds due to the on-line extraction method.

The early elution of very polar compounds was also observed applying an on-line LC-ESI-FT-ICR-MS method for the separation of OM.[44] As the hydrophilic fraction is co-eluting with salts, highly polar OM may not be detected with our method. Raeke et al. showed that when applying standard off-line SPE protocols, small and very polar

compound classes like carbohydrates have very low SPE recoveries and are also negatively biased in DI-ESI-FT-ICR-MS.[23]

Alternatively to OM extraction, also direct sample infusion in negative ion mode after dilution with methanol (i.e. without extraction) could be an option for OM analysis if the sample is not acidified with mineral acids (e.g. HCl).[21] Using our soil solution samples with approx. 2.5 g L^{-1} of salt DI-ESI-FT-ICR mass spectra were dominated by salt clusters. In contrast, our on-line nano-SPE method achieves a much cleaner spectrum and a larger number of OM-signals (Figure S13), demonstrating the necessity of an extraction step for soil solution samples.

Application of the method to soil solution samples from column experiments

The on-line nano-SPE-FT-ICR-MS method was applied to study chemical gradients of OM developing in the rhizosphere during plant growth in a soil column experiment. Two micro suction cup positions (MSC I and II) were selected based on the X-ray CT images (Figure 3A, B). According to X-ray CT maize roots developed in the proximity of both MSCs between day 7 and day 21 of the growth experiment, with a higher root density around MSC II (Figure 3C, D, Figure S14). For each sampling time, 2.5 µL soil solution collected from the MSCs were measured with on-line nano-SPE-FT-ICR-MS. Between 500 and 4300 MF were assigned within the mass range of 150 to 750 Da (Figure 3B). Although the on-line extraction only used 2.5 µL of soil solution sample, approximately twice as many formulas could be assigned as compared to published results using off-line extraction of rhizosphere soil.[24]

With increasing root biomass the composition of the OM shifted towards higher O/C and lower H/C ratios in both MSCs (Figure 3A). This trend was stronger for MSC II with a higher root length density (RLD; 5.94 cm cm^{-3}) than MSC I (1.49 cm cm^{-3}) at day 21 (Figure 3A). The detected differences in the aggregated elemental ratios regarding the time series and the different RLD were always larger than the replicate measurement variability (Table 2, Table S4).

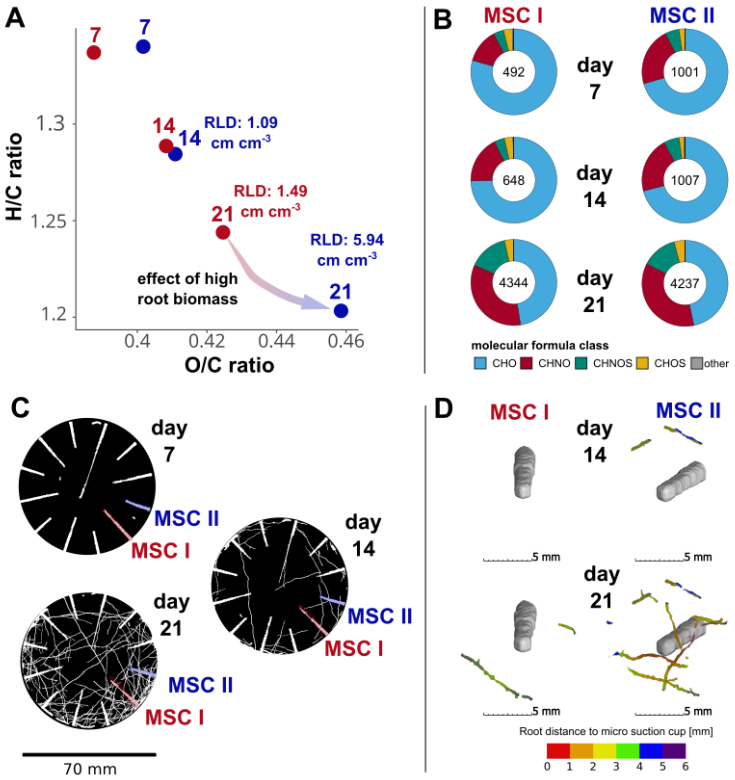

Figure 3: Soil solution OM gradient during plant growth: application of on-line nano-SPE A) Peak intensity weighted aggregated van Krevelen diagrams for soil solution samples of three time points (7 d, 14 d, 21 d, number above circles) and two MSCs (I: red, II: blue) of the same *Zea mays* plant. B) Relative ratio of molecular formula (MF) classes (CHO: blue, CHNO: red, CHNOS: cyan, CHOS: yellow, other: gray) for all six samples. The number in the center of the charts are the total number of MF. C) 3D X-ray CT-images with 2D maximum intensity projections in the soil layer defined by the MSCs at 16 cm below the soil surface. Roots and MSCs have the same X-ray attenuation and both structures appear white in the images. A larger version of the projections is available in the SI (Figure S14). D) 3D reconstruction of the roots close to the two MSC. Roots inside a cube (V = 1 cm^3) around the MSC tips are shown, and colors indicate the distance of the respective root segment to the center of the MSC.

The 5-fold difference of the local RLD in the soil volume accessible by the MSC I and II was mirrored in a distinct occurrence of MF assigned to MSC I and II samples (Figure 4). Expectedly also the intensity ratio of MF present in both MSC samples (I and II) showed the same trend towards higher O/C and lower H/C ratios (Figure 4) which could also be observed for the other soil solution samples (Figure S15).

Next to root-derived carbon, all soil solution samples also contained a background of complex soil solution OM with extensive isobaric overlap. However, the addition of oxygen-rich root-derived molecules was easily detected already at the nominal mass

level (Figure S16). Similarly, an addition of new, oxygen rich molecules (which were not present at day 7) to the soil solution was observed (Figure 3B, Figure 4, Table S5, Figure S17).

In addition, to the higher degree of oxygenation in rhizosphere OM, Kaplan et al.[24] also showed that rhizosphere OM had a higher WA molecular weight compared to soil less influenced by roots. We could observe a similar trend regarding the WA molecular weight over the growth period of three weeks. The high fraction of heteroatom containing MF in the rhizosphere also matched our findings (Figure 3B and Table S5).

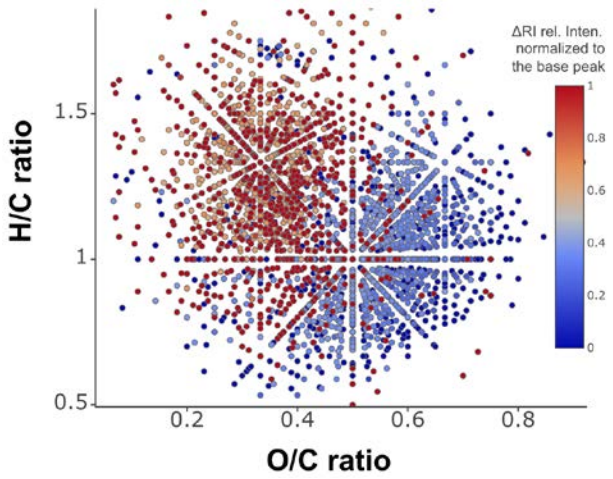

Figure 4: Overlay of van Krevelen diagrams for the two analyzed soil solutions from MSC I and II on day 21. Molecular formulas (MF) unique for a high root biomass in the proximity of the MSC (MSC II, dark blue, 701) and a lower root biomass (MSC I, dark red, 808) are highlighted. MF were the base peak normalized relative intensity is more than 50 % higher intensity in one sample is colored either: blue (MSC II, 979) or red (MSC I, 490). MF with no significant difference in the intensity (2067) are not shown.

The potential of the new method was demonstrated by showing trends in the soil solution OM composition related to RLD and root age. Despite the numerous analytical challenges like the background of soil OM, a high salt-to-OM ratio, and a low sample volume, our workflow revealed temporal and spatial trends in the molecular composition. The comparison of soil solutions from two nearby MSCs with contrasting root length density demonstrated the advantage of sampling a small soil solution volume to obtain new spatially resolved insights into rhizosphere processes.

Conclusions

We presented an on-line nano-solid phase extraction FT-ICR-MS workflow that can analyze OM from small sample volumes (down to 1 µL) without any additional sample preparation. The low pH of the eluent used in the method allows for a reproducible on-line extraction and MS measurement. To lower the overall salt content, samples can be diluted with the aqueous eluent without sacrificing sensitivity or spectral quality. Utilizing the potential to increase the IAT for FT-ICR-MS measurement makes it possible to detect thousands of MF in a single sample. The amount of carbon needed for an on-line extraction and measurement of OM was lowered by a factor of six as compared to DI-ESI measurements. More importantly, the low amount of consumed sample enables us to obtain high spatial precision and coverage of the root system.

Combining visualization of the root structure via X-ray CT with the analysis of soil solution OM by FT-ICR-MS, as demonstrated here, resulted in molecular insights into early rhizosphere development. The low sample consumption of our method allows resolving patterns of OM at spatial scales of the root system, which was not possible before due to much larger sample consumption for soil solution OM analysis or destructive sampling.

Our workflow enables the study of chemical gradients in space and time directly in a soil context. The low sample consumption of our method made it possible to also analyze the soil solution samples for nutrients (Table S5). We will now be able to link the release and transformation of OM with the nutrient status of the rhizosphere to gain a more complete picture of interlinked processes in the root-soil system. Additional mass spectrometric information may be generated by applying a nano-LC separation as well as additional measurements in positive ionization mode. Combining this analysis with the detailed structural insight provided by X-ray CT can deepen our understanding of the complex dynamics of SOM formation in the rhizosphere.

The combination of the on-line nano-SPE method with non-target or targeted analysis of other complex samples by FT-ICR-MS is a powerful tool, especially for fields like metabolomics. The method can be used where sample volume is limited and salt concentrations are high such as single-cell analysis[48] or sediment pore water.[49]

Supporting Information

Experimental details on the preparation of the soil column experiment, the soil solution sampling, and the X-ray CT measurement as well as additional tables and figures.

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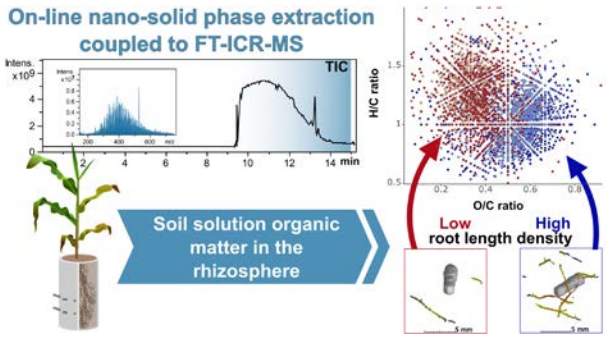
632 

633 For Table of Contents only

634

635