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Dissolved Fe(II/III) colorimetric assay using a plate reader (96-well plate)

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We are still developing and optimizing this protocol

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Abstract

This protocol describes the adaption of an Iron assay originally described in **Viollier 1999** and **Stookey 1970**, modified for use on a multi-mode plate reader spectrophotometer (BioTek, Synergy 2, Winooski, VT, USA), using standard **96-well plates** for rapid measurements of 1 mL water samples. This assay measures the concentration of both Fe(II) and Fe(III) ions in solution.

Samples for this assay should be filtered (**0.2 µm syringe filter**), measured or diluted to 1.20 mL volume and placed in **1.5 mL microcentrifuge tubes**. Care should be taken to avoid oxidation of Fe(II) ions by oxygen in the atmosphere, either by performing the steps quickly under a stream of N_2 gas right after sampling or by performing the first step of this assay inside an anaerobic chamber.

Guidelines

Working principle

Ferrozine binds to both Fe(II) and Fe(III) ions in solution and turns into a magenta-colored complex (note: Fe(II)-induced complex has ~60x stronger absorbance than Fe(III), thus constituting a larger part of the signal). Maximum absorbance is at **λ=562 nm** at pH 4-9. To determine the concentrations of Fe(II) and Fe(III) ions, two spectrophotometric absorbance measurements are made, termed A_1 and A_2, where A_1 is an initial measurement and A_2 after a reduction step to reduce all Fe(III) to Fe(II) ions.

After measuring A_1 and A_2, the following system of two equations is solved to find C_{FeII} and C_{FeIII}, the molar concentrations of Fe(II) and Fe(III) ions, respectively:
$A_1 = \beta_{Fe_{II}} C_{Fe_{II}} + \beta_{Fe_{III}} C_{Fe_{III}}$
$A_2 = \beta_{Fe_{II}} (C_{Fe_{II}} + C_{Fe_{III}}) \alpha$
where $\beta_x = \epsilon_x l$, x is the species to be measured, ϵ_x is the molar absorbance coefficient of the specific complex, l the path length. β can be interpreted as the instrument-specific absorbance coefficient and α is the dilution factor (0 < α < 1) due to the addition of reductant reagent to obtain A_2.

In order to solve for C_{FeII} and C_{FeIII}, all we need to determine are b_{FeII}, b_{FeIII}, and a (and estimate their uncertainties). We can obtain these values by analyzing a dilution series of standard solutions where the concentrations of Fe(II) and Fe(III) ions are known. A realistic α value (along with its uncertainty estimates) is obtained by doing the reduction step a second time to the standards only, obtaining A_3 (even though all Fe(III) has already been reduced). Hence $\alpha = A_3/A_2$. All analytical uncertainty estimates can be obtained by regression analysis after this step (usually linear regression, estimating error of the slope), using the same dataset already obtained.

After obtaining β_{FeII}, β_{FeIII}, and α, we may derive C_{FeII} and C_{FeIII} as the following:

$$C_{Fe_{II}} = \frac{A_2 - \alpha A_1}{\alpha(\beta_{Fe_{II}} - \beta_{Fe_{III}})}$$

$$C_{Fe_{III}} = \frac{\alpha\beta_{Fe_{II}} A_1 - \beta_{Fe_{III}} A_2}{\alpha(\beta_{Fe_{II}} - \beta_{Fe_{III}})\beta_{Fe_{II}}}$$

Measurement range

The range of measurement of this assay is between **0 - 80 µM Fe(II/III)**, thus for samples containing a higher amount of dissolved Fe, especially anoxic water samples, dilution is required. For unknown samples, it is useful to add a 10x dilution sample. For samples that may contain elevated Fe, an additional 100x dilution is recommended. An accurate way to perform dilution is by measuring mass instead of volume. A pipette (which measures volume) has about 1-3% accuracy, whereas an analytical balance (0.1-1 mg accuracy) is vastly better. Do not switch pipettes during colorimetric assay procedures and pay careful attention.

Materials

Materials

- 1.5 mL Eppendorf® Microcentrifuge Tubes
- Corning® 96-Well EIA/RIA Assay Microplate

Reagents

All reagents are prepared with nanopure water and stored in 4 °C fridge in the dark.

(A) **Ferrozine**: [M] 0.01 Molarity (M) *Ferrozine* (FW 492.47, 97%, **Aldrich #160601**) is prepared in [M] 0.1 Molarity (M) *ammonium acetate* (FW 77.08, 99.999%, **Aldrich #372331**) solution.

Preparation steps: add 200 mL nanopure water in a clean bottle, add 1.5416 g *ammonium acetate* powder, mix well, then add 0.98494 g *Ferrozine*, mix well and store at 4 ºC in the dark.

(B) **Reducing Agent**: [M] 1.4 Molarity (M) of *hydroxylamine hydrochloride* (FW 69.49, 99.99%, **Aldrich #379921**) is prepared in a solution of analytical grade *hydrochloric acid* at a final concentration of [M] 2.0 Molarity (M) (diluted from stock *hydrochloric acid* : HCl suprapur, 30%, [M] 9.46 Molarity (M) , **Aldrich #1.00318**).

Preparation steps: add 157.72 mL nanopure water in a clean bottle, add 42.28 mL (or by mass 48.63 g) 30% HCl. Add 19.4572 g *hydroxylamine hydrochloride*. Mix well.

(C) **Buffer**: [M] 10 Molarity (M) *ammonium acetate* (FW 77.08, 99.999%, **Aldrich #372331**) is prepared and adjusted pH to pH 9.5 with a solution of *ammonium hydroxide* (28-30%, **Aldrich #221228**).

Preparation steps: dissolve 38.54 g *ammonium acetate* in 50 mL of nanopure water. Adjust pH to pH 9.5 with a pH meter by adding concentrated *ammonium hydroxide* dropwise while keeping the solution mixed.

Standards

Standards are diluted from [M] 1000 Parts per Million (PPM) Fe(III) stock solution (prepared from [M] 0.0179 Molarity (M) of $FeCl_3$ (FW 162.20 **Aldrich #8.03945**) in 0.01 M *hydrochloric acid* (diluted from stock *hydrochloric acid*: HCl suprapur, 1 M (1 N), **Aldrich #1.09137**)). Series of Fe(III) standards are prepared and diluted in NaCl solution matching sample salinity. Salinity should be matched within 20%.

Preparation steps: add 200 mL nanopure water in a clean bottle, add 2.02 mL 1N HCl, mix well, add NaCl (amount to match expected/measured salinity of sample), then add 0.5807 g $FeCl_3$ powder. Mix well.

Safety warnings

Use safety goggles and nitrile gloves when performing the steps outlined in this assay. Dispose of left-over chemicals by evaporating off all liquids inside a chemical hood (96-well plate), or by collecting these liquids in designated chem-waste jars.

Before start

Samples for this assay should be filtered (**0.2 µm syringe filter**), measured or diluted to 1.20 mL volume and placed in **1.5 mL microcentrifuge tubes**. Care should be taken to avoid oxidation of Fe(II) ions by oxygen in the atmosphere, either by performing the steps quickly under a stream of N_2 gas right after sampling or by performing the first step of this assay inside an anaerobic chamber.

This assay has a limited detection range and therefore dilutions of the sample may be required. Refer to the Guideline section to understand the requirements.

1 Measure absorbance A_1: A_1 is measured *immediately* after adding 120 µL of *reagent A* to 1.2 mL of filtered/filtered-diluted samples or undiluted standard solutions. Mix the mixture well with the pipette by gently pipetting up and down a few times. Load three 200 µL mixtures onto a 96-well plate to make triplicate measurements on the spectrophotometer.

2 Reduction and measure absorbance A_2: collect 640 µL of the remaining mixture after step 1, then add 120 µL of *reagent B*. Mix with the pipette and begin the timer. The solution is allowed to react for 00:10:00 to complete the reduction from Fe(III) to Fe(II). At the 10 min mark, add 40 µL *reagent C*, mix with the pipette and *immediately* measure A_2 by loading triplicate 200 µL mixtures on the well plate. 10m

3 Steps 2 is repeated once only for the standards, from which A_3 is measured and α calculated. In the procedure above α should be close to 0.8. The goal of executing this step is to obtain a realistic α together with estimates of its uncertainty during the assay procedure. 10m