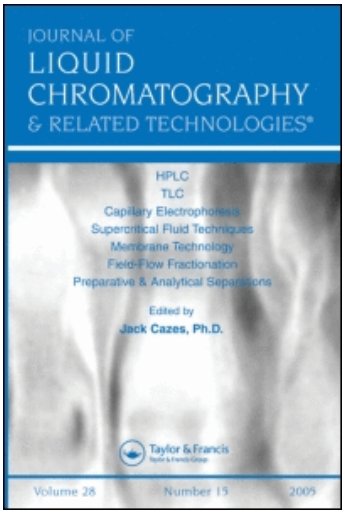

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DETERMINATION OF Fe(III) IN FERMENTATION BROTH BY ION-INTERACTION CHROMATOGRAPHY

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INTRODUCTION

Transition metals are essential in microbial metabolism. Iron, for example, is commonly found in iron-sulfur proteins and electron-transport proteins. Manipulation of transition metal concentration in fermentation media is often employed to retard microbial growth or influence secondary metabolism. For example, iron, calcium, magnesium, and zinc were found to stimulate growth of *Legionella pneumophilia*[1].

The chromatographic determination of Fe(III) has been accomplished by ion chromatography/post column derivatization[2,3], chelation ion chromatography[4-8], and ion-interaction[9-13] chromatography. In general, these methods were applied to relatively clean samples, such as river water. Ion-interaction chromatography is based upon pre-column derivatization with ethylenediaminetetraacetic acid, EDTA, to form Fe(III)EDTA$^-$. The resultant complex is eluted as an ion pair with tetrabutylammonium ion at a low pH value. In this work, ion-

interaction chromatography is applied to the rapid determination of Fe(III) in two common growth media, Mueller-Hinton and tryptic soy broths.

EXPERIMENTAL

Chemicals

Ethylenediaminetetraacetic acid (99+%), 40% tetrabutylammonium hydroxide, and sodium acetate were obtained from Aldrich Chemical Company (Milwaukee, WI) and used without further purification. Glacial acetic acid was purchased from Fisher Chemical Company (Fairlawn, NJ). Mueller-Hinton and tryptic soy broths were obtained from Sigma Chemical Company (St. Louis, MO). Acetonitrile and water used in the preparation of mobile phases were of HPLC grade and obtained from Fisher Chemical Company. Reference standard Fe(III) was purchased from Alltech Associates (Deerfield, IL) as a standard solution.

Instrumental

Ion-Interaction Chromatography A Dionex model DX300 liquid chromatograph (Dionex Corp., Sunnyvale, CA) interfaced with a Spectra-Physics model AS3500 autosampler (Spectra-Physics, Freemont, CA) was used. A Dionex spectral array UV-vis absorbance detector was used and data collected and analyzed with Dionex AI450 software. Unless otherwise specified, a Vydac 218TP54 column (Vydac Associates, Hesperia, CA) of dimensions 25 cm X 4.6 mm was used throughout method development. The pore diameter and particle size were 300Å and 5 μm, respectively.

Ion Chromatography with Post-Column Reaction Detection A Scientific Systems, Inc. model 350 pump was used to deliver the reagent solution to the above HPLC system. Post-column mixing of

reagent with mobile phase was accomplished using 10' of 0.010" Teflon tubing in a FIAtron FH40 heater set at 40°C.

Chromatographic Parameters

Ion-Interaction Chromatograhy The mobile phase was prepared by mixing 4.1 g sodium acetate, 2.75 mL of 40% tetrabutylammonium hydroxide, 3.75 g ethylenediaminetetraacetic acid (EDTA), and 5.00 mL glacial acetic acid per liter of HPLC grade water. The contents were thoroughly dissolved and filtered to 0.45 μm. The mobile phase was pumped at a rate of 1.00 mL/min, resulting in a back-pressure of about 1900 p.s.i. Unless otherwise specified, the injection volume and detection wavelength parameters were 20 μL and 320 nm, respectively. The column was periodically cleaned with a mobile phase consisting of a 70:29.9:0.1 mixture of acetonitrile, water, and trifluoroacetic acid. Mobile phases were filtered to 0.45 μm prior to use.

Ion Chromatography The mobile phase was prepared as described in reference (2) and consisted of 6 mM pyridine-2,6-dicarboxylic acid, 50 mM acetic acid, and and 50 mM sodium acetate. The post column reagent consisted of a 1% solution of EDTA in water. Mobile phase and reagent were filtered to 0.45 μm prior to use. Dionex HPIC-CG5 and -CS5 guard and analytical columns were used. The mobile phase and reagent flow rates were 1.0 and 0.5 mL/min, respectively. The detector was set at 520 nm.

Sample Preparation

Mueller-Hinton and tryptic soy broths were prepared per label instruction. Samples to be analyzed by ion-interaction chromatography were diluted with mobile phase 1:4 and filtered to 0.45 μm prior to injection. Samples to be analyzed by ion chromatography were diluted 1:4 with 0.05N HCl to achieve a final

composition of 0.01N HCl. These procedures appeared to stabilize iron in solution and improved peak shape. Prior to injection, samples were filtered with a syringe filter to 0.45 μm. Standards were prepared in HPLC grade water and diluted identically to the samples to minimize calibration bias.

RESULTS AND DISCUSSION

Figure 1 depicts chromatograms typical of fermentation broth. The Fe(III)EDTA$^-$ complex eluted as a symmetrical peak with a k′ value of about 0.8. The values of A_s and N were 1.2 and 1000 m^{-1}, respectively. Others have demonstrated that the retention of Fe(III)EDTA$^-$ in ion-interaction chromatography does not vary significantly with the concentration of organic

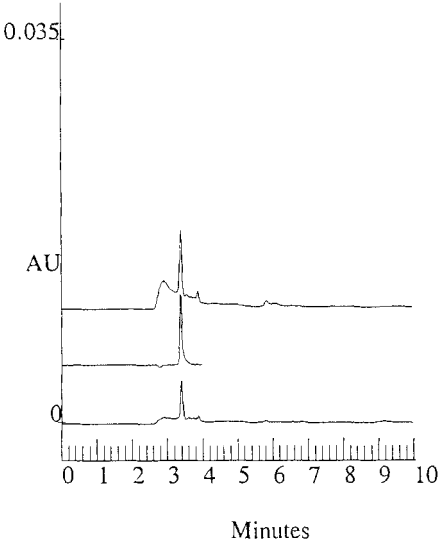

Figure 1. Typical chromatograms of Fe(III)EDTA$^-$ (top) Mueller-Hinton broth, (middle) 2.0 μg/mL standard, (bottom) tryptic soy broth.

modifier[9,13]. For this reason, no organic modifier was used in the mobile phase. As a result, few matrix components interfered with the analyte peak. Occasionally, the column needed to be stripped of absorbed matrix components with a mobile phase of stronger elutropic strength. This procedure was performed after approximately 50 sample injections.

The relationship of chromatographic response to the concentration of EDTA in the diluent was evaluated by diluting a concentrated solution of tryptic soy broth with mobile phase and water to achieve varying concentrations of EDTA at constant Fe(III) concentration. The results are shown in Figure 2. Below

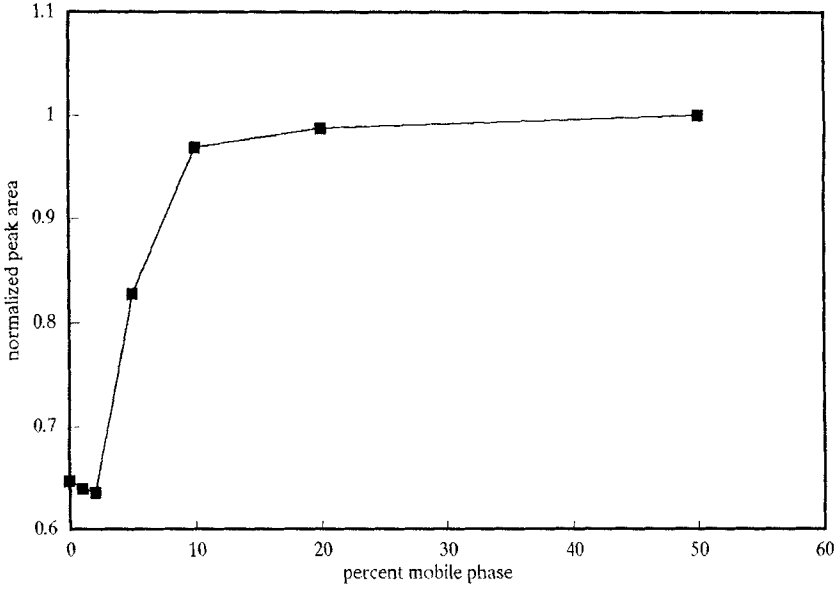

Figure 2. Plot of peak area of Fe(III)EDTA$^-$ complex (arbitrary units) as a function of mobile phase added to tryptic soy broth. The concentration of tryptic soy broth was held constant. Details of sample preparation are in text.

10% mobile phase (1.0 mM EDTA) the peak area is significantly affected by EDTA concentration. The sigmoidal response is suggestive of a competitive equilibrium of EDTA with other chelating agents in the medium. Dilution of the sample with mobile phase to a final composition of 20% mobile phase (2.0 mM EDTA) appears to be sufficient to minimize other interactions and provide a consistent response.

The precision of the assay is determined by the precision of the autosampler injection volume and reproducibility of sample preparation. The former value was evaluated by repetitive injection of the same solution of Fe(III) standard, approximately 1 μg/mL in concentration. Typically, the relative standard deviation of peak area measurement was below 2%. The precision of sample preparation was determined by repetitive preparation of a fermentation broth sample. The relative standard deviation (N=5) was approximately 4%.

The $Fe(III)EDTA^-$ peak area was linear over the concentration domain 0.2 to 12.0 μg/mL Fe(III), as shown in the lower trace in Figure 3. The value of r^2 was 0.9999, demonstrating excellent linear fit. The value of the concentration equivalent of the intercept was -0.02 μg/mL, indicating that Fe(III) was not leached from extraneous sources. Fe(III) was spiked into tryptic soy broth over the concentration domain 0.0 to 12.0 μg/mL. The upper trace of Figure 3 depicts the detector response as a function of the concentration of Fe(III) added. The value of r^2 was 0.9999, indicating excellent fit to a linear model. The concentration equivalent of the intercept was 0.73 μg/mL. The values of the slopes for standards and samples were 19601 and 19459 arbitrary units, respectively. The essentially equivalent slopes indicate the absence of matrix interactions with the analyte by the addition of EDTA to the sample.

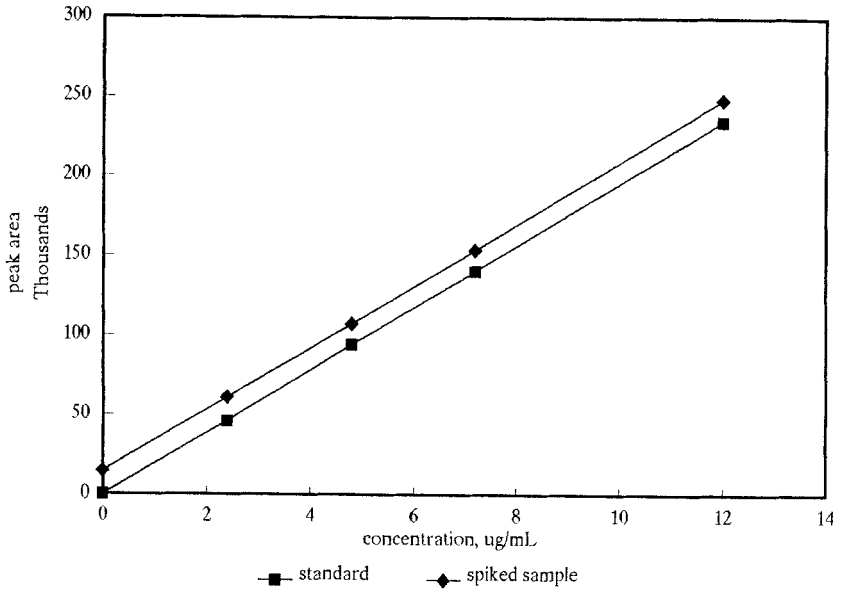

Figure 3. Plot of peak area for standard (bottom) and spiked tryptic soy broth (top). Equations of lines are given in text.

The purity of the Fe(III)EDTA$^-$ peak was evaluated by varying the detector wavelength with a constant sample of tryptic soy broth. The detector wavelengths used were 280, 320, and 350 nm. The apparent concentrations of Fe(III) were 8.41, 0.78, and 0.76 μg/mL, respectively. The values obtained at 320 nm and 350 nm are statistically equivalent, given a relative standard deviation of about 4% for each measurement. The high result obtained at 280 nm may indicate a proteinaceous interference, the effect of which is minimized at higher detector wavelengths. The relative peak areas for a standard at 280, 320, and 350 nm were 1.00, 0.51, and 0.20, respectively. These values are in excellent agreement with published spectra of the Fe(III)EDTA$^-$ complex[9,10].

For routine use, 320 nm was selected as a compromise in sensitivity in favor of selectivity.

The storage stability of fermentation broth samples was investigated by preparing samples of tryptic soy broth in 0.01N HCl and 20% mobile phase and analyzing the solutions periodically. The samples and a control, dissolved in water, were incubated at room temperature. Figure 4 shows the normalized peak area as a function of time. Significant degradation of the control and 0.01N HCl sample occured after 24 hours of storage. With increased storage time, adventitious microbial growth was evident. In contrast, the sample dissolved in 20% mobile phase was stable at least a week. No microbial

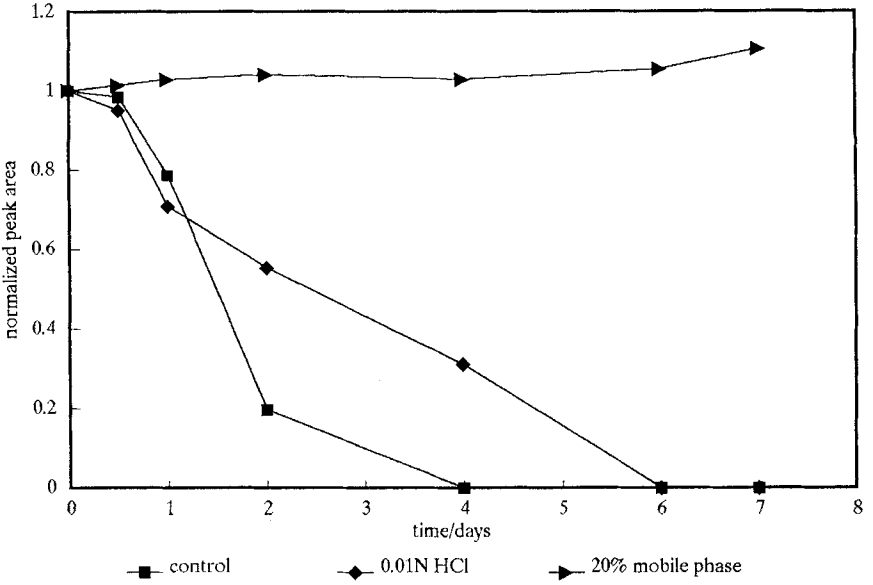

Figure 4. Plot of normalized peak area of Fe(III)EDTA complex in tryptic soy broth as a function of time. Broth concentrate was dissolved in ion-interaction chromatography mobile phase (top), 0.1N Cl (middle), and water (bottom) as a control.

infestation was evident. These observations are consistent with inhibition of microbial growth by metal chelating agents[1].

The accuracy of ion-interaction chromatography for the determination of Fe(III) in fermentation broth was evaluated by comparison of results obtained by ion-interaction chromatography to those obtained by ion chromatography for identical samples of fermentation broth. The results are compared in a Youden plot[14], given in Figure 5. Linear regression of the data gave the equation

$$IIC = 0.988*IC + 0.11 \quad r^2 = 0.998$$

where IIC and IC represent ion-interaction chromatography and ion chromatography, respectively. The values of the slope and intercept agree with the theoretical values of 1.00 and 0.00,

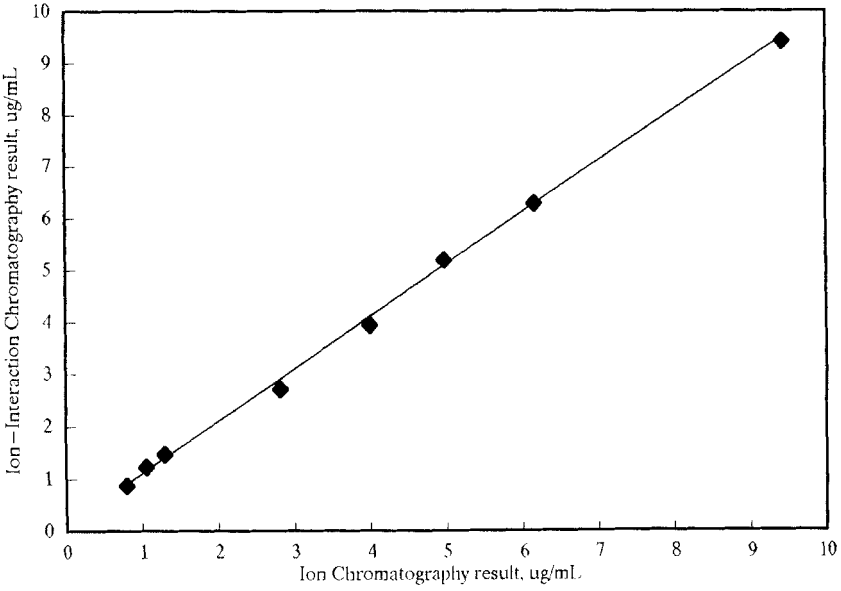

Figure 5. Comparison of results obtained for a series of spiked trypic soy broths by ion-interaction chromatography and ion chromatography. Equation of line is given in text.

respectively. The agreement of the two methods, which separate components by different retention mechanisms, implies that the assay is accurate within the concentration domain studied.

CONCLUSION

Ion-interaction chromatography has been applied to the determination of iron in fermentation broths. The method is sufficiently selective to avoid interference from matrix components and is useful in monitoring the uptake of Fe(III) during the course of fermentation.

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