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Quantitative determination of iron-siderophore complexes in peat by isotope-exchange size-exclusion UPLC - electrospray ionization high resolution accurate mass (HRAM) mass spectrometry

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ABSTRACT: A method was developed for the quantification of iron-siderophore complexes by electrospray high resolution accurate mass (HRAM) mass spectrometry (MS) without the need for authentic standards. The bulk of iron-siderophores complexes was purified by solid phase extraction (SPE) and concentrated by evaporation. The individual complexes were identified by fast size-exclusion chromatography (FastSEC) – Orbitrap MSⁿ on the basis of the exact molecular mass (± 1 ppm) and MS² or MS³ fragmentation. Their capability to exchange readily the natural ⁵⁶Fe for the added ⁵⁸Fe was demonstrated by SEC with ICP MS and ESI MS detection. The ESI MS response factor of each of the complexes was determined on the basis of the contribution of the exchanged stable isotope (⁵⁸Fe) to the total of exchanged iron determined by ICP MS. The method was applied to the analysis of peat sampled in the Eastern part of the French Pyrenean mountains. Nineteen siderophores belonging to four different classes were identified and quantified. The results were validated using ICP MS detection of iron by matching the sum of iron-complexes determined by isotope exchange - ESI MS within each peak observed by FastSEC-ICP MS.

INTRODUCTION

Iron is an essential micronutrient for microorganisms, plants and higher organisms.¹ However, despite its large abundance, its bioavailability in the environment is limited because of its presence in the form of scarcely soluble oxyhydroxides. The principal strategy of microorganisms to acquire iron is to synthesize diverse iron-chelating compounds called siderophores.²

More than 500 siderophores produced by various microorganisms, fungi, or plants have been described in a classical work of Hider and Kong which remains the most complete compendium up to now.² In soils and peatlands, most of iron is strongly but reversibly bound to dissolved organic matter (DOM) and is available for complexation by siderophore ligands.³ Nevertheless, iron acquisition by bacteria can be challenging even at high total iron concentrations in some environments, particularly at high pH where free iron solubility decreases, and aggregates, and other insoluble forms are formed.⁴ As the production of siderophores structures microbial ecosystems under pressure of iron scarcity, it is important to understand how iron deficiency affects metabolic rates and ecosystem community dynamics in carbon-rich habitats. The interest in peat is driven by the fact that the content of organic

matter is four times higher than in organics-rich soil, the concentration of iron can be as low as 0.1 $\mu\text{M}\cdot\text{g}^{-1}$ (dry weight),⁵ and its totality (> 97%) is present in non-characterized complexed forms. A recent effort to decipher the ligandosphere in a peatland led to the identification of an amino polycarboxylic acid rhizobactin B produced by *Pseudomonas* sp⁶ which can constitute as much as 7.5% of the peat prokaryotic community.

The recovery of siderophores for analysis can be problematic because of their adsorption to organic matter which is an extraordinarily complex matrix.⁷ Speciation of iron has been approached by spectroscopic,⁸ electrochemical,^{9, 10} or chemical (Chrome Azurol S assay)¹¹ methods. These methods are non-specific and measure bulk ligand concentrations of which siderophores represent usually only a subset.

The ultimate identification of individual metal-binding ligands can be achieved by NMR which, however, requires their relatively tedious isolation and purification.¹² Electrospray mass spectrometry (ESI MS) offers a much higher sensitivity and throughput than NMR. It provides information on the molecular mass of the siderophore ligand and/or its complex with iron. In addition, structural information can be obtained owing to the suite of fragments generated from the parent ions

by collision induced dissociation (MS²).¹³ Fourier-Transform Ion Cyclotron Mass Spectrometry (FT ICR MS) or Orbitrap MS can offer resolution permitting the isolation of an analyte *m/z* peak from a complex molecular system. The mass measurement accuracy (down to 50 ppb) yields a definitive molecular mass assignment of siderophores referenced in database and often allows the deconvolution of the empiric formula of unknown ligands.¹⁴ Targeted (1-2 Da window) high resolution MS² spectra can be routinely collected in LC - MS/MS^{3,15} where the presence of diagnostic fragments validates the detection of candidate siderophores. In practice, the low concentrations of iron-complexes present and the complexity of the matrix resulting in the ionization suppression require high-resolution chromatographic separation of the metal-complexes formed prior to their ESI MS detection. In the state-of-the-art protocols mass spectra were collected at a resolution of 450,000 (at *m/z* 200, 1 s transient) using Orbitrap MS^{15,16,17} or 600,000 (at *m/z* 400, 1-2 s transient) using FT-ICR MS.¹⁸ However, meaningful results were also obtained with lower resolutions (<100,000).^{3, 6, 19} An untargeted search for iron siderophores was possible using algorithms relying on the exact mass difference ($\Delta m = 1.995$) and natural relative abundance ratio ($R = 15.7$) for ⁵⁴Fe and ⁵⁶Fe.¹⁷ The use of bi-isotopic (⁵⁷Fe/⁵⁸Fe) enriched iron probes reacting with excess of siderophores was proposed to facilitate the data mining in the search of iron-binding siderophore ligands.¹⁹

Siderophores can be quantified by external calibration curves, but that requires authentic standards which are often hard to get. Alternatively, they can be quantified by HPLC-ICPMS, which offers a response factor independent of the molecular structure of the metal complex and has been frequently proposed for the quantification of iron complexes with biological ligands.²⁰ However, the separation of siderophores requires the use of organics-rich gradients which negatively affect the ICP MS detection and differentiate the response for iron as a function of the retention time. Also, the multitude of the siderophore complexes present results in their frequent co-elution and thus prevents the specific quantification of individual siderophores by ICP MS when no baseline separation of the iron complexes is achieved.

This work investigates UPLC size-exclusion chromatography (referred to as FastSEC) with ICP MS and ESI MS detection as a rapid technique for the screening for iron complexes with siderophores. The isocratic separation guarantees a stable, sensitive and quantitative detection by ICP MS over the duration of the chromatographic run and, after a post-column addition of an organic solvent, stable ionization conditions in ESI MS. The lack of the chromatographic baseline resolution can be compensated by high resolution mass spectrometry resulting in specific non-interfered iron isotopic pattern for each of the siderophore-complex.

The objective of this work was to develop a method for the quantification of iron-siderophore complexes by high resolution mass accurate (HRAM) mass spectrometry without the need for authentic standards. For this purpose, the ⁵⁸Fe isotopic exchange in kinetically labile (but thermodynamically stable) iron-siderophore complexes was explored to derive the relative electrospray MS sensitivities of the individual siderophores. The quantification was based on the ESI MS signal intensity of the ⁵⁸Fe saturated Fe-siderophore complexes while the total

exchanged iron was measured by isotope-exchange HPLC-ICP MS. The advantage of the enriched spike is that it allows one to account for the totality (both complexed and free) siderophore and, in the partial exchange conditions, creates a characteristic isotopic pattern facilitating data mining.

EXPERIMENTAL

Samples and standards. Two peat cores, each 50 cm in depth, were collected from two different zones in the Bernadouze peatland located at 1343 m.a.s.l. in the Eastern part of the French Pyrenean mountains (42.80273 N; 1.42361 E) 27 in November 2021 (samples A and B). At a distance of 11 cm from the top of the core, a 1-cm layer, destined for water extraction, was stored at -20 °C, and the subsequent 2-cm layer, destined for a biological enrichment, was kept at 4 °C before processing (Fig. SI-1).

Deionized water obtained from a Milli-Q® Type 1 system (Millipore, Belford, MA) was used throughout unless stated otherwise. Ammonium acetate (≥ 98% for molecular biology), 70% nitric acid (Fisher, Loughborough, UK), acetonitrile (≥ 99.9%), sodium hydroxide (≥ 97.0%, pellets, Sigma-Aldrich) and LC-MS grade methanol (≥ 98%, sigma Aldrich) were used. Citric acid (100 ppb) was used for column cleaning.

A standard solution (1000 ppm) of Fe (SCP, Villebon-sur-Yvette, France) was diluted accordingly. ⁵⁸Fe metallic iron (93%+) was purchased from STB Isotope (Germany) and dissolved in *aqua regia* to produce stock solutions of 50 mg/ml which were then diluted to produce working solution with concentrations in the order of ppm. The siderophore standards ferrichrome, ferrichrome A, and ferrirubin, were obtained from EMC microcollections (Tübingen, Germany).

Instrumentation and materials. The ICP MS spectrometer used was ICPMS 7500 (Agilent, Tokyo, Japan) equipped with a collision cell. Electrospray ionization mass spectrometer was Orbitrap Fusion Lumos Mass Spectrometer (Thermo Scientific, Bremen, Germany). The separations were carried out on a fastSEC column (Acquity UPLC protein BEH SEC column, 4.6 x 150 mm, Waters, Milford, CT) using an Agilent 1200 series (Agilent, Tokyo, Japan) connected to ICPMS or a Dionex ultimate 3000 RS (Thermo Scientific, Bremen, Germany) pump connected to ESI MS. A Superdex-75 10/300 GL SEC column (10 x 300 mm) (GE Healthcare) and reversed phase (Acquity UPLC BEH C18 column (2.1 x 150 mm x 1.7 μm, Waters, Ireland) were occasionally used for comparison experiments.

A Rotamax 120 mechanical shaker (Heidolph, Schwabach, Germany) and a Multifuge X3 FR centrifuge (Thermo scientific, Osterode am Harz, Germany) were used for peat (soil) extraction. 0.22-μm syringe filters (Millex-GP, Millipore, Bedford, MA) and HLB extraction cartridges (30 mg, Oasis® HLB, Waters, Milford, CT) were used for filtration and SPE preconcentration, respectively. A further preconcentration was achieved by solvent evaporation using a concentrator FSC400D, dri-block from TECHNE (Fisher Scientific, Illkirch, France).

Procedures

Water extraction of siderophores from peat. A peat (soil) sample (3 g) was suspended in 10-fold (*m/v*) excess of water and agitated for 2 h at 150 rpm in room temperature. Then, the sample was centrifuged at 4,500 rpm for 10 min at 4°C, and

filtered through a 0.22 µm syringe filter. The filtrates were stored at -20 °C.

Enrichment cultures. Biological enrichment was carried out according to Boiteau et al.¹⁸ In brief, a 250-mg sample of peat (soil) was suspended in 10 mL of iron-deficient M9 medium containing 1 mM MgSO₄, 0.2 mM CaCl₂, and 0.4% glucose. The peat was shaken for 20 min at 150 rpm at room temperature to obtain a homogenous inoculum. A 100-µL aliquot of this slurry was diluted with 25 mL of the M9 medium solution and incubated till the optical density reached 0.7. The enriched samples were then centrifuged at 4,500 rpm at 4 °C for 10 min. The supernatants were filtered through 0.22 µm (Millex-GP) filter and stored at -20 °C prior to analysis.

Solid-phase extraction (SPE). The supernatants were purified and pre-concentrated by solid phase extraction (SPE) as illustrated in Fig. SI-2. Prior to extraction, a cartridge was cleaned with 1 mL methanol (step 1), dried for 10 min following the manufacturer's protocol, and rinsed with 1 mL water to remove methanol (step 2). An aliquot of supernatant (after extraction from peat) was spiked with 10 ppm (⁵⁶Fe and diluted 5-fold with water to produce 1 ml of solution. pH was adjusted to 2-3 with 2% HNO₃ and the sample was passed through the SPE cartridge (step 3). The cartridge was rinsed with water (step 4) and eluted with 1 mL methanol (step 5). The solution was concentrated by evaporation (to 50 µL) under N₂ stream. The residue was dissolved to 100 µL of water to produce samples which were further analysed upon pH adjustment to 2-3 and ⁵⁸Fe addition (25, 50, 100, 200, 300 ppb) by FastSEC-ICPMS and FastSEC-ESI MS.

FastSEC - ICP MS/ESI MS conditions. The FastSEC separation of Fe-siderophore complexes was achieved with 10 mM ammonium acetate (pH 6.8) at 0.3 mL/min. The injection volume was 5 µL. Hydrogen gas (4 mL/min) was used as reaction gas. Other ICP MS parameters, such as RF power, RF matching, torch position, and carrier gas were adjusted daily according to the standard ICP MS optimisation protocol.

For FastSEC - ESI MS, a T-piece was installed between the column exit and electrospray source to allow the addition of 0.3 ml/min of acetonitrile to promote the ionization. ESI-MS parameters were set to a resolution of 240,000, scan range (m/z) of 150-2,000, and ionization energy of 3500 V. Carrier and shielding gas flow rates were 20 and 60 (arbitrary units), ion transfer tube and vaporizer temperature of 350 and 400 °C, RF lens of 30%, and maximum injection time of 100 ms. MS/MS analysis was performed for all species found in the sample. Ions were trapped using a ± 1.6 m/z isolation window and was fragmented using HCD collision energy of 30%.

Siderophores putative identification. The enrichment with ⁵⁸Fe modifies the Fe isotopic pattern which was introduced as the pattern scoring parameter in Compound Discoverer 3.3 (Thermo Scientific) to identify the sub-set of Fe-binding compounds. Based on the m/z, the formulae of the complexes with Fe were proposed. The Expected Compound parameter provided an opportunity to look for similarities between known siderophores and a group of Fe-rich compounds not found in the siderophores databases. MS² data were then used to compare the fragmentation data with the theoretical fragmentation spectra of the siderophore in question, or in the case of putative

siderophores, with a molecule that might be the parent compound (FISH Scoring parameter).

Quantification. ⁵⁶Fe was quantified by ICP MS in the sample prior to injection on the HPLC column, in the total column eluate (to determine the column recovery), and in the collected individual peak fractions (for the purpose of validation of ESI MS determinations of individual Fe-siderophore complexes) by spiking a sample aliquot with ⁵⁸Fe and comparing the ⁵⁶Fe intensity with that of ⁵⁸Fe spike. The employed formula was:

$$c(^{56}\text{Fe}) = I(^{56}\text{Fe}) / I(^{58}\text{Fe}) \times c(^{58}\text{Fe}).$$

For the determination of the concentration of the pool of ⁵⁶Fe-siderophore complexes by HPLC-ICPMS, aliquots of the extract after SPE were spiked with different concentrations of ⁵⁸Fe (25, 50, 100, 200, 300 ppb), equilibrated for 24 h, and chromatographed. The ⁵⁸Fe-signal signal intensity increased linearly until it reached a plateau indicating the complete replacement of the ⁵⁶Fe isotope by ⁵⁸Fe (cf. Fig. 3 lateron).

The concentration of the totality of ⁵⁶Fe-siderophore complexes was determined as a quotient of the ⁵⁸Fe intensity in the saturation conditions and the sensitivity derived from the slope of the linear range of the ⁵⁸Fe intensity as a function of the added concentration of the spike.

For the calculation of the concentrations of the individual ⁵⁶Fe-siderophore complexes by HPLC-ESI MS, aliquots of the extract after SPE were spiked with different concentrations of ⁵⁸Fe (25, 50, 100, 200, 300 ppb), equilibrated for 24 h, and chromatographed. The intensity of each ⁵⁸Fe-siderophore signal increased linearly until it reached a plateau indicating the complete replacement of the ⁵⁶Fe isotope by ⁵⁸Fe (cf. fig. 4 lateron).

The concentration of each siderophore complex c_i (expressed as iron) was calculated as the quotient of its signal intensity (I_i) at the plateau and the sensitivity s_i characteristic for each Fe-siderophore complex and dependent on the efficiency of its electrospray ionization

$$c_i = I_i / s_i$$

where s_i corresponds to the slope of the linear part of the function of ⁵⁸Fe-siderophore signal intensity vs. concentration of the ⁵⁸Fe added spike.

RESULTS AND DISCUSSION

Separation of iron-siderophore complexes by fastSEC – ICPMS. Iron-siderophore complexes were usually separated by reversed-phase (RP) HPLC using C₈ or C₁₈ HPLC stationary phases.^{7,19b,18} RP-HPLC can be readily coupled to ESI MS but the ICP MS detection is affected by the plasma quenching due to the organic solvent which affects the sensitivity.¹⁷ Also, the background of DOM being a continuum of compounds with different polarities often overlaps with siderophores increasing the background.²⁰ The RP chromatograms tend to be long (up to 100 min).¹⁷

Size-exclusion chromatography which separates the analytes principally according to the molecular size (or, more precisely, to hydrodynamic volume) has been largely used for the fractionation of metal complexes prior to ICP MS allowing a clear separation between the high molecular weight (HMW, >10 kDa) and the low molecular fraction (LMW, <10 kDa).^{21,22} Moreover, the existence of secondary specific adsorption

mechanism, however poorly understood, allows the separation of metal complexes within the LMW fraction.²⁴ A big advantage of SEC is the isocratic elution with purely aqueous buffers without the need for organic solvents that offers a uniform sensitivity over the duration of the chromatographic run.^{21,22} The coupling with ESI MS was achieved by a post-column addition of, usually acidified, organic solvent to promote the ionization.^{23,24}

Preliminary experiments using the most commonly used column for the separation of metal complexes, i.e. Superdex-75 (cross-linked agarose and dextran), showed the possibility of separation of several Fe-siderophore complexes with similar molecular weights. However, due to the column size (30 cm x 7.6 mm) the separations were long and the significant dispersion over the column negatively affected the detection sensitivity. Therefore, it was decided to replace the column by an ultrahigh-pressure liquid chromatography (UHPLC) column, referred to as FastSEC. The latter was recently proposed for the separation of metal complexes in plant extracts by Kińska et al.²⁵ The separation of Fe-siderophores is demonstrated in **Fig. SI-3** for a mixture of ferrichrome (MW = 741 Da), ferrichrome A (MW = 1053 Da), ferrirubin (MW = 1011 Da) and ferrichrocin (MW = 770 Da). The duration of the analysis was reduced 5-fold in comparison with the Superdex-75 column, at some expense of separation that can be compensated by molecule specific detection. The recovery, calculated as the ratio of the sum of HPLC-ICP MS peaks areas to the area of the iron peak obtained in the FIA mode without column, was 83 ± 0.3 %. The detection limit for iron (calculated as 3 times SD of noise) from ICP MS calibration/slope) was ca. 5.4 ppb. A post-column addition of acetonitrile allowed the specific detection of these four siderophores by ESI MS with detection limits about three times lower in comparison with those of ICPMS detection (ca. ≤ 1.5 ppb of Fe in the complex).

FastSEC-ICPMS analysis of native environmental samples: optimization of SPE. The direct analysis of supernatants obtained after leaching peat samples with water resulted in quasi-baseline chromatograms with hardly any peak detected (**Fig. 1a**, thin line). The high complexity of the matrix and the low concentrations of siderophores present made their detection difficult and required an improvement of sensitivity that could be achieved by preconcentration. Solid-phase extraction (SPE) using unipolar (C_8 - C_{18}) cartridges was reported elsewhere to allow the retention of hydrophobic siderophores while most of hydrophilic matrix constituents, including inorganic metal ions, were eliminated.²⁵ The retained siderophores (or Fe-siderophore complexes) are subsequently eluted with methanol, and concentrated by evaporation. The concentration factors to be obtained are limited by the matrix complexity and the risk of precipitation during evaporation of the eluate.

The retention of analytes under acidic conditions (pH 2-3) was carried out here following Waska et al.²⁶ HNO_3 was chosen for acidification instead of HCl to avoid interferences with ICP MS detection. According to the developed protocol, the theoretical preconcentration factor should be 20. It results from the concentration by evaporation, SPE serves to eliminate matrix compounds and purify the siderophore fraction. By comparing the peak area of the ferrichrome before and after SPE from flow injection analysis (FIA) results, the Fe recovery

of siderophore standard from the SPE column was calculated to be 95 ± 2 % and 86 ± 2 % in the absence and presence of sample matrix, respectively. These values are similar to the siderophore recovery value of 82.6 % reported by Waska et al.²⁶ At pH 8, the recoveries of iron ferrichrome and ferrichrome A complexes were about 70%.

The sensitivity of 5ppb in FastSEC-ICP MS allowed to detect siderophore-like peaks only in one sample. Therefore, an approach was adopted to grow siderophore-producing bacteria present in the samples which would lead to the enrichment of the sample with siderophores.^{26,4}

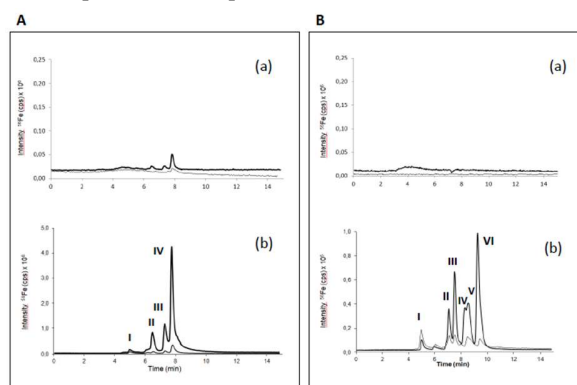


Figure 1. FastSEC – ICP MS chromatograms of peat extracts. a) native peat extract; b) biologically enriched peat extract before (thin-dash line) and after (bold-solid line) solid phase extraction. Left panels: sample A; right panels: sample B.

Detection and putative identification of siderophores after biological enrichment by FastSEC – ICP MS and FastSEC – ESI MS. The FastSEC-ICP MS chromatograms of the samples' supernatants analyzed before and after SPE are shown in **Fig. 1b**. They show a number of relatively well-defined peaks over a 5-10 min elution range and strongly indicate the possible co-elution. Note that the chromatogram after bacteria cultivation (**Fig. 1b**) reproduces the morphology of that of a native sample (sample A). Such a comparison was not possible for the second sample as no peaks were observed for the native sample, the cultivation represented the only way to produce a sufficient quantity of siderophores for their identification.

Eleven and 19 complexes could be identified by ESI MS/MS in samples A and B, respectively. The detailed mass spectrometric data are summarized in **Table SI-1**. They belong to several classes including rhizoferrin, ferrioxamine and pyoverdine (of which pseudobactin) illustrated in **Fig. SI-4**. Pyoverdines are a group of molecules consisting of a fluorescent chromophore attached to a peptide chain of 6-14 amino acids and an acyl side chain.²⁷ Pseudobactin was one of the first identified examples of pyoverdine, with the hexapeptide Lys-d-threo- β -OH-Asp-l-Ala-d-allo-Thr-l-Ala-d-N5-OH-Orn.²⁸ To date, more than 100 pyoverdines have been classified, the structures of over 60 of these were compiled by Hider and Kong.² Individual bacteria isolates typically synthesize compounds with the same peptide, but with several modifications – from the addition of acyl chains to the chromophore and sulfonation of the chromophore to formation of azotobactin (adding five-membered ring to the

chromophore).²⁹ Duckworth et al. showed that pyoverdines D47 and GB1 could undergo, besides methylation and sulfonation, hydroxyl (-OH) β -elimination but the reasons for the formation and biological relevance of sulfonated siderophores remained unknown.³⁰ Certain amino acids are susceptible to oxidative deamination to a ketone yielding α -keto acid. Consequently, such modification is possible for siderophores which contain several amino acids with free amine groups in their structure. In sample A, several compounds with formulae similar to pseudobactin were found. The mass differences between the parent compound and the compounds labelled as pseudobactin^{a-1} indicate that pseudobactin underwent transformations such as methylation, sulfonation, oxidation, oxidative deamination to alcohol, oxidative deamination to ketone or some of them simultaneously.

In sample B, compounds described by Inoue et al.³¹ as pyoverdines F-I, F-IIa and F-IIb were found. These siderophores are composed of 7 amino acids (Ser-Lys-Gly-FoOHOrn-Lys-FoOHOrn-Ser) and differ by the length of the acyl chain (R = CO-CH₂-CH₂-COOH, R = CO-CH₂-CH₂-CHNH₂-COOH and R = CO-CH₂-CH₂-CONH₂, respectively). The difference between F-I and F-IIb consists of an exchange of -OH to -NH₂ in the acyl chain. The same mass difference was observed later for the other pair of putative siderophores – designated in **Table 1** as pyoverdine^a and pyoverdine^d. These molecules, as well as pyoverdine^c, have not been assigned to any pyoverdines' F as the parent compound, so would be expected to have more amino acids in the peptide chain.

Pyoverdines bind iron through three bidentate functions – one catecholate and two hydroxamates. This results in their higher affinity for iron than that of ferrioxamines, which bind via three hydroxamate functions, but lower than that of enterobactin which uses three catecholates.²⁷ The stronger affinity of pyoverdines for iron, compared to ferrioxamines, would explain their significantly higher abundance in the analysed samples. Note that siderophore binding with iron is influenced strongly by pH and can be different between different siderophores. Also, the abundances of siderophores also reflect the growth/production of the producing organism relative to the uptake/degradation of the compound.

The tentative identification of the peaks on the basis of the exact molecular mass was confirmed by MS/MS spectra (**Table 2 - SI**). The presence of the detected siderophores was also confirmed by reversed-phase HPLC – ESI MS. Note that in reversed-phase HPLC the complex of Fe with rhizoferrin could hardly be observed because of the acidic pH conditions of the mobile phase (0.3% formic acid in 5mM ammonium formate). Indeed, rhizoferrin, a type of α -hydroxycarboxylate (hydrophilic negatively charged α -hydroxycarboxylate) siderophore, was reported to be unstable in acidic conditions.⁷ Note also that some aposiderophores were observed, e.g. bisucaberin, putrebactin and neocropogen, which indicates that not all the siderophores reacted with Fe³⁺ under the laboratory saturation conditions.

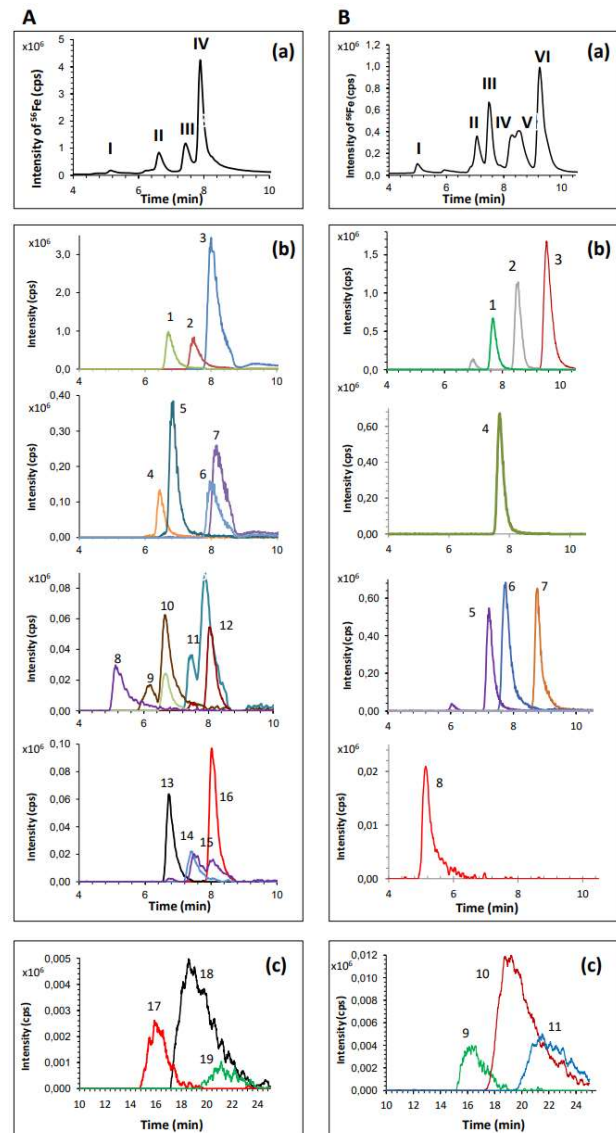


Figure 2. FastSEC chromatograms of the detected ⁵⁶Fe-siderophores complexes. Left panels: Sample A; right panels sample B. a) ICP MS detection; b) ESI MS detection corresponding to the a) chromatograms. Sample A: 1 - m/z 1043, 2 - m/z 1072, 3 - m/z 1042, 4 - m/z 1152, 5 - m/z 1122, 6 - m/z 1028, 7 - m/z 1025, 8 - m/z 490, 9 - m/z 1059, 10 - m/z 1154, 11 - m/z 1058, 12 - m/z 1091, 13 - m/z 1141, 14 - m/z 1088, 15 - m/z 497, 16 - m/z 1028; Sample B: 1 - m/z 1214, 2 - m/z 1369, 3 - m/z 1213, 4 - m/z 1214, 5 - m/z 1398, 6 - m/z 1242, 7 - m/z 1243, 8 - m/z 490; c) ESI MS chromatograms at retention time > 10 min where no ICP MS peaks can be seen: Sample A: 17 - m/z 626, 18 - m/z 640, 19 - m/z 654; Sample B: 9 - m/z 626, 10 - m/z 640, 11 - m/z 654. The Fe-siderophore complexes were grouped by panels as a function of their signal intensity for the sake of clarity of presentation.

The extracted ion chromatograms for all the individual Fe-siderophore complexes are summarized in **Fig. 2**. They show clearly that the majority of peaks observed in FastSEC - ICP MS are mixtures of different siderophores and cannot be individually quantified by ICP MS. On the other hand, the efficiency of electrospray ionization being species-dependent, they cannot be simply quantified by ESI MS using the peak intensity (height or area). In order to quantify these complexes

by ESI MS, the different ionization efficiencies of the different Fe-siderophores have to be taken into account. They can be evaluated by adding known amounts of the isotopic spike (^{58}Fe) and measuring the evolution of the intensity of the ^{58}Fe complex formed for each and every siderophore. The prerequisites are that i) the isotope exchange occurs, and ii) the peak intensity can be measured without interference which is only possible by high-resolution MS.

Isotope exchange of iron in Fe-siderophore complexes following the addition of ^{58}Fe . Fe-siderophore complexes have very high thermodynamic stability constants but can exchange the Fe isotopes if they are kinetically sufficiently labile and the Fe isotope replacing the native Fe is added in sufficient excess. In order to test this hypothesis, a preconcentrated SPE extract was spiked with $^{58}\text{Fe}^{3+}$ at various concentrations and the isotope exchange was monitored by FastSEC – ICP MS.

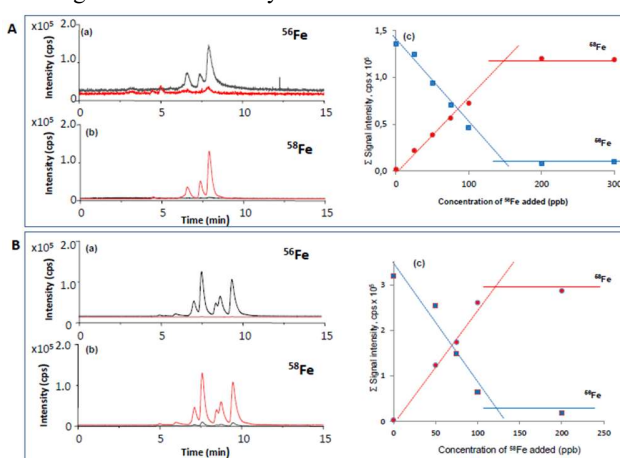


Figure 3. a,b) FastSEC - ICPMS chromatograms of iron-siderophore complexes in peat extracts after enrichment and SPE. Black line: ^{56}Fe ; red line: ^{58}Fe . a) before ^{58}Fe addition; b) 24 h after ^{58}Fe addition. c) effect of the ^{58}Fe amount added on the sum of the intensities of the Fe – containing peaks (after 24 h incubation). Top panels: Sample A; bottom panels: Sample B.

Figure 3 demonstrates that the originally present ^{56}Fe was completely exchanged after 24 h in the presence of excess of ^{58}Fe . The increase in the ^{58}Fe -siderophores signal was linear (till a saturation point) with the increasing concentration of added ^{58}Fe and was accompanied by a linear decrease of the ^{56}Fe signal. As the ICP MS detection integrates the co-eluting Fe-complexes, a similar saturation experiment was carried out with ESI MS detection, monitoring the replacement of the ^{56}Fe isotope in each and every Fe-siderophore complex by the added ^{58}Fe . A representative example showed in **Figure 4**.

For each siderophore a saturation point can be identified at the cross-section of the straight line representing the increase in intensity as a function of the added ^{58}Fe concentration and the straight line representing the plateau of saturation of the ligand with ^{58}Fe . The slope of the straight line connecting the zero point (no ^{58}Fe added, the natural ^{58}Fe (0.28%) is neglected) and the point of saturation (all ^{56}Fe has been displaced) is a measure of the electrospray sensitivity s_i (ionization efficiency) which may vary as a function of the compound. The linearity range is a function of the siderophore concentration and can be different for the different compounds.

The set of the identified siderophores is limited by the reactivity of the ligand with the added $^{56}\text{Fe}^{3+}$ spike before SPE. At the experimental conditions some siderophores may not form a complex with iron and thus would escape the analysis. This restricts the method applicability to the set of siderophore ligands which react with Fe^{3+} . This limitation is the same as that of the established data mining methods based on the use of bi-isotopic ($^{57}\text{Fe}/^{58}\text{Fe}$) iron probes proposed to facilitate the search for siderophore ligands.^{32,33}

The data demonstrate that the siderophores forming complexes with the excess of ^{56}Fe (prior to SPE), exchange them readily with the added ^{58}Fe spike. This observation may be in contradiction, at least for ferrioxamine E, with a very recent report reporting a very slow iron-ligand exchange kinetics for ferrichrome and ferrioxamine E.³⁴ It is possible that the kinetic stability of the naturally formed complex and that of the complex formed in the laboratory vary, possibly because of the different spatial conformation of the complex formed. There can be a pH effect as well, not discussed in the literature.

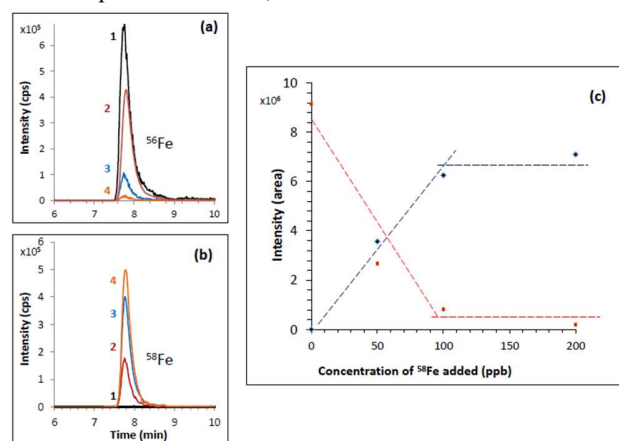


Figure 4. Effect of the ^{58}Fe addition on the ESI MS peak intensity of an iron-siderophore complex (example for pyoverdinec, m/z 1242) after 24 h incubation. a) ^{56}Fe -containing complex (peak height); b) ^{58}Fe -containing complex (peak height). 1 – no ^{58}Fe added; 2 – 50 ppb ^{58}Fe added; 3 – 100 ppb ^{58}Fe added; 4 – 200 ppb ^{58}Fe added. c) effect of the added ^{58}Fe amount on the Fe-siderophore complex peak area. Red line: ^{56}Fe ; black line ^{58}Fe .

Quantification of iron-siderophore complexes by isotope exchange – FastSEC - ESI MS.

Table 1 shows the results of the determination of the concentrations of the siderophores detected, expressed in concentrations of iron. Assuming that the molar ratio of iron to siderophore is 1 to 1, concentrations of the ligands can be easily calculated. Note that the method does not require an authentic standard for each of the siderophores knowledge, neither the exact identity of siderophore. Some unidentified iron-siderophore complexes were accounted for on the basis of their empiric formula showing the presence of one iron atom.

The FastSEC - ICP MS ^{56}Fe chromatograms shown in Fig. 3 allow a straightforward quantification of the bulk of Fe-siderophore complexes. However, the ICP MS peaks contain a number of co-eluting Fe-siderophore complexes as demonstrated by ESI MS. Also, the sensitivity of the ESI MS detection can be much higher for some compounds than that of ICP MS. Consequently, some siderophores may not have been

detected by ICP MS. Conversely, some Fe-siderophore complexes may have ESI MS detection but contribute to the ICP MS signal.

Nevertheless, it was possible to quantify the total iron by ICP MS within the ranges of elution time of iron-siderophores and compare it with the sum of the concentrations of Fe-siderophore complexes determined by isotope-exchange ESI MS. The results (Table 1) show an agreement within 20% between the

ESI MS and ICP MS values. The results obtained with ICP MS detection can be positively biased as some Fe-siderophore complexes may be not well ionized and remain undetected by ESI MS despite their potentially substantial concentration. The accuracy and reliability of the method can be improved by improving the resolution of the chromatographic sample introduction so that each siderophore arrives at the detector chromatographically pure while the parallel ICP MS detection eliminates the risk of false negatives.

Table 1. The concentrations of Fe-complexes (as Fe, ppb) in samples A and B determined on the basis of isotope exchange chromatograms using ESI MS and ICP MS detection.

	Peak*	RT (min)	Theoretical m/z	Natural ⁵⁶ Fe-complexes	ESI MS (ppb)	ICP MS (ppb)
SAMPLE A	I	5.1	490.051	Rhizoferrin	1.5 ± 0.2	ND
	II	6.42	1141.301	Non-identified Fe-complex	0.4 ± 0.0	20 ± 0.6
		6.57	1152.311	Pseudobactin ^a	1.4 ± 0.0	
		6.60	1154.326	Pseudobactin ^b	1.6 ± 0.0	
		6.70	1059.322	Pseudobactin ^c	0.2 ± 0.0	
		6.63	1043.328	Pseudobactin ^d	7.4 ± 0.2	
	III	6.80	1122.301	Pseudobactin ^e	3.8 ± 0.2	25 ± 2
		7.36	1088.350	Pseudobactin ^f	1.0 ± 0.0	
	IV	7.44	1072.354	Pseudobactin ^g	32 ± 0.0	53 ± 3
		7.86	1213.444	Pyoverdine F-Ib	0.3 ± 1.0	
		7.90	1042.342	Pseudobactin ^h	44 ± 0.3	
		7.95	1058.339	Pseudobactin ⁱ	3.5 ± 0.3	
		7.95	1025.317	Non-identified Fe-complex	4.0 ± 0.1	
		7.97	497.120	Non-identified Fe-complex	0.3 ± 0.0	
		8.01	1042.845	Non-identified Fe-complex	1.2 ± 0.0	
		8.10	1091.833	Non-identified Fe-complex	0.8 ± 0.0	
	9.40	1028.329	Non-identified Fe-complex	2.0 ± 0.0		
	ND	16.00	626.235	Ferrioxamine X1	0.02 ± 0.00	ND
	ND	18.50	640.251	Ferrioxamine D2	0.09 ± 0.003	ND
ND	21.50	654.266	Ferrioxamine E	0.01 ± 0.00	ND	
SAMPLE B	I	5.1	490.051	Rhizoferrin	0.3 ± 0.0	ND
	II	7.2	1370.446	Pyoverdine ^a	4.3 ± 0.3	16 ± 2
		7.2	1398.439	Pyoverdine ^b	9.5 ± 0.3	
	III	7.6	1214.428	Pyoverdine F-I	9.5 ± 0.1	23 ± 0.8
		7.6	1242.423	Pyoverdine ^c	13 ± 0.5	
	IV	8.6	1369.461	Pyoverdine ^d	22 ± 2	19 ± 4
	V	8.7	1243.455	Pyoverdine F-IIa	9.5 ± 0.5	8.50 ± 1.8
	VI	9.4	1213.445	Pyoverdine F-Ib	34 ± 0.8	28 ± 3
	ND	16.1	626.235	Ferrioxamine X1	0.22 ± 0.004	ND
	ND	19.0	640.251	Ferrioxamine D2	1.7 ± 0.1	ND
ND	21.9	654.266	Ferrioxamine E	0.9 ± 0.0	ND	

* peak from ICP MS, ND = not detected

Sample A. @ C₄₂H₅₇FeN₁₂O₁₆; a) C₄₃H₅₉FeN₁₂O₂₀S; b) C₄₃H₆₁FeN₁₂O₂₀S; c) C₄₂H₅₆FeN₁₁O₁₈; d) C₄₂H₅₆FeN₁₁O₁₇; e) C₄₂H₅₇FeN₁₂O₁₉S; f) C₄₃H₅₉FeN₁₂O₁₈; g) C₄₃H₅₉FeN₁₂O₁₇; h) C₄₂H₅₇FeN₁₂O₁₇; i) C₄₂H₅₄FeN₁₁O₁₆. **Sample B.** a) C₅₄H₇₄FeO₂₃N₁₆; b) C₅₅H₇₅FeO₂₄N₁₆; c) C₅₀H₇₀FeO₂₀N₁₄; d) C₅₄H₇₅FeO₂₂N₁₇.

CONCLUSION

FastSEC allows a rapid fractionation of Fe-siderophore complexes with sufficient resolution to enable their electrospray ionization and specific HRAM MS analysis. The observed iron-siderophores complexes were found to efficiently exchange the natural $^{56}\text{Fe}^{3+}$ for the isotopically enriched $^{58}\text{Fe}^{3+}$. This behavior opens the way to their quantification by isotope exchange fastSEC - ESI MS which has two major advantages: i) detection limits of ESI MS detection are generally much lower than those of ICP MS, and ii) the peak capacity of HR MS is much higher than that of HPLC which allows studies of a large number of species at the same time. The method implies that all the siderophores originally present in the sample have to be saturated with ^{56}Fe at the starting point. It will not account for the siderophores that do not take up iron in the laboratory experimental conditions. For the moment, the detection limits are hardly sufficient for large scale siderophore studies in the native environmental samples which requires the cultivation of bacteria present in the extract to increase the production of siderophores.

ASSOCIATED CONTENT

Supporting Information

Table SI-1. Fe-siderophore complexes identified in supernatants of peat samples. **Table SI-2.** MS/MS fragments. **Fig. SI-1.** Sampling. **Fig. SI-2.** Solid phase extraction procedure. **Fig. SI-3.** FastSEC chromatograms of Fe-siderophore complexes. **Fig. SI-4.** Representatives of the siderophore families present in the studied samples.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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