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Jul 13, 2020

Dissolved sulfide concentrations (H2S, HS-, S2-) colorimetric assay using a plate reader (96-well plate)

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In Development This protocol is published without a DOI.

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ABSTRACT

This protocol describes the adaption of a sulfide colorimetric assay originally described in Cline 1969, 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 < 400 µL of water samples. This assay measures the concentration of total sulfide species (H_2S, HS^-, S^{2-}) in solution.

Samples for this assay should be filtered (0.2 µm syringe filter) and placed in 1.5 mL Eppendorf tubes. Care should be taken to avoid oxidation of the sample by oxygen in the atmosphere, by completing the first step of the assay quickly right after sampling, which stabilizes the sample against further oxidation.

PROTOCOL CITATION

Jian Gong 2020. Dissolved sulfide concentrations (H2S, HS-, S2-) colorimetric assay using a plate reader (96-well plate). **protocols.io** https://protocols.io/view/dissolved-sulfide-concentrations-h2s-hs-s2-colorim-bd7ji9kn

KEYWORDS

Total dissolved sulfide, colorimetric assay, plate reader

CREATED

Mar 25, 2020

LAST MODIFIED

Jul 13, 2020

PROTOCOL INTEGER ID

34763

GUIDELINES

Working principle

N,N-dimethyl-p-phenylenediamine (diamine) and ferric ion together form a stable colored complex with zinc sulfide at pH 0.35, with maximum absorbance at **λ= 670 µm**. This property is used to design the colorimetric assay described here, useful to measure the concentration of sulfide in a large range of natural waters. This method is free from the interference effect of salts up to 40 ‰ and sulfite up to 100 µM. Thiosulfate is known to inhibit the color-development of this assay; this effect is also time-dependent.

Table 1. Suggested reagent concentrations and dilution factors to be used for the determination of sulfide-sulfur (modified from Cline 1969). Bold text indicates the concentrations applied in this assay.

Sulfide concen	Diamine concen	Ferric concen	Dilution factor	Path length
(µM)	(g/100 ml)	(g/100 ml)	(ml:ml)	(cm)
1-3	0.1	0.15	1:1	10

3-40	**0.4**	**0.6**	1:1	1
40-250	1.6	2.4	2:25	1
250-1000	4.0	6.0	1:50	1

Measurement range

Refer to Table 1 for the recommended preparation of diamine and ferric chloride reagent specific to the range of sulfide concentrations to be measured. Prepare standards and dilutions accordingly. Note that the spectrophotometer at the Bosak Lab uses a plate reader format, and the path length is roughly 4 mm when loading 200 µL wells, rather than the standard 10 mm on other spectrophotometers. Therefore, a dilution is applied to the sample (sample: reagent A = 4:10) to make the measurements consistent (Step 1 below). 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 TEXT

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) **Zinc acetate solution**: **[M]0.05 Molarity (M)** of *zinc acetate* ($Zn(CH_3COO)_2{\cdot}2H_2O$, FW 219.51, 98%, Aldrich #383058) is prepared in nanopure water and used for sample stabilization, dilution, as well as the preparation of sulfide standards.

(B) **Diamine reagent**: Referring to Table 1, the reagent is prepared from **0.4 g** *N,N dimethyl-p-phenyldiamine sulfate salt* ($(CH_3)_2NC_6H_4NH_2{\cdot}H_2SO_4$, FW 234.27, Aldrich #186384) and **0.6 g** of *anhydrous ferric chloride* ($FeCl_3$, FW 162.20, 97%, Aldrich #157740) dissolved in **100 mL** of 6 M (N) *hydrochloric acid* (diluted from stock *hydrochloric acid*: HCl suprapur, 30%, **[M]9.46 Molarity (M)** , Aldrich #1.00318). This reagent is prepared for detecting sulfide at a concentration between 3-40 µM (see Table 1) and is a good starting point if the concentration of sulfide is unknown in a given sample. Store this reagent at 4 °C in the dark.

(C) **Sulfide standard stock**: **[M]0.1 Molarity (M)** of *sodium sulfide nonahydrate* ($Na_2S{\cdot}9H_2O$, Aldrich #431648, 99.99%) is prepared in anaerobic nanopure water that is previously allowed to boil at 100 °C. The sulfide standard solution is kept in a hermetically sealed serum bottle. The headspace of the bottle was flushed with CO_2: N_2 (20:80 vol) atmosphere using standard anaerobic techniques to maintain anoxic conditions. The exact concentration of Na_2S stock solution can be verified by precipitating an exact volume of Na_2S with an excess volume of 0.3 M *silver nitrate* (AgNO3, Aldrich #209139, 99%).

Standards

Fresh standard solutions are prepared around the same time of sampling, by diluting *reagent C* with *reagent A*. This series of standard solutions should be prepared to cover the range of sulfide concentrations expected.

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 STARTING

Samples for this assay should be filtered (0.2 µm syringe filter) and placed in 1.5 mL Eppendorf tubes. Care should be taken to avoid oxidation of the sample by oxygen in the atmosphere, by completing the first step of the assay

quickly right after sampling, which stabilizes the sample against further oxidation.

1 **Sample stabilization**: Add **400 µL** of liquid sample in **1 ml** of *reagent A* upon sampling. This step makes *zinc sulfide* and stabilizes the sample from further oxidation (should be already done during sampling). Here 400 µL corresponds roughly to a detection range at 3 - 40 µM sulfide-sulfur. This amount can be further reduced (for example, adjusted to 200 µL) if higher sulfide concentrations are expected. This step dilutes the sample while also stabilizes the sample.

2 **Colored-complex development**: Transfer **900 µL** of the stabilized mixture to a new tube and **15 µL** of *reagent B* is added. Mix by pipetting up and down a few times. The reaction is timed for **20 minutes**, **in the dark.**

3 **Measurement**: Measure absorbance at **670 nm**. The system at the Bosak Lab is a multi-mode plate reader spectrophotometer (BioTek, Synergy 2, Winooski, VT, USA). Measure by loading quadruplicate **200 µl** mixtures on a 96-well plate.