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RESEARCH PAPER

Determination of Siderophores in Seawater by High Performance Liquid Chromatography-Tandem Mass Spectrometry Coupled with Solid Phase Extraction

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Abstract: Siderophores are produced and secreted by marine microorganisms as a kind of specific iron binding organic ligand with high affinity. In this study, a liquid chromatography-tandem mass spectrometric method coupled with solid phase extraction pretreatment was developed for the determination of siderophores in seawater. The seawater samples were filtered through a 0.22-μm PES membrane, extracted with an ENVI-18 extraction cartridge, and eluted with methanol. The eluted sample was separated on a reversed phase SB-C_{18} column using a gradient elution program with 0.1% (*V/V*) formic acid and methanol as the mobile phases. Qualitative analysis was performed with mass spectrometry under multiple-reaction monitoring mode. Good linearity ($R^2 > 0.99$) was obtained for pyoverdines-Fe, ferrichrome and ferrioxamine E at linear concentration ranges of 0.001–3.00 μg mL^{-1}, 0.005–15.00 μg mL^{-1}, 0.001–3.00 μg mL^{-1}, respectively. The instrumental detection limits of the three analytes were 0.08, 1.76 and 1.36 ng mL^{-1}, and the limits of quantification were 0.27, 5.87 and 4.53 ng mL^{-1}, respectively. As for the standard addition experiments in seawater, the relative standard deviations of the three analytes were lower than 12%, while the recoveries were 12.1%–18.6% for pyoverdines-Fe, 82.0%–97.7% for ferrichrome and 70.0%–98.3% for ferrioxamine E.

Key Words: Siderophore; Solid phase extraction; High performance liquid chromatography-tandem mass spectrometry; Seawater

1 Introduction

Concentration of bioavailable iron in the ocean surface is extremely low, usually down to nM level. As a group of organic chelators with relatively low molecular weight (0.5–1.5 kDa)[1], siderophores have high chelating affinity toward iron. In some open oceans where iron is limited, siderophores are produced and secreted by certain marine bacteria and fungi. Siderophores can specifically combine with iron in seawater, and the formed complex can be recognized and transported by highly efficient transport systems in the cell membrane, through which the iron is transferred into microbial cells to meet the demands of the organisms[1–3]. Based on their functional groups, siderophores can be divided into three types, i.e., hydroxamates, phenolates or catecholates and citrates. Since siderophores are key factors affecting marine biomass, the qualitative and quantitative analyses of siderophores are very important to corresponding researches.

Siderophores are difficult to be analyzed because of their complicated structures and low concentrations in seawater[4–6], and usually, a large amount of seawater sample is needed and specific techniques for extraction and enrichment are required. Traditional treatment methods such as liquid-liquid extraction and column chromatography[6–8] suffer the disadvantages such as complex operation procedure and poor reproducibility. On the other hand, solid phase extraction (SPE) has been applied to the extraction of siderophore[9,10] with the advantages including good reproducibility, small reagent consumption and high efficiency. Spectrophotometric techniques have been widely adopted for the analysis of most siderophores. For example, Chrome azurol S test method[11] is used for the analysis of most siderophores secreted by bacteria and fungi.

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Additionally, some specific analytical methods have also been developed, including Cskay method[12] for determination of hydroxylamines, and Arnow method[13] for detection of catecholate-type siderophores. However, these methods are usually influenced by matrix effects[1], resulting in poor recovery and low sensitivity. Kliz *et al*[14] applied high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) technique to the screening of pyoverdin-type siderophores with high analytical speed, but only qualitative analysis was done in their work.

The existing analytical methods cannot cover all types of siderophores, together with low recovery. In natural seawater, only hydroxamates have been determined[9,15], while siderophores with catechol functional groups cannot be detected in aqueous samples probably due to their high lipophilic property.

This study aimed to develop a method for analysis of siderophores in seawater with typical hydroxamate siderophores as the target analytes. Siderophores in seawater samples were preconcentrated using SPE technique and analyzed by HPLC-tandem MS. The method proposed here was easy to operate with less matrix effect, and could provide molecular structural information. The recoveries of target siderophores were found to be higher than those previously reported.

2 Experimental

2.1 Instruments and reagents

Agilent 6490 Triple Quadrupole tandem mass spectrometer (Agilent, USA) equipped with Agilent 1290 HPLC with ESI ionization source. Milli-Q water purification system (Millipore, USA) was used to produce ultrapure water. 0.22-μm polyether sulfone (PES) syringe filter and 0.22-μm PES membrane were from Pall Corporation, USA.

Chromatographic grade methanol and acetonitrile were obtained from Tedia, USA. 96% formic acid and NaOH (both ACS grade) were from Sigma, USA. NaCl (GR grade) was from Sinopharm Chemical Reagents, China. Super pure HCl was purchased from Kunshan Jincheng Reagents, China. SPE cartridges, including 500 mg per 6 mL Supelclean LC-18, 500 mg per 6 mL Supelclean ENVI-18 and 1 g per 6 mL Supelclean ENVI-Carb from Supelco (USA), 200 mg per 6 mL Waters OASIS HLB (Waters, USA) and 100 mg mL^{-1} Isolute ENV+ (Biotage, Sweden) were used in the experiments.

Three target siderophores, ferrichrome (FC, iron free, CAS #F8014), ferrioxamine E (FO E, iron complex, CAS #38266) and pyoverdines-Fe (PVDs-Fe, iron complex, CAS #P8374), were supplied by Sigma, USA. The chemical structures could be found in the corresponding data sheet. FC and FO E are typical hydroxamate siderophores with a cyclic structure. PVDs-Fe, with linear structure, was provided as siderophore mixtures, with hydroxamate and catecholate functional groups. Each of the three siderophores complexed with iron in a ratio of 1:1.

2.2 Preparation of solutions

Stock solutions of siderophore standards, including 200 μg mL^{-1} PVDs-Fe, 500 μg mL^{-1} FC and 200 μg mL^{-1} FO E, were prepared using ultrapure water. The stock solutions were stored at –20 °C and diluted to the required concentration before use. Three concentration levels were used in the experiment. The low-concentration standard solution contained 0.01 μg mL^{-1} PVDs-Fe, 0.05 μg mL^{-1} FC, and 0.01 μg mL^{-1} FO E. The medium-concentration standard solution contained 0.10 μg mL^{-1} PVDs-Fe, 0.50 μg mL^{-1} FC, and 0.10 μg mL^{-1} FO E. While the high-concentration level standard solution contained 1.00 μg mL^{-1} PVDs-Fe, 5.00 μg mL^{-1} FC, and 1.00 μg mL^{-1} FO E.

Artificial seawater with mixed siderophores was prepared by dissolving the required amount of NaCl in 1–10 L ultrapure water, in which the pH was adjusted by 0.01 M HCl and 0.01 M NaOH, and the final concentrations of PVDs-Fe, FC and FO E were 3.00, 15.00 and 3.00 μg mL^{-1}, respectively.

2.3 Pretreatment of samples

The natural seawater samples were filtered using a 0.22-μm membrane, stored at –20 °C, and thawed before use. The synthetic seawater sample was analyzed without filtration. The ENVI-18 cartridge was conditioned with 5 mL of methanol and 5 mL of ultrapure water in sequence. The samples were passed through the cartridge at a flow rate of 5 mL min^{-1}. The cartridge was rinsed with 5 mL of ultrapure water, and eluted with 4 mL of methanol. The eluent was reduced to less than 100 μL using nitrogen, and then diluted to 1.00 mL with 0.1% (*V/V*) formic acid and filtered with a 0.22-μm membrane prior to analysis with HPLC-MS^2.

2.4 Analysis with HPLC-MS^2

HPLC parameters including ZORBAX SB-C_{18} separation column (50 mm × 2.1 mm, 1.8 μm, Agilent, USA), injection volume of 10 μL, 0.1% (*V/V*) formic acid solution as mobile phase A, and methanol as mobile phase B, and flow rate at 0.25 mL min^{-1}, were adopted in the HPLC analysis. Gradient elution program was as follows: 0–3 min, 5% B; 3–18 min, 5%–100% B; 18–20 min, 100% B; 20–20.01 min, 100%–5% B; 20.01–23 min, 5% B.

The mass spectrometry parameters were as follows: ESI ionization source; multiple-reaction monitoring (MRM) mode; capillary voltage, 2886 V; sheath gas temperature, 200 °C; gas flow rate, 6 L min^{-1}; dry gas temperature, 300 °C; dry gas