

CHARACTERIZATION OF MICROBIAL SIDEROPHORES BY MASS SPECTROMETRY

Tomáš Pluháček,^{1,2} Karel Lemr,^{1,2} Dipankar Ghosh,³ David Milde,¹ Jiří Novák,² and Vladimír Havlíček^{1,2*}

¹Department of Analytical Chemistry, Faculty of Science, Regional Centre of Advanced Technologies and Materials, Palacky University, 17. listopadu 12, 771 46, Olomouc, Czech Republic

²Institute of Microbiology, AS CR v.v.i., Videnska 1083, CZ 142 20, Prague 4, Czech Republic

³Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067, India

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Siderophores play important roles in microbial iron piracy, and are applied as infectious disease biomarkers and novel pharmaceutical drugs. Inductively coupled plasma and molecular mass spectrometry (ICP-MS) combined with high resolution separations allow characterization of siderophores in complex samples taking advantages of mass defect data filtering, tandem mass spectrometry, and iron-containing compound quantitation. The enrichment approaches used in siderophore analysis and current ICP-MS technologies are reviewed. The recent tools for fast dereplication of secondary metabolites and their databases are reported. This review on siderophores is concluded with their recent medical, biochemical, geochemical, and agricultural applications in mass spectrometry context. © 2015 Wiley Periodicals, Inc. Mass Spec Rev.

Keywords: iron; siderophores; mass spectrometry; biomarkers; dereplication

I. INTRODUCTION

This review is dedicated to mass spectrometry analysis of microbial siderophores, important natural iron chelators. Iron is a highly vital essential nutrient both in microbes and mammals. Consequently, competition for iron is often a conserved feature in most host-pathogen interactions (Haas, 2012). Not surprisingly, both microbes and mammals have evolved numerous strategies for iron fitness. Mammals have learnt to always limit availability of free iron and instead conceal this element in complexes with iron binding proteins such as transferrin, lactoferrin, ferritin, and others, or incorporate iron into compounds such as heme in haemoglobin (Morgenthau, Beddek, & Schryvers, 2014). In order to overcome limited bioavailability of free iron in their hosts, pathogens have evolved many different

energy-dependent iron acquisition systems from mammalian complexes. One class of iron acquisition systems is represented by siderophores, secretory low-molecular-weight iron-chelating compounds, used by microbes to scavenge iron from host iron-binding proteins (Haas, Eisendle, & Turgeon, 2008). Subsequently, the iron-siderophore complexes are transported into the microbes via specific receptors on the microbial cell surface and the iron is released. The ligating groups contain mostly oxygen atoms (of hydroxamate, catecholate, α -hydroxy- and α -keto-carboxylic acids) as well as nitrogen atoms. The latter case is represented e.g., by maduraferriin, in which iron binding nitrogen of hexahydropyridazine-3-carboxylic acid is negatively charged (Kellerschierlein et al., 1988). On the other hand, microbial biosynthetic machinery also involves diverse products including crystalline metal nanoparticles (Siponen et al., 2013). Recently, transferring bacterial magnetosome gene clusters to easily handled microorganisms enabled synthetic production of monodisperse single magnetic domain nanoparticles at ambient temperature (Kolinko et al., 2014). In addition to ferromagnetic Fe₃O₄ or Fe₃S₄ nanoparticles, some bacteria yeasts and fungi produce gold, ZnS, CdS, zirconium, and silver nanoparticles (Asmathunisha & Kathiresan, 2013). Metal nanoparticle producers can also be found among important human fungal pathogens (Bhainsa & D'Souza, 2006). Therefore, extracellular scavenging and acquisition of essential elements using energy dependent protein complexes is an evolutionary conserved fitness factor for all pathogens and consequent target for potential therapeutic strategies.

In addition to biochemical applications, siderophores enjoy considerable potential in biotechnology and pharmaceutical sector. The obligator significance of siderophores in pathogens may allow using siderophores as *Trojan horses*, representing a novel class of antibiotics with considerable therapeutic potential (Bassetti et al., 2013; Fernandes & de Carvalho, 2014; Miller et al., 2009). Growing pool of evidence indicates that siderophores and their semisynthetic analogues may be used in anti-cancer therapeutic strategies (Brard, Granai, & Swamy, 2006; Keeler & Brookes, 2013). Siderophores have also been demonstrated to enjoy potential in magnetic resonance imaging (MRI) (Ding, Helquist, & Miller, 2008) or positron emission tomography reagents (Petrik et al., 2010). Microbial siderophores can also be used as specific disease biomarkers in cases when ribosomal protein fingerprinting is difficult (Bertrand

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*Correspondence to: Vladimír Havlíček, Institute of Microbiology, AS CR v.v.i., Videnska 1083 CZ 142 20, Prague 4, Czech Republic. E-mail: vlhavlic@biomed.cas.cz

et al., 2009; Ye et al., 2013). They also have wide applications in bioremediation (Dimkpa et al., 2009a) and geochemistry (Duckworth et al., 2009). Biocontrol involving plant-pathogenic fungi interaction is another fascinating application area of siderophores (Tortora, Diaz-Ricci, & Pedraza, 2011).

The analysis of siderophores in natural samples (seawater, soil, biological fluids) presents many unique challenges. In natural samples, siderophores are often present in low concentrations and exist in multi-protein complexes. In order to address concentration issues, most analytical strategies for siderophores usually employ pre-concentration of samples by solid phase extraction (SPE) with columns packed with reversed-phase or ion-exchange sorbents and subsequently isolated by high performance liquid chromatography (HPLC). In nuclear magnetic resonance (NMR) spectroscopy characterization, the metal is generally removed from the complex prior to data acquisition, as Fe^{3+} causes line broadening of NMR signals (Birus et al., 1995). To obtain NMR spectra of siderophores in metal-chelated form, usually the diamagnetic Ga^{3+} or Al^{3+} siderophore analogs are employed. In this review we summarize enrichment procedures and mass spectrometry armory capable to trace siderophores and characterize their structure. The critical evaluation is focused to mass data filtering, isotopic structure and iron localization using separations followed by mass spectrometry.

II. CHARACTERISTICS OF MICROBIAL SIDEROPHORES

Siderophores involve hydroxamate, catecholate, phenolate, α -hydroxycarboxylate, or mixed-function groups capable to form coordination complexes with very high affinity and selectivity for ferric ions (Hider & Kong, 2010). The biosynthesis of microbial secondary metabolites is manifested through sophisticated molecular machinery involving diverse ribosomal, hybrid, or non-ribosomal polyketide synthases (NRPS) (Franke, Ishida, & Hertweck, 2014; Kalb, Lackner, & Hoffmeister, 2013). Siderophores are mostly biosynthesized in NRPS (Bunet et al., 2014; Inglis et al., 2013), encompassing various functional catalytic domains responsible for the activation of the amino acid, propagation of the growing peptide chain, condensation of the amino acids and the release of peptides by hydrolysis or macrocyclization, etc. (Lee et al., 2010; Yin et al., 2013). The products of non-ribosomal synthesis generally contain atypical building blocks and their molecular weights span from 400 to 1600 Da (Meyer et al., 2008).

There are many analytical techniques for characterizing siderophores. These include CAS (Chrome azurol S) assays (Milagres, Machuca, & Napoleão, 1999), growth stimulation tests, ^{59}Fe uptake assays and isoelectrofocusing. Although they are fairly functional, these assays fail to give structural information and can easily be misinterpreted. In contrast siderophore analysis using mass spectrometry, NMR spectroscopy and/or X-ray diffraction (Bottcher & Clardy, 2014) allow for detailed structure analysis. In addition to screening for known siderophores, the HPLC/ESI-MS approach is extremely useful for the detection of biogenetic precursors of siderophores. As illustrative example, pyoverdins are biosynthesized by large NRPSs involving at least 12 different proteins, starting in the cytoplasm and ending in the periplasm (Parker et al., 2014). More than 60 pyoverdins have been described so far (Schalk &

Guillon, 2013) and represent very complex branch-cyclic structures (Fig. 1).

Table 1 summarizes selected important microbial siderophores with their physico-chemical characteristics. Molecular formulas and IUPAC mass values of the desferri- and ferriforms (Fe^{3+}) of siderophores can be used for initial orientation in complex fermentation broths or clinical samples. Table 1 also reports the microbial source and original references, from which actual structures can be traced. At present, our in-house siderophore libraries contain secondary metabolites of genera *Aspergillus*, *Alternaria*, *Emericella*, *Pseudallescheria*, *Cladosporium*, *Fennellia*, *Eurotium*, *Trichothecium*, *Phoma*, *Metarhizium*, *Beauveria*, and *Candida*. This collection also contains 600 annotated microbial siderophore entries and can be provided upon request in xml or xls formats. Xml format is used in mMass open source tool, which gained reputation in lipid and proteomic analysis (Strohalm et al., 2010). The software supports general data formats and can be downloaded for free from www.mmass.org. For a typical computer screen snapshot see Figure 2. In this particular example siderophores were identified in an externally calibrated MALDI mass spectrum of a urinal sample. Mass tolerance was set to 2 ppm and no adduct formation (or iron presence) was selected. Hence, ferric form of Ferrioxamine E (m/z 654.2680) was not labeled in this case. Positive hits were annotated by green circles in the mass spectrum and listed in green in identified compound list.

For general microbial compounds, the most comprehensive commercial database is AntiBase (Wiley-VCH, Weinheim, Germany) the 2012 version of which contains 40,000 recorded compounds (<http://www.wiley-vch.de/stmdata/antibase.php>, access date June 26, 2014). In addition, there are many other useful repositories (KEGG, Metlin, human metabolomics database, Scifinder, Antimarin database, dictionary of natural products). However, not all of them are freely accessible from the web. Scripps center for metabolomics claims more than 240,000 metabolite entries (<http://metlin.scripps.edu/index.php>, access date June 27, 2014). The in-house siderophore library can also be obtained from R. Schuhmacher group, which recently published a comprehensive work on *Trichoderma* siderophores (Lehner et al., 2013). Free online siderophore library contains 262 structures with microbial source information (http://bertrandsamuel.free.fr/siderophore_base/siderophores.php, access date June 26, 2014). Chemistry and biology of siderophores covering 1950 up to the end of 2009 was reviewed by R. Hider and X. Kong. For structures see also the supplementary file available from the internet (Hider & Kong, 2010). In addition to the main document, product ion mass spectra and a lot of useful information can also be found in supplementary material from P. Dorrestein's work (Moree et al., 2012). Some commercial software tools for fast dereplication of natural products were reviewed recently (Klitgaard et al., 2014).

III. ENRICHMENT OF SIDEROPHORES

Siderophore overproducing strains with substantial industrial importance can be found in patent literature (Weber et al., 2010). With the exception of fermentation broths the concentrations of siderophores in other real samples are low and masked by complex matrix. The removal of interfering compounds as well as pre-concentration of siderophores represent a standard step not only in clinical diagnostics (Willemse-Erix et al., 2010).

TABLE 1. Mass spectrometry data of selected naturally occurring siderophores

Siderophore	Molecular formula	Theoretical masses (m/z)		Strain or source	Ref.
		[M+H] ⁺	[M+Fe-2H] ⁺		
Amphibactin D	C ₃₈ H ₆₉ N ₇ O ₁₃	832.5026	885.4141	Marine water	(Gledhill et al., 2004; Mawji et al., 2008; Mawji et al., 2011)
Amphibactin E	C ₄₀ H ₇₁ N ₇ O ₁₃	858.5183	911.4297	Marine water	
Anguibactin	C ₁₅ H ₁₆ N ₄ O ₄ S	349.0965	402.0080	<i>Vibrio anguillarum</i>	(Naka, Actis, & Croso, 2013)
Bacillibactin	C ₃₉ H ₄₂ N ₆ O ₁₈	883.2628	936.1743	<i>Bacillus cereus</i> , <i>Bacillus anthracis</i>	(Zawadzka et al., 2008)
Bisucaberin	C ₁₈ H ₃₂ N ₄ O ₆	401.2395	454.1509	<i>Vibrio alginolyticus</i> B522, <i>Shewanella algae</i> B516	(Boettcher & Clardy, 2014)
Bisucaberin B	C ₁₈ H ₃₄ N ₄ O ₇	419.2500	472.1615	<i>Tenacibaculum mesophilum</i>	(Fujita, Nakano, & Sakai, 2013)
Coelichen	C ₂₁ H ₃₉ N ₇ O ₁₁	566.2780	619.1895	<i>Streptomyces</i> spp.	(Dimkpa et al., 2008)
Coprogen	C ₃₅ H ₅₆ N ₆ O ₁₃	769.3978	822.3093	<i>Laccaria laccata</i> , <i>Laccaria bicolor</i> , <i>Pseudomonas aeruginosa</i> , <i>Aspergillus fumigatus</i>	(Haselwandter et al., 2013; Hayen & Volmer, 2005)
Corynebactin	C ₁₈ H ₂₆ N ₂ O ₁₄	495.1457	548.0571	<i>Corynebacterium diphtheriae</i>	(Zajdowicz et al., 2012)
Desferrioxamine A _{1a} Desferrioxamine A _{1b}	C ₂₄ H ₄₆ N ₆ O ₈	547.3450	600.2565	<i>Salinispora tropica</i> CNB-440	(Ejje et al., 2013)
Desferrioxamine A ₂	C ₂₃ H ₄₄ N ₆ O ₈	533.3293	586.2408	<i>Salinispora tropica</i> CNB-440	
Desferrioxamine B	C ₂₅ H ₄₈ N ₆ O ₈	561.3606	614.2721	<i>Streptomyces</i> spp., <i>Pseudomonas aeruginosa</i> , <i>Aspergillus fumigatus</i>	(Dimkpa et al., 2008; Ejje et al., 2013; Gledhill et al., 2004; Hayen & Volmer, 2005; Mawji et al., 2011; McCormack, Worsfold, & Gledhill, 2003; Petrik et al., 2014)
Desferrioxamine D ₁	C ₂₇ H ₅₀ N ₆ O ₉	603.3712	656.2827	<i>Salinispora tropica</i> CNB-440	(Ejje et al., 2013)
Desferrioxamine D ₂	C ₂₆ H ₄₆ N ₆ O ₉	587.3399	640.2514	Marine water	(Ejje et al., 2013; Mawji et al., 2011)
Desferrioxamine E	C ₂₇ H ₄₈ N ₆ O ₉	601.3556	654.2670	<i>Streptomyces</i> spp., <i>Aspergillus fumigatus</i> , marine water	(Dimkpa et al., 2008; Ejje et al., 2013; Mawji et al., 2008; Mawji et al., 2011; Petrik et al., 2014)
Desferrioxamine G	C ₂₇ H ₅₀ N ₆ O ₁₀	619.3661	672.2776	<i>Pseudomonas stutzeri</i> , marine water	(Essen et al., 2007; Gledhill et al., 2004; Mawji et al., 2008; Mawji et al., 2011)
Desferrioxamine G ₂	C ₂₆ H ₄₈ N ₆ O ₁₀	605.3505	658.2619	<i>Pseudomonas stutzeri</i>	(Essen et al., 2007)
Desferrioxamine N	C ₂₆ H ₅₀ N ₆ O ₈	575.3763	628.2878	<i>Salinispora tropica</i> CNB-440	(Ejje et al., 2013)
Desferrioxamine X ₁	C ₂₅ H ₄₄ N ₆ O ₉	573.3243	626.2357	<i>Pseudomonas stutzeri</i>	(Essen et al., 2007)
2,3-Dihydroxybenzoic acid	C ₇ H ₆ O ₄	155.0339	207.9453	<i>Escherichia coli</i> , <i>Bacillus DET9</i>	(Ma & Payne, 2012; Patel et al., 2010)
2,3-Dihydroxybenzoylglycine	C ₉ H ₉ NO ₅	212.0553	264.9668	Strain ST3	(Ahire et al., 2011b)
2,3-Dihydroxybenzoylserine	C ₁₀ H ₁₁ NO ₆	242.0659	294.9774	Strain ST2	(Ahire et al., 2011a)
Dimerumic acid	C ₂₂ H ₃₆ N ₄ O ₈	485.2606	538.1721	<i>Scedosporium apiospermum</i> , <i>Trichoderma</i> spp.	(Bertrand et al., 2009; Lehner et al., 2013)
Enterobactin	C ₃₀ H ₂₇ N ₅ O ₁₅	670.1515	723.0630	<i>Escherichia coli</i> , Strain ST2, <i>Pseudomonas aeruginosa</i> , <i>Klebsiella pneumoniae</i>	(Ahire et al., 2011a; Bachman et al., 2011; Hayen & Volmer, 2005; Ma & Payne, 2012)

Epichloënin A	C ₄₆ H ₇₄ N ₁₂ O ₁₈	1083.5317	1136.4431	<i>Epichloë festucae</i>	(Koulman et al., 2012)
Epichloënin B	C ₄₄ H ₇₁ N ₁₁ O ₁₇	1026.5102	1079.4217	<i>Epichloë festucae</i>	
Ferricrocin	C ₂₈ H ₄₇ N ₉ O ₁₃	718.3366	771.2481	<i>Laccaria laccata</i> , <i>Laccaria bicolor</i> , <i>Aspergillus fumigatus</i> , <i>Trichoderma</i> spp.	(Haselwandter et al., 2013; Lehner et al., 2013; Petrik et al., 2014)
Ferrichrome	C ₂₇ H ₄₅ N ₉ O ₁₂	688.3260	741.2375	<i>Pseudomonas aeruginosa</i> , <i>Aspergillus fumigatus</i> , marine water	(Hayen & Volmer, 2005; McCormack, Worsfold, & Gledhill, 2003; Petrik et al., 2014)
Ferrirhodin	C ₄₁ H ₆₇ N ₉ O ₁₇	958.4728	1011.3842	<i>Pseudomonas aeruginosa</i> , <i>Fusarium sacchari</i>	(Hayen & Volmer, 2005; Munawar et al., 2013)
Fusarinine A	C ₂₂ H ₃₈ N ₄ O ₉	503.2712	556.1826	<i>Trichoderma</i> spp.	(Lehner et al., 2013)
Fusarinine B	C ₃₃ H ₅₆ N ₆ O ₁₃	745.3978	798.3093	<i>Laccaria laccata</i> , <i>Laccaria bicolor</i> , <i>Fusarium roseum</i>	(Haselwandter et al., 2013)
Fusarinine C (cyclic fusigen)	C ₃₃ H ₅₄ N ₆ O ₁₂	727.3873	780.2987	<i>Laccaria laccata</i> , <i>Laccaria bicolor</i> , <i>Fusarium roseum</i> , <i>Aureobasidium pullulans</i> HN6.2, <i>Aspergillus fumigatus</i> , <i>Trichoderma</i> spp.	(Haselwandter et al., 2013; Lehner et al., 2013; Petrik et al., 2014; Wang et al., 2009)
Mirubactin	C ₂₆ H ₃₂ N ₆ O ₁₁	605.2202	658.1316	<i>Actinosynnema mirum</i>	(Giessen et al., 2012)
Mugineic acid 2'-epi-mugineic acid	C ₁₂ H ₂₀ N ₂ O ₈	321.1292	374.0407	rhizosphere	(Dell'mour et al., 2010; Dell'mour et al., 2012)
2'-deoxymugineic acid	C ₁₂ H ₂₀ N ₂ O ₇	305.1343	358.0458	rhizosphere	(Dell'mour et al., 2010; Dell'mour et al., 2012; Weber, von Wiren, & Hayen, 2006)
N ⁶ -methyl coprogen B	C ₃₄ H ₅₆ N ₆ O ₁₂	741.4029	794.3144	<i>Scedosporium apiospermum</i>	(Bertrand et al., 2009)
Petrobactin	C ₃₄ H ₅₀ N ₆ O ₁₁	719.3610	772.2725	<i>Bacillus cereus</i> , <i>Bacillus anthracis</i> , <i>Bacillus subtilis</i>	(Bugdahn et al., 2010; Zawadzka et al., 2008)
Pseudobactin	C ₄₂ H ₆₀ N ₁₂ O ₁₆	989.4323	1042.3438	<i>Pseudomonas fluorescens</i> WCS374r	(Djavaheri et al., 2012)
Pseudomonine	C ₁₆ H ₁₈ N ₄ O ₄	331.1400	384.0515	<i>Pseudomonas fluorescens</i> WCS374r	
Putrebactin	C ₁₆ H ₂₈ N ₄ O ₆	373.2082	426.1196	<i>Shewanella putrefaciens</i>	(Soe & Codd, 2014)
Quinolobactin	C ₁₁ H ₉ NO ₄	220.0604	272.9719	<i>Pseudomonas fluorescens</i> ATCC 17400	(Mossialos et al., 2000)
Rhizoferrin	C ₁₆ H ₂₄ N ₂ O ₁₂	437.1402	490.0517	<i>Francisella tularensis</i>	(Sullivan et al., 2006)
Rhodobactin	C ₃₆ H ₅₀ N ₁₀ O ₁₃	831.3632	884.2746	<i>Rhodococcus rhodochrous</i>	(Dhungana et al., 2007)
Rhodotorulic acid	C ₁₄ H ₂₄ N ₄ O ₆	345.1769	398.0883	<i>Pseudomonas aeruginosa</i> , marine water	(Hayen & Volmer, 2005; McCormack, Worsfold, & Gledhill, 2003)
Salicylic acid	C ₇ H ₆ O ₃	139.0390	191.9504	<i>Pseudomonas fluorescens</i> WCS374r	(Djavaheri et al., 2012)
Salmochelin S1	C ₂₆ H ₃₀ N ₂ O ₁₆	627.1668	680.0783	<i>Escherichia coli</i>	(Caza et al., 2008)
Salmochelin S2	C ₄₂ H ₄₉ N ₃ O ₂₆	1012.2677	1065.1792	<i>Escherichia coli</i>	
Salmochelin S4	C ₄₂ H ₄₇ N ₃ O ₂₅	994.2571	1047.1686	<i>Escherichia coli</i>	
Salmochelin SX	C ₁₆ H ₂₁ NO ₁₁	404.1187	457.0302	<i>Escherichia coli</i>	

Scabichelin	C ₂₆ H ₄₉ N ₉ O ₁₀	648.3680	701.2795	<i>Streptomyces antibioticus</i> NBRC 13838, <i>Streptomyces scabies</i> JCM 7914, <i>Streptomyces turgidiscabies</i> JCM 10429	(Kodani et al., 2013)
Staphyloferrin A	C ₁₇ H ₂₄ N ₂ O ₁₄	481.1300	534.0415	<i>Staphylococcus aureus</i>	(Pandey, Jarvis, & Low, 2014)
Staphyloferrin B	C ₁₆ H ₂₄ N ₄ O ₁₁	449.1514	502.0629	<i>Ralstonia solanacearum</i>	(Bhatt & Denny, 2004)
Synechobactin A	C ₂₂ H ₄₀ N ₄ O ₉	505.2868	558.1983	<i>Synechococcus sp.</i> PCC 7002	(Boiteau et al., 2013)
Synechobactin B	C ₂₄ H ₄₄ N ₄ O ₉	533.3181	586.2296	<i>Synechococcus sp.</i> PCC 7002	
Synechobactin C	C ₂₆ H ₄₈ N ₄ O ₉	561.3494	614.2609	<i>Synechococcus sp.</i> PCC 7002	
N,N',N''-triacylfusarinine C	C ₃₉ H ₆₀ N ₆ O ₁₅	853.4189	906.3304	<i>Laccaria laccata</i> , <i>Laccaria bicolor</i> , <i>Aspergillus fumigatus</i>	(Haselwandter et al., 2013; Petrik et al., 2014)
Turgichelin	C ₂₄ H ₄₄ N ₈ O ₁₁	621.3207	674.2322	<i>Streptomyces antibioticus</i> NBRC 13838, <i>Streptomyces scabies</i> JCM 7914, <i>Streptomyces turgidiscabies</i> JCM 10429	(Kodani et al., 2013)
Vanchrobactin	C ₁₆ H ₂₃ N ₅ O ₇	398.1670	451.0785	<i>Vibrio anguillarum</i>	(Naka, Lopez, & Crosa, 2008)
Vicibactin	C ₃₃ H ₅₄ N ₆ O ₁₅	775.3720	828.2835	<i>Rhizobium leguminosarum</i> ATCC14479	(Wright et al., 2013)
Yersiniabactin	C ₂₁ H ₂₇ N ₃ O ₄ S ₃	482.1236	535.0351	<i>Klebsiella pneumoniae</i>	(Bachman et al., 2011)

Table 2 summarizes literature data indicating the application of solid phase extraction of specific molecular structures. The most popular sorbents were polystyrene-divinylbenzene copolymers represented by Amberlite XAD-2, Amberlite XAD-4, Amberlite XAD 16 commercial cartridges. For some structures modified polystyrene-divinylbenzene copolymers were found superior. For eluents suitable for hydroxylated (Isolute ENV+ cartridge), copolymeric divinylbenzene and N-vinylpyrrolidone (Oasis HLB 12 cc cartridges), acrylic ester resins (Amberlite XAD-7 cartridge) or silica based bonded phases (Sep-Pak C₁₈ plus cartridge) see Table 2 and references cited therein. For explorative SPE for accelerated microbial natural product discovery see references (Mansson et al., 2010; Nielsen et al., 2011).

Significantly, currently there is paucity of enrichment strategies targeting specific valence of iron utilizing more specific iron bonding. An affinity ferrous-form siderophore capture could potentially be carried out by Smopex-102 fibers acting as metal scavengers (Karppi et al., 2009). Chopped fibers weak acid cation exchangers based on acrylic acid grafted polyolefins (exchange capacity 7–10 mmol/g) can also be potentially tested (<http://www.scavengingtechnologies.com/>, access date June 25, 2014).

IV. CHARACTERIZATION OF SIDEROPHORES BY MOLECULAR MASS SPECTROMETRY

The applications of mass defect data filtering was reviewed by Sleno (2012). In ESI or MALDI mass spectra both desferric and ferric forms of siderophores can be observed (Fig. 3, insets). The iron signature can be inferred even from low resolution mass spectrum due to stable isotope profile [naturally occurring isotopes: ⁵⁴Fe (5.8%), ⁵⁶Fe (91.8%), ⁵⁷Fe (2.1%), and ⁵⁸Fe (0.3%)]. In iron specific case interpreter tools search for 1.99533 or 52.91146 m/z differences between isotopic (⁵⁴Fe, ⁵⁶Fe) signals or desferric and ferric form of a siderophore, respectively (Yin et al., 2013). Siderophore structural details are then obtained from the product ion scanning. Figure 1 indicates dissociation of amidic bonds in ferric form of ferrioxamine B. For m/z values of protonated molecules and [M+Fe-2H]⁺ ions of selected siderophores see Table 1.

Figure 4 illustrates the hydrogen index as a function of the oxygen/carbon (oxygen index) and nitrogen/carbon atomic ratios of carbon-containing both desferric and ferric forms of siderophores. Van Krevelen plots were generated *in silico* from the siderophore set listed in Table 1. Desferric forms have higher indexes than their ferric forms from definition. Note the non-constant differences between the ferric and desferric form

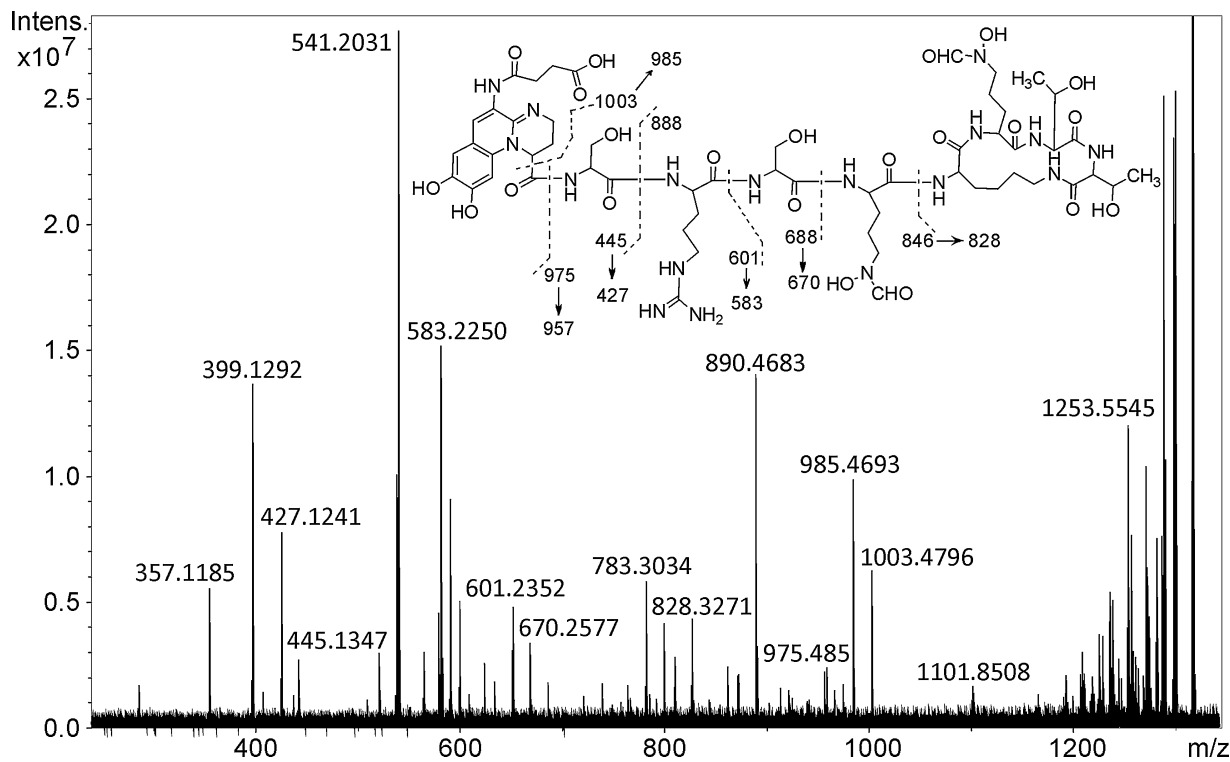


FIGURE 1. Fragmentation of Pyoverdins Pa A.

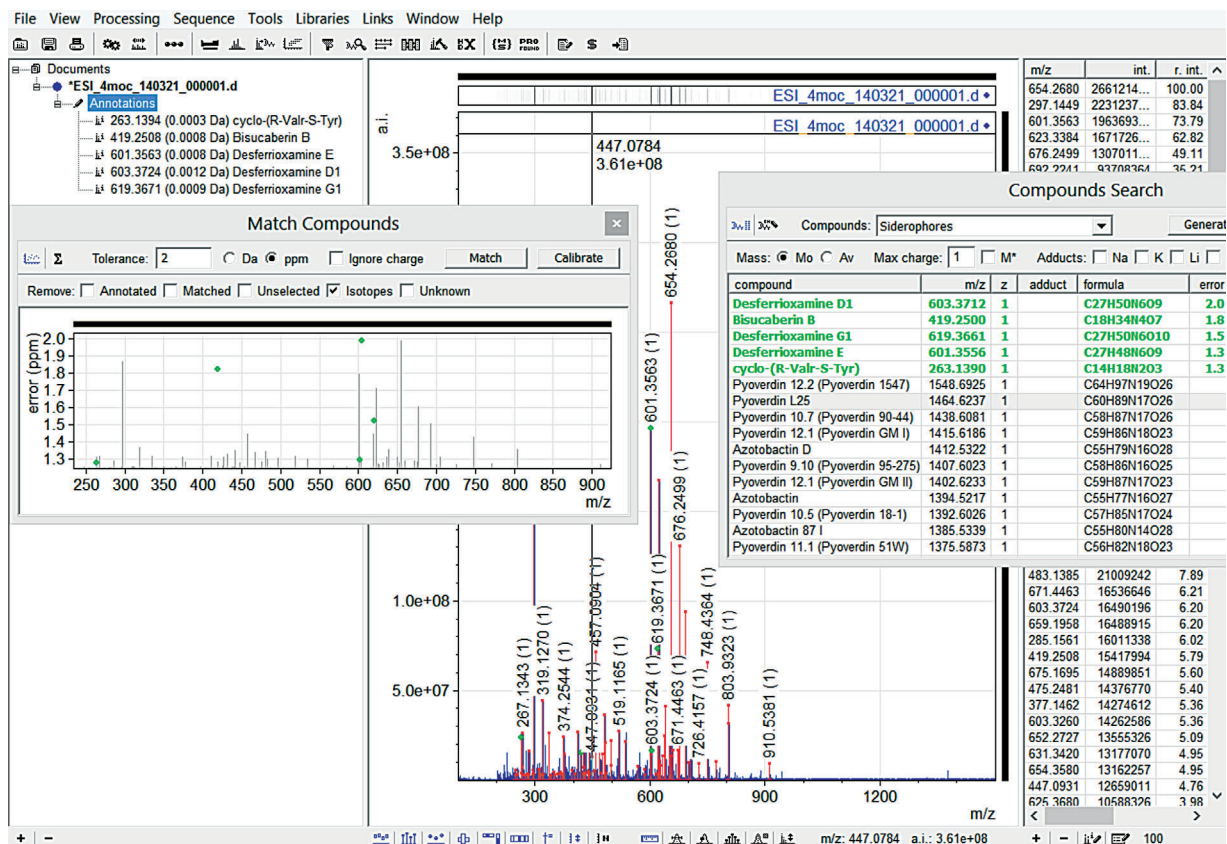


FIGURE 2. Snapshot of mMass application.

TABLE 2. Protocols for solid phase extraction of microbial siderophores

SPE cartridge	Analyte	Eluent	Ref.
Isolute ENV+	epichloënin A	methanol	(Lee et al., 2010)
	epichloënins A, B	formic acid : water : methanol 1:20:80 (v/v/v)	(Koulman et al., 2012, Lee et al., 2010)
	synechobactins A, B, C ferrioxamine E, ferrichrome	methanol	(Boiteau et al., 2013)
	amphibactins D and E, ferrioxamine B, ferrioxamines D ₂ , E, G	acetonitrile : propan-2-ol : water : formic acid 81:14:5:1 (v/v/v/v)	(Mawji et al., 2008, Mawji et al., 2011)
	amphibactins D, and E ferrioxamine B, and G	formic acid : water : methanol 1:20:80 (v/v/v)	(Gledhill et al., 2004)
	ferrichrome, ferrioxamine B, rhodotorulic acid	formic acid : water : methanol 1:20:80 (v/v/v)	(McCormack, Worsfold, & Gledhill, 2003)
Amberlite XAD-2	coprogen, ferricrocin, fusarinine B, fusarinine C, N,N',N"-triacetyl fusarinine C	methanol	(Haselwandter et al., 2013)
	ferrioxamines A _{1a} , A _{1b} , A ₂ , B, D ₁ , D ₂ , E, N	50 % methanol and 100 % methanol	(Ejje et al., 2013)
Amberlite XAD-7	anguibactin, vanchrobactin	methanol	(Naka, Lopez, & Crosa, 2008)
	rhodobactin	1 % TFA in 10 % methanol	(Dhungana et al., 2007)
Amberlite XAD-16	“Hydroxamate-type” siderophores	methanol	(McMillan et al., 2010, Velasquez et al., 2011)
Amberlite XAD-4	pyoverdins	50 % methanol	(Murugappan et al., 2012)
Sep-Pak C ₁₈ plus	ferrioxamines B, E, G, G ₂ , X ₁	methanol	(Lee et al., 2010)
Oasis HLB 12 cc	coprogen, enterobactin, ferrioxamine B, ferrichrome, ferrirhodin, pyochelin, rhodotorulic acid	2 % formic acid in methanol	(Hayen & Volmer, 2005)

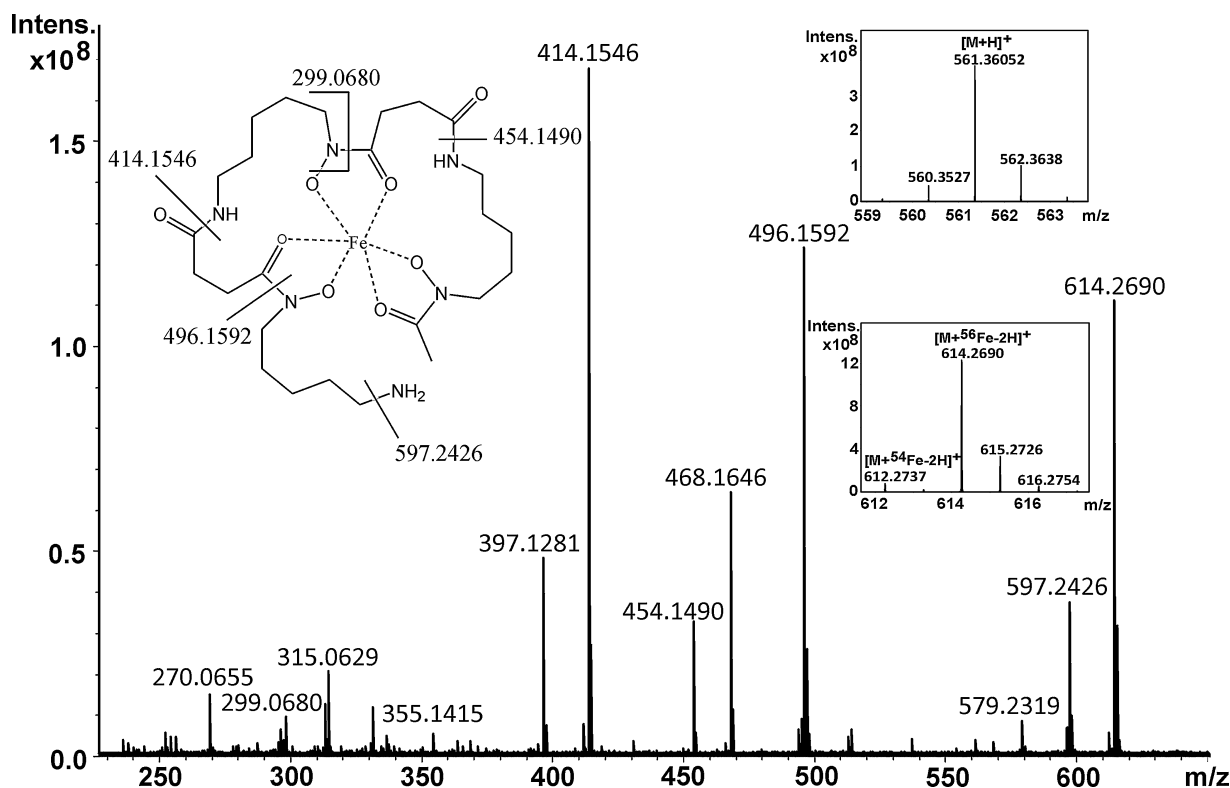


FIGURE 3. The product ion spectrum of ferrioxamine B $[M+^{56}\text{Fe}-2\text{H}]^+$ ion (m/z 614.2721, isolation window 5 Da). The insets show details of molecular regions of desferric (top) and ferric forms (bottom) recorded in the conventional mass spectrum.

of a selected siderophore. Clustering the siderophores into families according to oxygen index is depicted in Figure 5. The broad distribution of bactins (1) well corresponds to their structural diversity. On the contrary, ferrioxamines (2) are more homogeneous in their elemental composition (Table 1). Van

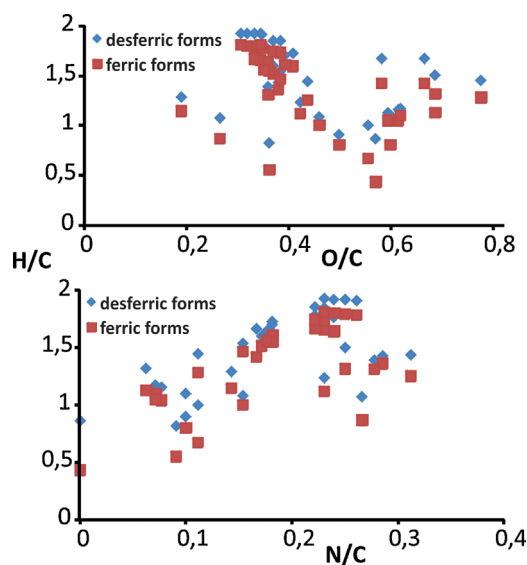


FIGURE 4. Dirk Willem van Krevelen's H/C over O/C (top) and N/C (bottom) diagrams illustrating the distribution of desferric and ferric forms in siderophore space. Individual siderophores were extracted from Table 1.

Krevelen plots can be used for initial orientation in and dataset presentation of complex fermentation broths.

In addition to stable isotopes, ^{59}Fe -labeling was also used in biochemical studies (Expert, Boughammoura, & Franza, 2008). The monitoring the ^{59}Fe -labeled fermentation broths can be directly carried out by liquid chromatography with radio-detection (Budzikiewicz, Schafer, & Meyer, 2007). The total number of references related to ^{59}Fe -labeling is rather low and may be attributed to the introduction of inductively coupled plasma mass spectrometry (ICP-MS) applications.

V. THE CONTRIBUTION OF ICP-MS AND ATOMIC SPECTROMETRY TECHNIQUES TO SIDEROPHORE CHARACTERIZATION

Inductively coupled plasma mass spectrometry offers many unique advantages over other techniques for analysis of metal-complexed biomolecules like siderophores. The first advantage is high temperature ionization (approximately 5,500 K) over low-temperature ion sources, resulting in decomposition of all chemical bonds in the analyte plasma. Therefore, the data acquired from a plasma ion source represent the total content of an element in the sample, enabling simple and accurate multielement quantitation. Conventional ICP-MS generates mass spectra in the mass-to-charge (m/z) range 5–257. A second advantage of ICP-MS is the analyte plasma has the high ion density, provides excellent collision rates and therefore also higher sensitivities than some other ion sources. The ICP ion source is also less vulnerable to the salts and solvents usually

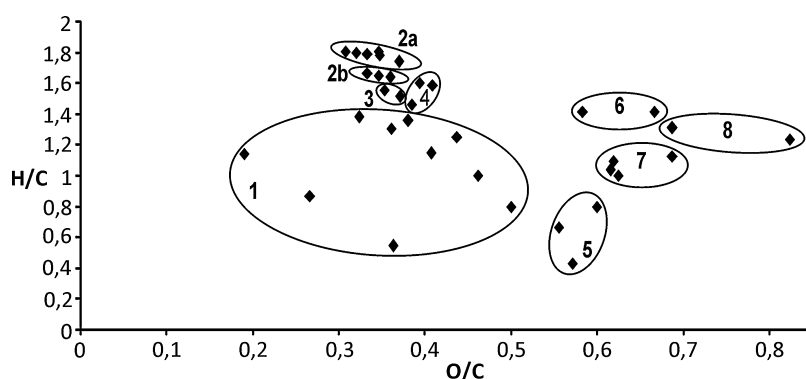


FIGURE 5. Two-dimensional van Krevelen's diagram indicating the clustering of siderophore families extracted from Table 1: 1-bactins, 2a-linear ferrioxamines, 2b-cyclic ferrioxamines, 3-coprogens, 4-fusarinines, 5-2,3-dihydroxy derivatives (benzoic acid, glycine, and serine), 6-mugineic acids, 7-salmochelins, 8-staphyloferrins.

present in biological samples. In combination with HPLC the ICP-MS thus represents a good tool for locating ferric forms of siderophores in complex sample mixtures.

According to recent report sensitive detection (<1 picomole Fe) of siderophores can be achieved by using iron-free HPLC systems to reduce background Fe levels (Boiteau et al., 2013). In that report the $^{40}\text{Ar}^{16}\text{O}^+$ interferences were minimized on ^{56}Fe by application of a collision cell and introducing oxygen into the sample carrier gas to prevent the formation of carbon deposits that decrease sensitivity. HPLC-ICP mass spectrometry in low organic solvent systems can also be used for ratio determination of bound and free iron in clinical samples. The addition of pyridine-2,6-dicarboxylic acid into the mobile phase and the resulting anionic complexes were separated by ion exchange chromatography (IonPac Cs-5A column) followed by ICP-MS detection (Hu, 2011). An older alternative based on reductive displacement of iron by gallium was performed using ascorbate as the reducing agent to increase the sensitivity. Nanomolar detection limits were achieved in this case (Moberg et al., 2004).

Six free metals [Co(II), Cu(II), Fe(III), Mn(II), Ni(II), and Zn(II)] together with their complexes with phytosiderophores (mugineic acid, epi-mugineic acid and deoxymugineic acid and their diastereomers) were analyzed by capillary electrophoresis (CE) and sector field ICP-MS (Dell'mour et al., 2010). CE separation was realized at a voltage of +25 kV employing a background electrolyte containing 20 mM ammonium bicarbonate at pH 7.2. Limits of detection of $[\text{M}-4\text{H}+\text{Fe}(\text{III})]^-$ ions were in 0.9–2.2 μM range. Slightly better sensitivity was achieved in negative ion mode for the corresponding desferric forms (0.8–1.4 μM).

Phytoremediation is another important area of siderophore industrial applications. Inductively coupled plasma mass spectrometry was used to measure iron and cadmium contents in the bacterium *Streptomyces tendae* and in sunflower plant grown in Cd-amended soil (Dimkpa et al., 2009b). It was concluded that siderophores can help reduce toxic metal uptake in bacteria, while simultaneously facilitating the uptake of such metals by plants.

High-precision isotopic analyses by multicollector ICP-MS were used to investigate the mass-dependent fractionation of Fe isotopes during bacterial metal assimilation in experiments with *Azotobacter vinelandii*. *A. vinelandii* is a diazotroph with high

demand for iron during nitrogen fixation. The growth medium became isotopically lighter as Fe was removed from solution. The experimental data were interpreted in terms of Rayleigh fractionation, yielding fractionation factor of 1.0011 for iron (Wasylenki et al., 2007).

Alternatively, iron content in siderophore samples can be determined by graphite furnace atomic absorption spectrometry (Lehner et al., 2013). Eighteen different iron chelators isolated from *Trichoderma* spp. were detected and ten structures characterized by mass spectrometry.

VI. MASS SPECTROMETRY IN MEDICINAL APPLICATIONS OF SIDEROPHORES

Resistance to antibiotics was revealed by the monitoring of characteristic mass shifts of microorganism biomarkers in mass spectra of vegetative cells and spores grown with/without antibiotics in a ^{13}C -labeled/non-labeled media (Demirev et al., 2013). The presence of small acid-soluble spore proteins generated in the presence of an antibiotic in 2 hr frame was a resistance indicator. In tackling antibiotic resistance the three siderophore-related approaches were developed: competitive iron chelation, blocking of siderophore biosynthesis, and finally the development of siderophore-drug conjugates called *Trojan horse antibiotics* as new promising drugs capable to combat infections caused by resistant bacteria and fungi (Costa Mattos et al., 2013; Ji & Miller, 2012; Wencewicz et al., 2009). The considerable antibacterial activity against ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Escherichia coli*) was revealed by desferrioxamine, sideromycins or staphyloferrin A conjugates (Milner et al., 2013; Wencewicz & Miller, 2013; Flanagan et al., 2011; Tomaras et al., 2013). Baulamycins A and B were identified as inhibitors of siderophore biosynthesis in *Staphylococcus aureus* and *Bacillus anthracis*. Their excellent antibacterial activity against Gram-positive and Gram-negative bacteria represented the potential of siderophores as broad-spectrum antibiotics (Tripathi et al., 2014).

Mass spectrometry imaging (MSI) experiments provide direct access inhibition constants in antimicrobial susceptibility studies (Moree et al., 2013). The interaction between combating microorganisms through siderophore equipment was also

assessed by MSI (Moree et al., 2012; Yang et al., 2011). Mass spectrometry imaging was applied for the determination of secondary metabolites of worldwide collected yeast, filamentous fungi, pathogens, and marine-derived bacteria including *Streptomyces coelicolor*, *Bacillus subtilis*, *Pseudomonas aeruginosa* (Hsu et al., 2013). The determination and visualization of metabolites of *Pseudomonas aeruginosa* and *Aspergillus fumigatus* causing polymicrobial infection in patients with cystic fibrosis was another exciting application of MSI (Moree et al., 2012).

In fully sequenced microorganisms many siderophore receptors have been characterized by mass spectrometry (Mathavan et al., 2014; Podkowa et al., 2014). The receptor proteins are synthesized in response to iron limitation and may become major outer membrane proteins with copy numbers up to 10^5 . Siderophore-transport processes may or may not involve the permeation of the complex in ferric form through the cell membrane into cytoplasm. The desferric siderophore is rapidly excreted making it an attractive diagnostic biomarker molecule (Petrik et al., 2014).

To conclude, siderophores represent very exciting family of metal chelators that have extremely broad spectrum of biological activities and functions with an array of important medical, biochemical, geochemical, and agricultural applications. Although not possessing simple organic structures, mass spectrometry has contributed significantly to the fields of siderophore inorganic and molecular analytical chemistries in past five years. This paper represents the first mass spectrometry review in the field of microbial siderophores highlighting their emerging applications and medical importance.

VII. ABBREVIATIONS

CAS	chrome azurol S
CE	capillary electrophoresis
ESI-MS	electrospray ionization mass spectrometry
FOXE	ferrioxamine E
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
MC-ICP-MS	multicollector inductively coupled plasma mass spectrometry
MRI	magnetic resonance imaging
MSI	mass spectrometry imaging
NMR	nuclear magnetic resonance
SPE	solid phase extraction
TAFC	triacetylufusarinine C

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