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硕 士 学 位 论 文

海水中铁载体的固相萃取预处理和
色谱/质谱分析

Pretreatment with solid phase extraction and analysis with
liquid chromatography/mass spectrometry for siderophores
in seawater

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目 录	
摘 要	I
ABSTRACT	III
第一章 绪论	1
1.1 铁对海洋生物生长的意义	1
1.2 海水中铁的赋存形态及生物有效性	2
1.2.1 颗粒态铁.....	3
1.2.2 溶解态铁.....	3
1.3 铁载体概述	5
1.3.1 铁载体的定义、微生物摄铁机制及特点.....	5
1.3.2 铁载体的结构.....	6
1.3.3 海洋环境中铁载体的浓度.....	13
1.4 铁载体的分析方法	13
1.4.1 铁载体的萃取.....	14
1.4.2 铁载体的分离纯化.....	15
1.4.3 铁载体的定量检测方法.....	16
1.4.4 铁载体结构的鉴定及表征方法.....	17
1.5 课题的提出与研究目标及内容	18
1.5.1 课题的提出.....	18
1.5.2 研究目标及内容.....	19
第一章参考文献	20
第二章 铁载体的高效液相色谱分离-二级管阵列检测器测定方法研究	27
2.1 引言	27
2.2 实验部分	27
2.2.1 研究目标物的选择.....	27
2.2.2 主要试剂与材料.....	30

2.2.3 实验仪器.....	30
2.2.4 仪器参数.....	32
2.3 实验结果与讨论	32
2.3.1 检测波长的选择.....	32
2.3.2 色谱柱的选择.....	33
2.3.3 流动相的选择及洗脱程序的优化.....	38
2.3.4 标准工作曲线的绘制.....	40
2.4 本章小结	41
第二章参考文献	41
第三章 海水中铁载体的固相萃取预处理方法研究.....	43
3.1 前言	43
3.2 实验部分	43
3.2.1 仪器与试剂.....	43
3.2.2 仪器参数.....	45
3.2.3 样品预处理方法.....	45
3.2.4 正交实验设计.....	46
3.3 实验结果与讨论	46
3.3.1 正交实验结果.....	46
3.3.2 正交实验结果的验证.....	52
3.4 本章小结	54
第三章参考文献	54
第四章 海水中铁载体的高效液相色谱分离-质谱测定方法研究	56
4.1 前言	56
4.2 实验部分	56
4.2.1 仪器与试剂.....	56
4.2.2 仪器参数.....	57
4.2.3 样品预处理方法.....	58
4.2.4 实验方法.....	58
4.3 实验结果与讨论	58

4.3.1 质谱参数的优化.....	58
4.3.2 质谱分析.....	61
4.3.3 液相色谱参数的优化.....	65
4.3.4 基质效应的考察.....	66
4.3.5 工作曲线、检出限和加标回收率.....	67
4.3.6 实际海水样品分析.....	68
4.4 本章小结	68
第四章参考文献	69
第五章 结语与展望	71
5.1 研究总结与贡献	71
5.2 不足与展望	72
攻读硕士学位期间发表的论文	73
致 谢.....	74

Table of Contents

ABSTRACT (in Chinese)	I
ABSTRACT (in English)	III
Chapter 1 Preface	1
1.1 Significance of iron in the growth of marine organisms	1
1.2 The species of iron in seawater and its bioavailability	2
1.2.1 Particulate iron	3
1.2.2 Dissolved iron	3
1.3 Siderophore overview	5
1.3.1 The definition, uptake mechanism and characteristics of siderophore ...	5
1.3.2 Structures of siderophore	6
1.3.2 Siderophore concentration in marine system	13
1.4 Analytical methods for siderophore	13
1.4.1 Extraction of siderophore.....	14
1.4.2 Separation and purification of siderophore.....	15
1.4.3 Quantitative analysis for siderophore	16
1.4.4 Characteristic identification for siderophore	17
1.5 Objectives and contents of the research	18
1.5.1 Research background.....	18
1.5.2 Research objectives and contents	19
References for Chapter1	20
Chapter 2 Detection method of siderophore using high performance liquid chromatography-diode array detector	27
2.1 Introduction	27
2.2 Experimentation	27
2.2.1 Selection of the targets	27
2.2.2 Reagents and materials	30
2.2.3 Instruments.....	30

2.2.4 Instrumental parameters.....	32
2.3 Results and discussion	32
2.3.1 Selection of detection wavelength	32
2.3.2 Selection of chromatographic column	33
2.3.3 Optimization of mobile phase and elution program	38
2.3.4 Calibration curve.....	40
2.4 Summary of Chapter 2	41
References for Chapter 2.....	41
Chapter 3 Development of the solid phase extraction pretreatment method for siderophore in seawater.....	43
3.1 Introduction.....	43
3.2 Experimentation.....	43
3.2.1 Apparatus and reagents	43
3.2.2 Instrumental parameters.....	45
3.2.3 Sample pretreatment method	45
3.2.4 Orthogonal experimental design.....	46
3.3 Results and discussion	46
3.3.1 Results of orthogonal experiment	46
3.3.2 Result validation	52
3.4 Summary of Chapter 3	54
References for Chapter 3.....	54
Chapter 4 Detection method of siderophore using high performance liquid chromatography coupled with electrospray tandem mass spectrometry	56
4.1 Introduction.....	56
4.2 Experimentation.....	56
4.2.1 Apparatus and reagents	56
4.2.2 Instrumental parameters.....	57

4.2.3 Sample pretreatment	58
4.2.4 Experimental design	58
4.3 Results and discussion	58
4.3.1 Optimization of MS parameters.....	58
4.3.2 MS analysis.....	61
4.3.3 Optimization of liquid chromatographic parameters	65
4.3.4 Matrix effects	66
4.3.5 Calibration curve, detection limits and recovery	67
4.3.6 Application on natural seawater.....	68
4.4 Summary of Chapter 4	68
References for Chapter 4.....	69
Chapter 5 Conclusion and perspective	71
5.1 Summary and contribution of the research.....	71
5.1 Shortage of the research and perspective	72
Publications during master graduate study	73
Acknowledgements.....	74

摘要

铁在生物的生命活动中扮演着重要角色,如含铁蛋白在生物的光合作用、呼吸作用、硝酸盐和亚硝酸盐的还原、氮的固定及 DNA 的合成等过程中都起着不可替代的作用。但是海洋表层的生物可利用态铁浓度极低,影响甚至限制着浮游植物的生长。在铁限制条件下,海洋中的一些细菌和真菌能够合成并分泌一种与铁有极强螯合能力的低分子量有机配体——铁载体。铁载体被释放到细胞外后有效地与铁结合,通过细胞膜上特定铁载体受体的识别和转运,将铁运输到微生物细胞内,以满足生物体对铁的需求。

由于铁载体结构复杂多样,在海洋环境中浓度低,分析相对困难。传统的前处理方法存在着操作繁琐、重现性较差等缺点;经典的铁载体测定方法易受基底干扰,回收率和灵敏度均较差。针对现有方法存在的不足,本研究建立了回收率较高、重现性好、检出限较低、定性准确、操作简单的海水中氧肟酸型铁载体的分析方法。主要内容和结果如下:

(1) 建立了铁载体的高效液相色谱(HPLC)-二极管阵列检测器(DAD)测定方法,考察了不同类型的色谱柱、流动相组成、梯度洗脱程序等参数,以标准物质的分离度、峰形及响应强度为标准,优化分离检测条件。结果显示,C₁₈液相色谱柱对目标物的分离效果优于凝胶色谱柱。Pyoverdines-Fe(PVDs-Fe)在 0.20-8.00 μg/mL、Ferrichrome(FC)在 3.00-20.00 μg/mL、Ferrioxamine E(FO E)在 0.20-5.00 μg/mL 范围内线性良好,仪器检出限为 PVDs-Fe 0.015 μg/mL、FC 0.155 μg/mL、FO E 0.061 μg/mL,定量限为 PVDs-Fe 0.050 μg/mL、FC 0.517 μg/mL、FO E 0.203 μg/mL。

(2) 优化了海水中铁载体的固相萃取(SPE)预处理参数。分别以人工海水和天然海水为基质,建立了铁载体的 SPE 预处理方法。采用正交实验,考察了 SPE 柱型、洗脱溶剂种类和用量、样品 pH、上样体积等因素对萃取效果的影响。选择的 SPE 条件为:ENVI-18 为萃取柱,100% 甲醇为洗脱溶剂,洗脱溶剂体积 4 mL,不调节盐度和 pH,上样体积设为 1.0 L。以 1.0 L 近岸海水进行加标实验,得到三种目标物方法的平均萃取效率为 PVDs-Fe 39.6%,FC 144%,FO E 76.1%;相对标准偏差(RSD)(n=3)分别为 12.1%、6.50%、7.70%。

(3) 建立了海水中铁载体的高效液相色谱-串联质谱 (HPLC-MS/MS) 测定方法。在 HPLC-DAD 方法的基础上, 对 HPLC-MS/MS 分离条件进行优化, 提高分析速度、灵敏度、定性准确性。三种铁载体化合物标准物质 PVDs-Fe、FC、FO E 的测定线性范围分别为 0.001-3.00 $\mu\text{g/mL}$ 、0.005-15.00 $\mu\text{g/mL}$ 、0.001-3.00 $\mu\text{g/mL}$, 相关系数 $R^2 > 0.99$; 仪器检出限分别为 0.08 ng/mL 、1.76 ng/mL 和 1.36 ng/mL ; 定量限分别 0.27 ng/mL 、5.87 ng/mL 和 4.53 ng/mL 。结合所建立的 SPE 前处理方法, 进行方法回收率实验, 三种目标物四次测定的 RSDs $< 12\%$, 方法回收率分别为 PVDs-Fe 12.1%-18.6%, FC 82.0%-97.7%, FO E 70.0%-98.3%。分析取自厦门近岸海域海水中的铁载体, 未检出类铁载体物质。本研究尝试利用质谱图对铁载体目标物进行初步结构解析, 为未知铁载体的结构分析提供基础。

关键词: 铁载体; 分析; 固相萃取; 高效液相色谱-串联质谱; 海水

ABSTRACT

Iron plays an important role in life activities. For example, iron-containing metalloenzymes are essential for many life processes, including photosynthesis, respiration, nitrate and nitrite reduction, nitrogen fixation and DNA synthesis. However, the concentration of bioavailable iron in the ocean surface is extremely low that limits phytoplankton growth. In some open oceans where iron is limited, marine microorganisms are able to produce and secrete low-molecular-weight, high affinity iron binding ligands named as siderophores. The siderophores can specifically combine iron in seawater, and be recognized and transported by the highly efficient transport systems. In this way the iron is transferred into microbial cells to meet the demand of organisms.

The siderophores are difficult to analyze due to their complicated structures and low concentrations in seawater. Traditional treatment methods suffer shortages of complex operation and poor reproducibility, while the detection methods widely used for siderophores have matrix effects, resulting in poor recoveries and sensitivity. To compensate of existing analytical methods, the present study aimed to develop methods for determination of three representative hydroxamate siderophores in seawater, which possessed the features of higher recoveries, good reproducibility, high sensitivity and accuracy. The contents and results of the study are summarized as the follows:

(1) An instrumental analysis for siderophores was established, using high performance liquid chromatography (HPLC) equipped with diode array detector (DAD). The LC parameters, including separation columns, composition of the mobile phases, gradient elution program, were optimized based on the resolution, peak symmetry and signal intensity. The results showed that C₁₈ reversed phase column was better for retention and separation of the siderophores than gel columns. The standards had good linearity within the concentrations of 0.20-8.00 µg/mL for Pyoverdines-Fe (PVDs-Fe), 3.00-20.00 µg/mL for Ferrichrome (FC), 0.20-5.00

$\mu\text{g/mL}$ for Ferrioxamine E (FO E). The instrumental detection limits and limits of quantification for PVDs-Fe, FC, FO E, were $0.015 \mu\text{g/mL}$, $0.155 \mu\text{g/mL}$, $0.061 \mu\text{g/mL}$; $0.050 \mu\text{g/mL}$, $0.517 \mu\text{g/mL}$, $0.203 \mu\text{g/mL}$, respectively.

(2) A solid-phase extraction (SPE) pretreatment method for the siderophores in seawater was investigated in detail. The method was developed with matrixes of synthetic seawater and natural seawater. In present study the parameters affecting SPE were investigated, which included the selection of SPE cartridges, concentration of methanol in the eluting solution, volume of the eluting solution, volume of the seawater sample, sample salinity, and sample pH. An orthogonal experiment was designed for optimization of SPE parameters. From the results, parameters for extraction were selected as: ENVI-18 cartridge for SPE, 4 mL 100% methanol for elution, sample volume of 1.0 L, and the sample could be extracted without adjusting salinity and pH. The method was applied to analyze 1.0 L seawater collected from Xiamen coast, and the average extraction efficiencies were 39.6%, 144%, 76.1% for PVDs-Fe, FC, FO E. The relative standard deviations (RSDs) ($n=4$) of the extraction efficiencies for PVDs-Fe, FC and FO E were 12.1%, 6.50%, 7.70%.

(3) An analytical method for siderophores in seawater was established, using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Based on the HPLC-DAD method, LC parameters were further optimized for HPLC-MS/MS. The analytical speed, sensitivity and qualification were enhanced. The linearities were good ($R^2 > 0.99$) for PVDs-Fe, FC, FO E at the concentrations of $0.001\text{-}3.00 \mu\text{g/mL}$, $0.005\text{-}15.00 \mu\text{g/mL}$, $0.001\text{-}3.00 \mu\text{g/mL}$, respectively. The instrumental detection limits and limits of quantification for the three analytes were 0.08, 1.76, 1.36 ng/mL; 0.27, 5.87, 4.53 ng/mL, respectively. Combined the SPE pretreatment method, recoveries were 12.1%-18.6% for PVDs-Fe, 82.0%-97.7% for FC, 70.0%-98.3% for FO E, while the RSDs ($n=4$) were lower than 12%. A seawater sample collected from Xiamen coast was analyzed with the proposed method for siderophores, and no target siderophores could be detected. In the study, MS^2 analysis of PVDs-Fe, FC, FO E was carried out, providing the reference information for analysis of unknown siderophores.

Keywords: siderophore; analysis; solid phase extraction; high performance liquid chromatography-tandem mass spectrometry; seawater

厦门大学博硕士学位论文摘要库

第一章 绪论

铁 (Fe) 是动物、植物、微生物生长的必要微量营养元素。铁对生物的重要性还归因于其良好的氧化还原性质、配位能力及激活氧的能力, 这些性质使铁参与生命活动的电子传递等新陈代谢过程^[1]。20 世纪 80 年代末, 随着大洋高营养盐低叶绿素 (High Nutrient and Low Chlorophyll, HNLC) 海区的发现, 铁限制海洋初级生产力的观点也被提出并证实, 海洋中的铁及其生物可利用性开始受到广泛的关注^[2-5]。铁在地壳中的丰度排名第四, 但海水中铁的浓度却不高。在海水表层氧化性和近中性 pH 条件下, 铁以溶度积低达 10^{-38} ^[6-8] 的 Fe(III) 的氧化物的形式存在, 也易形成沉淀或吸附于颗粒物上, 导致海水表层溶解态铁 ($<0.4 \mu\text{m}$ 或 $<0.2 \mu\text{m}$) 的浓度极低, 仅为 $0.02\text{-}1 \text{ nmol/L}$ ^[7]。

20 世纪 80 年代起, 科学家在对自然环境中有有机络合态铁的配体进行研究时发现, 有一类有机配体与微生物、藻类的生物量有很高的关联性^[9-11], 其在铁胁迫条件下由海洋中的一些细菌和真菌分泌并释放, 具有极强的 Fe(III) 螯合能力。此物质即是铁载体。

在 HNLC 海区, 铁载体对于生物可利用铁具有重要贡献, 也是海区生物量的重要影响因子, 因而铁载体的研究得到越来越多的重视。科学家在铁载体研究的相关领域开辟了多个方向, 而分析方法是铁载体研究中不可忽视的技术支撑, 准确获取定性定量分析数据是相关研究的关键。

1.1 铁对海洋生物生长的意义

对大多数海洋生物来说, 铁的缺乏并不是因为环境中总铁含量很低, 而是由于生物可利用铁的缺乏。铁在海水中的形态极大地影响铁的溶解度^[12]、氧化还原活性^[13]和生物可利用性^[14-16], 进而影响海区的初级生产力、物种组成和浮游群落的营养结构。

HNLC 海区拥有丰富的营养元素如氮、磷、硅等, 但叶绿素含量却不高, 即生物量较低。研究同时发现, 这些海区的溶解态铁含量很低。在大洋中, 大约有 30% 的区域处于 HNLC 状态, 包括赤道太平洋、副热带太平洋、南大洋等^[17]。针对这些区域的低铁情况, 研究者进行了中尺度的铁加富实验^[18-20]。研究发现铁

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