

Methylene blue derivatization then LC–MS analysis for measurement of trace levels of sulfide in aquatic samples

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Abstract The methylene blue method has been widely used for analysis of sulfide for more than 100 years. Direct measurement of methylene blue at nanomolar concentrations is impossible without a preconcentration step, however. In this study the response of LC–MS with electrospray ionization (ESI) to methylene blue was evaluated. HPLC with simple isocratic elution was followed by ESI–MS quantification, which was compared with traditional UV–visible detection. The limit of detection for sulfide was approximately 50 ng L^{-1} , or 1.5 nmol L^{-1} . Analysis time was substantially reduced by use of isocratic elution. Interfering compounds produced by side reactions can be eliminated by use of the mass filter. A polysulfide sample was also analyzed to determine which products are formed and whether or not polysulfides react stoichiometrically with methylene blue reagent. It seems that polysulfides do not react quantitatively with methylene blue and so cannot be quantified reliably by use of this method.

Keywords Sulfide · Polysulfides · Water · Methylene blue · LC–MS

Introduction

Cycling of sulfur in the environment affects all aspects of the biosphere from bacteria to plants and has received much

attention because of its profound affect on the cycling of heavy metals. Reduced sulfur in the form of sulfide or organic thiols tends to form strong bonds with type B metal ions, for example lead and mercury [1]; it is also critically important to the fate of iron and several other trace elements. Type B metals have a highly polarizable valence-electron shell and tend to form strong bonds with anions which are also highly polarizable, for example reduced sulfur. Excess sulfide has been shown to increase the solubility of cinnabar, traditionally regarded as a sink for mercury in the biosphere [2, 3]. Polysulfides, formed by direct reaction of sulfide with S^0 [4], have also been shown to increase the solubility of cinnabar [5]. It has, in fact, been suggested that under anoxic conditions the chemistry of mercury is mainly controlled by sulfide [6, 7], although other studies suggest that dissolved organic matter (DOM) may also be important [8, 9]. Sulfide has also been shown to affect the speciation of the highly toxic methylmercury. Craig and Moreton [10] and Hintelmann et al. [11] report significant correlations between methylmercury and sulfide.

Accurate and sensitive determination of sulfide is therefore critical to assessment of heavy metal speciation. Methods for detection and quantification of sulfide are numerous and span almost all aspects of analytical chemistry, including gravimetric methods, iodometric titration, electrochemical methods, and UV–visible and fluorescence techniques [12]. One of the methods most commonly used for sulfide analysis employs the ion-selective electrode (ISE). The sulfide electrode has a detection limit of approximately $0.1 \text{ } \mu\text{mol L}^{-1}$, is selective for sulfide, and has few interferences. It does, however, require addition of sulfide antioxidant buffer (SAOB), a highly caustic solution, to keep the sulfide reduced and in the S^{2-} form [13]. Samples must also be analyzed within 2 h, because the rate of degradation of sulfide has been reported to be $6\% \text{ h}^{-1}$ [14].

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Probably the most popular and most enduring technique for measurement of sulfide is the methylene blue method. This was published by Fischer [15] when it was realized that visual determination of methylene blue was much more sensitive than gravimetric analysis by precipitation with lead, the prevailing method of the time. Although initial results using this technique were somewhat erratic, Cline [16] determined the effects of changes in pH, reagent strength, and salinity, and optimized the conditions to achieve precision of $\pm 2\%$. The method is rapid, highly selective for sulfide [17], and methylene blue is more stable than free sulfide, thus extending the time available between sampling and analysis [18]. The methylene blue method has been used under similar experimental conditions since then.

The concentration of sulfide in the oxygenated surface waters of freshwater lakes is extremely low, at the nanomolar level or lower, because sulfide is rapidly degraded in the presence of oxygen [19, 20]. Any sulfide present in oxic waters occurs as a result of kinetic stabilization, because of binding to metals such as zinc [21, 22]. It may, however, still have an important effect on the cycling of mercury and other heavy metals, because of high complexation constants for mercury and sulfide [23, 24]. Direct colorimetric measurement of methylene blue at the nanomolar level is not possible, and preconcentration is required. Tang and Santchi [18] used an SPE procedure and HPLC separation to achieve a detection limit of approximately 1 nmol L^{-1} . Bowles et al. [24] used a purge-and-trap procedure to concentrate sulfide using a sodium hydroxide trapping solution; the sulfide was subsequently derivatized to form methylene blue. Although both methods have been used to achieve subnanomolar detection limits, extraction procedures tend to be time-consuming and can add significant uncertainty to the analysis.

Liquid chromatography coupled to mass spectrometry with electrospray ionization has much lower detection limits than UV–visible spectroscopy. LC–ESI–MS requires the analyte to be charged, however, or at least somewhat polar to create gaseous ions in the electrospray process. By coincidence, the methylene blue molecule is a good candidate for use with electrospray ionization—it is a charged molecule, irrespective of pH, with a mass of 284 amu, which is sufficiently large to avoid the interference encountered from solvent ionization and from contaminants.

In this experiment the conditions required for analysis of methylene blue by electrospray LC–MS were determined and a simple chromatographic technique was developed for sensitive determination of sulfide in lake water samples. To validate the new method several samples were measured by LC–MS and HPLC with UV–visible detection to compare mass spectrometric detection with the more traditional technique.

It has been suggested [19] that the methylene blue technique will work with polysulfides, in addition to free sulfide, although the exact product of the reaction, and its efficiency, remain unknown. To determine the nature of the product, if any, formed, a mixture of potassium polysulfides was also derivatized and analyzed by LC–MS.

Methods

N,N-*p*-Phenylenediamine sulfate, iron(III) chloride, sodium sulfide nonahydrate, aqueous ammonia solution, ammonium acetate, ammonium formate, and heptafluorobutyric acid were obtained from Sigma–Aldrich. HCl and acetonitrile (optima grade) were purchased from Fisher Scientific. Solutions (25 mmol L^{-1}) of the *N,N*-*p*-phenylenediamine sulfate and iron chloride were prepared separately in 6 mol L^{-1} HCl and mixed (1:1 v/v) just before use [16, 18]. All sulfide standards were prepared in an oxygen-free glove box. Sulfide stock solution was prepared weekly by dissolving 0.5 g sodium sulfide nonahydrate in 100 mL deionized water, which was deoxygenated by vigorous bubbling with ultra-pure N_2 gas for at least 30 min and standardization by iodometric titration [25]. Working standards were prepared fresh before each analysis. Calibration standards were generated by adding the appropriate amount of sodium sulfide stock solution to a 25-mL volumetric flask followed by 1.67 mL mixed diamine reagent (12.5 mmol L^{-1}) and diluting to volume with deoxygenated, deionized water. This corresponds to a 1:15 ratio of mixed diamine reagent to sample volume as recommended by Mylon et al. [19]. Serial dilutions covered the concentration range from 500 to $0.1 \mu\text{g L}^{-1}$.

Separations were performed using an Alliance 2695 HPLC coupled to a Micromass Quattro triple quadrupole mass spectrometer. Full-scan mass spectra were acquired between 100 and 300 *m/z* units to confirm the mass of the methylene blue molecule. Capillary and cone potentials were 3 kV and 50 V, respectively, the source block temperature was 80 °C, and the desolvation temperature was 180 °C. A sample was prepared, using the method described previously, resulting in a 1 mg L^{-1} methylene blue solution (with respect to sulfide) and the sample was introduced by use of a syringe pump at $10 \mu\text{L min}^{-1}$. The sample was monitored for 1 min with an interscan delay of 0.1 s in positive-ion mode. A product-ion spectrum was then also obtained for each of the peaks in the initial scan. For this the capillary and cone potentials were 3 kV and 35 V, respectively, argon was the collision gas and the collision energy was 30 eV.

When the MS conditions had been determined the mass spectrometer was interfaced with the Alliance 2695 HPLC unit. Chromatographic separation was performed with a

Phenomenex C₁₈ column (100 mm×4.6 mm, 3 μm particle size) at a flow rate of 0.6 mL min⁻¹. Solutions of aqueous ammonia and ammonium acetate, and ammonium formate (15 mmol L⁻¹) and ammonium heptafluorobutyrate (5 mmol L⁻¹) were evaluated to find the optimum mobile phase. Initially, the mobile phase comprised 70% acetonitrile and 30% buffer and was varied to 60% acetonitrile and 40% buffer, depending on the separation. The flow rate was 0.6 mL min⁻¹ and the flow was split such that half of the flow was directed into the MS and half into a Waters 2870 variable-wavelength UV–visible detector. Flow splitting was necessary because flow rates should not exceed 0.3 mL min⁻¹ when using the electrospray ion source.

For most of the samples quantification was performed by selected-ion monitoring (SIM) in which the quadrupole mass filter was operated at unit resolution and set to $m/z=284$, 285, and 286. Capillary and cone potentials of 3 kV and 49 V, respectively, were selected and the desolvation temperature was increased to 250 °C. After development of the SIM procedure an MS–MS method was also developed, using the most intense product ion transition. Capillary and cone voltages were maintained as above and the collision energy was varied from 25 to 55 eV, to determine the optimum value, i.e. that resulting in the highest signal intensity. Injection volumes were varied from 20 to 60 μL, depending on the sample.

To test the accuracy of this method a series of lake-water samples, taken and derivatized previously, were tested with the LC–MS procedure described above and retested by HPLC with UV–visible detection, using a method similar to that described by Tang and Santchi [18]. Samples were injected using a Waters 600S HPLC with acetonitrile and aqueous buffer (50 mmol L⁻¹ ammonium acetate, 1 mmol L⁻¹ pentane sulfonate) as mobile phase. A gradient was used, starting with 30% acetonitrile, which after one minute was ramped to 70% within 4 min and reduced again to 30% after 15 min analysis time. Detection was performed using a Waters 2487 dual-wavelength UV–visible detector set at 668 nm; the injection volume was 100 μL.

To evaluate any possible products of the methylene blue reaction with the polysulfide mixture, a 1000 mg L⁻¹ solution of polysulfide was prepared by crushing 0.05 g potassium polysulfide and dissolving in 50 mL deoxygenated DI water. An aliquot of the resulting solution was diluted by a factor of 250 and infused into the Micromass Quattro LC–MS system, by use of a syringe pump, at 10 μL min⁻¹. A full-scan mass spectrum from m/z 100 to 200 in negative-ion mode was acquired to determine the composition of the polysulfide solution. An aliquot of the original solution, then 1.67 mL of the diamine reagent described above, were pipetted into a 25-mL flask; the solution was then diluted to volume with deoxygenated DI water. The solution was left to stand for 30 min to enable

full color development and then infused using the syringe pump procedure described above.

Before collection of lake-water samples for sulfide measurements a combination temperature and dissolved-oxygen probe was lowered into the lake. Readings were taken at 1-m intervals to determine the position of the oxycline. Sulfide is rapidly degraded in the presence of oxygen, so samples taken at or below the oxycline must be derivatized immediately after sampling to preserve in-situ sulfide concentrations. Unfiltered lake water was pumped from depth into 10-mL glass serum vials using a peristaltic pump. The vials were allowed to overflow three times the container volume and a rubber septum was then inserted into the vial. Next, a steel needle was inserted through the septum and the septum was squeezed into the vial; this serves to remove any air still trapped below the septum. Another syringe was quickly inserted into the septum and 0.67 mL of the mixed diamine reagent described above, was injected through the septum. This technique enables derivatization of anoxic sulfide samples in the field while minimizing exposure of the sample to oxygen. All samples were then kept cool until the time of analysis, when samples were injected directly into the LC–MS with no additional preparation or preconcentration.

Results and discussion

Figure 1 shows a full-scan mass spectrum obtained from a 1 mg L⁻¹ solution of methylene blue, infused at 10 μL min⁻¹ by use of syringe pump. The peak at $m/z=284$ corresponds to the M⁺ molecular ion of methylene blue. The peak at $m/z=286$, resulting mostly from S³⁴, is approximately 6% of the $m/z=284$ peak, which is the predicted ratio for the molecular structure of methylene

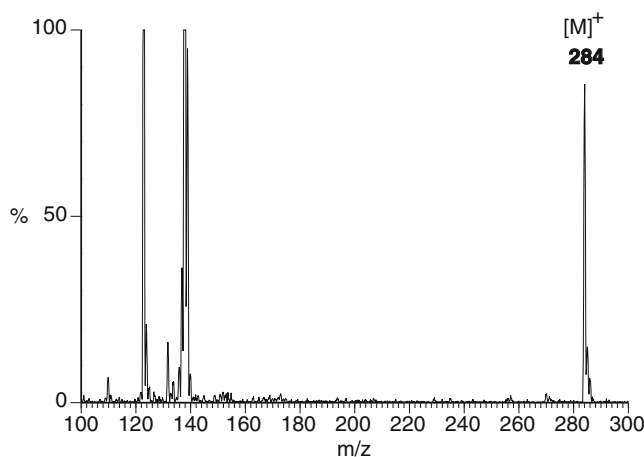


Fig. 1 ESI mass spectrum obtained from 1 mg L⁻¹ methylene blue introduced by means of a syringe pump (positive-ion scan, 10 μL min⁻¹)

blue resulting from a combination of S^{34} and C^{13} isotopes. The peak at $m/z=285$ is a result of C^{13} isotopes in the methylene blue molecule. Van Berkel et al. [26] report a mass spectrum of methylene blue obtained by single quadrupole ESI-MS showing a peak at $m/z=270$, which was hypothesized to occur as a result of oxidative demethylation in the electrospray process. The ion was not observed in this study, however. Scheifers et al. [27] analyzed methylene blue by secondary-ion MS. They found a peak at $m/z=268$ as a result of the loss of methane from the parent compound, which is consistent with this study.

An MS–MS spectrum was generated by setting the initial quadrupole to exclude all masses except $m/z=284$. The sample was infused at $10 \mu\text{L min}^{-1}$ by use of a syringe pump. After 1 min, the collision gas (argon, 30 eV collision energy) was activated and the second quadrupole was set to scan for the resulting fragments. Figure 2 shows the product ion spectra recorded. The large peak at $m/z=268$ corresponds to loss of methane (CH_4) from the molecule. The peak at $m/z=254$ results from the loss of two methyl groups and the peak at $m/z=240$ is a result of the removal of one of the dimethylamine groups from the methylene blue molecule.

At this stage, the mass spectrometer was interfaced with a Waters 2695 HPLC unit, using the Phenomenex C_{18} column, as described above. The flow rate was maintained at 0.6 mL min^{-1} and the flow was split such that 0.3 mL min^{-1} flowed into the mass spectrometer and 0.3 mL min^{-1} was directed into the Waters 2487 UV–visible detector. Figure 3 shows chromatograms obtained from a 1 mg L^{-1} solution of methylene blue ($10 \mu\text{L}$ injection volume) by use of mobile phases comprising 70% acetonitrile and different buffer components. The best peak shape and shortest retention time were achieved by use of 5 mmol L^{-1} heptafluorobutyric acid. The acetonitrile fraction was

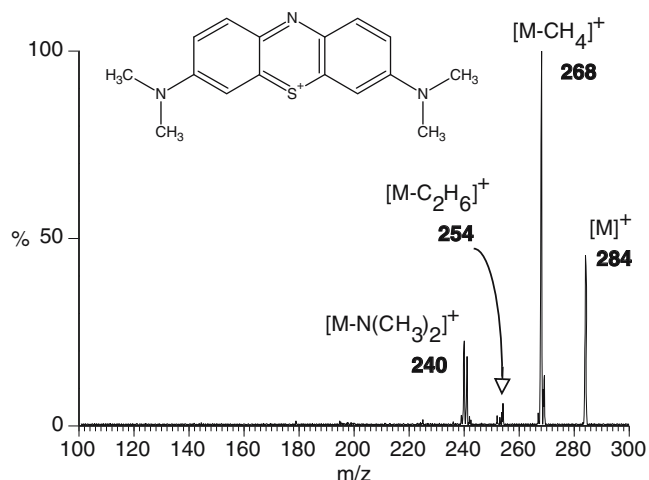


Fig. 2 Product ion obtained by ESI-MS–MS for $[\text{MH}]^+$ of methylene blue ($10 \mu\text{L min}^{-1}$, 30 eV collision energy)

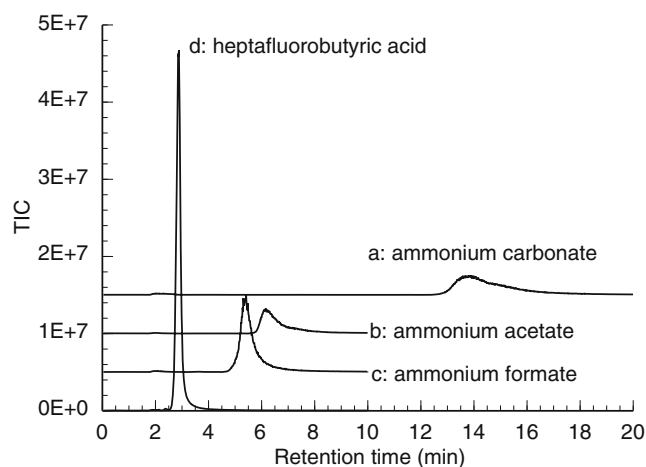


Fig. 3 Chromatograms obtained from 1 mg L^{-1} methylene blue in 70:30 acetonitrile–buffer with the modifiers: (A) 15 mmol L^{-1} ammonium carbonate, (B) 15 mmol L^{-1} ammonium acetate, (C) 15 mmol L^{-1} ammonium formate, and (D) 5 mmol L^{-1} heptafluorobutyric acid. The y-axis offsets are: B, 0.5×10^7 counts; C, 1×10^7 counts; D, 1.5×10^7 counts

reduced to 60% to ensure complete retention of the compound, resulting in a retention time of 3.2 min. This mobile phase was used for subsequent experiments.

To determine the figures of merit for this technique, calibration standards from 500 to $0.1 \mu\text{g L}^{-1}$ were prepared by serial dilution. The resulting calibration plot shows linearity is good over this range ($R^2=0.9995$). Replicate measurement of a $25 \mu\text{g L}^{-1}$ sulfide solution gave an RSD of 6.3% ($n=3$). Quality-control standards of $25 \mu\text{g L}^{-1}$ are routinely measured and typically return a recovery that is within 5% of its nominal concentration. The response to measurement of low sulfide concentrations using the SIM mode is illustrated in Fig. 4. A series of blank solutions was prepared by adding the mixed diamine reagent to deoxygenated DI water without sulfide. The limit of detection

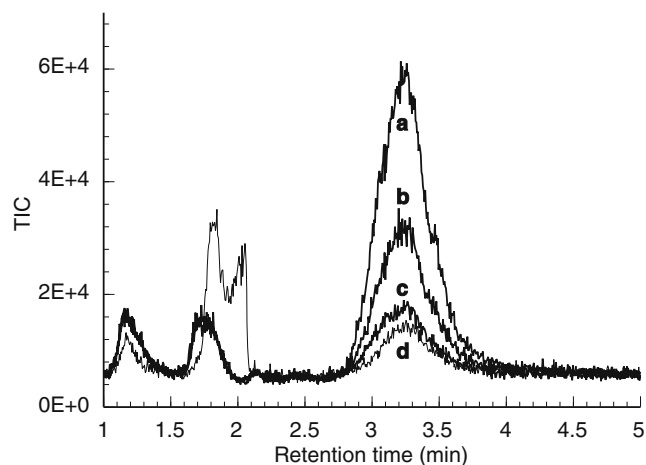


Fig. 4 Chromatograms obtained from methylene blue at concentrations of (A) $1 \mu\text{g L}^{-1}$, (B) $0.5 \mu\text{g L}^{-1}$, (C) $0.1 \mu\text{g L}^{-1}$, and (D) blank, measured in SIM mode with the mobile phase 60:40 acetonitrile– 5 mmol L^{-1} HPBA

was determined, on the basis of $3 \times \text{SD}$ for a series of blank measurements [28], to be approximately 50 ng L^{-1} (1.5 nmol L^{-1}) sulfide. In contrast, the UV–visible signal was indistinguishable from the baseline at concentrations $\leq 1 \text{ } \mu\text{g L}^{-1}$.

A series of lake water samples, taken in duplicate, was tested using the LC–MS (SIM) procedure described above; in Fig. 5 they are compared with results obtained by HPLC with UV–visible detection at 668 nm, based on a method similar to that of Tang and Santchi [18]. Results for low sulfide concentrations ($< 50 \text{ } \mu\text{g L}^{-1}$) scatter around the 1:1 response line. The deviation at higher sulfide concentrations ($> 150 \text{ } \mu\text{g L}^{-1}$) was attributed to the different calibration standards used, as measurements were conducted on different days. Owing to the instability of sulfide at low concentrations standards have to be prepared fresh every day. Because sulfide standards are very difficult to reproduce exactly, the observed deviation indicates the method is not limited by the accuracy of the measurement, but by the accuracy with which standards can be prepared. Overall, the two techniques are in good agreement with each other.

MS–MS detection mode frequently results in improved detection limits. For complex matrices, in particular, interferences are eliminated and the signal to noise ratio of the resulting mass spectra is superior to that for SIM techniques. To evaluate MS–MS performance, a series of standards was measured using the ion transition from $m/z=284$ to $m/z=268$. Collision energy was varied from 25 to 55 eV and it was determined that 30 eV resulted in the highest response to methylene blue. Inspection of the peaks seems to indicate, however, that the sensitivity of the MS–MS method is similar to that of the SIM method for measurement of sulfide standards, although the MS–MS technique may provide better results in analysis of a more complex sample matrixes.

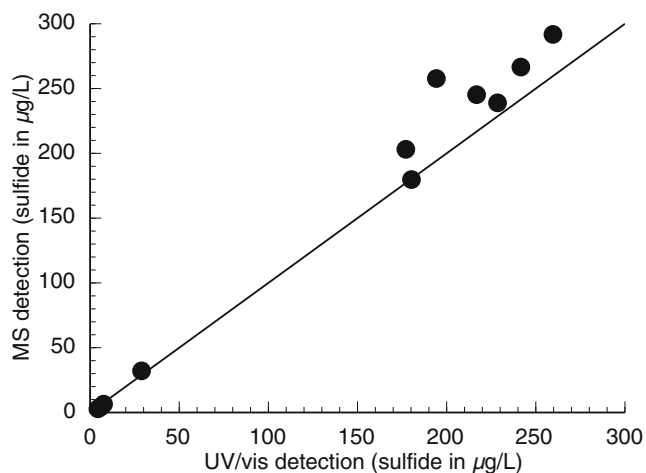


Fig. 5 Comparison of results obtained by LC–UV–visible and LC–MS

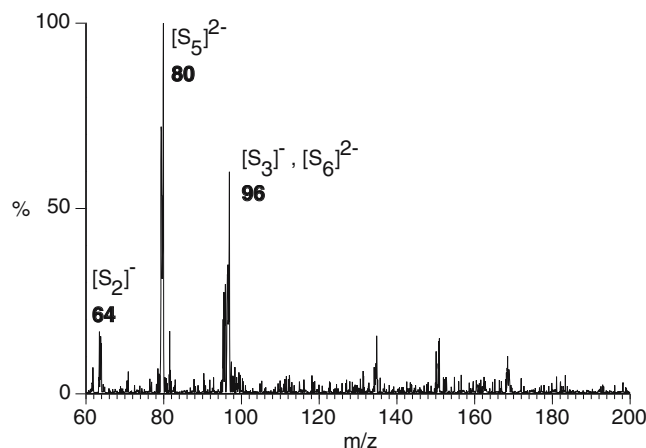


Fig. 6 Full ESI-MS scan of a potassium polysulfide solution used for the methylene blue reaction

Figure 6 shows the full-scan mass spectrum obtained from a polysulfide solution in negative-ion mode. Peaks are consistent with trisulfide (singly charged, $m/z=96$), tetrasulfide (double negative charge, $m/z=64$), pentasulfide (double negative charge, $m/z=80$), and hexasulfide (double negative charge, $m/z=96$) compounds in solution. To investigate the potential formation of methylene blue from polysulfides this solution was diluted to $158 \text{ } \mu\text{g L}^{-1}$ (as sulfur) and spiked with the mixed diamine reagent. The measured concentration of derivatized methylene blue was approximately $4.7 \pm 0.001 \text{ } \mu\text{g L}^{-1}$ ($n=10$), which corresponds to a concentration of 0.15 mmol L^{-1} in the original solution. It seems the mixed diamine reagent only reacts with approximately 3% of the polysulfides present in the solution.

Conclusions

The methylene blue reaction remains a useful technique for measuring sulfide in surface and sediment pore water. While not necessarily readily available in all research laboratories, use of LC–MS for separation and detection of methylene blue has several advantages over traditional UV–visible detection and is a powerful extension of this established technique. Detection limits are greatly improved and analysis times are reduced compared to those reported for HPLC–UV–visible techniques, because simple isocratic elution is now possible. Problems associated with interfering side reactions, for example those reported in Bowles et al. [24] are eliminated, because the interfering product can be filtered out by the mass spectrometer. Although the MS–MS technique did not significantly improve the sensitivity compared with the SIM technique, MS–MS is widely regarded to be superior for quantification and may yield better results if a more complex sample matrix is analyzed.

Although there are reports in the literature [19, 24] that the methylene blue reaction detects acid volatile sulfides, including HS^- , ZnS, and polysulfides, this study shows that only a small fraction of the total polysulfide in solution reacts with the diamine reagent to form methylene blue. It therefore seems the methylene blue technique cannot reliably be used to estimate the concentration of polysulfides.

It should also be noted that the use of an internal standard in LC–MS analysis is often useful to overcome the effects of ion suppression or enhancement, which are common in electrospray ionization. Although significant ion suppression was not evident in this study, it may become a problem if this technique is used for analysis of sulfide in a more complex matrix. An isotopically labeled methylene blue compound would be ideal for this type of analysis; no such compound was available at the time this study was conducted, however. Until such a time, a compound similar in structure to that of methylene blue, for example 1,9-dimethylmethylene blue (CAS# 23481-50-7), which is available commercially, could prove useful in overcoming ion suppression or enhancement, which would eliminate the need to matrix-match standards.

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