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Novel approach to the determination of structurally similar hydroxamate siderophores by column-switching capillary liquid chromatography coupled to mass spectrometry

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Abstract

In this study a new approach to determine three different siderophores (ferrichrome, ferrichrysin, ferricrocin) in natural soil solutions as well as in cultures of fungi is presented. The method includes enrichment of the analytes on a short pre-column, packed with C_{18} material, and subsequent highly selective separation of the analytes on a capillary porous graphitic carbon (PGC) column. In contrast to normal C_{18} packing materials, porous graphitic carbon offers chromatographic resolution between the three very similar analytes. The selectivity of the method is enhanced even further by the electrospray ionization (ESI) mass spectrometric detection. The combination of a short pre-column and a packed capillary separation column results in a method with high sensitivity. Reported detection limits, defined as the concentration giving the signal-to-noise ratio 3:1, is 27.7 pM for ferrichrome, 46.1 pM for ferricrocin and 37.4 pM for ferrichrysin.

Keywords: Porous graphitic carbon; Column-switching; Soil; Siderophores; Ferrichrome; Ferricrocin; Ferrichrysin; Hydroxamates

1. Introduction

It is well known that trace levels of different metals are essential for growth and proliferation of cells. Higher order organisms may acquire this metal supply, like iron, through feeding on lower organisms that already have incorporated iron. On the other hand, lower organisms like microbes, fungi and some plants utilise siderophores in their acquisition of iron. Siderophores are strong iron chelators of low molecular mass ($M_r < 1500$), and most siderophores can be classified as either hydroxamates or phenolates–catecholates [1]. The siderophores are excreted during iron-limited conditions and form water-soluble complexes with Fe^{III}.

Siderophores are believed to contribute to the weathering of minerals [2,3]. Mycorrhizal fungi associated with coniferous trees might exude siderophores to facilitate etching and weathering of primary minerals delivering mineral nutrients to the trees. To investigate these processes it is important to have accurate and sensitive methods to determine different siderophore concentrations in field soil solutions and in laboratory plant growth experiments. It is also of great importance to have reliable techniques to study

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excretion of siderophores from different species of fungi and microbes in cultured samples. This would facilitate the understanding of the mechanisms behind the weathering processes. The obtained knowledge could also be very helpful in the understanding of plant nutrition.

Isolation and purification of siderophores is commonly achieved with liquid–liquid extraction using an organic solvent [1] or with adsorption chromatography using polymeric XAD-based resins [4,5]. Solid-phase extraction (SPE) has also been evaluated as a sample pre-treatment technique for siderophores [6]. Separation of hydroxamate siderophores in culture filtrate is traditionally accomplished using C_{18} stationary phases and acetonitrile or methanol gradients [4,5,7–13]. Detection of siderophores can be achieved with different bioassays, for instance by growth stimulation studies, or by chemical assays, like the Arnow and Csáky test [14]. Instrumental techniques like UV-Vis, NMR and MS have also been utilised for this purpose [14]. Both fast atom bombardment and electrospray ionization (ESI) has proven applicable together with MS for successful detection and characterisation of hydroxamate siderophores [14]. Most experimental methods reported are developed for identification rather than quantification purposes and therefore the detection limit has not been a major concern. If siderophores are to be analysed in natural samples, on the other hand, the method has to provide enhanced sensitivity. Even though many LC methods have been developed for qualitative purposes UV-Vis has often been applied for detection. This detection technique does not offer any selectivity in the detection of different siderophores, and hence a chromatographic system with high resolution is needed to ensure unambiguous identification. The shortcoming of using C_{18} materials is the disability to separate highly similar compounds, like siderophores differing in only one residue [7].

An on-line mass spectrometric approach to analyse low levels of various siderophores in complex samples would be ideal in the sense that it would be both fast and selective. For complex samples that contain a variety of components, like field soil solutions, a column-switched LC system could offer pre-concentration and cleaning of the sample on-line. The combination of efficient pre-concentration using a capillary SPE column and subsequent sufficient separation of the analytes on a separation column coupled to mass spectrometric detection would provide a simultaneous sensitive and selective method.

As already mentioned, the challenge to accomplish sufficient separation of various siderophore structures is difficult to fulfil using conventional C_{18} column materials. To separate very similar compounds a really selective packing material, like porous graphitic carbon (PGC), is preferred. The surface of PGC is composed of hexagonally attached carbon atoms arranged in layers [15]. Its unique selectivity is explained by the layered flat surface of PGC, that has a more ordered architecture than the brush-type surface associated with C_{18} materials. Hence PGC is well suited for separation of highly similar compounds, a fact that has been exploited in separations of geometric isomers and enantiomers [16]. PGC is also considered to be very physically and chemically stable and can be used over the entire pH range.

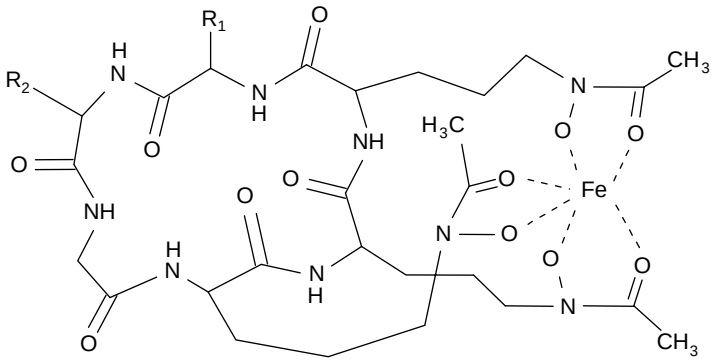
Compared to conventional columns, the use of capillary chromatography has a number of advantages that could be of vital importance for successful analysis of siderophores in natural samples [17,18]. When sample volume is limited, capillary columns offer higher sensitivity than conventional LC columns. In addition, when LC is combined with ESI-MS it is desirable to have narrow columns, since this implies reduced flow rates [19]. With low flow rates a higher ion sampling rate can be achieved and hence increased sensitivity. It is also possible to increase ionization efficiency through miniaturisation of the electrospray interface.

In this study a new approach to the analysis of siderophores is presented. The analytical method is based on a column-switched capillary LC system with ESI-MS detection. The use of PGC as packing material in the separation step was investigated and different mobile phases and on-line switching events were tried. The method was evaluated for three closely related siderophores belonging to the hydroxamate family, namely ferrichrome, ferrichrysin and ferricrocin (Fig. 1).

2. Experimental

2.1. Chemicals

Methanol and acetonitrile of LiChrosolv grade together with formic acid, iron(III) chloride-6-hydrate



	R_1	R_2
Ferrichrome	H	H
Ferricrocin	H	CH_2OH
Ferrichrysin	CH_2OH	CH_2OH

Fig. 1. Chemical structure of ferrichrome, ferricrocin and ferrichrysin.

and trichloroethylene of analytical grade were purchased from Merck (Darmstadt, Germany). Ferrichrome, isolated from cultures of Ustilago Sphaerogena, magnesium sulphate-7-hydrate and diiodomethane were obtained from Sigma–Aldrich (Munich, Germany). Ammonium formate of analytical grade and D-(+)-glucose were purchased from BDH (Poole, UK) and sodium chloride from Acros organics (NJ, USA). Agar was purchased from Acumedia Manufacturers (Baltimore, MD, USA), potassium dihydrogen phosphate and di-ammonium hydrogen phosphate from Riedel–de Haën (Seelze, Germany) and calcium dichloride-2-hydrate from Fluka (Buchs, Switzerland). Ferrichrysin and ferricrocin were kindly provided from University Professor Dr. K. Haselwandter. All solutions were prepared with water purified with a MilliQ-system (Millipore, Bedford, MA, USA).

2.2. Samples and sample preparation

The organic mor layer, the O-horizon, of a podzolized soil was sampled at Heden, Svartberget research park, 70 km NW of Umeå, Sweden (64°14′N, 10°46′E). The site is forested with Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) that are about 70 years old. At the sites a pit of about 1 m^3 was excavated. The mor organic soil layer was removed and collected. After removing the green part from the mor layer it was filled into cylindrical sampling cups. The sampling cups were made of a poly(vinyl chloride) (PVC) tube with an inner diameter of 4.6 cm and a volume of about 120 ml. The 10–15 cm depth in the mineral soil of a podzolized soil was sampled at Horröd, 10 km SW of Hässleholm, Sweden (56°05′N, 13°39′E). The site is forested with Norway spruce (*P. abies*) about 80 years old and the soil was treated with dolomite, 3250 kg/ha, in 1994. The samples were taken by driving the PVC soil-sampling cups horizontally into the profile. After sampling, the cups were sealed with tight-fitting polyethylene lids. Within 24 h after sampling, the stored soil samples were centrifuged at a speed of 16,500 g for 30 min using the centrifugation drainage technique described by Giesler and Lundström [20]. A Beckman J2-HS centrifuge with JA-14 rotor (Spinco Division, Palo Alto, CA, USA) was used for this purpose.

The mycorrhizal species *Suillus variegatus* were subcultured on agar in Petri dishes and thereafter a nutrient solution without iron was inoculated with a small part removed from the agar culture. The culture was incubated at room temperature in the dark on a rotary shaker before sampling.

The centrifugates from the field soil solutions and the culture solutions were filtered through a hydrophilic 0.45 μm filter (Millex-HV, Millipore). To the iron-deficient culture media iron(III) chloride-6-hydrate was added during stirring until a proper colour was formed. To remove excess concentration of humic substances from the field soil solutions, 50 ml of each solution were ultrafiltered using a stirred cell,

Amicon 8050 (Beverly, MA, USA) with a $M_r = 1000$ cut-off filter (PES 4, Intersep, Wokingham, UK). The filtration was stopped when the filter became dry.

2.3. Preparation of capillary columns

The columns used throughout this study were packed in-house. The pre-columns were packed with 5 μm, 120 Å C_{18} particles (ODS A, YMC Europe, Schermbeck/Weselerwald, Germany) and the separation columns with 5 μm, 250 Å PGC particles (Hypercarb, Thermo Hypersil-Keystone, Cheshire, UK). All columns were slurry packed in 200 μm i.d. fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) at a constant pressure of 320 bar for an hour. Fibreglass frits (GF/A, Whatman, Maidstone, UK) were used to retain the packing material. Acetonitrile was used as both push and slurry media in the case of PGC columns. A mixture of trichloroethylene–diiodomethane (3:2 (v/v)) was used as slurry media and acetonitrile as push media in the case of C_{18} columns. After packing the columns they were cut to desired length, 10 and 150 mm for the pre- and separation column, respectively. Each pre-column was constructed by fitting the small piece of column between two empty 50 μm i.d. fused silica capillaries (Polymicro Technologies). To keep the column and the capillaries together Teflon® tubing was used as sleeves.

2.4. Chromatography

A buffer containing 6 mM ammonium formate and 4 mM formic acid in Milli-Q water, pH 4.0, was prepared. Two different mobile phases were obtained by mixing the buffer with organic modifier. Mobile phase A was a mixture of buffer–methanol (99:1 (v/v)), and mobile phase B a mixture of buffer–acetonitrile (85:15 (v/v)). Two six-port injection valves (Valco Instruments, Houston, TX, USA), one mechanic and one electric, were used in the experimental set-up. In one valve a 30 μl injection loop was fitted and in the other the pre-column and the separation column were fitted. In the loading position, the pre-column was pumped with mobile phase A from a Jasco PU-980 pump (Jasco, Tokyo, Japan) using a flow rate of 5 μl/min. At the same time, the separation column was pumped with a Rheos 2000 pump (Flux Instruments, Basel, Switzerland) operating at a flow rate of 2 μl/min. When the valve was switched to unload sample, both the pre- and separation column was pumped with the Rheos pump at 2 μl/min.

All chromatograms reported were drawn in Excel (Version 97, Microsoft) and MacQuan 1.6 (MDS Sciex, Concord, Canada) was utilised for integration of peak areas and determination of signal-to-noise ratios.

2.5. Mass spectrometry

All results reported were performed on a Sciex API III tandem quadrupole mass spectrometer (MDS Sciex) equipped with a pneumatically assisted electrospray ionization interface. The ion spray and orifice voltages were 4300 and 70 V, respectively. The nebulizer gas flow was set to approximately 276 kPa and the spraying was performed off-axis. For all analytes the signal from the proton adduct of the molecular ion were recorded using selected ion monitoring, corresponding to the following ions *m*/*z* 741.0 for ferrichrome, *m*/*z* 771.1 for ferricrocin and *m*/*z* 801.1 for ferrichrysin. Attempts were also made to fragment the analytes in the collision cell of the mass spectrometer. The analytes were found to be very stable in the gas phase, and hence no characteristic fragments that could be used for a more selective detection were found. During all LC-ESI-MS experiments the connection between the LC column and the electrospray interface was grounded. The software Tune 2.5 (MDS Sciex) was utilised for data acquisition.

3. Results and discussion

The goal was to develop a method to determine siderophore content in water samples of different origins. The method was to be compatible with ESI-MS, and hence volatile mobile phases were used throughout all experiments. Different parts of the chromatographic system were developed in parallel and the analytical method was evaluated with ferrichrome, ferrichrysin and ferricrocin.

3.1. Pre-concentration

In this study we used an approach with on-line SPE to wash away interfering matrix components and to pre-concentrate our analytes. Traditional silica-based

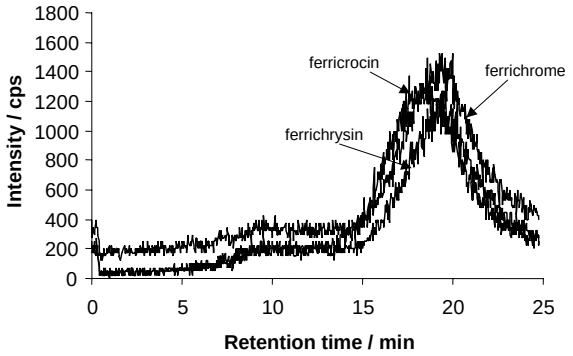


Fig. 2. Injection of a mixture of ferrichrome, ferricrocin and ferrichrysin on a pre-column. Sample: 23.1 nM ferrichrome, 34.7 nM ferricrocin and 23.3 nM ferrichrysin in Milli-Q. Conditions: mobile phase A loads 30 μl sample onto the pre-column (C_{18} 10 mm × 0.2 mm) at a flow rate of 5 μl/min, ESI-MS with selected ion monitoring is applied for detection.

C_{18} packing material was used in the pre-column. Different injection volumes (20, 30 and 40 μl) and mobile phase compositions were tried. Since the intention was to retain the analytes as much as possible a mobile phase with low organic modifier content was preferred. A mixture of ammonium formate buffer–methanol (99:1 (v/v)) was chosen, corresponding to mobile phase A in the experimental section. The injection volume was determined to be 30 μl, ensuring complete separation between the injection plug and the eluting analytes. The injection of a standard solution of the three analytes in Milli-Q water onto a pre-column is shown in Fig. 2. The concentrations were 23.1, 34.7 and 23.3 nM for ferrichrome, ferricrocin and ferrichrysin, respectively. The fact that the samples injected and the mobile phase used contained low organic modifier content led to a risk of collapse of the packing material, consequently regeneration by the injection of 30 μl methanol was performed between every run.

3.2. Separation

In contrast to earlier reports where C_{18} packing materials have been used for the separation of hydroxamate siderophores; for the first time the applicability of a PGC packing material was investigated. In our hands different C_{18} phases did not offer enough resolution between the analytes and subsequently another separation material was needed. The PGC material turned out to have promising features and a screening of suitable mobile phases were performed (details not reported here). Short time of analysis and successful separation of the analytes was ensured using a mobile phase containing a mixture of ammonium formate buffer–acetonitrile (85:15 (v/v)), corresponding to mobile phase B in the experimental section. The elution order from the PGC column followed decreased polarity and the order was ferrichrysin, ferricrocin and ferrichrome. The exchange of one or two glycines against a serine in the peptide backbone of the siderophore makes it more polar and consequently ferrichrysin and ferricrocin are less retained than ferrichrome on the column.

3.3. Column-switching

Different on-line switching events were tried and it was determined that switching the pre-column 10 min after injection and then switching it back again after another 5 min enabled a short total time of analysis. From Fig. 3 it can be seen that it takes approximately 9 min for the injection plug to go through the pre-column and that the analytes starts to elute approximately 13.5 min after injection. Consequently the first on-line switching event should take place some-

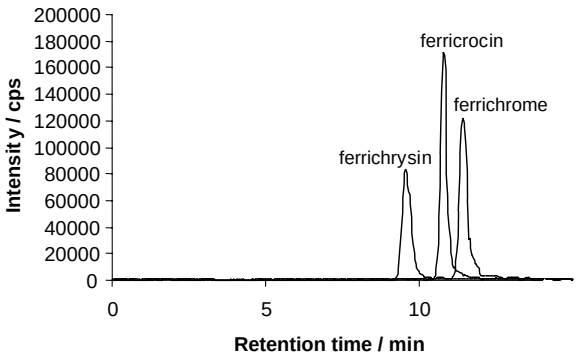


Fig. 3. Separation of ferrichrome, ferricrocin and ferrichrysin on the column-switched capillary LC system. Sample: 23.1 nM ferrichrome, 34.7 nM ferricrocin and 23.3 nM ferrichrysin in Milli-Q water Conditions: mobile phase A loads 30 μl sample onto the pre-column (C_{18} 10 mm × 0.2 mm) at a flow rate of 5 μl/min, mobile phase B elutes the analytes from the pre-column onto the separation column (PGC 150 mm × 0.2 mm) at a flow rate of 2 μl/min. Detection: selected ion monitoring of the protonated molecular ions using ESI-MS.

where between 9 and 13.5 min following injection. To maximise the pre-concentration efficiency one should switch the pre-column as early as possible and hence 10 min was chosen. Switching the valve back after another 5 min made it possible to start regeneration of the pre-column during a run in progress and the total time of analysis was thus reduced accordingly.

In Fig. 3, a chromatogram resulting from the injection of 30 μl standard solution containing 23.1, 34.7 and 23.3 nM of ferrichrome, ferricrocin and ferrichrysin, respectively, onto the column-switched system is shown. As expected, the same elution order of the analytes is obtained as in the direct injection onto a PGC column.

The applicability of the method was also investigated by drawing calibration curves. Standard solutions with concentrations ranging from less than 1 nM to more than 30 nM were injected, see calibration results in Table 1. From these results it was easily recognised that this approach has promising features in quantification of low nanomolar concentrations of the analytes.

Table 1
Calibration results, limit of detection (LOD), limit of quantification (LOQ) and *r*-values

Analyte	Concentration range (nM)	LOD[a] (pM)	LOQ[b] (nM)	*r*-value
Ferrichrome	0.61–30.77	27.7	0.09	1.000
Ferricrocin	0.92–46.22	46.1	0.15	0.999
Ferrichrysin	0.62–31.1	37.4	0.12	0.999

Results from the injection of four different standard solutions containing mixtures of ferrichrome, ferricrocin and ferrichrysin in Milli-Q water, every standard was injected two times.
[a] LOD = 3:1 S/N.
[b] LOQ = 10:1 S/N.

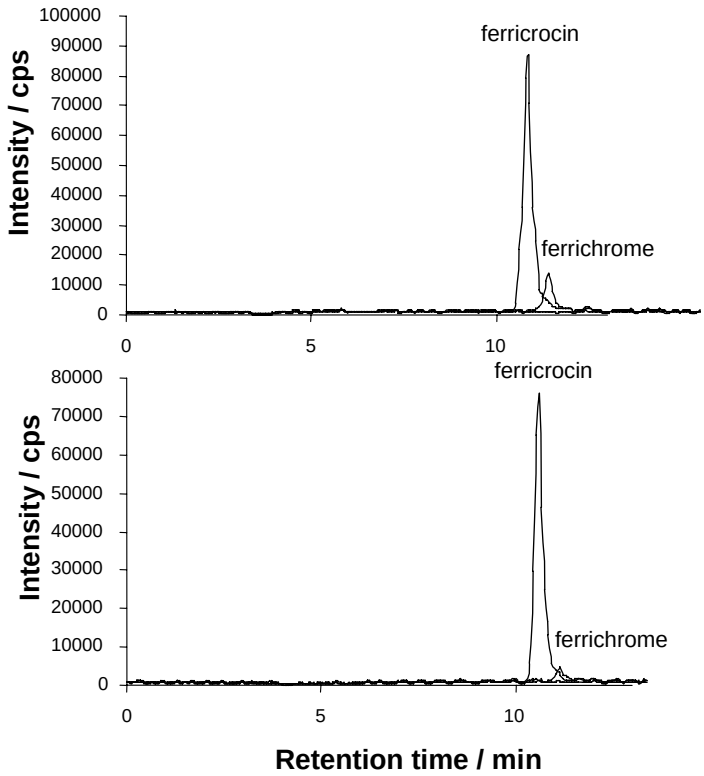


Fig. 4. Injections of 30 μl real samples onto the column-switched capillary LC system. The top chromatogram is from the injection of a cultured sample of *S. variegatus* mycorrhiza. The bottom chromatogram is from a field soil solution sample from a podzolized soil. For further information on chromatographic conditions, see Fig. 3. Selected ion monitoring of the protonated molecular ions is used for detection.

The pre-concentration ability of the system was determined by comparison between the concentration of analyte at the peak maximum and the concentration during injection. This was done for the calibration solution with the lowest concentration of analyte and the system was considered to be concentration sensitive as a whole. The result showed that all analytes were pre-concentrated approximately 24 times within the system.

Injections of real samples were further performed to verify the applicability of the described method. In Fig. 4, the top chromatogram is from a culture sample of the mycorrhiza species *S. variegatus* and the bottom chromatogram is from a field soil solution sample from Heden, Svartberget research park. As can be seen, both samples contain ferricrocin and ferrichrome, and there are no disturbing peaks in the two chromatograms that would hurt the analysis. The predicted concentration of ferricrocin in a field soil solution from Horröd using external calibration was also compared to the concentration predicted using standard additions to the sample. No significant difference between the two predictions was found, thus no pronounced suppression of the signal from possible co-eluting compounds was observed for this particular sample.

4. Conclusions

As is evident in this report, we have developed an alternative method to determine the content of siderophores by using a novel approach to separate and detect low amounts in both cultures of fungi and natural soil solution samples. The procedure established allows the sample to be concentrated and analysed with minimal effort, a feature that is often requested. The method also shows potential to be effective in quantitative analyses. As shown, it can be used to determine the production of siderophores by different fungi and microbes in cultures and the abundance of siderophores in soil solution. In this context, the potential role of siderophores for the weathering process and plants nutrient uptake by mycorrhiza might be elucidated by this analytical technique. However these are only some suggestions of known area of application. Due to the high selectivity of the method it will also be useful when trying to identify new siderophores which are not known of as of today.

Acknowledgements

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