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Separation of siderophores by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) was applied as a fast method of siderophore separation. Siderophores are iron binding and regulating cell products, which facilitate iron transport into cells. A fast and efficient method of siderophore analysis is important for better understanding of the iron pathways in a sea environment or marine organisms. The best results of CE analysis were obtained using free zone CE in 25 m*M* phosphate buffer at basic pH using a constant voltage of 20 kV. Under these conditions it was possible to detect the presence of siderophores in seawater. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Siderophores; Ferrioxamines; Iron

1. Introduction

Iron is required in many biochemical processes such as photosynthesis, respiration, synthesis of DNA and chlorophyll, and reduction of nitrates. However, due to the insolubility of iron(III) oxides and hydroxides at neutral pH, iron occurs in seawater at subnanomolar concentrations. To sequester iron from the environment, microorganisms produce high-affinity ferric chelating compounds termed siderophores [3]. Siderophore are defined as iron binding, iron regulating low-molecular-mass cell products, which facilitate the transport of ferric iron into cells. They have very high iron(III) affinity constants (10^{23}–10^{52}) and their biosynthesis is repressed in high iron concentration in the environment [4]. In samples from a sea environment (cyanobacteria, algae) concentrations of siderophores varied from 0.1 to 100 n*M* [1,2].

Desferrioxamines comprise one large hydroxamate family. Their iron complexes are called ferrioxamines. They are characteristic of the *Streptomyces* and *Actinomycetes* species, fungi and some enterobacteria. Bacterial siderophores from the family of ferrioxamines and the producing organisms are listed in Table 1 [3]. These ferrioxamines occur as both linear and cyclic compounds. Desferrioxamine B, trade-named Desferal, became the drug of choice for the treatment of transfusional iron overload. Ferrioxamines E (nocardamine) and D_2 are cyclic compounds whereas all other ferrioxamines are linear. Rhodotorulic acid is a dipeptide of acetylated N-hydroxyornithine, which mediates iron uptake in the yeast *Rhodotorula* species [3]. The structures of four hydroxamate-type siderophores are shown in Fig. 1. Literature reports suggest that siderophores bind iron and promote iron transport to the cells,

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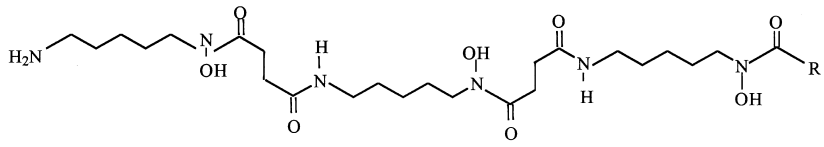
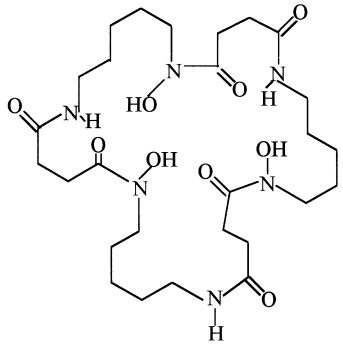
Table 1
Bacterial siderophores from the ferrioxamine family

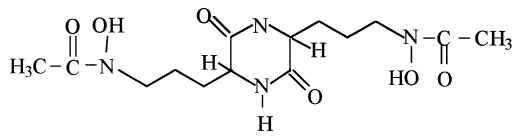
Siderophore	Producing organism
Ferrioxamine (A_1, A_2, B, C, D_1, D_2, E, F, G, H)	*Streptomyces pilosus, S. griseus, S. griseoflavus, S. olivaceus, S. aureofaciens, S. glilaeus, S. Lavendule*
Ferrioxamine B	*Arthrobacter simplex*
Ferrioxamine E, D_2, B	*Erwinia herbicola*
Ferrioxamine E	*Pseudomonas stutzeri*
	Chromobacterium violaceum
Ferrioxamine G	*Hafnia alvei*



Desferrioxamine B R = CH_3
Desferrioxamine G R = $(CH_2)_2CO_2H$



Desferrioxamine E, Nocardamine



Rhodotorulic acid

Fig. 1. Structure of hydroxamate siderophores.

which excreted them. Siderophores display a specific activity only in relation to the organisms from which they originate. During cyanobacterial blooms, other algae can be completely suppressed owing to the lack of iron, since they are unable to use the iron transported to the cell [5]. Siderophores such as rodotorulic acid, retro-(Et)-arthrobactin, schizokinen, desferrioxamine B, can modify physiological processes in populations of cyanobacteria and green algae cells [6,7]. Certain derivatives of ferrioxamines such as ferriomycin A_1, danomycin A and danomycin B show antibiotic activity. These siderophores are produced by most *Actinomycetes* [8]. Marine and freshwater phytoplankton (prokaryotic and eukaryotic) grown at low iron levels produce hydroxamate-type siderophores, but the only one, which has been structurally identified, is schizokinen (citrate-hydroxamate siderophore) [9]. It seems that iron chelators may play a key role in phytoplankton growth and succession. Until now standard chromatographic methods as thin-layer chromatography, ion-exchange chromatography and reversed-phase high-performance chromatography have been used for siderophore separation [10,11]. Microorganisms isolated from coastal and oceanic water have been shown to produce siderophores in iron deficient culture media [12,13]. Soluble iron–siderophore complexes have been identified in these samples, although little effort has been directed toward their detection. The main objective of CE application was to find a fast and efficient method of analysing siderophore complexes. Another objective was to use CE to detect siderophores in seawater. The migration behavior of each siderophore was studied separately. Fast analysis of siderophores by CE and its detection in seawater is described.

2. Material and methods

2.1. Siderophore samples

Desferrioxamine B was purchased from the Ciba-Giegy (Basle, Switzerland) as a desferrioxamine methansulfonic (Desferal). Ferrioxamines E and G were obtained as a gift from Dr. R. Reissbrodt (Robert Koch-Institute, Wernigerode, Germany). Rhodotorulic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Samples of subsurface seawater (~50 dm^3) were collected in May 1996 at the Gdansk Deep station (54°34'N; 19°10'E) in the Baltic Sea. A schematic of the procedure for isolation of siderophore-like substances is shown in Fig. 2 [14,15]. The siderophore–iron(III) complex was prepared by adding of 10% excess of iron(III) chloride to the siderophore solution. The excess was neutralized with 0.1 *M* sodium hydroxide and the solution was lyophilized.

2.2. Capillary electrophoresis

A Beckman (Palo Alto, CA, USA) P/ACE System 2100 capillary electrophoresis instrument with the cathode on the detection side was employed. Free zone (FZCE) and micellar electrokinetic chromatographic (MEKC) methods were used. All solutions were filtered through a 22-μm pore membrane filter. Siderophore samples were analyzed as iron(III) complexes. The capillary cassette used was fitted with a fused-silica uncoated capillary, 57 cm (50 cm to the detector)×50 μm I.D. Runs were made at a constant voltage of 20 kV. Pressure injection of the sample for 4 s was used. The temperature of the capillary was maintained at 30°C. The separation effect was monitored at 214 nm. Analyses were performed using three different buffers: buffer A, 25 m*M* phosphate buffer, pH 7.0; buffer B, 25 m*M* phosphate buffer, pH 12.5; buffer C, 33 m*M* sodium tetraborate, 113 m*M* boric acid, 100 m*M* sodium dodecyl sulfate (SDS), pH 8.6. All samples investigated were dissolved in water. The chromatography data were acquired using SYSTEM GOLD software (Beckman).

3. Results and discussion

Because of low concentration of siderophores and the complex composition of biological material from sea environment, CE was applied as a fast and efficient method of siderophore analysis. The different structures and hydrophobic properties of siderophores were the reason for using the MEKC method. Using buffer C (containing SDS) as a separation medium, partial separation of siderophores was achieved (Fig. 3). A very broad, unhomogeneous

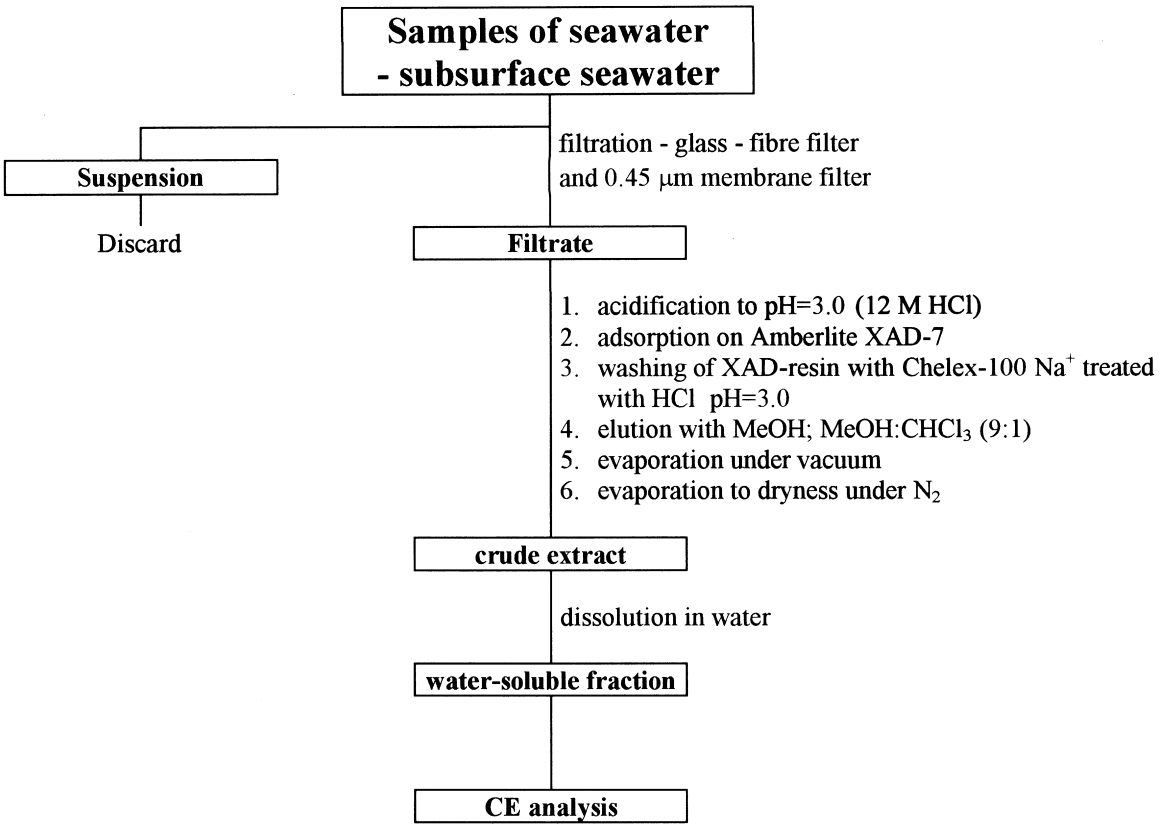


Fig. 2. Schematic procedure for siderophore isolation.

peak of rhodotorulic acid and incomplete separation of ferrioxamines G and E were observed. With buffer A, ferrioxamines B, E, and G were unrecognizable and migrated as one peak (Fig. 4). All the siderophores tested were resolved by FZCE using buffer B (Fig. 5). At this pH, complete and fast separation of siderophores was observed. Nonetheless, a small broadening of rhodotorulic acid peak was perceptible. Using buffer B a seawater sample was analyzed (Fig. 6). Ferrioxamines E and G were found. A blank test did not show the presence of siderophores (data not shown). Peaks with a migration time about 7.5 min were unrecognizable. They migrated close to the rhodotorulic acid peak but sharp peak shapes were completely different from the wide peak of rhodotorulic acid. Coinjection of the natural sample with the siderophores mixture used as a standard showed excellent covering of ferrioxamines E and G peaks (Fig. 7). The two highest peaks visible in the seawater sample (with migration time about 7.5 min) disappeared in the coinjected sample. The wide peak of rhodotorulic acid probably masked them. A new peak, with high intensity and a sharp shape and with migration time of ~8 min appeared in the coinjected sample. It was clear that the two peaks with migration time of ~8 min in the coinjected sample were different compounds.

4. Conclusion

CE is a useful method for separation of siderophores. Using FZCE at basic pH all siderophores investigated were resolved. CE can be recommended for the detection of and behavior of siderophores and tracing the iron pathways in sea environment. Our results demonstrate the presence of siderophores ferrioxamine family in the subsurface seawater.

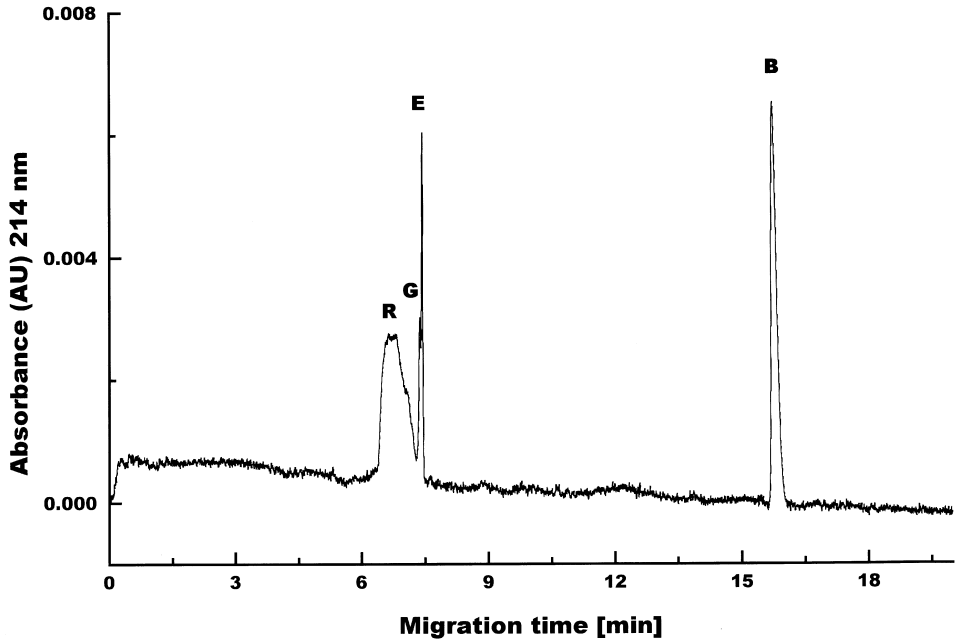


Fig. 3. MEKC analysis of siderophore mixture. Electrophoresis conditions: 57 cm (50 cm to the detector)×50 μm uncoated fused-silica capillary, buffer C, constant voltage 20 kV, temperature 30°C, pressure injection for 4 s. Siderophore labels: ferrioxamine B, B; ferrioxamine G, G; ferrioxamine E, E; rhodotorulic acid, R. Siderophores concentration was about 0.1 mg/ml.

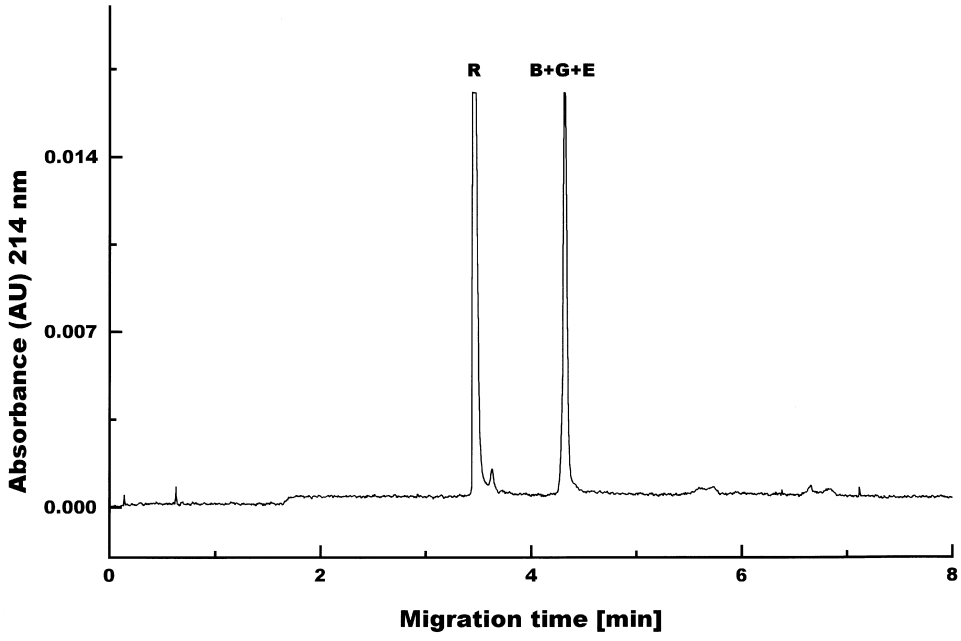


Fig. 4. FZCE analysis of siderophore mixture at neutral pH. Electrophoresis conditions and siderophore labels as in Fig. 3, except that buffer A was used.

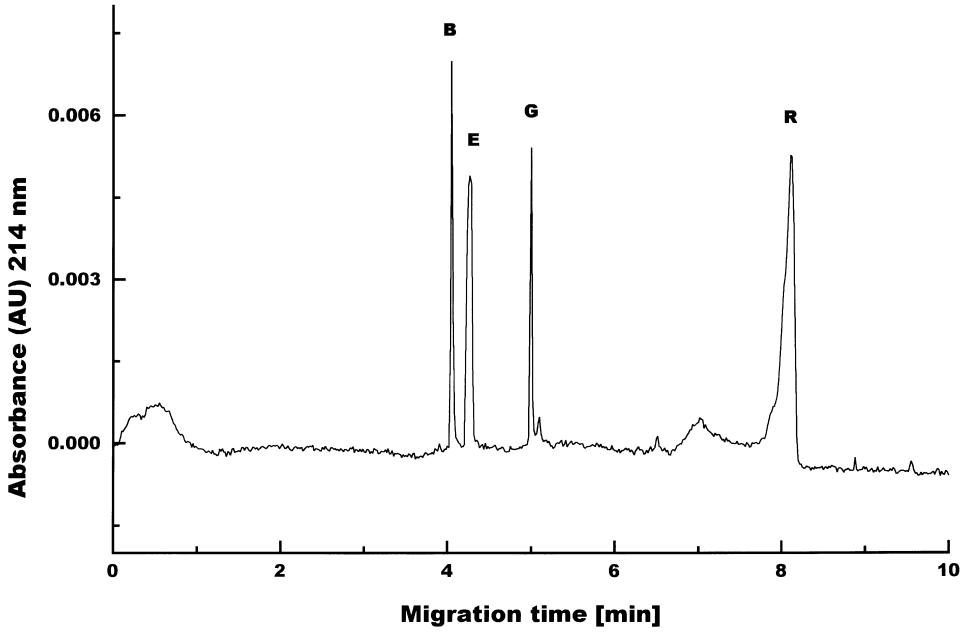


Fig. 5. FZCE analysis of siderophore mixture at basic pH. Electrophoresis conditions and siderophore labels are as in Fig. 3, except that buffer B was used.

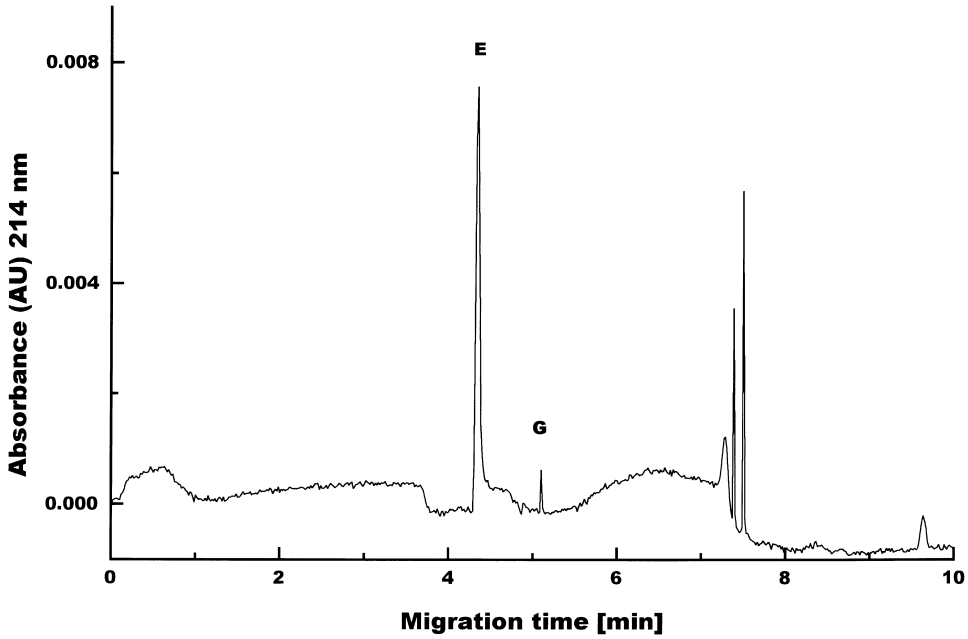


Fig. 6. FZCE analysis of siderophore sample from seawater. Electrophoresis conditions and siderophore labels are as in Fig. 3, except that buffer B was used.

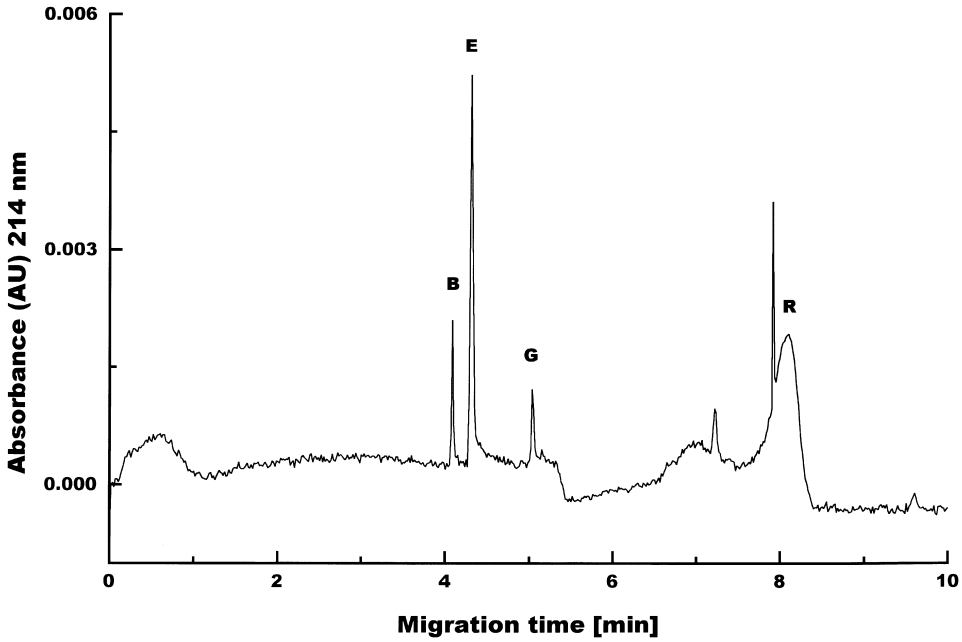


Fig. 7. FZCE analysis of seawater sample coinjected with siderophore mixture. Electrophoresis conditions and siderophore labels are as in Fig. 3, except that buffer B was used.

These compounds have not been previously detected in seawater (Baltic seawater).

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