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Article

A New Approach to Characterize Siderophore-Type Ligands in Seawater by Solid Phase Synthesis and SPE-HPLC-ESI-MS/MS Analysis

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Abstract: Siderophores are organic ligands involved in dissolved iron (dFe) speciation in the oceans. Their study is crucial for a better understanding of the biogeochemical cycle of Fe in the marine environment, particularly in certain areas, such as the Southern Ocean, where Fe deficiency limits marine productivity. In this study, an analytical method is proposed for the extraction and pre-concentration of siderophores from seawater samples by solid phase extraction (SPE) and subsequent analysis by high-performance liquid chromatography—electrospray ionization—mass spectrometry (HPLC-ESI-MS/MS). Two siderophores were used as standards: Ferrioxamine E, a commercially available hydroxamate siderophore, and a staphyloferrin A-like compound with two citric acid units, synthesized in our laboratories by solid-phase peptide synthesis. A central composite design, considering different pH (2, 3.5, and 5) and sample loading volume (50, 125 and 200 mL) as variables, was used to optimize the extraction yield with SPE C18 cartridges. Tests were conducted on samples of artificial seawater spiked with siderophore standards. Ferrioxamine E showed high extraction yields in all tests carried out. On the contrary, the extraction of staphyloferrin A-like compound was significantly affected by both pH and loading volume.

Keywords: siderophore; iron speciation; HPLC-ESI-MS/MS; solid phase extraction; central composite design; solid phase peptide synthesis

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1. Introduction

Iron (Fe) is the most important trace element in the ocean, being a micronutrient required for phytoplankton growth and thus involved in marine primary productivity and carbon export [1]. The dissolved Fe (dFe) is the most bioavailable fraction, and its solubility is increased above the solubility product of Fe-(hydr)oxides by complexation with dissolved organic ligands (L) [2–4]. L is generally classified as strong (L1-2-type) or weak (L3-4-type) ligands based on Fe binding affinities through conditional stability constant values (logK'Fe'L) obtained by competitive ligand exchange-adsorptive cathodic stripping voltammetry (CLE-AdSV) analysis [3]. L consists of a pool of different compounds such as polysaccharides, hemes, humic substances, and siderophores [3].

Siderophores are produced by microorganisms to sequester and assimilate dFe under low Fe conditions [3,5,6] and form particularly stable complexes with Fe (logK'Fe'L > 12). Thus, they belong to class L1 [3,5–8]. Siderophores coordinated to insoluble Fe(III) are first transported into microbial cells by membrane-bound Fe-siderophore receptors. Afterwards, Fe is released from siderophores, typically via the reduction of Fe(III) to Fe(II) by microbe-mediated redox processes [3,5–8].

Several laboratory and field studies have provided evidence that the variety and complexity of siderophores depends on a number of factors, including environmental conditions, biological activity, and the concentration of macro- and micronutrients [6,9–15].

Salinity, pH, and temperature are among the environmental variables that can affect Fe speciation in seawater and, consequently, the synthesis of siderophores by marine organisms.

To date, more than 80 siderophores have been isolated from marine microorganisms [6]. According to their functional groups, siderophores are divided into three main categories (hydroxamates, catecholates, and carboxylates), to which other classes are added not falling into the above-mentioned categories owing to their different functional groups (e.g., mixed hydroxamates/α-hydroxycarboxylates, mixed hydroxamates/catecholates, mixed hydroxamates/catecholates) and which are generally referred to as "complexones" [6,16].

The study of siderophores is crucial to forming a better understanding of the biogeochemical cycle of Fe in the marine environment, especially in some oceanic areas, such as the Southern Ocean, where Fe deficiency limits marine productivity. However, the characterization of these compounds is very difficult due to their complex structures and low concentrations in seawater.

The analytical methods reported in the literature use large sample volumes (order of liters) to extract and pre-concentrate the siderophores [17–21].

Moreover, most studies on siderophores focus on the analysis of bacterial culture supernatant extracts using spectrophotometric assays, such as the Chrome Azurol Sulphonate (CAS) assay, which can only be used for high concentrations of these analytes [2,16,22–24]. However, these assays do not provide information on the structure of the molecules.

Solid phase extraction (SPE) and high-performance liquid chromatography—electrospray ionization—mass spectrometry (HPLC-ESI-MS/MS) could be a valid approach to extract and identify the siderophores from seawater samples, overcoming the limitations outlined above [3,12,18,21,25].

In this manuscript, a new approach is proposed for the extraction, pre-concentration, and identification of siderophore-type ligands from seawater samples by SPE, followed by HPLC-ESI-MS/MS analysis.

Thanks to the approach of experimental design, it was possible to optimize the SPE procedure with a limited number of experiments, taking into account potential variables influencing pre-concentration. The optimization was carried out on artificial seawater samples spiked with siderophore standard molecules commercially available or synthesized in our laboratories.

We synthesized a derivative with a scaffold-like staphyloferrin A. Staphyloferrin A is an α-hydroxy carboxylate siderophore, thus consisting of two citric acid units, and it belongs to the category of "complexones" [16,26].

Our derivative has the same portion of α-hydroxy carboxylate (two units of citric acid), but it differs for the linker between the two citric units, respectively 2,5-diaminopentaenoic acid for staphyloferrin A and 2,6-diaminohexanoic acid for our molecule (see Figure 1 for comparison of the chemical structures).

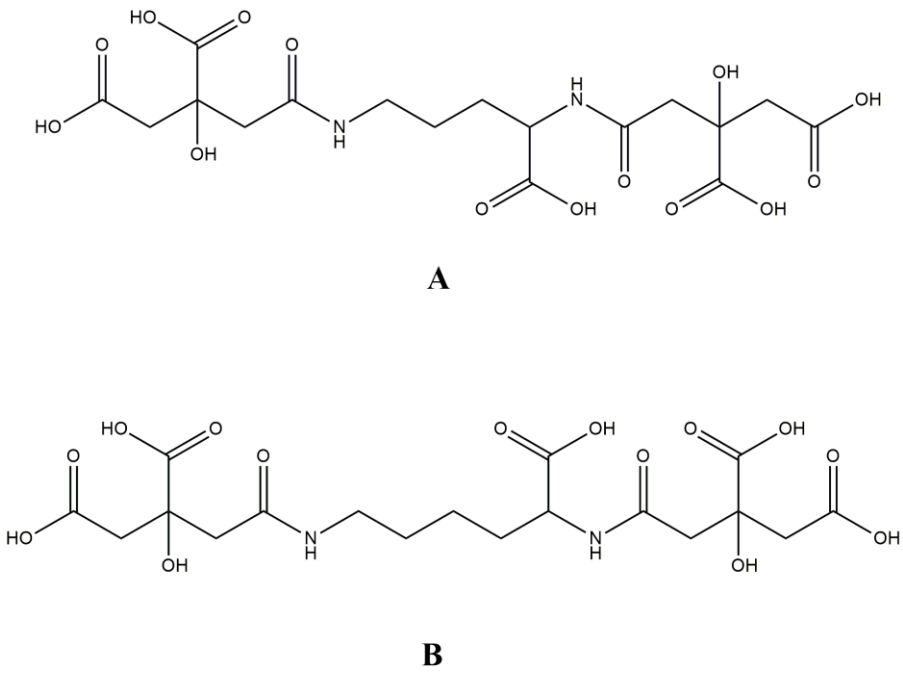

Figure 1. Structure of staphyloferrin A (**A**) and of derivative synthesized in this study (**B**).

2. Materials and Methods

2.1. Artificial Seawater

Tests for the development of the SPE and HPLC-ESI-MS/MS method were carried out using artificial seawater (ASW). The composition of ASW with a salinity of 35 g L^{-1} is given in Table 1. All constituents were purchased from Merck, Darmstadt, Germany.

Table 1. Composition of artificial seawater of salinity 35 g L^{-1} (weights based on 4 L of H_2O).

Constituent	Molar Concentration (Mol L^{-1})	Weight (g)
NaCl	0.47	109.87
$Na_2SO_4 \times 10\ H_2O$	1.87×10^{-2}	24.10
KCl	6.25×10^{-3}	1.864
$CaCl_2 \times 2\ H_2O$	6.25×10^{-5}	2.934
$NaHCO_3$	1.58×10^{-3}	0.531
KBr	5.63×10^{-4}	0.268
H_3BO_3	3.83×10^{-4}	0.080
$SrCl_2$	8.20×10^{-5}	0.052

2.2. Siderophore-Type Standards

Ferrioxamine E (FOE, $C_{27}H_{45}FeN_6O_9$) was purchased from Merck, Darmstadt, Germany. It is a hydroxamate siderophore with a cyclic structure [10,27,28]. 1 mg L^{-1} stock solution was made in MQ-water (Millipore, El Paso, TX, USA) and stored at +4 °C.

The single steps of the entire synthesis of our derivative ($C_{18}H_{26}N_2O_{14}$) are reported in Schemes A1 and A2 described in Appendix A. 1 mg L^{-1} stock solution was made for the derivative in MQ-water and stored at +4 °C.

2.3. Central Composite Design

Central composite design (CCD) was applied using CAT software [29] and following the instructions reported in [30]. CCD allows the estimation of linear and quadratic effects of the factors and their interactions.

The model was validated considering different pH (2, 3.5, and 5) and sample loading volume (50, 125, and 200 mL) as variables. Three levels were selected for each variable

(the lower coded as −1, the intermediate coded as 0, and the upper coded as +1), and 11 experiments were conducted by combining the different experimental conditions (Table 2). Three experiments at the central point of the experimental domain were added to the design to estimate the variability associated with the performance of the experiments. The experiments were performed in random order to avoid systematic errors due to the time effect.

Table 2. Central composite design and levels of variables for optimization of the solid phase extraction.

Experiment	Factor 1	Factor 2	pH	Sample Volume (mL)
1	−1	−1	2	50
2	+1	−1	5	50
3	−1	+1	2	200
4	+1	+1	5	200
5	0	+1	3.5	200
6	0	−1	3.5	50
7	+1	0	5	125
8	−1	0	2	125
9	0	0	3.5	125
10	0	0	3.5	125
11	0	0	3.5	125

2.4. Extraction and Preconcentration Procedure

A Waters Millipore Sep-pak Vacuum manifold was used. Reversed-phase C18 SPE cartridges (Supelclean™ ENVI™ – 18, Supelco®500 mg, 3 mL) were used to extract and preconcentrate the analytes. 3 mL of methanol (MeOH, HPLC grade, VWR, Radnor, PA, USA) and 3 mL of Milli-Q water, acidified at pH~2 with HCl (Merck,, Darmstadt, Germany), were used for the conditioning step. 10 mL of acidified Milli-Q water was used to wash the solid phase. The analytes were eluted using 6 mL of MeOH based on the tests performed on the different fractions collected. The scheme of the optimized extraction procedure employing 180 mL of acidified sample is shown in Figure 2.

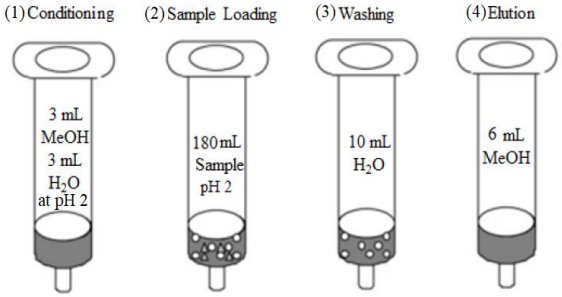

Figure 2. Scheme of the extraction and preconcentration procedure by SPE method using the optimized conditions.

The samples were dried by N_2 flow and stored at −20 °C until analysis. Before the analysis, the sample was taken up with 50 µL of a 0.1% (*v*/*v*) formic acid (Carlo Erba Reagents, Milan, Italy) solution in water and centrifuged for 5 min at 13.000 rpm.

Tests were conducted on samples of ASW spiked with siderophore standards at concentrations like those present in real samples (~20 ppb). ASW was used as a procedural blank.

2.5. HPLC-ESI-MS/MS Conditions

A micro HPLC-ESI-MS/MS, consisting of an Agilent 1100 Series HPLC System (Santa Clara, CA, USA) equipped with an autosampler and an Agilent Technologies XCT trap LC/MSD mass spectrometer provided with a high-capacity ion trap, was used.

Phenomenex Jupiter 5 µm C18 300 Å 150 × 1 mm column (Torrance, CA, USA) was used for the chromatography. The flow rate was held at 50 µL/min, and the injection

volume was 8 µL for both samples and standards. The mobile phase was composed of (A) 0.1% (*v*/*v*) formic acid in water and (B) 0.05% (*v*/*v*) formic acid in acetonitrile. A mobile phase consisting of a 0.1% (*v*/*v*) solution A of formic acid in water and a 0.05% (*v*/*v*) solution B of formic acid in acetonitrile was used. The programmed gradient was: 0 min: A (100%), B (0%), 5 min: A (100%), B (0%), 30 min: A (0%), B (100%), 35 min: A (0%), B (100%), 35.01 min: A (100%), B (0%). The chromatographic system was equipped with a diode array UV detector. The wavelengths (λ) used during the analysis were 220–280–320–450–650 nm.

The HPLC system was directly interfaced to the mass spectrometer using an ESI source. Capillary temperature and voltage were optimized to 360 °C and −3800 V, respectively. The analysis was carried out with positive ionization, performing a scan from 100 to 1500 *m*/*z*, and the MS/MS spectra were acquired at values lower than 1200 to avoid difficult-to-interpret spectra.

A calibration curve with height concentration levels for ferrioxamine E and with five concentration levels for staphyloferrin A-like compound was made to relate the peak area obtained to the concentration of the compound. The concentration range was between 0.75 and 100 ppm in the case of FOE and between 1.25 and 20 ppm in the case of staphyloferrin A-like compound. Linearity was evaluated with the R^2 coefficients.

3. Results

3.1. Synthesis

Our molecule standard was obtained by a combined liquid and solid-phase synthesis using the method previously published [31] with significant modifications. Synthesis is essentially composed of two steps. In the first phase, tert-butyl citrate, a monomer derived from citric acid, in which the central carboxylic group is protected (via ester formation), is obtained with a method previously described with slight modifications [32]. In this way, the conjugation reaction between the amine groups of lysine occurs selectively with one of the two terminal acid groups of citric acid and not also with the central one, preventing the formation of a mixture of isomers. The resulting derivative is conjugated by the classical Fmoc solid-phase peptide synthesis to a particular lysine protected on Nε of the side chain with 4-methyltrityl group (Mtt). This procedure allows the selective introduction of the monomers of citric acid derivatives on the lysine backbone. The protection on the central carboxylic group of citric acid will be removed in an acidic medium during the detachment of the product from the resin. Eventually, the product is purified in preparative HPLC and lyophilized. The overall yield was about 30%.

3.2. HPLC-ESI-MS/MS Analysis

The optimization of the method started with the acquisition of chromatograms and spectra of the standards.

Figure 3 shows the chromatogram of the synthesized siderophore of the FOE standard mixture and the relative extracted ionic currents. Figure 4 shows the chromatogram of the procedural blank (no spiked ASW).

No interferences were observed in no spiked ASW in the retention time of the analytes, demonstrating the selectivity of the method.

Figure 5 shows the MS spectra and MS/MS spectra of the two compounds. The concentration of both standards was 20 mg L^{-1}, and separation was improved using the elution gradient as reported in the materials and methods section. Signal acquisition in TIC mode allowed us to assess that the chromatographic conditions used efficiently separate the two compounds.

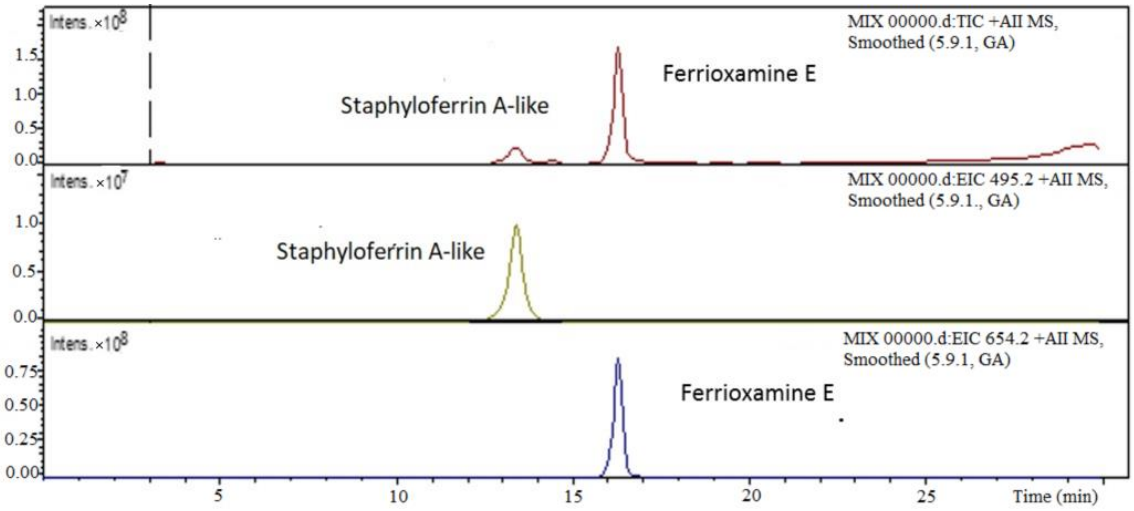

Figure 3. Chromatogram obtained from analysis of the standard mixture of staphyloferrin A-like compound and ferrioxamine E in TIC mode, and ionic currents extracted for staphyloferrin A-like compound ($[M+H]^+$ 495.0) and ferrioxamine E ($[M+H]^+$ 654.2).

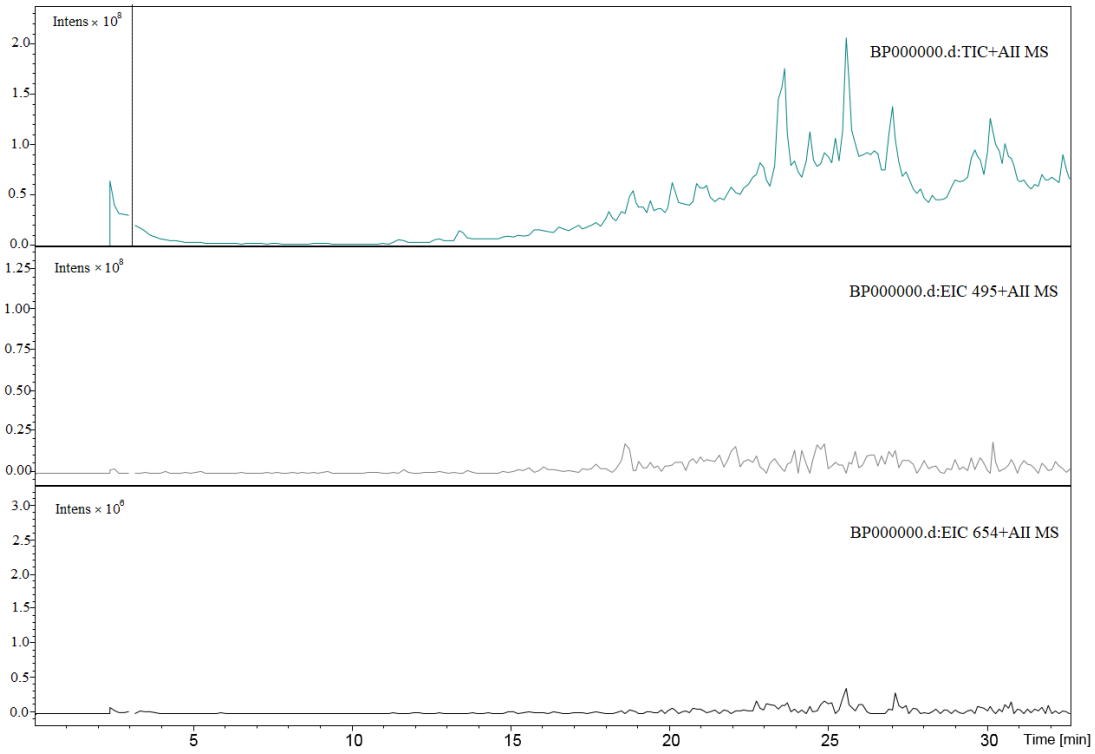

Figure 4. Chromatogram of the procedural blank.

The mass relative to the base peak of the synthesized compound corresponds to the pseudo-molecular ion $[M+H]^+$ 495.0 (Figure 5A). Some assumptions about the fragments generated have been made based on the diagnostic fragments and on the most frequent mass losses. The following diagnostic fragments were identified in the MS/MS spectra (Figure 5B): $[M+H]^+$ 477.1, 431.1, 321.2 and 257.1. The following mass losses were evaluated: 18 attributable to a loss of H_2O, 46 attributable to a loss of CH_2O_2 or C_2H_6O, 64 attributable to a loss of C_5H_4, and 110 attributable to a loss of C_6H_8NO.

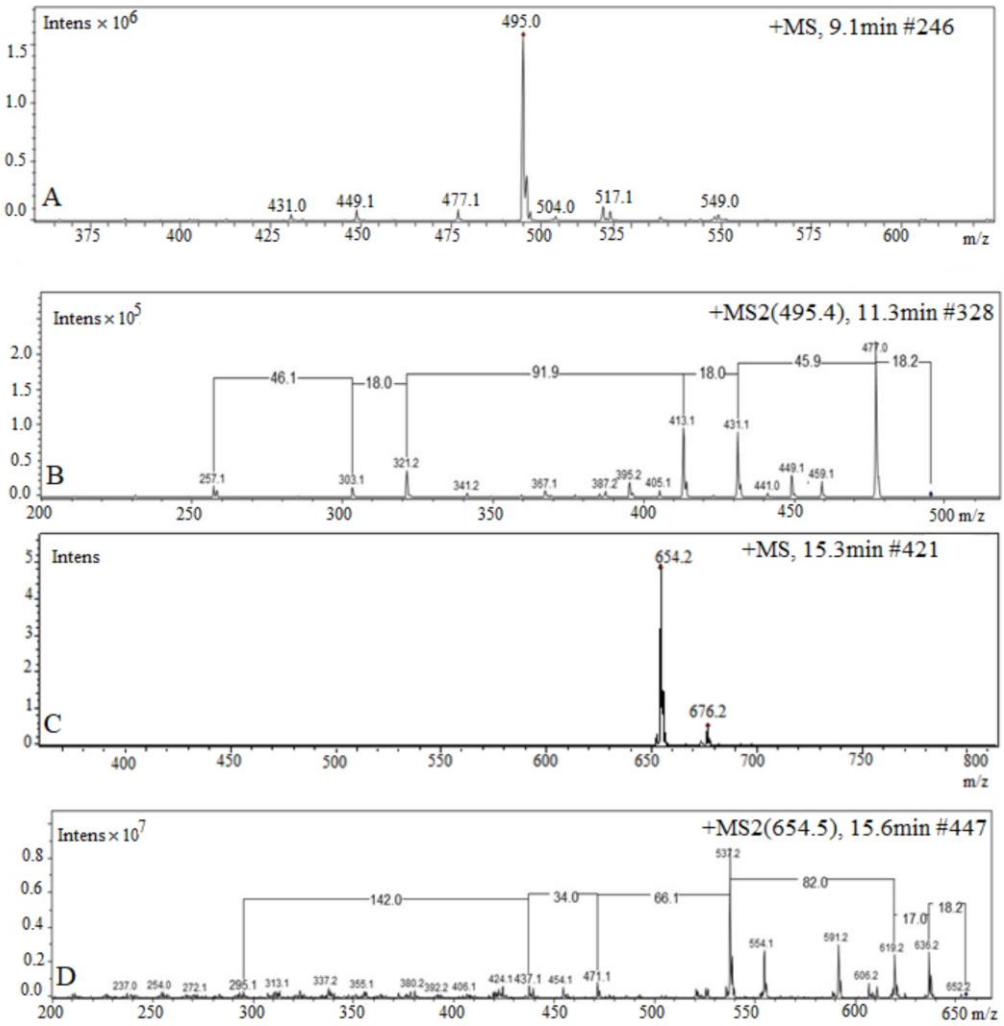

Figure 5. MS and MS/MS spectra related to chromatographic peaks of staphyloferrin A-like compound (**A**,**B**) and ferrioxamine E (**C**,**D**).

The mass of the base peak of FOE corresponds to the pseudo-molecular ion $[M+H]^+$ 654.2 (Figure 5C). Several works have been found in the literature related to the study of fragmentation in mass spectrometry of FOE and, more generally, of hydroxamate siderophores. Fragmentations obtained in this study from FOE analysis agreed with those reported in the literature [18,33,34]. In particular, the following diagnostic fragments were identified in the MS/MS spectra (Figure 5D): $[M+H]^+$ = 636.3 ($-H_2O$); 619.2 (N(1)-C(2), C(33)-N(1), (N(7)-O)-Fe, and N(7)-O cleavage); 537.2 (C(6)-N(7), C(11)-N(12) and (N(7)-O)-Fe cleavage); $[M+H]^+$ = 471.2 ($C_{18}H_{33}N_5O_6Fe^+$); $[M+H]^+$ = 437.1244, (resulted from cleavage at three positions: (1) between the α-methylene group (C2) and N(1), (2) the amide at C(22)-N(23), and (3) (N(29)-O)-Fe, for the neutral loss of 217.1432 mass unit corresponding to $C_9H_{19}N_3O_3$) and $[M+H]^+$ = 295.1 ($C_{12}H_{10}N_2O_3Fe^+$).

The linearity was checked by means of the coefficients of determination (R^2) that were 0.9911 for FOE and 0.9995 for staphyloferrin A-like compound, respectively, meaning good linearity within the established concentration ranges (Figure 6).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to 3·S/N and 10·S/N, respectively, where S/N is the signal/noise ratio. The LODs were 0.39 and 0.62 mg L^{-1} for FOE and staphyloferrin A-like compound, respectively. The LOQs were 0.78 and 1.25 mg L^{-1} for FOE and staphyloferrin A-like compound, respectively.

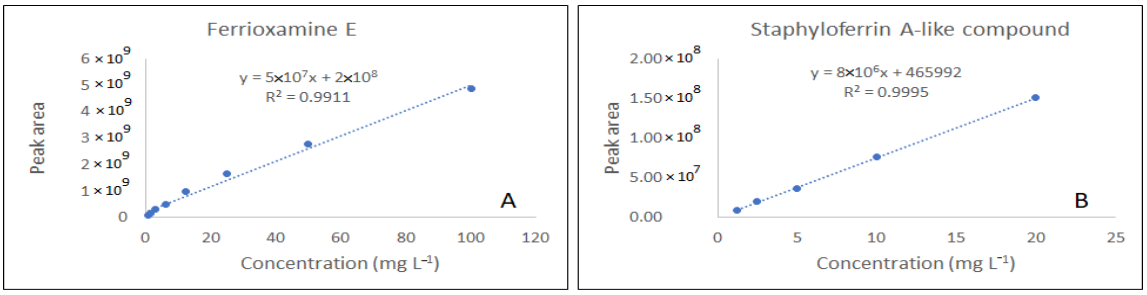

Figure 6. Calibration curve of ferrioxamine E (**A**) and staphyloferrin A-like compound (**B**).

3.3. Optimization of the Solid Phase Extraction by Central Composite Design

The processed results obtained by the 11 experiments showed that in the case of ferrioxamine E, the chosen variables (pH and loading volume) were not significant since high extraction yields (around 85%) were obtained in each experimental condition. pH and sample loading volume, on the other hand, were significant for our compound with an explained model variance of 78%, which showed extraction yields of around 10% under the optimized conditions of 180 mL and pH 2 (Figure 7).

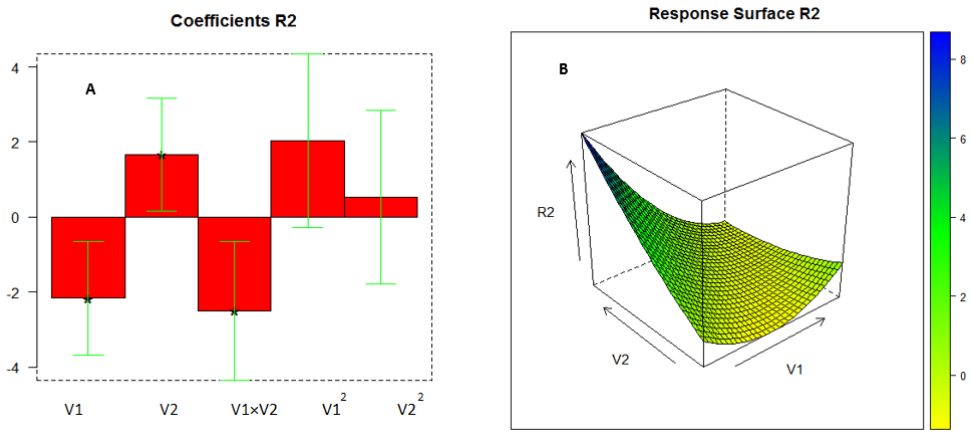

Figure 7. A bar plot representing the coefficients of the computed model (**A**) and response surface for staphyloferrin A-like compound (**B**).

4. Discussion

Siderophore-type are the main Fe—low molecular weight organic ligands in seawater [3,6,35–40]. It is well known that many microorganisms produce siderophores to compete for iron under iron-limited aerobic growth conditions in the marine environment.

The study of these ligands is crucial in order to better understand the biogeochemical cycle of Fe in the marine environment with special regard to its speciation and bioavailability. The study and characterization of siderophores in the marine environment are particularly complex from an analytical point of view due to their low concentrations and different molecular structures. Large volume of samples (in the order of liters) is often reported for the identification of siderophore ligands [18,21]. This condition is not always fulfilled during multidisciplinary oceanographic campaigns in the Southern Ocean when different chemical and biological parameters are determined on the same sample aliquots [4].

Far fewer structures of marine siderophores have been identified ferrioxamine species as hydroxamate siderophores in seawater samples collected in the Atlantic Ocean [18]. Six siderophore-type compounds with hydroxamate functionality were detected in both New Zealand coastal and sub-Antarctic surface and subsurface samples [21,25].

A class of marine siderophores contains α hydroxycarboxylic acid moieties in the form of β hydroxyaspartic acid or citric acid, that, when coordinated to Fe(III), is photoreactive in the natural sunlight conditions of the mixed layer of the upper ocean has been reported [41].

While commercial standards for testing the optimization of extraction and identification methods are available for ferrioxamine, there are none for siderophores containing α hydroxycarboxylic acid moieties.

Therefore, we synthesized a derivative with a scaffold similar to staphyloferrin A containing the α-hydroxy carboxylate (two units of citric acid) linked with a 2,6-diaminohexanoic acid portion combining a liquid and solid-phase synthesis. Our molecule differs from staphyloferrin A only in the number of carbon atoms in the main chain, but it has the same functional groups complexing Fe. This synthesis has allowed us to produce adequate amounts of the siderophore with a simple protocol in a relatively short time and with an overall yield of about 30%.

In particular, the backbone is indeed modified during the solid phase synthesis. In fact, the orthogonally-protected Fmoc-L-Lys(Mtt)-OH allows the selective introduction of the monomers of citric acid derivatives on the lysine backbone.

Furthermore, this side chain protection avoids the use of the allyloxycarbonyl (Alloc), as previously published [31]. The allyl-protecting group in N-Fmoc-Lys(Alloc)-OH is deprotected using a Pd(PPh3)4 catalyst. This reaction is reagent and time-intensive, requiring special care to remove all atmospheric oxygen (use of argon gas), which may poison the palladium catalyst, leading to undesirable side reactions. Palladium is a heavy metal, and traceless removal of it from the final structure could be quite complicated. Moreover, since our molecule is to be utilized as a siderophore, the presence of any heavy metals in the synthetic strategy could cause inaccuracy in the biological study.

The HPLC-ESI-MS/MS conditions were developed from those used in previous work for the identification of other organic iron ligands by slightly modifying the composition and gradient of the mobile phase [42]. The parameters evaluated in the method's setup (selectivity, linearity, LOD, and LOQ) resulted in adequate identification and the quantification of the two considered standard molecules.

SPE is the most widely used method for the extraction of water samples with various solid-phase sorbents. SPE is an effective technique for the extraction of DOM from seawater, freshwater, and glacial environments [20,43,44]. Different types of sorbents were tested, obtaining recovery rates exceeding 40% of bulk DOM from the marine environment [19]. Complexes of Cu and Fe with natural DOM have been successfully extracted using both polystyrene-divinylbenzene polymers (SDVB) or bonded silica (C18) [18,21,45]. These two types of sorbents display some differences in their extraction mechanisms: C18 retains compounds based on van der Waals forces and hydrogen bonding. SDVB polymers additionally retain compounds based on π–π interactions between the aromatic structures of the compounds and the sorbents [45].

The C18 sorbent was used as a stationary phase in SPE extraction in this study based on the data reported by [45], who found that the recoveries of Cu and Fe-DOM obtained with C18 sorbent were three times higher than those obtained with other types of sorbent in seawater samples.

Moreover, SPE allows the complete desalting of the eluted sample, thus reducing the matrix interferences during the HPLC-ESI-MS/MS analysis. Our optimized SPE procedure takes only a few hours to obtain a ready-to-analyze sample, which is a great advantage with respect to other procedures employing dialysis membranes [42].

The SPE extraction yield was optimized by CCD. CCD is a widely used and long-known experimental design pattern for modeling and surface response optimization because it allows the estimation of the nonlinearity of the responses in the provided data set, it helps to predict the curvature in the obtained continuous responses, and it provides the maximum information in a minimal experimental trial by reducing the number of trials required to estimate the squared terms in the second-order model [30,46].

The optimization had the aims of minimizing the sample loading volume (50–200 mL) and evaluating the pH effect on the extraction, which has been previously reported in the literature [3].

Regarding sample loading volume, test results showed that, due to the efficiency of the SPE technique, analytes can be extracted and preconcentrated to be detected in mass spectrometry, even using small sample volumes. Our optimized conditions require only 180 mL of the sample, showing the main advantage of using a smaller sample volume than other procedures reported in the literature [18,21]. This is the great advantage of our method because it will allow smaller volumes of water to be collected during sampling voyages and consequently reduce the time for both sampling and sample preparation.

The pH of the sample can significantly affect the interaction between the analytes and functional groups of the stationary phase of the SPE columns and, thus, the extraction yield. The pH will have different effects on each type of siderophore, as the classes are extremely heterogeneous and characterized by high structural variety and functional groups [3,42].

In particular, at seawater pH (~8), siderophore complexes of carboxylate and catecholate types are deprotonated and negatively charged, with low affinity for the functional groups of the stationary phases of commonly used SPE (e.g., C18, polystyrene divinylbenzene, XAD) [3]. However, acidification of the sample could also cause hydrolysis or precipitation of siderophores, particularly for catecholate siderophores [21].

The results obtained showed that ferrioxamine E, being a hydroxamate siderophore, is less affected by the effect of pH than those of the catecholate and carboxylate types, showing high extraction yields in all tests carried out at different pH. For staphyloferrin A-like compound, on the other hand, the effect of pH is particularly significant, allowing its extraction only at acidic pH (2).

Under the optimized conditions, the SPE method has a pre-concentration factor of 3000. Considering the data reported in the literature concerning the FOE concentration (~10 pM, [18]), the pre-concentration factor is adequate for the application of the method to the analysis of real seawater samples, even when the siderophore concentration is low. With our SPE-optimized conditions, we obtained better FOE recoveries than those achieved by McCormack et al. from both synthetic and natural seawater with an Isolute ENV+ cartridge [17]. The recovery of staphylopherrin A -like compound, which had not been reported before, was relatively low, but it was comparable with other reported siderophore recoveries from seawater samples [17].

5. Conclusions

Our study provides a method for studying siderophores in seawater, which are important ligands for dFe speciation, particularly in iron-limited areas such as the Southern Ocean.

The use of SPE and HPLC-ESI-MS/MS proved to be an efficient method for extraction, preconcentration, and identification of siderophore-type ligands in seawater. In particular, the optimized conditions with CCD allowed satisfactory extraction yields for the identification of ferrioxamine E and staphyloferrin A-like compound, greatly reducing the sample volumes required.

Our method resulted in a significant decrease in sample preparation time, which will be very advantageous during oceanographic campaigns due to the smaller amount of seawater to be sampled.

The chemical synthesis of the staphyloferrin A-like derivative is quite simple, starting from a commercially available molecule like citric acid and employing a traditional Fmoc strategy using an orthogonal protocol with a common and not expensive Fmoc-Lys(Mtt)-Wang resin. In our protocol, the reaction is much faster, allowing for the removal of the protecting groups without undesirable reactions.

The study of MS fragmentation has yielded various structural information and is a good starting point for using this method as a qualitative investigation technique in real samples by comparing MS spectra.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The single steps of the entire synthesis of our derivative are described in Schemes A1 and A2, reported in Appendix A.

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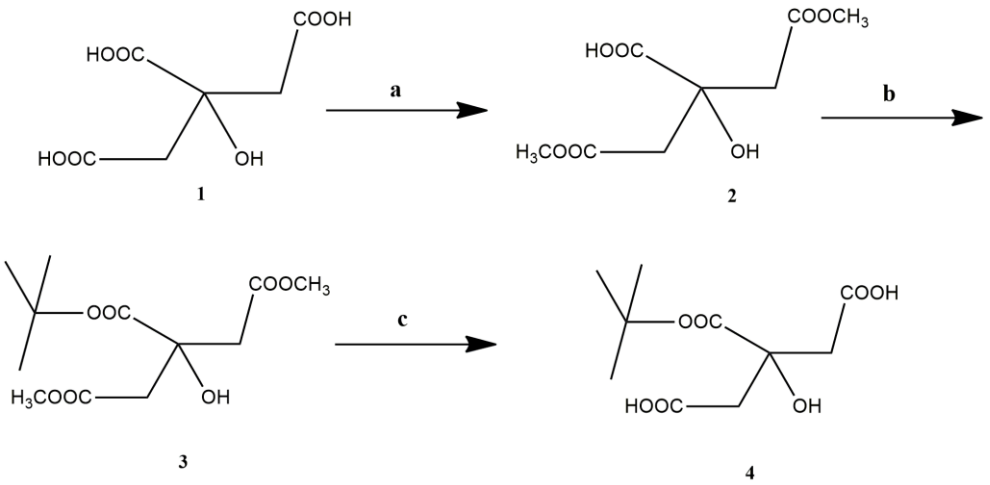
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A



Scheme A1. Reagents: (a) Concentrated H_2SO_4, MeOH, refluxed, 1 h; (b) perchloric acid 60%, tert-butyl acetate, RT, 4 days; (c) 2M aqueous NaOH in MeOH, RT, 3 h. Numbers 2 to 4 refer to the intermediate and final compounds of the synthesis shown in Scheme A1.

1,5-Dimethyl citrate monohydrate (2-hydroxy-4-methoxy-2-(2-methoxy-2-oxoethyl)-4-oxobutanoic acid) 2.

Citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) **1** (770 mg, 4 mmoL) was dissolved in methanol (10 mL) together with 98% sulfuric acid (1 mL). The mixture was refluxed for about 1 h, then allowed to cool to room temperature, and cold water (5 mL) was added with stirring. The solution was neutralized with solid calcium carbonate to remove the excess of citric and sulfuric acids as their insoluble calcium salts. The filtrate was concentrated down under a vacuum to give a pale-yellow residue. The residue was dissolved in acetonitrile, filtered, and lyophilized to give 1,5-dimethyl citrate as a yellow oil (181 mg, 21%). ESI-MS: m/z 221.2 $[M+H]^+$.

3-tert-Butyl 1,5-dimethyl citrate (2-Tert-butyl 1,3-dimethyl 2-hydroxypropane-1,2,3-tricarboxylate) 3.

1,5-Dimethyl citrate monohydrate **2** (181 mg, 0.82 mmoL) was suspended in tert-butyl acetate (3 mL), and 60% perchloric acid solution (30 µL) was carefully added to the mixture. The mixture was stirred at room temperature for 96 h. After this time, the reaction mixture was slowly poured into a saturated aqueous solution, bringing the solution to pH 6. The

organic layer was separated. The aqueous layer was extracted three times with diethyl ether (3 × 5 mL). The organic layers were combined, dried over anhydrous Na_2SO_4 and filtered. The organic mixture was concentrated in a vacuum to afford a pale-yellow oil. The residue was then dissolved in hot hexane (5 mL) with rapid stirring. The solution was allowed to cool to room temperature and then placed in a refrigerator until a white precipitate (probably trimethylcitrate) formed. The precipitate was filtered and discarded. The clear filtrate was evaporated to yield give 3-tert-butyl-1,5-dimethyl citrate as a colorless viscous oil (163 mg, 72%). ESI-MS: m/z 277.3 $[M+H]^+$.

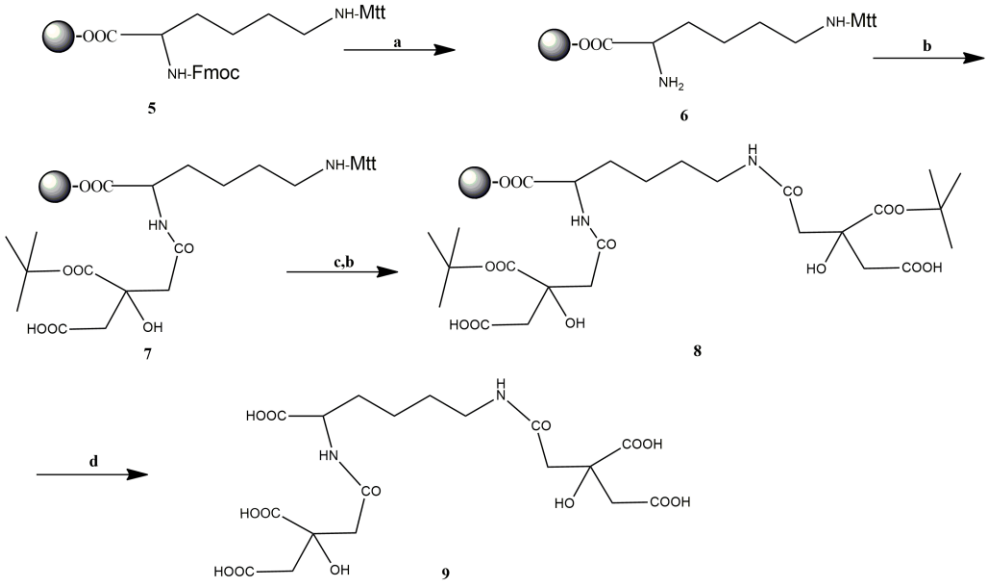
3-tert-Butyl citrate (3-(tert-butoxycarbonyl)-3-hydroxypentanedioic acid) 4.

3-tert-Butyl-1,5-dimethyl citrate **3** (163 mg, 0.59 mmoL) was dissolved in methanol (3 mL). 2 M sodium hydroxide (2 mL) was slowly added to this solution at 0 °C. The mixture was warmed to room temperature and stirred for about 3 h.

The volume of the mixture was then reduced on a rotary evaporator, and the resulting aqueous solution was acidified to pH 3 using 2 N HCl.

The product was then extracted with ethyl acetate (3 × 5 mL), and the extracts were combined. The organic layers were dried over anhydrous Na_2SO_4 and filtered. The solution was concentrated in a vacuum to afford a white solid product.

The final product was then purified by preparative HPLC. The peak of interest was concentrated and lyophilized to obtain the title compound as a grey powder (44 mg, 30%). ESI-MS: m/z 249.2 $[M+H]^+$.



Scheme A2. Reagents: (a) 20% piperidine/DMF, T = 40°, 30 min; (b) 4, HBTU, DIPEA, NMP, T = 40°, 2 h; (c) TFA 1%/DCM, RT, 30 min; (d) TFA:TIPS:H2O 95:2.5:2.5, RT, 2 h. Numbers 5 to 9 refer to the intermediate and final compounds of the synthesis shown in Scheme A2.

2-(2-((5-carboxy-5-(3-(carboxymethyl)-3,4dihydroxy-4-oxobutanamido)pentyl)amino)-2-oxoethyl)-2-hydroxysuccinic acid 9.

The final backbone **9** was manually synthesized using the standard method of solid-phase peptide synthesis according to the 9-fluorenylmethoxycarbonyl(Fmoc) strategy.

Commercially available Fmoc-Lys(Mtt)-Wang resin was rinsed with anhydrous dichlorometane (DCM) overnight.

Fmoc deprotection of **5** was carried out using 20% piperidine in N, N-dimethylformamide (DMF) for 30 min to liberate the primary amine on the resin. First, coupling between amine **6** and acid **4** was carried out using O-benzotriazol-N,N,N′,N′tetramethyluronium hexafluorophosphate (HBTU) (1eq), N,N-diisopropilethylamine (DIPEA) (2eq), at 0.2 M final concentration in anhydrous N-methylpyrrolidone (NMP) for 2 h at T = 40 °C.

The mono-protected derivative of citric acid **4**, was prepared in three steps as previously described. After completion of the reaction, the 4-methyltrityl (Mtt) moiety was removed from compound **7** by incubating the resin in a cocktail of DCM and trifluoroacetic acid 1% (TFA) for 30 min. The resin containing the liberated ε-amino group was then coupled with the same citric acid derivative for approximately 2 h, in a similar manner as the first coupling reaction of citric acid derivative **4,** to obtain the derivative **8**.

After completion of the synthesis, the resin was rinsed with DCM and dried overnight. The final cleavage of the product from the solid support and the removal of all protecting groups was carried out using a mixture consisting of 95% TFA, 2.5% water, and 2.5% triisopropylsilane (TIPS) for about 2 h.

The crude compound **9** was precipitated in ice-cold diethyl ether and then purified by preparative reverse phase HPLC on an Agilent 1260 Infinity preparative HPLC equipped with a Phenomenex C18 Luna column (21.20 × 250 mm). The separation was obtained with a gradient starting with 5% solvent B for 5 min, linearly increasing to 70% solvent B in 30 min and up to 100% B in 10 min. The solvents used were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The peak of interest was then collected, evaporated under a vacuum and then lyophilized. The identity and purity of the compound were verified by mass spectrometry analysis using an Agilent 1100 series LC/MSD ion trap instrument. ESI-MS: m/z 495.1 $[M+H]^+$.

References

1. Ibisanmi, E.; Sander, S.G.; Boyd, P.W.; Bowie, A.R.; Hunter, K.A. Vertical distributions of iron-(III) complexing ligands in the Southern Ocean. *Deep Sea Res. II* **2011**, *58*, 2113–2125. [CrossRef]
2. Barbeau, K.; Rue, E.L.; Trick, C.G.; Bruland, K.W. Photochemical reactivity of siderophores produced by marine heterotrophic bacteria and cyanobacteria based on characteristic Fe(III) binding groups. *Limnol. Oceanogr.* **2003**, *48*, 1069–1078. [CrossRef]
3. Gledhill, M.; Buck, K.N. The organic complexation of iron in the marine environment: A review. *Front. Microbiol.* **2012**, *3*, 69. [CrossRef]
4. Rivaro, P.; Ardini, F.; Grotti, M.; Aulicino, G.; Cotroneo, Y.; Fusco, G.; Mangoni, O.; Bolinesi, F.; Saggiomo, M.; Celussi, M. Mesoscale variability related to iron speciation in a coastal Ross Sea area (Antarctica) during summer 2014. *Chem. Ecol.* **2019**, *35*, 1–19. [CrossRef]
5. Reid, R.T.; Livet, D.H.; Faulkner, D.J.; Butler, A. A siderophore from a marine bacterium with an exceptional ferric ion affinity constant. *Nature* **1993**, *366*, 455–458. [CrossRef] [PubMed]
6. Chen, J.; Guo, Y.; Lu, Y.; Wang, B.; Sun, J.; Zhang, H.; Wang, H. Chemistry and biology of siderophores from marine microbes. *Mar. Drugs* **2019**, *17*, 562. [CrossRef] [PubMed]
7. Hunter, K.A.; Boyd, P.W. Iron-binding ligands and their role in the ocean biogeochemistry of iron. *Environ. Chem.* **2007**, *4*, 221–232. [CrossRef]
8. Vraspir, J.M.; Butler, A. Chemistry of marine ligands and siderophores. *Annu. Rev. Mar. Sci.* **2009**, *64*, 43–63. [CrossRef]
9. Bundy, R.M.; Boiteau, R.M.; McLean, C.; Turk-Kubo, K.A.; McIlvin, M.R.; Saito, M.A.; Van Mooy, B.A.S.; Repeta, D.J. Distinct siderophores contribute to iron cycling in the mesopelagic at station ALOHA. *Front. Mar. Sci.* **2018**, *5*, 61. [CrossRef]
10. Laglera, L.M.; Tovar-Sanchez, A.; Sukekava, C.F.; Naik, H.; Naqvi, S.W.A.; Wolf-Gladrow, D.A. Iron organic speciation during the LOHAFEX experiment: Iron ligands release under biomass control by copepod grazing. *J. Mar. Syst.* **2020**, *207*, 103151. [CrossRef]
11. Ahmed, E.; Holmström, S.J.M. Siderophores in environmental research: Roles and applications. *Microb. Biotechnol.* **2014**, *7*, 196–208. [CrossRef]
12. Boiteau, R.M.; Mende, D.R.; Hawco, N.J.; Mcilvin, M.R.; Fitzsimmons, J.N. Siderophore-based microbial adaptations to iron scarcity across the eastern Pacific Ocean. *Proc. Natl. Acad. Sci. USA* **2016**, *13*, 14237–14242. [CrossRef] [PubMed]
13. Moore, C.M.; Mills, M.M.; Arrigo, K.R.; Bopp, L.; Boyd, P.W.; Galbraith, E.D.; Geider, R.J.; Guieu, C.; Jaccard, S.L.; Jickells, T.D.; et al. Processes and patterns of oceanic nutrient limitation. *Nature* **2013**, *6*, 701–710. [CrossRef]
14. De Serrano, L.O.; Camper, A.K.; Richards, A.M. An overview of siderophores for iron acquisition in microorganisms living in the extreme. *Biometals* **2016**, *29*, 551–571. [CrossRef]
15. Boukhalfa, H.; Lack, J.; Reilly, S.D.; Hersman, L.; Neu, M.P. Siderophore production and facilitated uptake of iron and plutonium in *P. putida*. *AIP Conf. Proc.* **2003**, *673*, 343–344. [CrossRef]
16. Zajdowicz, S.; Haller, J.C.; Krafft, A.E.; Hunsucker, S.W.; Mant, C.T.; Duncan, M.W.; Hodges, R.S.; Jones, D.N.M.; Holmes, R.K. Purification and structural characterization of siderophore (Corynebactin) from *Corynebacterium diphtheriae*. *PLoS ONE* **2012**, *7*, e34591. [CrossRef]
17. McCormack, P.; Worsfold, P.J.; Gledhill, M. Separation and detection of siderophores produced by marine bacterioplankton using high-performance liquid chromatography with electrospray ionization mass spectrometry. *Anal. Chem.* **2003**, *75*, 2647–2652. [CrossRef]

18. Mawji, E.; Gledhill, M.; Milton, J.A.; Tarran, G.A.; Ussher, S.; Thompson, A.; Wolff, G.A.; Worsfold, P.J.; Achterberg, E.P. Hydroxamate siderophores: Occurrence and importance in the Atlantic Ocean. *Environ. Sci. Technol.* **2008**, *42*, 8675–8680. [CrossRef]
19. Dittmar, T.; Koch, B.; Hertkorn, N.; Kattner, G. A simple and efficient method for the solid-phase extraction of dissolved organic matter (SPE-DOM) from seawater. *Limnol. Oceanogr. Methods* **2008**, *6*, 230–235. [CrossRef]
20. D'Andrilli, J.; Dittmar, T.; Koch, B.P.; Purcell, J.M.; Marshall, A.G.; Cooper, W.T. Comprehensive characterization of marine dissolved organic matter by Fourier transformation cyclotron resonance mass spectrometry with electrospray and atmospheric pressure photoionization. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 643–650. [CrossRef]
21. Velasquez, I.; Nunn, B.L.; Ibisanmi, E.; Goodlett, D.R.; Hunter, K.A.; Sander, S.G. Detection of hydroxamate siderophores in coastal and Sub-Antarctic waters off the Southeastern Coast of New Zealand. *Mar. Chem.* **2011**, *126*, 97–107. [CrossRef]
22. Konetschny-Rapp, S.; Jung, G.; Meiwes, J.; Zahner, H. Staphyloferrin A: A structurally new siderophore from staphylococci. *Eur. J. Biochem.* **1990**, *74*, 65–74. [CrossRef] [PubMed]
23. Boiteau, R.M.; Fitzsimmons, J.N.; Repeta, D.J.; Boyle, E.A. Detection of iron ligands in seawater and marine cyanobacteria cultures by High-Performance Liquid Chromatography—Inductively Coupled Plasma-Mass Spectrometry. *Anal. Chem.* **2013**, *85*, 4357–4362. [CrossRef] [PubMed]
24. Manck, L.E.; Park, J.; Tully, B.J.; Barbeau, K.A.; Poire, A.M.; Bundy, R.M.; Dupont, C.L. Petrobactin, a siderophore produced by *Alteromonas*, mediates community iron acquisition in the global ocean. *ISME J.* **2021**, *16*, 358–369. [CrossRef] [PubMed]
25. Velasquez, I.B.; Ibisanmi, E.; Maas, E.W.; Boyd, P.W.; Nodder, S.; Sander, S.G. Ferrioxamine siderophores detected amongst iron binding ligands produced during the remineralization of marine particles. *Front. Mar. Sci.* **2016**, *3*, 172. [CrossRef]
26. Budzikiewicz, H. Bacterial citrate siderophores. *Mini-Rev. Org. Chem.* **2005**, *2*, 119–124. [CrossRef]
27. Berner, I.; Konetschny-Rapp, S.; Jung, G.; Winkelmann, G. Characterization of ferrioxamine E as the principal siderophore of *Erwinia herbicola* (Enterobacter agglomerans). *Biol. Met.* **1988**, *1*, 51–56. [CrossRef]
28. Kingsley, R.A.; Reissbrodt, R.; Rabsch, W.; Ketley, J.M.; Tsolis, R.M.; Everest, P.; Dougan, G.; Bäumler, A.J.; Roberts, M.; Williams, P.H. Ferrioxamine-mediated Iron(III) utilization by *Salmonella enterica*. *Appl. Environ. Microbiol.* **1999**, *65*, 1610–1618. [CrossRef]
29. Leardi, R.; Melzi, C.; Polotti, G. Chemometric Agile Tool (CAT). 2017. Available online: http://gruppochemiometria.it/index.php/software (accessed on 15 June 2023).
30. Benedetti, B.; Caponigro, V.; Ardini, F. Experimental design step by step: A practical guide for beginners. *Crit. Rev. Anal. Chem.* **2022**, *52*, 1015–1028. [CrossRef]
31. Pandey, R.K.; Jarvis, G.G.; Low, P.S. Chemical synthesis of staphyloferrin A and its application for *Staphylococcus aureus* detection. *Org. Biomol. Chem.* **2014**, *12*, 1707–1710. [CrossRef]
32. Guo, H.; Naser, S.A.; Ghobrial, G.; Phanstiel, O. Synthesis and biological evaluation of new citrate-based siderophores as potential probes for the mechanism of iron uptake in mycobacteria. *J. Med. Chem.* **2002**, *45*, 2056–2063. [CrossRef] [PubMed]
33. Gledhill, M. Electrospray ionisation-mass spectrometry of hydroxamate siderophores. *Analyst* **2001**, *126*, 1359–1362. [CrossRef] [PubMed]
34. Sidebottom, A.M.; Karty, J.A.; Carlson, E.E. Accurate mass MS/MS/MS analysis of siderophores Ferrioxamine B and E1 by Collision-Induced Dissociation Electrospray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2015**, *26*, 1899–1902. [CrossRef] [PubMed]
35. Wells, M.L.; Price, N.M.; Bruland, K.W. Iron chemistry in seawater and its relationship to phytoplankton: A workshop report. *Mar. Chem.* **1995**, *48*, 157–182. [CrossRef]
36. Macrellis, H.M.; Trick, C.G.; Rue, E.L.; Smith, G.; Bruland, K.W. Collection and detection of natural iron-binding ligands from seawater. *Mar. Chem.* **2001**, *76*, 175–187. [CrossRef]
37. Achterberg, E.P.; Holland, T.W.; Bowie, A.R.; Mantoura, R.F.C.; Worsfold, P.J. Determination of iron in seawater. *Anal. Chim. Acta* **2001**, *442*, 1–14. [CrossRef]
38. Vong, L.; Laës, A.; Blain, S. Determination of iron–porphyrin-like complexes at nanomolar levels in seawater. *Anal. Chim. Acta* **2007**, *588*, 237–244. [CrossRef]
39. Hopkinson, B.M.; Morel, F.M.M. The role of siderophores in iron acquisition by photosynthetic marine microorganisms. *BioMetals* **2009**, *22*, 659–669. [CrossRef]
40. Longhini, C.M.; Sá, F.; Neto, R.R. Review and synthesis: Iron input, biogeochemistry, and ecological approaches in seawater. *Environ. Rev.* **2018**, *27*, 125–137. [CrossRef]
41. Butler, A. Marine siderophores and microbial iron mobilization. *Biometals* **2005**, *18*, 369–374. [CrossRef]
42. Rivaro, P.; Ardini, F.; Grotti, M.; Vivado, D.; Salis, A.; Damonte, G. Detection of carbohydrates in sea ice extracellular polymeric substances via solid-phase extraction and HPLC-ESI-MS/MS. *Mar. Chem.* **2021**, *228*, 103911. [CrossRef]
43. D'Andrilli, J.; Cooper, W.T.; Foreman, C.M.; Marshall, A.G. An ultrahigh-resolution mass spectrometry index to estimate natural organic matter lability. *Rapid Commun. Mass Spectrom.* **2015**, *29*, 2385–2401. [CrossRef] [PubMed]
44. Zhang, L.; Yuan, D.X.; Fang, K.; Liu, B.M. Determination of siderophores in seawater by high performance liquid chromatography-tandem mass spectrometry coupled with solid phase extraction. *Chin. J. Anal. Chem.* **2015**, *43*, 1285–1290. [CrossRef]

45. Waska, H.; Koschinsky, A.; Ruiz Chancho, M.J.; Dittmar, T. Investigating the potential of solid-phase extraction and Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) for the isolation and identification of dissolved metal–organic complexes from natural waters. *Mar. Chem.* **2015**, *173*, 78–92. [CrossRef]
46. Bhattacharya, S. Central Composite Design for Response Surface Methodology and Its Application in Pharmacy. In *Response Surface Methodology in Engineering Science*; IntechOpen: London, UK, 2021. [CrossRef]