Rapid quantification and isotopic analysis of dissolved sulfur species

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RATIONALE: Dissolved sulfur species are of significant interest, both as important substrates for microbial activities and as key intermediaries in biogeochemical cycles. Species of intermediate oxidation state such as sulfite, thiosulfate, and thiols are of particular interest but are notoriously difficult to analyze, because of low concentrations and rapid oxidation during storage and analysis.

METHODS: Dissolved sulfur species are reacted with monobromobimane which yields a fluorescent bimane derivative that is stable to oxidation. Separation by Ultra-Performance Liquid Chromatography (UPLC) on a C18 column yields baseline resolution of analytes in under 5 min. Fluorescence detection (380 nm excitation, 480 nm emission) provides highly selective and sensitive quantitation, and Time-of-Flight Mass Spectrometry (TOF-MS) is used to quantify isotopic abundance, providing the ability to detect stable isotope tracers (either 33S or 34S).

RESULTS: Sulfite, thiosulfate, methanethiol, and bisulfide were quantified with on-column detection limits of picomoles (μM concentrations). Other sulfur species with unshared electrons are also amenable to analysis. TOF-MS detection of 34S enrichment was accurate and precise to within 0.6% (relative) when sample and standard had similar isotope ratios, and was able to detect enrichments as small as 0.01 atom%. Accuracy was validated by comparison to isotope-ratio mass spectrometry. Four example applications are provided to demonstrate the utility of this method.

CONCLUSIONS: Derivatization of aqueous sulfur species with bromobimane is easily accomplished in the field, and protects analytes from oxidation during storage. UPLC separation with fluorescence detection provides low-μM detection limits. Using high-resolution TOF-MS, accurate detection of as little as 0.01% 34S label incorporation into multiple species is feasible. This provides a useful new analytical window into microbial sulfur cycling. Copyright © 2017 John Wiley & Sons, Ltd.
Ion chromatography (IC) has been employed to separate multiple anions of sulfur (i.e., \( \text{SO}_4^{2-}, \text{SO}_3^{2-}, \text{HS}^-, \text{H}_2\text{S} \)) [16,17]. However, IC eluent chemistry can alter sulfur speciation; some sulfur species, e.g. polysulfides, are too reactive for accurate quantification with IC; further, IC provides no isotopic information, although it has been combined with multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) to achieve this [18,19]. Derivatization of sulfur species with methyl trifluoromethanesulfonic acid (methyl triflate) is capable of stabilizing polysulfides with quantification down to approximately 25 μM by UV absorption spectrophotometry coupled to high-performance liquid chromatography (HPLC); however, with extraction and evaporation post-extraction, lower limits are reported [20].

Drawbacks of the methyl triflate method include the toxicity of the reagent, multistep extraction procedure, addition of sulfur to the derivatized product, and applicability only to reduced sulfur species. Cyclic voltammetry and in situ probes have also been used to measure and quantify many of the reactive sulfur species in environmental samples at relatively high spatial resolutions (mms) [19,21,22]. The limitations of cyclic voltammetry are that it cannot distinguish between the isotopes of sulfur.

Here, we adapt a previously described method for preserving aqueous nucleophilic sulfur species as bimane derivatives, and update their separation and detection to modern instrumentation. Key features of the derivatization are that (1) reaction with bromobimane (mBBr) is quantitative thus causing no isotopic fractionation; (2) the resulting bimane derivatives are very stable towards subsequent oxidation, providing a convenient means to preserve samples in the field; and (3) bimane is strongly fluorescent, enabling non-destructive fluorescence detection [23-26]. The quantitative mBBr reaction proceeds as a nucleophilic substitution of sulfur compounds containing an unshared pair of electrons (Fig. 1), which includes sulfite (\( \text{SO}_3^{2-} \)), thiosulfate (\( \text{S}_2\text{O}_3^{2-} \)), organic thiols (R-SH), bisulfide (\( \text{HS}^- \)), and polysulfides (\( \text{S}_n^- \)) but not elemental sulfur (\( \text{S}^0 \)) or sulfate (\( \text{SO}_4^{2-} \)). mBBr is a neutral molecule capable of crossing the cellular membrane, enabling reactions with both intra- and extracellular sulfur pools. Bimane derivatives have been previously used to quantify sulfur species from a range of diverse samples including hydrothermal vent fluids, sulfur-oxidizing bacterial cultures (\( \text{SO}_3^{2-}, \text{SO}_4^{2-} \)), HS\(^-\) in human blood, and cysteine and HS\(^-\) in methanogens [27-31].

To improve on the separation of derivatized sulfur species by HPLC, which takes ~60 min per sample [24,25], we employ Ultra-Performance Liquid Chromatography (UPLC) for rapid (<5 min) separation of derivatized sulfur species. For detection and quantitation, we evaluated both a conventional fluorescence detector and a state-of-the-art Time-of-Flight (TOF) mass spectrometer (TOF-MS). Fluorescence detection is less expensive and more sensitive than MS, and nondestructive so it can be coupled with preparative fraction collection of individual sulfur species, e.g. for subsequent high-precision analysis of natural abundance isotope ratios. However, fluorescence itself provides no isotopic information, which is the main impetus for using MS. The high-resolution TOF-MS employed here allows us to distinguish isobaric interferences in the molecular ions, and accurately quantify \( \text{^33S} \) enrichment down to levels of ~0.6%. The same methodological approach could presumably be used for \( \text{^33S} \) enrichments. We note that while TOF-MS was used in this study, quadrupole or other low-resolution MS could be an alternative, with the caveat that isotopic discrimination might suffer.

To refine and test this updated methodology, we focused on four sulfur analytes: \( \text{SO}_4^{2-}, \text{SO}_3^{2-}, \text{CH}_3\text{SH}, \text{HS}^- \). Other sulfur species are also potentially accessible with this approach. These analytes were measured in standard solutions (both a dilution series of decreasing concentration, and a series of varying isotopic composition), then characterized in four separate proof-of-principle applications: (1) measurement of sulfite and thiosulfate concentrations in marine sediment pore-waters, where they are present at low μM concentrations; (2) measuring \( \text{^34S} \) appearance in bisulfide in a pure culture of sulfate-reducing bacteria amended with \( \text{^34SO}_4^{2-} \) tracer; (3) measuring \( \text{^34S} \) in bisulfide in methane seep sediments incubated with \( \text{^34SO}_4^{2-} \) tracer; and (4) identifying previously unreported intermediate sulfur species in a culture of sulfur-oxidizing bacteria.

**Figure 1.** The nucleophilic substitution reaction occurs where a nucleophilic sulfur molecule replaces bromide from bromobimane. The reaction is buffered by HEPES at a pH of 8 and EDTA is used as a chelating agent. In the case of bisulfide, two moles of bromobimane are reacted per one mole of bisulfide. However, most other nucleophilic sulfur molecules form a terminal group at the end of a single bimane.
EXPERIMENTAL

Derivatization protocol
Monobromobimane (hereafter just bromobimane or mBBr) powder (≥97%; Sigma Aldrich, St. Louis, MO, USA) was dissolved in acetonitrile (ACN) to produce a working stock solution of 50 mM mBBr. This was diluted prior to use to a concentration of ~2-fold stoichiometric excess relative to sulfur analytes. Some sulfur species have multiple reaction sites, e.g. HS− binds two bimanes, necessitating the excess reagent. On the other hand, too much mBBr creates an HPLC peak that can coelute with bimane thiols and produce large amounts of background fluorescence; thus we avoided adding a large excess. A typical mBBr reaction mixture was prepared by combining the 50 mM working stock solution of mBBr 1:1 with 50 mM HEPES and 5 mM EDTA in MilliQ water (at pH 8). An aliquot of 300 µL from the reaction mixture was added to 100 µL of sample (e.g. a ratio of 3:1 v/v). Samples were reacted with mBBr for 1 h in the dark at room temperature. The reaction was then quenched with 600 µL 65 mM methanesulfonic acid in MilliQ water to 400 µL of sample and bromobimane reaction mixture (e.g. ratio of 3.2 v/v). Samples were stored in amber glass vials at −20°C until analysis. Both the mBBr reaction mixture and methanesulfonic acid were bubbled with N2 gas prior to use to remove O2, and the entire derivatization procedure was carried out in an anaerobic chamber to minimize oxidation of analytes. Similar precautions to minimize oxidation during field sampling and derivatization are strongly recommended.

Liquid chromatography
Separation of the bimane derivatives was conducted on an ACQUITY I-Class UPLC® system (Waters, Milford, MA, USA) equipped with a flow-through needle autosampler. Analytes were separated on an ACQUITY UPLC® BEH C18 column (2.1 mm x 50 mm, 1.7 µm, 130 Å) maintained at 45°C using an injection volume of 1 or 2 µL. The elution profile contained a mobile phase comprised of 0.1% formic acid (LCMS grade), 1% ACN (LCMS grade), and 98.9% MilliQ water (solvent A) and 100% ACN (LCMS grade) (solvent B) (Table 1).

Effluent from the UPLC system was directed sequentially through an ACQUITY fluorescence (FLR) detector and a Xevo G2-S TOF mass spectrometer (both Waters). The FLR detector was operated at an excitation wavelength of 380 nm and an emission wavelength of 480 nm.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Initial</td>
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<td>90.00</td>
</tr>
<tr>
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<td>0.40</td>
<td>90.00</td>
</tr>
<tr>
<td>3</td>
<td>3.25</td>
<td>0.40</td>
<td>75.00</td>
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<td>0.40</td>
<td>20.00</td>
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<tr>
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<td>3.95</td>
<td>0.40</td>
<td>20.00</td>
</tr>
<tr>
<td>6</td>
<td>4.00</td>
<td>0.40</td>
<td>90.00</td>
</tr>
<tr>
<td>7</td>
<td>4.50</td>
<td>0.40</td>
<td>90.00</td>
</tr>
</tbody>
</table>

Table 1. Optimized elution program for separation of bimane derivatives of inorganic sulfur species

Mass spectrometry
The TOF mass spectrometer employed electrospray ionization (ESI) in both negative and positive ion mode, optimized as follows: source temperature 120°C, desolvation temperature 500°C, desolvation gas flow 800 L/h, capillary voltage 2.5 kV, cone voltage 50 V, cone gas flow 40 L/h, and flight tube 9.0 kV. Collision energy was 6.0 eV. Acquisition mass range was 50–800 Da and the calibration mass range was 91.183–1993.753 Da. A lock mass of 556.2771 Da was used to verify mass accuracy of ±3 mDa. The scan time was 0.2 s with an interscan time of 0.014 s from 0.0 to 4.5 min. Data was collected in centroid format. Sulfur species were quantified using the molecular ion.

Quantification of sulfur species
Calibration standards for quantification were created by serial dilution of sodium sulfite, sodium thiosulfate, sodium sulfide and sodium thiomethoxide (all from Sigma-Aldrich) in N2-bubbled MilliQ water. Concentrations ranged from 3.335 nM to 6.667 mM. The integrated mass spectral peak area and integrated fluorescence peak area were recorded for each standard and sample using MassLynx® and QuanLynx® software, which produced linear regressions of integrated peak area vs. concentration with average R² values ≥0.99.

Quantification of stable sulfur isotope enrichment
Several approaches for extracting isotopic enrichment data from LC/MS spectra are possible. Here we adopt the approach of measuring the molecular ion and ~7–10 isotope peaks (depending on level of 34S enrichment) for each analyte species, in both the sample and one or more standards. For each analyte, the average molecular weight of the bimane derivative was calculated as the weighted mean of the molecular ion and isotopic peaks, using peak height as the weighting factor for abundance because peak area is not available for data collected in centroid mode:

\[
\overline{M} = \sum_{n=1}^{10} M_n H_n / \sum H_n
\]

where \( M_n \) and \( H_n \) are the accurate mass and height, respectively, of a given mass spectral peak. \( \overline{M} \) can then be compared between sample and standard to calculate the shift in mean molecular weight of the sample:

\[
\Delta M = \overline{M}_{\text{sample}} - \overline{M}_{\text{std}}
\]

Although this analysis confounds contributions from isotopes of S, C, H, and N, the latter three are entirely derived from atoms in the bimane derivative, which is the same for both sample and standard and so cancel in the calculation of \( \Delta M \). Isotopes of oxygen are not necessarily identical, e.g. in \( \text{SO}_3^2- \) or \( \text{SO}_4^{2-} \), but with a difference of 26 mDa between \( ^{18}\text{O}^{16}\text{O} \) and \( ^{34}\text{S} \), the TOF mass spectrometer employed here easily resolves these two isobars. \( \Delta M \) can thus be interpreted directly as the change in average mass due to sulfur isotope substitution, and is linearly proportional to the fractional incorporation of isotope label. The slope of the relationship (between \( \Delta M \) and atom% label) is ~1 if \( ^{33}\text{S} \) is the label and
-2 if $^{34}\text{S}$ is the label. For most applications then, a straightforward approach is simply to generate a linear calibration curve for $\Delta M$ using multiple standards of known isotopic abundance.

To be able to compare data collected by LC/MS to isotope ratios measured by isotope-ratio mass spectrometry (IRMS), we first converted $\Delta M$ into the absolute (fractional) abundance of $^{34}\text{S}$ using:

$$34F = \left( \frac{\Delta M + \overline{M}_{\text{VCDT}}}{1.98759} \right) - 16.08997$$

where $\overline{M}_{\text{VCDT}}$ is the mean atomic weight of sulfur in the VCDT (Vienna Canyon Diablo Troilite) international reference material, and the constants 1.98759 and 16.08997 derive from the masses and abundances of sulfur isotopes in that reference material. The isotope ratio was then calculated from the following equation:

$$\frac{34^{R}\text{def}}{32^{S}} = \frac{34F}{1 - 34F}$$

and an enrichment factor (EnrF, i.e., fractionation factor) was calculated as:

$$\alpha_{A/B} = \frac{34^{R}_{A}}{34^{R}_{B}}$$

analogous to the calculation typically employed by natural-abundance IRMS measurements.

Sequentially diluted sulfur standards with concentrations and isotopic compositions that bracketed those of the samples were used to quantify $S$ isotope enrichment. To determine the effect that non-bracketed standards have on the determined EnrF of a sample, we performed the isotopic analysis described above with standards of bisulfide that were more than three orders of magnitude less concentrated than sample concentrations. This exercise produced an additional 6.2% decrease in the recorded EnrF of the sample; however, as standard concentrations approach and overlap sample concentrations, this additional error is greatly decreased. Peak heights and exact masses were recorded either with MassLynx® or using a partially automated analysis with MAVEN (Metabolomics Analysis and Visualization Engine).[32,33] As a test of precision, a zero-enrichment experiment (i.e. comparison of two aliquots of the same sample) was repeated 10 times to yield an enrichment factor of $4.27\%^{34}\text{S}$ sul. This exercise produced an additional 6.2% decrease in the recorded EnrF of the sample; however, as standard concentrations approach and overlap sample concentrations, this additional error is greatly decreased. Peak heights and exact masses were recorded either with MassLynx® or using a partially automated analysis with MAVEN (Metabolomics Analysis and Visualization Engine).[32,33] As a test of precision, a zero-enrichment experiment (i.e. comparison of two aliquots of the same sample) was repeated 10 times to yield an enrichment factor of $4.27\%^{34}\text{S}$ sul.

**Isotope-ratio mass spectrometry (IRMS)**

Sulfur isotope ratios and weight percentage for precipitated ZnS weighed into tin capsules (5 × 7 mm) were determined on a Delta Plus XL Plus isotope-ratio mass spectrometer (Thermo Quest) attached to a NC2500 elemental analyzer (EA; CE Instruments). All sulfur isotopic compositions are reported as per mil variations relative to VCDT following the conventional delta notation:

$$\delta^{34}\text{S} = \left( \frac{^{34}\text{S}}{^{32}\text{S}} \right)_{\text{Sample}} - \left( \frac{^{34}\text{S}}{^{32}\text{S}} \right)_{\text{VCDT}} \times 1000$$

Values of $\delta^{34}\text{S}$ were calibrated relative to an internal standard of Ag$_2$S$_2$ (18.03‰); precision and accuracy for these measurements are estimated as $<0.2%$.

To achieve cross-calibration of the two methods, two aliquots from seven sulfide samples with increasing atomic percentages of $^{34}\text{S}$, ranging from 4.27 to 17.4%, were measured by isotope-ratio mass spectrometry (IRMS), in an anaerobic chamber, 9.8 mL of a 23 mM 4.27% $^{34}\text{S}$ sul sulfide solution was used. In a 185 mM 100% $^{34}\text{S}$ sul sulfide solution, this solution was treated with aliquots of the 4.27% $^{34}\text{S}$ sul sulfide solution to produce solutions of intermediate $^{34}\text{S}$ abundance. Parallel aliquots of these standards were derivatized with mBBr and measured by UPLC/TOF-MS, and precipitated as zinc sul ond for measurement by EA/IRMS.

**Experiments with sulfate-reducing and sulfur-oxidizing bacterial cultures**

Stocks of *Desulfovoccus multivorans* DSM 2059 and *Sulfurovum lithotrophicum* DSM 23290[34] were obtained from the German Collection of Microorganisms (DSMZ) and initially propagated in the lab using the recommended media. Triplicate anaerobic cultures of *D. multivorans* were grown in 30 mL butyl rubber stoppered serum bottles at 28°C to exponential phase in a modified MJ medium[34] (g/L): NaCl 30.0, K$_2$HPO$_4$ 0.434, CaCl$_2$•2 H$_2$O 0.28, MgSO$_4$•7 H$_2$O 3.40, MgCl$_2$•6 H$_2$O 4.18, KCl 0.33, NH$_4$Cl 0.35, NiCl$_2$•6 H$_2$O 0.50 mg, Na$_2$SeO$_3$•5 H$_2$O 0.50 mg, FeCl$_3$ 0.01, NaHCO$_3$ 1.5, Na$_2$S$_2$O$_3$•5 H$_2$O 1.5, NaNO$_2$ 2.8, with the addition of 10 mL DSM 141 Trace Element solution and 10 mL of DSM 141 vitamin solution. The medium was supplemented with pyruvate to a final concentration of 10 mM, and sulfate solution with a $\delta^{34}\text{S}_{\text{VCDT}}$ value of $+2000\%$ was added to a final concentration of 0.28 mM. The medium was kept anaerobic by saturation with 80:20 N$_2$:CO$_2$ gas, followed by adjustment of the pH to 7.0. During exponential phase (47.5 h after inoculation), 100 μL from each culture was collected into a 1 mL syringe and transferred after filtration through a 0.2 μm acrodisc filter into 300 μL of the mBBr reaction mixture.

Continuous culturing of *S. lithotrophicum* was carried out in a BioFlo115 fermentor/bioreactor (New Brunswick Scientific). Oxidant availability, in this case the concentration of nitrate (NO$_3^-$), was the growth-limiting factor. A working volume of 1.75 L was maintained with an agitation rate of 150 rpm. A constant pressure of 0.5 bar from a 80:20 N$_2$:CO$_2$ gas mixture. Temperature was maintained at 28°C. In the exponential phase in a modified MJ medium[34] (g/L): NaCl 30.0, K$_2$HPO$_4$ 0.434, CaCl$_2$•2 H$_2$O 0.28, MgSO$_4$•7 H$_2$O 3.40, MgCl$_2$•6 H$_2$O 4.18, KCl 0.33, NH$_4$Cl 0.35, NiCl$_2$•6 H$_2$O 0.50 mg, Na$_2$SeO$_3$•5 H$_2$O 0.50 mg, FeCl$_3$ 0.01, NaHCO$_3$ 1.5, Na$_2$S$_2$O$_3$•5 H$_2$O 1.5, NaNO$_2$ 2.8, with the addition of 10 mL DSM 141 Trace Element solution and 10 mL of DSM 141 vitamin solution. The medium was supplemented with pyruvate to a final concentration of 10 mM, and sulfate solution with a $\delta^{34}\text{S}_{\text{VCDT}}$ value of $+2000\%$ was added to a final concentration of 0.28 mM. The medium was kept anaerobic by saturation with 80:20 N$_2$:CO$_2$ gas, followed by adjustment of the pH to 7.0. During exponential phase (47.5 h after inoculation), 100 μL from each culture was collected into a 1 mL syringe and transferred after filtration through a 0.2 μm acrodisc filter into 300 μL of the mBBr reaction mixture.
reached steady state, 25 mL aliquots were removed from the vessel; from these a 100 μL aliquot was collected into a 1 mL syringe and filtered through a 0.2 μm acrodisc filter into 300 μL of the bromobimane reaction mixture.

Sediment incubations
Sediment samples were collected on May 9, 2013, from the Santa Monica Basin, offshore California, as part of an expedition led by the Monterey Bay Aquarium Research Institute on the R/V Western Flyer. Using the ROV Doc Ricketts (Dive DR463), sediment push cores PC 61 and PC 55 were collected in an extensive white microbial mat overlying an active methane seep at a water depth of 860 m and in situ temperature of 4°C (lat. 35.473431 N, long. 118.4007 W). Upon collection shipboard, PC 55 was processed shipboard in 1 cm intervals according to Orphan et al.[38] Pore-water aliquots from each depth horizon were collected using an argonpressurized Reeburgh-type squeezer, preserved for geochemical analyses, and stored at -20°C. PC 61 was sectioned into two 6 cm intervals and heat sealed under anoxic conditions in a mylar bag flushed with argon and stored at 4°C until processing for microcosm incubation experiments (established 40 days after collection).

Stable isotope probing (SIP) experiments were conducted at 10°C in 5 mL butyl stoppered serum bottles with 1 mL of sediment slurry (0–6 cm PC 61) mixed with anoxic artificial seawater containing 5 mM unlabeled sulfide and 28 mM 34SO4 for 40 days. Residual unlabeled SO4 was in the sediment slurry diluted the tracer to a level of ~80% 34S, based on initial sampling. The incubation was allowed to settle before time points of the supernatant were collected. The supernatant was centrifuged at 20,000 g for 5 min. A 50 μL aliquot of the overlying liquid was added to the mBBr reaction mixture and allowed to react as described above. Before analysis this solution was thawed and diluted 1:10 in 20:80 ACN:65 mM methanesulfonic acid.

RESULTS AND DISCUSSION
Quantitation of dissolved sulfur analytes
The use of bimane derivatives for quantitation of dissolved sulfur species has many benefits. Experimental testing of this assay showed reactivity with a broad range of aqueous sulfur species, and provided rapid, quantitative measurement with the addition of a ~2-fold molar excess of the bromobimane (mBBr) reagent, and resulted in no isotopic fractionation. Bromobimane is reactive towards most sulfur species with unpaired electrons, and so has the ability to target a wide variety of trace, labile species in addition to those studied here (Table 2). Sulfate and elemental sulfur are notable exceptions that do not react with mBBr. The reagent, while moderately expensive, is safe and easily deployed in the field to preserve samples immediately after collection.[24,25,27] Although we did not quantitatively study the stability of bimane derivatives, qualitative evidence suggests they are highly stable when stored at ~20°C. Mixtures of derivatized standards stored in this way were measured with no loss in signal for at least 3 months (longer times were not tested).

Marine pore-water samples, derivatized from each depth horizon were collected using an argon-pressurized Reeburgh-type squeezer, preserved for geochemical analyses, and stored at -20°C. Mixtures of derivatized standards stored in this way were measured with no loss in signal for at least 3 months (longer times were not tested).

Quantification of dissolved sulfur analytes

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular formula</th>
<th>Reaction with mBBr</th>
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<tbody>
<tr>
<td>Bisulfide</td>
<td>HS−</td>
<td>+</td>
</tr>
<tr>
<td>Sulfite</td>
<td>SO32−</td>
<td>+</td>
</tr>
<tr>
<td>Thiosulfate</td>
<td>S2O32−</td>
<td>+</td>
</tr>
<tr>
<td>Polysulfides</td>
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<tr>
<td>Cysteine</td>
<td>C2H2NO2S</td>
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<tr>
<td>Glutathione</td>
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</tr>
<tr>
<td>Coenzyme A</td>
<td>C21H39N10O14P3S</td>
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</tr>
<tr>
<td>Coenzyme M</td>
<td>C4H8O3S2−</td>
<td>+</td>
</tr>
<tr>
<td>Organic Thiols</td>
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</tr>
<tr>
<td>Sulfate</td>
<td>SO42−</td>
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</tr>
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</table>

+ indicates positive reaction, 0 indicates reaction will not occur
R indicates alkyl or organic substituent

Data sourced from Fahey and Newton[27] and Zopfi et al.[29]

Table 2. List of representative sulfur compounds and their predicted reaction with bromobimane

Collection and analyzed 29 months later, yielded HS− concentrations that were identical within error to those obtained shipboard by the Cline assay, and SO32− and S2O32− concentrations that were similar to previous reports (data not shown). Underivatized sulfide and sulfate do not last nearly this long in the presence of O2, even when stored at ~20°C.

The optimized UPLC elution gradient described above and in Table 1 was able to fully resolve the bimane derivatives of SO32−, S2O32−, CH2SH, and HS− within 4.5 min (Fig. 2(A)), making this a very rapid separation method. However, the composition of the eluent was found to influence the separation and required modification depending on the species of interest. For example, LC peaks representing SO32− bimane and S2O32− bimane were observed to co-elute under conditions containing >90% of the polar eluent (0.1% formic acid, 1% ACN, and 98.9% MilliQ water). Similarly, >70% organic eluent (100% ACN) was required to separate CH2S2− bimane from S2− dibimane by 3.75 min. Additionally, as the peak of unreacted mBBr elutes close to sulfide dibimane, excessively large reagent concentrations can lead to a broad peak area that can influence the sulfide measurement. Limiting the mBBr reagent to a 2-fold excess largely avoided this problem, while still enabling quantitative preservation of sulfur intermediates.

Performance characteristics for quantitation of analytes by either FLR or TOF-MS are given in Table 3. Comparison of the two methods revealed a higher degree of sensitivity using fluorescence detection over TOF-MS. The TOF-MS method was found to be sensitive down to the low micromolar range (sample concentration before dilution in the reaction mixture); based on an injection volume of 1 μL, equivalent to <1 pmol injected on-column. Both the FLR and TOF-MS detectors became saturated at ≥3 mmol sulfur on-column, yielding a dynamic range of ~9 orders of magnitude. Both detectors
Figure 2. Chromatography of sulfur species using bromobimane preservation and UPLC/TOF-MS analysis showing a separation of a mixture of sulfur standards consisting of SO$_3$$^-$$^-$, S$_2$O$_3$$^-$$^-$, CH$_3$SH, and HS$^-$$^-$ each at 250 $\mu$M. (A) Extracted ion chromatogram of 1: sulfite bimane 0.55 min 271.0389 Da, 2: thiosulfate bimane 0.96 min 303.0609 Da, 3: methanethiol bimane 2.97 min 239.0854 Da, and 4: sulfide dibimane 3.25 min 415.1440 Da. (B–E) Mass spectra with the chemical structure, chemical formula, and isotopic distribution for the negative ion of sulfite bimane (B), negative ion of thiosulfate bimane (C), positive ion of methanethiol bimane (D), and positive ion of sulfide dibimane (E). [Color figure can be viewed at wileyonlinelibrary.com]

Table 3. Performance characteristics for FLR and TOF-MS detection of bimane derivatives

<table>
<thead>
<tr>
<th></th>
<th>Sulfite bimane</th>
<th>Thiosulfate bimane</th>
<th>Sulfide dibimane</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLR Sensitivity a (S/N)</td>
<td>916:1</td>
<td>1333:1</td>
<td>1833:1</td>
</tr>
<tr>
<td>TOF-MS Sensitivity a (S/N)</td>
<td>165:1</td>
<td>369:1</td>
<td>371:1</td>
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<tr>
<td>FLR Precision (relative %)</td>
<td>2.35</td>
<td>6.10</td>
<td>3.95</td>
</tr>
<tr>
<td>TOF-MS Precision (relative %)</td>
<td>4.71</td>
<td>3.81</td>
<td>4.00</td>
</tr>
<tr>
<td>FLR Accuracy a (± $\mu$M)</td>
<td>0.63</td>
<td>0.86</td>
<td>2.32</td>
</tr>
<tr>
<td>TOF-MS Accuracy a (± $\mu$M)</td>
<td>3.31</td>
<td>3.64</td>
<td>4.04</td>
</tr>
</tbody>
</table>

aQuantified at 33.35 $\mu$M.
yielded linear calibration curves over this range. Precision and accuracy were similar for the two detectors, typically a few percent relative to sample concentration. In summary, both detectors were shown to be capable of quantitation of dissolved sulfur species. Quantitation of sulfur species by TOF-MS is more expensive and complex than the FLR detection but has the advantage of enabling positive identification of unknown sulfur species, while the nondestructive FLR detector is ideally suited for preparative fraction collection.

Measurement of sulfur isotopic enrichment

Several different strategies were tested for quantifying isotopic abundance based on the relative intensity of isotopic peaks of the molecular ion (Figs. 2(B)–2(E)). These included calculation of M + 2/M ion abundance ratios, Σ(M + 2/M + ...M + 7/M) ion abundance ratios, and fitting the complete envelope of isotopic peaks with a forward model. In the end, the calculation of a weighted-mean mass shift (as described in the Experimental section) was both the most complete envelope of isotopic peaks with a forward model. Presumably, the deviations of measured data from correct isotope ratios therefore reflect instrumental fractionations that do not scale uniformly with isotopic composition. Such effects are well known in measurements of natural-abundance isotopes where they are often referred to as "scale compression/expansion". For this reason, two-point calibration of isotopic compositions is almost universally recommended.[36–38] However, in our case no 34S-enriched reference materials exist, so we are unable to explicitly test accuracy across the range of isotopic compositions to a level that approaches measurement precision. Nevertheless, the linearity and reproducibility of the responses of both instruments in Fig. 3 strongly suggest that – with calibration

Further validation of method accuracy is complicated by the fact that the standard analytical technique, IRMS, is not designed to measure the relatively large isotopic enrichments being targeted by our LC/MS method. Certified international reference materials for calibration span a range of only a few tens of permil,[35] whereas an artificial enrichment in 34S of only 1% (absolute abundance) constitutes a shift in δ34S of nearly 250‰. These issues notwithstanding, we measured the same series of sulfide isotopic standards (see Experimental section) using both UPLC/TOF-MS and EA/IRMS (Fig. 3).

Seven samples ranging from 4.27 to 17.4 atom percent 34S were measured. Data from both instruments were highly reproducible and linear over the entire range of isotopic compositions. However, both yielded apparent 34S abundances significantly lower than those calculated for the standards based on mixing volumes. The measured values averaged 98.8% and 90.4% of the theoretical values for the TOF-MS and IRMS data, respectively (Fig. 3(A)). Comparison of the two datasets with each other is also highly linear (Fig. 3(B)).

Both datasets (i.e., from TOF-MS and IRMS) were collected by comparing unknown, 34S-enriched samples with a single isotopic standard with a natural abundance of 34S. Presumably, the deviations of measured data from correct isotope ratios therefore reflect instrumental fractionations that do not scale uniformly with isotopic composition. Such effects are well known in measurements of natural-abundance isotopes where they are often referred to as "scale compression/expansion". For this reason, two-point calibration of isotopic compositions is almost universally recommended.[36–38] However, in our case no 34S-enriched reference materials exist, so we are unable to explicitly test accuracy across the range of isotopic compositions to a level that approaches measurement precision. Nevertheless, the linearity and reproducibility of the responses of both instruments in Fig. 3 strongly suggest that – with calibration

![Figure 3](Image)

Figure 3. Cross-calibration of the same samples of HS- with increasing atomic percentage of 34S derivatized with bromobimane and precipitated with zinc acetate run using UPLC/TOF-MS and EA/IRMS, respectively: (A) Atomic percentage of 34S in HS- plotted against enrichment factor (red circles) (EnrF = –0.1077 + 0.2613*%34S, R² = 0.999), as determined by the UPLC/TOF-MS methodology, and δ34SVCDT (δ34S = –1027.3 + 244.0*%34S, R² = 0.999), as measured by EA/IRMS (blue squares). (B) A systematic and reproducible linear cross-calibration (gold circles) was established linking our enrichment factors (EnrF) with conventional IRMS δ34SVCDT measurements of sulfur (EnrF = 0.9935 + 0.001071*δ34S, R² = 0.999). The solid black line represents the theoretical relationship between atom percentage of 34S, enrichment factors, and δ34SVCDT. [Color figure can be viewed at wileyonlinelibrary.com]
standards having suitable $^{34}$S-enriched compositions – accuracy for isotopic abundance should approach measurement precision, i.e., 0.6% relative.

Specific reasons for the deviation of TOF-MS data at high $^{34}$S enrichments are not currently known. Fractionations during ionization, transmission, and detection are all possible. Ion shading, in which the earlier-arriving $^{34}$S ions induce a slight electric field at the detector that repels the later-arriving $^{34}$S ions, is a possibility specific to the TOF that would clearly scale nonlinearly with isotope ratio.

**Application 1: Quantitation of pore-water SO$_3^{2-}$ and S$_2$O$_3^{2-}$ in methane seep sediments**

As an initial test of the method’s ability to quantify trace sulfur species in complex natural samples, UPLC separation with FLR and TOF-MS detection were used to compare environmental pore-water concentrations of SO$_3^{2-}$ and S$_2$O$_3^{2-}$ in a depth profile of sediments from the Santa Monica Basin. The sediments were collected within an active methane seep, where microbial sulfur metabolism associated with anaerobic oxidation of methane and chemosynthetic sulfide oxidation is enhanced. Both the FLR and TOF detectors were used to quantitate SO$_3^{2-}$ and S$_2$O$_3^{2-}$; we present the FLR data for SO$_3^{2-}$ and the TOF S$_2$O$_3^{2-}$ concentrations highlighting the abilities of both instruments for accurate quantitation (Fig. 4(A)). The FLR and TOF detectors produced S$_2$O$_3^{2-}$ and SO$_3^{2-}$ values that were inconsistent with previous reports. SO$_3^{2-}$ and S$_2$O$_3^{2-}$ reached maximum concentrations of 12.5 and 42.5 μM at 1.5 and 3.5 cm depth, respectively. SO$_3^{2-}$ concentrations were below detection limits by 4.5 cm depth. S$_2$O$_3^{2-}$ concentrations varied across much of the 18.5 cm core. Conventional analysis of HS$^-$ by the Cline assay and SO$_3^{2-}$ by ion chromatography show typical profiles of HS$^-$ building with depth to a maximum of 12.5 mM by 10.5 cm and SO$_3^{2-}$ decreasing from 25 mM at the surface down to 2 mM at depth (Fig. 4(B)). We did not observe an increase in SO$_3^{2-}$ or S$_2$O$_3^{2-}$ concentrations at the HS$^-$ and SO$_4^{2-}$ transition as might be expected. The concentrations of SO$_3^{2-}$ and S$_2$O$_3^{2-}$ were reproducible and fall within the range of previously reported environmental marine sediments (see Zopfi et al.[39] for review).

**Application 2: Tracing $^{34}$S-labeled sulfate in a sulfate-reducing bacterial culture**

The second experimental demonstration of this method was to track the conversion of $^{34}$S-labeled sulfate into $^{34}$S-sulfide, using batch cultures of the sulfate-reducing bacterium (SRB) *Desulfococcus multivorans*. Cultures were amended with 280 μM labeled sulfate ($^{34}$FSO$_4$ = 12.4%) and unlabeled thiosulfate (6.44 ± 0.15 mM; $^{34}$FS$_2$O$_3$ = 4.2%). Here we demonstrate the utility of the UPLC/TOF-MS analytical methodology for tracking the production of isotopically enriched sulfur species (Fig. 5(A)), and through the addition of $^{34}$S-enriched sulfate, we examine the potential for sulfur disproportionation and preferred sulfur substrate usage by *D. multivorans*. Previous reports suggested the potential for thiosulfate reduction in addition to the canonical sulfate reduction by *D. multivorans*, but the specific preference of sulfur substrate by this organism has not been directly tested.[40]

As the *D. multivorans* cultures reached the exponential phase of growth, HS$^-$ accumulated to concentrations of 1.66 ± 0.49 mM. Abiotic control bottles for this experiment had no quantifiable HS$^-$ or SO$_3^{2-}$ and an average thiosulfate concentration of 6.44 ± 0.15 mM, indistinguishable from the amount added. The triplicate experimental bottles had final thiosulfate concentrations of 5.42 ± 1.35 mM. Significantly more HS$^-$ was produced than the 280 μM SO$_4^{2-}$ provided, which implies a contribution of both SO$_3^{2-}$ and S$_2$O$_3^{2-}$ reduction to the HS$^-$ produced. The mass balance of sulfur was conserved, within error; however, we cannot rule out the possibility of a minor amount of non-enriched HS$^-$. 

![Figure 4](https://wileyonlinelibrary.com/journal/rcm) Pore-water profile from methane seep sediments underlying sulfide-oxidizing microbial mat (push core PC55) recovered from the Santa Monica Basin. PC55 was sectioned at 1 cm intervals for the upper 6 cm, then at 3 cm intervals down to 18.5 cm: (A) Concentrations, with depth, of bromobimane-preserved intermediate sulfur species (sulfite and thiosulfate) measured by FLR and TOF detectors, respectively. (B) Colorimetric analysis (Cline assay) of sulfide concentrations and IC measurements of sulfate and dissolved inorganic carbon (DIC) measured from aliquots of the same pore-water samples. [Color figure can be viewed at wileyonlinelibrary.com]
transferred with the initial inoculum. Assuming that all of the \( ^{34}\text{SO}_4^{2-} \) was converted into \( \text{HS}^- \), more than 1.3 mM of \( \text{HS}^- \) had to come from unlabeled \( \text{S}_2\text{O}_3^{2-} \). There were also μM levels of \( \text{SO}_3^{2-} \) detected in the experimental bottles, which might have been produced via a number of different pathways. Notably, there was no detectable enrichment of \( ^{34}\text{Si} \) in \( \text{S}_2\text{O}_3^{2-} \), indicating it did not derive from the reduction of \( ^{34}\text{SO}_4^{2-} \) substrate. At exponential phase, \( ^{34}\text{FHS} \) was 5.4 ± 0.4% and \( ^{34}\text{F}_{\text{SO}_3} \) was 4.5 ± 0.5%. Approximately one-third of the \( ^{34}\text{S}-\)enriched \( \text{HS}^- \) was created by \textit{D. multivorans} reducing labeled sulfate, but the mechanism resulting in enrichment of the \( \text{S}_2\text{O}_3^{2-} \) pool remains unclear. The minor incorporation of the \( ^{34}\text{S} \) label into thiosulfate could suggest that \( \text{SO}_4^{2-} \) is being incompletely reduced to \( \text{S}_2\text{O}_3^{2-} \) during sulfate reduction, or possibly that thiosulfate is being slowly formed from \( \text{HS}^- \).

**Application 3: Tracing \(^{34}\text{S}\)-labeled sulfate in methane seep sediment incubations**

To test the utility of the sulfur SIP method in environmental samples, we conducted 40-day incubations with labeled sulfate (28 mM, \( \text{FSO}_4 = 1 \), i.e., 100% \(^{34}\text{S} \) label) added to methane seep sediments. UPLC chromatograms of the mBBd-derivatized supernatant contained many more peaks relative to the experiments with pure cultures of \textit{D. multivorans},

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Figure 5. (A) Representative negative ionization chromatogram from triplicate cultures of \textit{Desulfooccus multivorans}, a sulfate-reducing bacterium, incubated with \(^{34}\text{S}\)-labeled sulfate. Peaks shown in the chromatogram are labeled with the compound, elution time, and most abundant ion, and include 1: HEPES 0.31 min 237.095 Da; 2: sulfite bimane 0.52 min 271.042 Da; 3: thiosulfate bimane 0.86 min 303.015 Da; 6: bromine cluster 2.89 min 375.607 Da, 7: sulfide dibimane 3.11 min 413.130 Da. (B) Positive ionization chromatogram from a methane seep sediment incubation with \(^{34}\text{SO}_4^{2-} \); as this is a complex natural sample, more compounds were detected relative to pure cultures or standards. Peaks are 1: HEPES 0.31 min 239.111 Da, 3: thiosulfate bimane 0.89 min 305.030 Da, 4: bimane 1.51 min 193.102 Da, 5: \(^{34}\text{S} \) sulfide bimane 2.67 min 227.063 Da, 6: bromobimane 2.89 min 271.012 Da, 7: \(^{34}\text{S} \) sulfide dibimane 3.11 min 415.146 Da, 8: disulfide dibimane 3.46 min 469.098 Da. (C) Mass spectrum of the positive ion of sulfide dibimane from methane seep sediment incubations (peak 7 in (B)). The peak heights for \(^{32}\text{S} \) (415.1455 Da) and \(^{34}\text{S} \) (417.1416 Da) were approximately equal due to the extensive conversion of \(^{34}\text{SO}_4^{2-} \) into sulfide (\(^{34}\text{SH}^- \)) presumably associated with sulfate-coupled anaerobic oxidation of methane. [Color figure can be viewed at wileyonlinelibrary.com]
including several peaks that were clearly not \( \text{SO}_3^{2-} \), \( \text{S}_2\text{O}_3^{2-} \), \( \text{CH}_3\text{S}^- \), or \( \text{HS}^- \). Each peak in the environmental samples was subsequently identified through comparisons with standards or de novo determination from the mass spectral data (Fig. 5(B)). Two separate bimane derivatives of \( \text{HS}^- \) were observed: sulfide monobimane and sulfide dibimane. We infer that the amount of \( \text{mBBr} \) added to these samples (at the time of collection) was insufficient to fully react with bisulfide due to other reactive sulfur species. For natural samples precise concentrations of total reactive sulfur species are likely to be unknown; however, if an estimation can be provided, then a two-fold stoichiometric excess of \( \text{mBBr} \) can still be supplied. This caused considerable problems for quantitation because generation of a sulfide monobimane calibration curve is not straightforward. Nevertheless, based on sulfide dibimane we observed the build-up of 4.35 mM sulfide with \( \text{FSO}_4 = 0.498 \) (i.e., 50% \(^{34}\text{S} \) label) (Fig. 5(C)). Sulfate reduction was clearly prevalent in these sediments, likely coupled to the anaerobic oxidation of methane (AOM). In addition to large quantities of \( \text{HS}^- \), the sediment incubations also contained 1.65 mM \( \text{S}_2\text{O}_3^{2-} \) with no detectable \(^{34}\text{S} \) enrichment (\( \text{FS}_2\text{O}_3 = 0.042 \)). Disulfide dibimane, corresponding to the polysulfide \( \text{HS}_2^- \), was also detected in the incubations. The zero valent (\( \text{HS}_2^- \)) has been hypothesized as the chemical intermediate in the AOM microbial syntrophy between a sulfate-reducing methanotrophic ANME archaea and a disulfide-disproportionating deltaproteobacteria.\(^{12}\) Milucka et al.\(^{12}\) used the methyl triflate technique to measure polysulfides from 3-x \( \text{S} \) chain length; however, this method was incapable of directly measuring the primary proposed intermediate, \( \text{HS}_2^- \). Here we show that the TOF-MS method for measuring bromobimane derivatives of sulfur is capable of \( \text{HS}_2^- \) detection; however, to develop this into a quantitative assay, further work and reliable standards for \( \text{HS}_2^- \) are needed as we are currently unable to reliably create and derivatize individual chain-length polysulfides with bimane.

![Figure 6](https://wileyonlinelibrary.com/journal/rcm)

**Figure 6.** (A) Negative ionization chromatogram from slow growth and oxidant limited continuous culture of *Sulfurovum lithotrophicum*, a sulfur-oxidizing epsilonproteobacterium. The three resolvable peaks are 1: thiosulfate bimane 1.38 min 303.0114 Da, 2: disulfanemonosulfonate (\( \text{S-S-SO}_3^- \)) bimane 2.06 min 334.9837 Da, 3: trisulfanemonosulfonate (\( \text{S-S-SO}_3^- \)) bimane 2.94 min 366.9554 Da. (B) Isotope model of the negative ion of trisulfanemonosulfonate (\( \text{S-S-SO}_3^- \)) bimane with theoretical mass and isotopic distribution. (C) Mass spectrum of the negative ion of trisulfanemonosulfonate (\( \text{S-S-SO}_3^- \)) bimane from the oxidant-limited chemostat experiment (peak 3 in (A)). [Color figure can be viewed at wileyonlinelibrary.com]
Application 4: Identification of novel sulfur species in a continuous culture of the sulfur-oxidizing bacterium *Sulfurovum lithotrophicum*

Coupling bimane derivatization to mass spectral analysis should enable the identification of potentially novel and important intermediate redox species of sulfur. In this application, we studied the sulfur-oxidizing bacterium *Sulfurovum lithotrophicum* in oxidant-limited continuous culture, with thiosulfate as the supplied sulfur source. We hypothesized that partial oxidation of thiosulfate could result in the accumulation of unusual and potentially important sulfur species of intermediate redox state. Chromatograms of mBBr-derivatized samples from these cultures revealed two unidentified peaks (Fig. 6(A)) in addition to thiosulfate. Using *de novo* analysis of the mass spectral data, these two unknown bimane-derivatized sulfur compounds were tentatively identified as disulfanemonosulfonate (‘S-S-SO3H) bimane (mass 334.9837 Da) and trisulfanemonosulfonate (‘S-S-S-SO3H) bimane (mass 366.9553 Da) (Figs. 6(A), 6(B) and 6(C)). These compounds appeared to be produced during S2O32− oxidation by *S. lithotrophicum* and were not observed in the standard solutions or in any of the other samples we analyzed. Quantitation and identification, via liquid chromatography and FLR detection, of these compounds was not possible as authentic standards are not available to construct calibration curves and retention times.

The chemistry of sulfanemonosulfonic acids has been previously described and the formation of these acids is proposed to be a result of the nucleophilic degradation of sulfur chains.[41] The proposed reaction mechanism involves either SO3− or S2O32− interacting with elemental sulfur or polysulfide chains. Through microscopic examination of *S. lithotrophicum* cells, structures consistent with intracellular sulfur globules were visible (data not shown).[42,43] Sulfanemonosulfonic acids were previously detected in the cell pellet of *Allochromatium vinosum*, a purple photosynthetic sulfur oxidizer, but these sulfur compounds were not detected in the supernatant.[28] Due to the analytical limitations of XANES spectroscopy, that study was unable to determine the chain length of these molecules. The experiments with *S. lithotrophicum* here demonstrate the added advantage of using TOF-MS, with its high mass resolving capabilities, for untargeted identification and resolution of unknown sulfur compounds.

The detection of sulfanemonosulfonic acids in two distantly related sulfur-oxidizing bacteria points to the potential importance of these compounds. One possibility is that intracellular sulfanemonosulfonic acids may represent a pathway by which microorganisms first begin to utilize their stored reserves of sulfur globules (elemental sulfur/polysulfides). Indeed, previous experiments with *A. vinosum* revealed a correlation between intracellular sulfur and the relative concentration/presence of sulfanemonosulfonic acids.[28] The reaction mechanism originally proposed by Meyer et al.[41] would be consistent with the ability of dissimilatory sulfite reductase (Dsr) subunits to produce SO3− which could then interact with the intracellular elemental sulfur/polysulfides creating potentially important intracellular sulfanesulfonic acids.

**CONCLUSIONS**

We present a method for preservation, separation, and quantitative analysis of dissolved sulfur-containing analytes. It is based on derivatization with monobromobimane (mBBr), which is rapid, quantitative, and easily adapted to field conditions. Qualitative evidence suggests that mBBr-derivatized samples are stable relative to unreacted samples, with minimal oxidation over the course of months to years. Baseline separation of four key analytes, sulfite, thiosulfate, methanethiol, and bisulfide, was achieved by UPLC in just 5 min, making this a high-throughput analytical approach. Detection by either fluorescence or time-of-flight mass spectrometry provided highly sensitive, precise, and accurate quantitation. Using TOF-MS, we demonstrate the ability to quantify 34S isotopic enrichments with ~0.6% relative precision, over a dynamic range from natural abundance (4.2%) to at least 50%. In the proof-of-principle applications described here, we (1) demonstrated thiosulfate utilization during sulfate reduction by pure cultures of the sulfate reducing bacterium *D. multivorans*; (2) detected zero valent disulfide (H2S2) in methane seep sediments, a polysulfide species that has been historically challenging to measure with other methods; and (3) resolved the identity of two unknown sulfur metabolites as disulfanemonosulfonate and trisulfanemonosulfonate produced by *S. lithotrophicum* during thiosulfate oxidation under oxidant limited conditions.

The methodology demonstrated here has numerous potential applications. The ability to quantify reactive, low-abundance species in complex natural samples will be extremely useful in studies of biogeochemical sulfur cycling, especially in environments supporting rapid sulfur cycling such as oxygen minimum zones,[5,6] below the sulfate reduction zone in marine sediments,[7,8] sulfur-metabolizing microbial symbioses,[9,42] and freshwater sediments.[10,11] Moreover, coupling UPLC with nondestructive FLR detection to preparative fraction collection provides an analytical route to high-precision IRMS-based isotopic analysis of these compounds at natural isotopic abundance. The ability to detect a large dynamic range of isotopic enrichments is also extremely useful for stable-isotope probing (SIP)-type experiments, either in environmental sample incubations or in culture. This is likely to have applications in microbial ecology, biochemistry, and even biomedicine.[45]

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Quantification and isotopic analysis of dissolved sulfur species


