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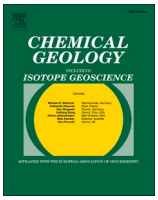

Analysis of total thiol concentrations in environmental samples using ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS)

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ABSTRACT

In this study, we developed an ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) approach to measure the concentration of total reactive thiol binding sites in a range of types of environmental samples by monitoring the decrease of monobromo-(trimethylammonio)-bimane (qBBr) caused by thiol-qBBr binding reactions. We first examined the stability of qBBr and potential side reactions that could reduce the accuracy of the UHPLC-MS approach. We found that significant loss of qBBr occurs in the absence of thiols in aqueous solutions after 2–4 h due to qBBr degradation and other side reactions, such as a chloride-for-bromide substitution reaction. To address these issues, in our approach we conduct the thiol-qBBr binding reaction in the presence of excessive qBBr for only 1 h, and we compare the MS signal of unbound qBBr in a thiol-free qBBr standard with that in a thiol-bearing qBBr standard in order to calculate the decrease in the concentration of free qBBr in solution that is caused by thiol-qBBr binding. Using 100–600 nM of N-Acetyl-L-cysteine (ACYS) as a model thiol in a 2 μM qBBr standard, we found that the measured decrease in the MS signal of unbound qBBr linearly correlates to the concentration of added ACYS, with a R^2 of 0.988, demonstrating that monitoring the decrease in unbound qBBr can be used to calculate the concentration of total thiol sites in a sample. We demonstrate that the new UHPLC-MS approach yields accurate results when measuring a series of single and mixed thiol standards in the 200–500 nM range, with measured values ranging from 86% to 109% of the known concentration of thiols. In addition to the above control tests, we also used the UHPLC-MS approach to measure dissolved thiol concentrations in several lake water samples. These measurements revealed that each lake contained low μM levels of total thiols, not only demonstrating the ability of the analytical approach to determine thiol site concentrations in natural waters, but also suggesting the widespread importance of these sites in controlling chalcophile element behavior in these systems. The UHPLC-MS approach not only can be used to determine the concentration of dissolved thiol sites at nM levels, but can also be adapted to measure the concentration of thiol binding sites on other environmental samples such as soil, sediment, and solid-phase organic matter surfaces.

1. Introduction

Sulfhydryl binding sites on organic compounds (thiols) are ubiquitous in natural and engineered surface waters, groundwater, and soil settings, and they can be present on dissolved organic molecules, solid phase organic matter, and microbial cell surfaces (Liem-Nguyen et al., 2017; Leclerc et al., 2015; Joe-Wong et al., 2012; Yu et al., 2014). Compared with other types of binding sites (e.g., amine, carboxyl and phosphoryl sites) on organic compounds, thiols have a much higher affinity to bind chalcophile elements such as Hg, Cd, Au, Zn, Cu, Se and As (Guine et al., 2006; Mishra et al., 2010; Yu et al., 2018; Yu and Fein, 2017a). Thiols can also react with quinones via nucleophilic reactions, and their reaction rates are much faster than those between quinones and other nucleophiles such as amines (Li et al., 2016; Jameson et al., 2004). Therefore, although thiols are typically less abundant than other binding sites in environmental samples (Joe-Wong et al., 2012; Yu et al., 2014), they likely play a more important role in controlling the fate, transport and bioavailability of chalcophile elements and quinones in the environment. For example, Boulegue et al., 1982 measured the speciation of Cu in sediment pore waters from a salt marsh environment

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and found that in some samples >90% of the Cu in solution was present bound to thiols. Similarly, the environmental behavior of Hg in contaminated soil systems typically is controlled by its binding to thiols on soil humic acids (Leterme and Jacques, 2015). Despite the importance of thiols in controlling the environmental behavior of chalcophile elements and quinone-containing organic compounds, measurements of thiol concentrations in natural systems are rare, largely because total thiol concentrations in environmental samples are likely in the pM to lower μM range (Leclerc et al., 2015) and it has been an analytical challenge to quantify thiol concentrations at these low levels. An approach that is able to accurately quantify the total thiol concentrations in environmental samples is crucial for not only yielding more accurate models of the fate and transport of chalcophile elements in the environment, but also for aiding the development of more effective and efficient remediation approaches for waters and soils contaminated by toxic chalcophile elements, such as Hg, Se, and Cd.

Analysis of the concentration of small thiol molecules in aqueous solution typically involves the selective derivatization of thiols with thiol-specific labeling reagents, followed by chromatographic separation of the thiol derivatives for UV/vis or fluorescence measurements (Dalle-Donne and Rossi, 2009; Hansen and Winther, 2009). However, the chromatography technologies that are required in this approach, such as liquid chromatography (LC) (Fahey and Newton, 1987), are not suitable for directly measuring many environmental samples, such as those containing bacterial cells, solid organic matter, or large polymer molecules. In order to measure the concentration of thiols on bacterial cell surfaces, thiol analysis methods have been developed recently using fluorescence or potentiometric titration measurements that do not involve subjecting the sample to chromatographic separation (Joe-Wong et al., 2012; Yu et al., 2014; Rao et al., 2014). Although these methods work well for determining the concentration of total thiol sites on bacterial cell surfaces, they are still not suitable for the analysis of most environmental samples. The potentiometric titration approach (Yu et al., 2014) requires samples to contain at least 10 g/L of biomass and it has a detection limit of approximately 10 μmol of thiols per gram of biomass, which is significantly higher than the nM or lower level of total thiols likely to be present in most environmental samples (Sander et al., 2013). The fluorescence approach can detect thiol concentrations in ultrapure water as low as 0.1 μM (Joe-Wong et al., 2012; Bakour et al., 2023; Worms et al., 2022). However, some common constituents of realistic complex environmental samples (e.g., dissolved organic matter, etc.) can strongly affect the background fluorescence of a sample, thereby increasing the detection limit significantly (Joe-Wong et al., 2012; Worms et al., 2022). X-ray absorption spectroscopy can be used to measure metal-thiol binding even under very low concentrations (Mishra et al., 2010; Sarret et al., 1998; Mishra et al., 2009; Guiné et al., 2006), but this method only measures the thiol sites that bind with specific metals, and does not yield a total thiol site concentration.

Huynh et al., 2020 recently developed a titration approach with a thiol-labeling reagent, monobromo-(trimethylammonio)-bimane (qBBr) for determining the total thiol site concentration in aqueous samples. The approach uses high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) to measure the residual qBBr concentrations after a wide range of concentrations of qBBr are introduced to a thiol-bearing sample. The total thiol concentration in a sample corresponds to the added qBBr concentration at which the residual qBBr concentration is zero, and can be determined by extrapolation of the linear part of the 'residual qBBr' vs 'added qBBr' curve to the x-axis. Because this approach measures the signal of the thiol-labeling reagent, qBBr, rather than directly measuring the thiol signal, it can be applied to a variety of types of samples for determining total thiol site concentrations in aqueous solutions or on microbial cell or other surfaces. More importantly, mass spectrometry has significantly lower detection limits for organic compounds than UV/vis or fluorescence spectrometry, making it possible in theory to precisely measure the thiol site concentration in samples that contain nM levels of thiols. Therefore, this LC-MS based method represents the most promising means for measuring total thiol concentrations in the wide range of samples of environmental interest.

In this study, we first tested the accuracy of the approach described by (Huynh et al., 2020), and found several severe limitations of the method due to the instability and reactivity of the qBBr molecule. We then examined the stability of qBBr and potential side reactions during its reaction with thiols. We found that it is crucial to optimize the reaction between qBBr and thiols, balancing the time requirement for the qBBr-thiol binding reaction to go to completion with the need to minimize the loss of qBBr through degradation. To address these issues, we developed an ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) approach for the analysis of the concentration of total reactive thiol binding sites in different environmental samples by monitoring the decrease of unbound qBBr caused by the thiol-qBBr reaction. In this method, we measure the decrease in the MS signal of the unbound qBBr between a thiol-free qBBr standard and a thiol-bearing sample with the same initial qBBr concentration. Because our new approach quantifies the decrease in the MS signal of unbound qBBr rather than quantifying the absolute concentration of unbound qBBr as is done in the Huynh et al. (2020) approach, the effect of qBBr degradation is minimized because a similar extent of qBBr degradation occurs in the qBBr standard and in the corresponding thiol-bearing sample if they are handled identically. In contrast, the Huynh et al. (2020) approach requires the analysis of a minimum of 6–8 solutions per sample, each with different qBBr concentrations and potentially each with a different extent of qBBr degradation, making it impossible to quantify the extent of qBBr degradation that occurs in each solution. Our approach circumvents the need to quantify qBBr degradation by analyzing standard/sample solution pairs for which qBBr degradation is constant. Furthermore, using UHPLC reduces the qBBr analysis time by approximately 50% relative to the HPLC approach, and because our approach requires only two qBBr measurements in order to determine the thiol concentration in a sample, the potential degradation loss of qBBr is reduced dramatically. These changes significantly improve the accuracy and precision of LC-MS based thiol analyses. We demonstrate that the UHPLC-MS approach can measure dissolved thiol concentrations in standards as low as 100 nM, and can be successfully applied to measure lake water samples that contain low μM levels of total thiols.

2. Materials and methods

2.1. Materials

Monobromo(trimethylammonio)bimane bromide (qBBr) was purchased from Toronto Research Chemical, Inc. Two model thiols, N-Acetyl-L-cysteine (ACYS) and Glutathione (GLU), were purchased from VWR International. LC-MS grade acetonitrile with 0.1% formic acid was purchased from Honeywell – Burdick & Jackson. All the qBBr and thiol stock solutions were freshly prepared before each batch of experiments, using a phosphate buffer that contained 1.8 mM Na_2HPO_4, 18.2 mM NaH_2PO_4 and 50 mM $NaNO_3$ (NaCl in a few specified cases) and its pH was adjusted to 7.0 ± 0.1. To minimize light-induced qBBr degradation, all the experiments were conducted in a dark area with lights off, and all the solution containers were fully covered with aluminum foil prior to MS analysis.

2.2. Analysis of qBBr using UHPLC-MS

Analyses of the concentrations of qBBr and of the products of its reaction with thiols were carried out using an ultra-high performance liquid chromatography-mass spectrometry system (UHPLC-MS). We used a Dionex Ultimate 3000 UHPLC system with a Waters ACQUITY UPLC HSS T3 column (50 × 2.1 mm) with a 1.8 μm particle size (cat# 186003538), coupled to a Bruker microTOF-Q II electrospray ionization time-of-flight quadrupole mass spectrometer that was set in positive

mode. The spectrometer used a capillary voltage of 2000 V and capillary temperature of 180 °C. The nebulizing gas was set to 7.0 L/min. The injection volume for each sample was 5 μL. UHPLC separations were achieved by elution for 5 min using a solution of 97.4% ultrapure water, 2.5% acetonitrile and 0.1% formic acid at a flow rate of 0.4 mL/min. After data collection, the extracted ion chromatograms (EICs) of interest were processed and peak areas were integrated using Bruker Compass DataAnalysis 4.2. The EIC peak at $m/z = 328.07 \pm 0.05$ was used for calculating the peak area corresponding to the unbound qBBr cation, and the EIC peak at $m/z = 411.17 \pm 0.05$ was used for calculating the peak area corresponding to the bound qBBr-ACYS reaction product.

2.3. Tests for qBBr stability

Two sets of control experiments were conducted in order to evaluate the stability of qBBr during the qBBr-thiol binding reaction. In the first set of experiments, we investigated the effect of chloride on the stability and reactivity of the qBBr molecule. In these experiments, 20 mL of a 20 μM qBBr stock solution that contained 1.8 mM Na_2HPO_4, 18.2 mM NaH_2PO_4 and 50 mM NaCl as a buffer was prepared and the pH was adjusted to 7.0 ± 0.1 before the experiments. This qBBr solution was then rotated for 1 h and its qBBr concentration was measured using UHPLC-MS at 0 h and 1 h. Because this initial experiment indicated that Cl^- reacts with qBBr, we then tested a series of 2 μM qBBr stock solutions that contained 1.8 mM Na_2HPO_4, 18.2 mM NaH_2PO_4, 50 mM $NaNO_3$ and a range of NaCl concentrations from 0 to 46 mM. These qBBr solutions were rotated for 1 h and their qBBr concentration were then measured using UHPLC-MS. Because the first set of experiments indicated that NO_3^- does not react with qBBr (see Results and Discussion below), we conducted a second set of control experiments to evaluate the stability of qBBr in the presence of $NaNO_3$. The buffer solution used in these experiments was the same as that used in the first set of controls, but with $NaNO_3$ replacing the NaCl. The initial qBBr concentrations in the $NaNO_3$ control experiments were 2, 4, 8 and 20 μM, and the qBBr concentrations were measured using UHPLC-MS at 0 h, 1 h, 2 h and 4 h.

2.4. Quantification of ACYS using the Huynh et al (Mishra et al., 2009) approach

In our next set of experiments, we tested the accuracy of the approach described by (Huynh et al., 2020). That is, we created thiol-bearing ACYS standards containing a range of qBBr concentrations and measured the concentration of free qBBr after reaction between the qBBr and the ACYS. In theory, free qBBr concentrations should be equal to zero at total qBBr concentrations up to the concentration of the ACYS in solution because of complete binding of all of the added qBBr with the thiol site of the ACYS. At higher qBBr concentrations than the concentration of the ACYS, excess qBBr should remain in solution after the binding of qBBr and ACYS occurs. Therefore, following the method of (Huynh et al., 2020), we measured the qBBr concentration of a series of test solutions. A 100 μM ACYS stock solution and a 100 μM qBBr stock solution were prepared in separate buffer solutions composed of 1.8 mM Na_2HPO_4, 18.2 mM NaH_2PO_4 and 50 mM $NaNO_3$ and the pH of each was adjusted to 7.0 ± 0.1. The two stock solutions were then mixed at different ratios with additional buffer solution to yield buffer solutions that contained 4 μM ACYS and 1 to 40 μM qBBr. In order to determine the reaction kinetics, the concentrations of the remaining qBBr and the reaction products were sampled and analyzed at 0, 1, 2, and 4 h, and the concentration of the remaining qBBr in each solution was determined using UHPLC-MS.

2.5. Quantification of thiol concentrations by monitoring qBBr decrease

Because our test of the approach of (Huynh et al., 2020) illustrated several significant limitations of their analytical method, we developed a new approach that avoids those limitations. Our approach uses the magnitude of the qBBr decrease that accompanies the qBBr-thiol reaction to quantify the thiol concentration in a sample, and this new approach requires only two measurements for each sample: 1) a thiol-free qBBr solution as a reference, followed by 2) an identical qBBr solution that contains the sample solution (with unknown thiol concentration). In order to construct a calibration curve for our approach, we analyzed a series of 2 μM qBBr solutions that contain 100–600 nM of ACYS as standards with known thiol concentrations using UHPLC-MS, with an analysis of thiol-free 2 μM qBBr reference before each measurement. From these measurements, we constructed a calibration curve that relates the decrease in the unbound qBBr peak area (the peak area for the thiol-free qBBr solution minus the peak area for the thiol-bearing qBBr solution) to the known thiol concentration of each ACYS standard sample. To determine the thiol concentration in an unknown sample, we first dilute the sample 10 times using the phosphate-buffered $NaNO_3$ solution to minimize the matrix difference between the sample and the reference solution. We then measure the area of the unbound qBBr peak for the sample solution and for the thiol-free reference solution, each containing 2 μM qBBr. We use these measurements to calculate the decrease in peak area, and applying this to the calibration curve relationship yields the thiol concentration in the sample.

In order to test our approach, we measured the thiol concentration of a range of thiol-bearing solutions containing known concentrations of model thiols, ACYS and/or GLU, treating these solutions as samples with unknown thiol concentrations. In these experiments, a 5 μM ACYS and/or GLU stock solution and a 20 μM qBBr stock solution were prepared separately in the same phosphate-buffered $NaNO_3$ solution as described earlier, and the two stock solutions were then mixed at different ratios with additional $NaNO_3$ buffer solution to yield solutions that contain 200–400 nM total thiols and 2 μM qBBr. The concentrations of total thiols in these solutions were determined as described above by measuring the decrease in the unbound qBBr peak area relative to a thiol-free 2 μM qBBr standard, and the measured concentrations were compared with the known thiol concentrations as a test of the approach.

In addition to testing our new approach with ACYS and/or GLU solutions of known thiol concentrations, we also used our approach to measure aqueous thiol concentrations in complex lake samples. Lake surface samples were collected in acid-washed polypropylene sample bottles from six lakes at the University of Notre Dame Environmental Research Center (UNDERC) near Land O'Lakes, Wisconsin, U.S.A. (89.32′W, 42.13′N). Lake characteristics, including morphology and water chemistry, have been described by (West et al., 2016). The UNDERC lakes are situated in watersheds dominated by wetlands (11–79%) and forest (6–75%). Samples were collected in May 2022, with one repeat sampling of one lake (Crampton Lake) in October 2022, using acid-washed polypropylene sample bottles, and the surface water samples were centrifuged and filtered through a 0.2 μm membrane so that only aqueous thiols were measured. To measure the total concentration of thiol sites in the lake samples, 0.5 mL of each sample was added to 4.5 mL of the phosphate-nitrate buffer (described earlier) that contained 2 μM of qBBr to create a 1:9 lake water:buffer dilution. The dilution step is added in order to keep the matrix in the lake water-qBBr sample similar to that of the thiol-free qBBr standard in order to minimize potential matrix effects on the qBBr analysis. Each diluted solution was then allowed to react for 1 h, and the decrease in the unbound qBBr peak area in each sample was then analyzed using UHPLC-MS and was used to calculate the total thiol concentration in each sample. The October 2022 Crampton Lake sample did not contain a detectable concentration of thiols using our method. In order to verify the accuracy of our method and to test for potential matrix effects on the method from lake water, we added 300 nM or 500 nM of ACYS and 2 μM of qBBr to two solutions: 1) a phosphate-nitrate buffer solution that contained October 2022 Crampton Lake water at a 1:9 (lake water:buffer solution) dilution, and 2) undiluted October 2022 Crampton Lake water. After 1 h of reaction, the unbound qBBr peak area for each these solutions was measured using UHPLC-MS and was compared with the unbound qBBr

peak area from the UHPLC-MS EIC for a thiol-free phosphate-nitrate buffer solution that also contained 2 μM of qBBr, and the difference in these peak areas was used to calculate the total thiol concentrations in these solutions.

3. Results and discussions

A phosphate-buffered NaCl solution has been used in previous studies involving qBBr (Huynh et al., 2020; Kosower and Kosower, 1995; Yu and Fein, 2017b) in order to keep the qBBr-thiol reaction at the optimal pH of 7. However, when we analyzed a freshly prepared qBBr solution in a phosphate-buffered NaCl solution using UHPLC-MS, two peaks were present in the chromatogram of the qBBr solution (Fig. 1a), suggesting that two compounds were present in the qBBr solution. qBBr dissolves as an unbound aqueous cation that contains one Br atom. Based on its characteristics, Compound 2, with a retention time of ~3.0 min, corresponds to this unbound qBBr cation. Because Br has two stable isotopes, ^{79}Br and ^{81}Br, and because these two isotopes have relative abundances of 50.69% and 49.31% respectively, two primary peaks with similar intensities occur at *m/z* of 328.066 and 330.065 in the mass spectrum of the unbound qBBr cation, and which can be observed in both the measured and the simulated mass spectra of qBBr (Fig. 1c and e, respectively). In contrast, the mass spectrum of Compound 1, with a retention time of ~2.4 min, has only one primary peak that occurs at *m/z* of 284.117 (Fig. 1b), indicating that it is a signal from a molecule that does not include a Br atom. Because the mass difference between Compound 1 and the qBBr cation (Compound 2) matches the mass difference between Cl and Br, we conclude that Compound 1 results from the replacement of the Br on the qBBr cation by a Cl atom, and we simulated the mass spectrum of this reaction product (the qBCl cation, Fig. 1d). The measured mass spectrum of Compound 1 matches well with the estimated mass spectrum of the qBCl cation. This finding indicates that the presence of high concentrations of chloride ions (e.g., in this case 50 mM Cl^- vs 10 μM qBBr) can reduce the concentration of qBBr by partially converting qBBr to qBCl.

The reaction between qBBr and Cl^- is dependent on the concentration of Cl^-. For example, we analyzed 2 μM qBBr solutions in the presence of varied NaCl concentrations using UHPLC-MS, and found that the qBCl peak is undetectable when the Cl^- concentration is 4.6 mM (163 ppm) or lower (Fig. 2). It is noteworthy that 4.6 mM of Cl^- is still 3

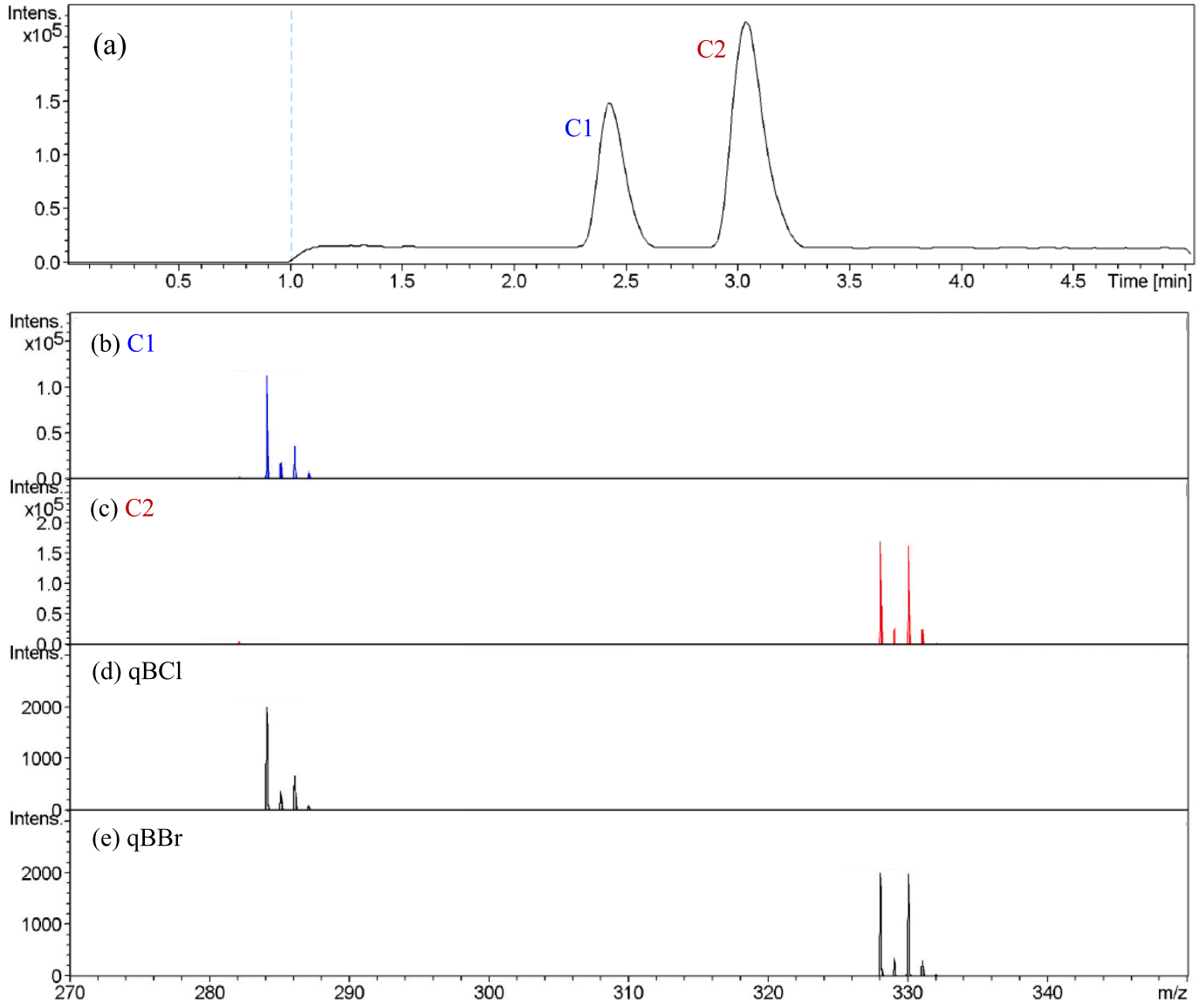

Fig. 1. (a) Base peak chromatogram of a 10 μM qBBr solution in the presence of 50 mM NaCl. C1 and C2 represent the two compounds detected; (b) the measured mass spectrum of Compound 1 with a retention time of ~2.4 min; (c) the measured mass spectrum of Compound 2 with a retention time of ~3.0 min; (d) the simulated mass spectrum of $C_{13}H_{19}ClN_3O_2^+$ (the qBCl cation); (e) the simulated mass spectrum of $C_{13}H_{19}BrN_3O_2^+$ (the qBBr cation).

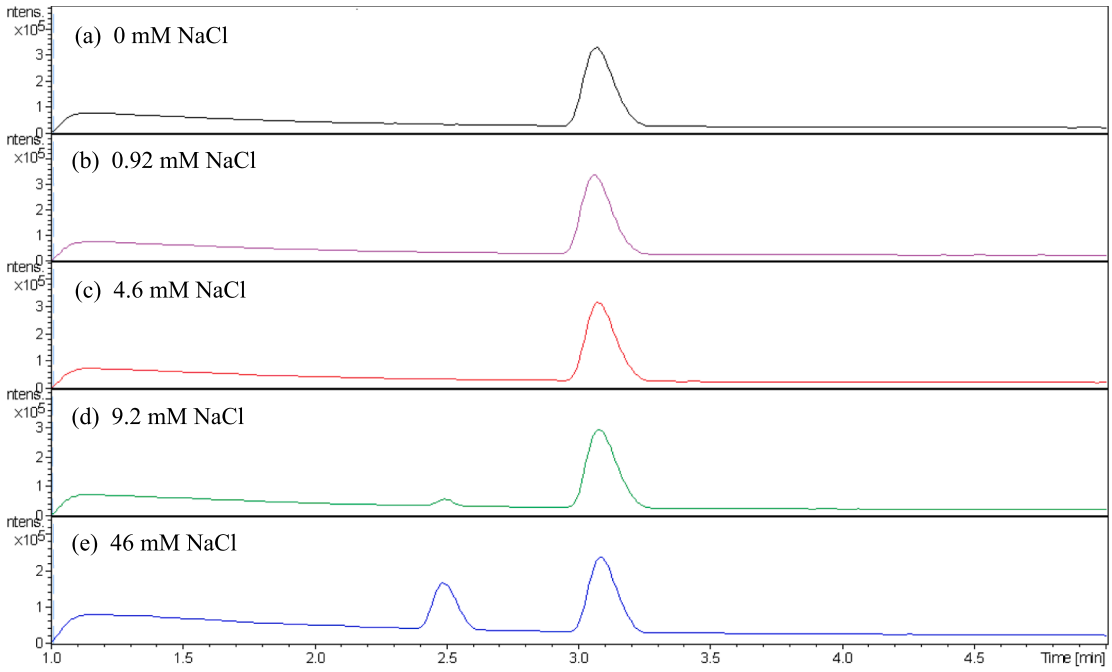

Fig. 2. Base peak chromatogram of a 2 μM qBBr solution in the presence of 50 mM of $NaNO_3$ and varied concentrations of NaCl: (a) 0 mM; (b) 0.92 mM; (c) 4.6 mM; (d) 9.2 mM; (e) 46 mM. Compound 1 with a retention time of ~2.5 min represents the qBCl cation, and Compound 2 with a retention time of ~3.1 min represents the qBBr cation.

orders of magnitude higher than the 2 μM of qBBr that is present in solution. This indicates that the reaction between qBBr and Cl^- is either very slow or only occurs at high Cl^- concentrations, e.g., ≥ 9.2 mM of Cl^- for 2 μM qBBr in this case. However, it is still crucial to minimize the use of NaCl in a buffer solution of qBBr in order to accurately quantify the decrease in qBBr concentration caused by the reaction between qBBr and thiols. After we replaced the NaCl with the same concentration of $NaNO_3$, we found that only the qBBr peak was present in the chromatogram of the qBBr solution (Fig. 2a), suggesting that NO_3^- does not react with qBBr, and that the presence of NO_3^- does not affect the qBBr measurement.

Compared to the results of (Huynh et al., 2020), our approach significantly improves the linearity of qBBr calibration curves (Fig. 3). One likely reason for the improvement is that our use of UHPLC rather than HPLC, reduces the analysis time from approximately 12–17 min per measurement to 5 min for each measurement, thereby minimizing the inevitable degradation of qBBr that occurs in solution. Another reason for the improvement in calibration linearity is that we eliminated the influence of Cl^- by using $NaNO_3$ as an electrolyte buffer. As shown in Fig. 3b, our approach yields a good linear relationship between the concentration of added qBBr and its MS signal in a range of 0.25–4.0 μM with a R^2 value of 0.997. The 1σ uncertainties of the MS signals of the selected qBBr standards (0.25, 2, 4 μM) are 2–4%, as determined by five repeat analyses of each sample. In order to further test the detection limit of qBBr concentration of our approach, we measured a series of qBBr standards in a range of 0.02–0.2 μM with an increased sample injection volume (25 μL compared with the 5 μL volume used above) (Fig. 3b). These tests also produced a good linear relationship between the concentration of the added qBBr and its MS signals in this qBBr concentration range ($R^2 = 0.999$, Fig. 3c). These results suggest that our approach is capable of accurately measuring qBBr to at least as low as 0.02 μM (20 nM), thereby quantifying thiol concentrations in a sample at nM levels.

To demonstrate the limitations of the (Huynh et al., 2020) approach of using the residual qBBr concentration directly to yield a thiol concentration in a sample, we used that approach to analyze a series of samples each containing 4 μM of a model thiol, ACYS, but each with a different concentration of qBBr, ranging from much less qBBr than ACYS to much more. We allowed each system 2 h for the qBBr-thiol binding reaction to occur. After the 2 h reaction period, we detected a significant concentration of residual unbound qBBr even when the added qBBr concentration was much lower than the ACYS concentration (Fig. 4). For

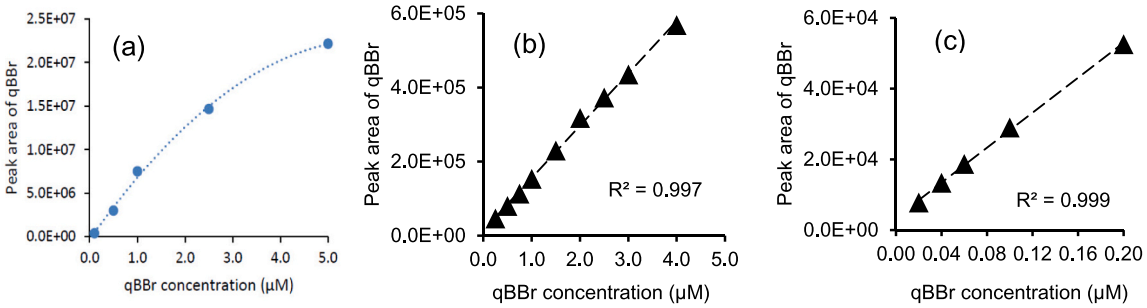

Fig. 3. (a) Calibration curve of qBBr standards using HPLC-MS/MS from (Huynh et al., 2020); (b) Calibration curve of qBBr standards using UHPLC-MS from this study with a 5 μL sample injection volume; (c) Calibration curve of qBBr standards using UHPLC-MS from this study with a 25 μL sample injection volume. The y axes values correspond to the peak areas on the EICs at $m/z = 328.07 \pm 0.05$, the unbound qBBr peak.

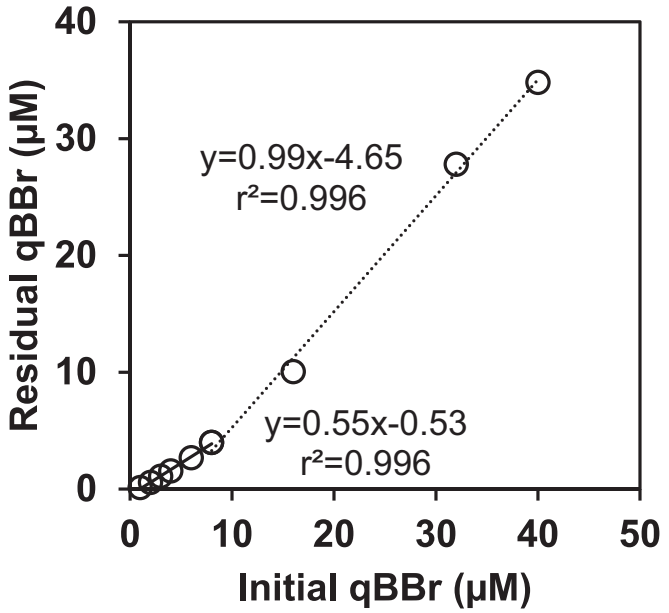

Fig. 4. UHPLC-MS analyses of 4 μM ACYS solutions containing between 1.0 and 40 μM qBBr. The residual free qBBr concentrations were measured after 2 h of reaction. The lower equation and r^2 value correspond to the 0.25–8.0 μM initial qBBr data; the upper equation and r^2 value correspond to the >8.0 μM initial qBBr data.

example, in a system with 2 μM qBBr and 4 μM ACYS, 0.6 μM unbound qBBr remained in solution after the 2 h reaction period. Huynh et al., 2020 indicate that the residual free qBBr concentration should be negligible at qBBr concentrations less than or equal to the thiol concentration of the sample due to qBBr-thiol binding, and that residual free qBBr should only be present when qBBr concentrations exceed the thiol concentration in the sample. We found that this ideal response does not occur. In our experiments, the measured residual free qBBr concentrations increase linearly with the concentration of added qBBr from 0.25 to ~8 μM qBBr, and increase with a steeper slope above 8 μM (Fig. 4). It is unclear why (Huynh et al., 2020) measured negligible free qBBr when [qBBr] ≤ [Thiol] and we did not, but our results suggest that at least 8 μM qBBr is required to complete the reaction with 4 μM ACYS in our experiment. Our data suggest that the qBBr-thiol binding reaction may be kinetically or sterically controlled, with the reaction rate decreasing when both the concentrations of ACYS and qBBr are low.

To test if extending reaction time can promote the extent of reaction between qBBr and ACYS, we monitored the concentration of the qBBr-ACYS reaction product as a function of time and initial qBBr concentration. The results confirm that the 2 h reaction time that we used is sufficient for the reaction to be completed as the MS signal of reaction product did not increase significantly after 1 h in any of the experiments (Fig. 5). In fact, extending the reaction time likely hampers the measurement of the thiol concentration in a sample by promoting qBBr degradation. For example, our controls show that the concentration of qBBr stock solutions decreased significantly after 2–4 h even though no thiol was added to the solutions, and longer times and higher qBBr concentrations generally resulted in greater qBBr loss (Fig. 6). After 4 h, the 2 μM and 4 μM qBBr stock solutions lost ~10% of their original qBBr concentrations, and the 8 μM and 20 μM stock solutions lost ~20% of their qBBr. These results suggest that accurate quantification of qBBr concentration can only be accomplished within 1–2 h of the introduction of fresh a qBBr solution to a sample, and that qBBr degradation likely also contributes to the loss of qBBr during the qBBr-thiol reaction if left for longer times. Therefore, determining the thiol concentration in a sample based directly on the measured residual free qBBr concentration leads to high analytical uncertainty due to factors such as the solution

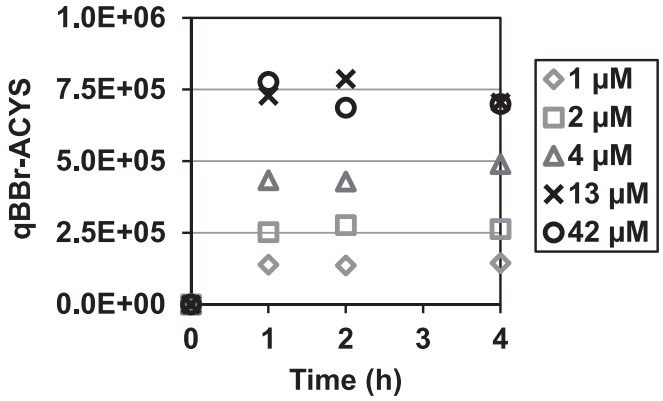

Fig. 5. Effect of time on the reaction between 4 μM of ACYS and 1–42 μM of qBBr. The y-axis shows the peak area of the reaction product of ACYS and qBBr, and the EIC peak at m/z = 411.17 ± 0.05 was used for calculating the peak areas.

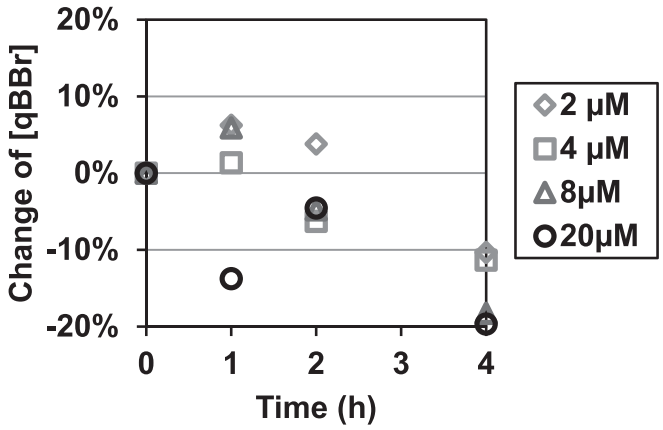

Fig. 6. Stability test results for freshly prepared qBBr standard solutions that contain 2, 4, 8 or 20 μM of qBBr. The graph shows the percentage change in qBBr concentration as a function of time since the solution preparation.

matrix (e.g., Cl^-), the qBBr-thiol reaction time, and the qBBr degradation that occurs during the waiting time between sample preparation and LC-MS analysis.

Compared with the work of (Huynh et al., 2020), our approach applies several improvements that minimize the loss of qBBr that is not related to the qBBr-thiol binding reaction, and likely which explain our consistent observation of non-zero concentrations of residual free qBBr when [qBBr] ≤ [Thiol]. Our data are consistent with incomplete qBBr-thiol binding and hence incomplete removal of free qBBr when [qBBr] ≤ [Thiol]. It is likely that (Huynh et al., 2020) only observed negligible free qBBr concentrations when [qBBr] ≤ [Thiol] due to other factors than thiol binding (qBBr degradation, chloride-substitution for Br, etc.) which dropped the qBBr concentration to be below detection in their study for these samples with low qBBr concentrations. In fact, most of the experiments in (Huynh et al., 2020) show significant qBBr loss that is not related to qBBr-thiol reaction, e.g., < 10 μM, and values as low as 1–2 μM, of residual qBBr after adding 20 μM qBBr to 4 μM thiols when 16 μM of unbound qBBr should remain in solution (results shown in their Fig. S5).

Our findings suggest that directly using the curve of the residual free qBBr concentration versus the added qBBr concentration to determine the thiol concentration in a sample (as depicted in Fig. 4) fails for two reasons: (1) quantification of qBBr is time-sensitive due to the instability of qBBr, especially when high concentrations of chloride are present; (2) excessive qBBr is required to fully react with the thiols in a sample. To

address these issues, we designed a new approach to measure the thiol concentration in a sample by adding a small volume of the sample to a qBBr solution and monitoring the decrease of qBBr after qBBr-thiol reaction. The qBBr decrease in a sample is calculated by comparing the unbound qBBr peak area at $m/z = 328.07 \pm 0.05$ of a thiol-free qBBr reference solution and a sample-bearing qBBr solution that are measured consecutively using UHPLC-MS. Because the reference and the sample qBBr solutions contain the same initial concentration of qBBr, are prepared using the same buffer solution at the same time and are measured consecutively after the reaction, the extents of qBBr degradation should be similar in the two solutions. Therefore, the measured qBBr decrease using our new approach is primarily due to the qBBr-thiol reaction and is only partially affected by the minor difference in qBBr degradation between the reference and sample qBBr solutions. The accuracies of both the (Mishra et al., 2009) approach and our approach are affected by qBBr degradation. However, the accuracy of the (Huynh et al., 2020) approach is affected by the relatively large absolute extent of qBBr degradation in a sample solution, while the accuracy of our approach is affected by the relatively small difference in the extents of qBBr degradation between two similar solutions. Therefore, the uncertainties that arise from qBBr degradation are much smaller using our approach compared to the (Mishra et al., 2009) approach.

In order to build a calibration curve for our new approach, we added 100–600 nM of a model thiol, ACYS, to a series of 2 μM qBBr solutions containing the phosphate-buffered $NaNO_3$ solution as described earlier, and measured the decrease in the unbound qBBr peak area using UHPLC-MS after 1 h of reaction. That is, we measured the area of the unbound qBBr peak for the thiol-free reference solution and subtracted the area of the unbound qBBr peak in the signal for each of the thiol-bearing standards, yielding a value for the decrease in the qBBr signal for each thiol-bearing standard. As shown in Fig. 7, the magnitude of the decrease of the unbound qBBr MS signal shows a strong linear relationship with the concentration of the added ACYS ($R^2 = 0.988$), and therefore can be used as a calibration curve to calculate the total concentration of thiol sites in a sample with unknown thiol concentration. The measured decrease of the unbound qBBr MS signal when 100, 200 or 600 nM of ACYS was added has a 1σ uncertainty ($n = 5$) of 23.7%, 21.0% and 3.8%, respectively. Because the MS signal of the 2 μM qBBr solution exhibits a 1σ uncertainty (n = 5) of ~4% in the absence of thiol and because the thiol concentrations were calculated by qBBr decreases, the thiol detection limit of this method is estimated to be approximately 80 nM when 2 μM qBBr is used as a reference solution. The 100 and 200 nM ACYS sample analyses exhibited relatively high uncertainties likely because their thiol concentrations were relatively close to the detection limit of the approach. In contrast, because 600 nM is much higher than the detection limit, the 600 nM ACYS sample analysis exhibited a much smaller uncertainty. It is noteworthy to point out that it took ~5 h to analyze all of the samples and their replicates that are depicted in Fig. 7. Compared to the 10% qBBr loss that we measured for the 2 μM qBBr standard after 4 h (Fig. 6), the small uncertainty (3.8%) of the qBBr decrease for the 600 nM ACYS sample suggests that measuring the qBBr decrease rather than the absolute qBBr concentration for thiol analyses significantly improves the time-sensitivity of the approach. Although we did not measure a sample containing <100 nM of ACYS in this study, the detection limit of this method might be lowered by using a lower qBBr reference solution concentration, e.g., 0.5 or 1 μM qBBr, thereby potentially extending the method to analyze samples with thiol concentrations lower than 100 nM.

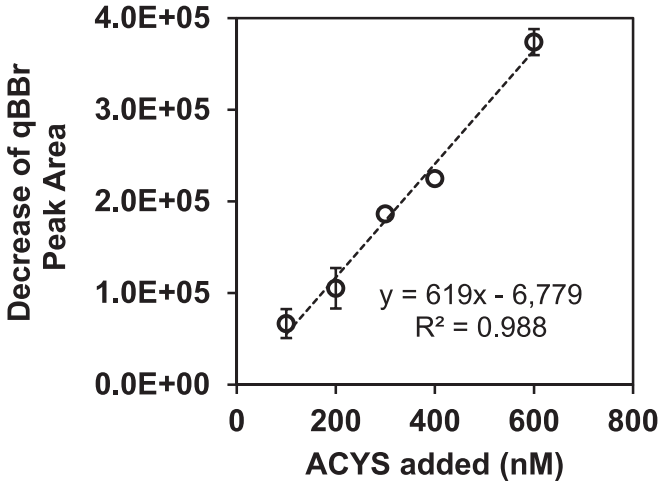

Fig. 7. The decrease in the peak area of unbound qBBr in solutions with 2 μM qBBr after adding 100–600 nM of a model thiol, ACYS, and after 1 h of reaction. The EIC peak at $m/z = 328.07 \pm 0.05$ was used for calculating the peak area corresponding to the unbound qBBr cation. The error bars represent 1σ uncertainties ($n = 5$).

To validate the accuracy of our UHPLC-MS approach, we measured the thiol concentrations of several standard solutions (200–500 nM ACYS and/or GLU in either the phosphate-buffered $NaNO_3$ solution alone, in a 1:9 Lake water/Buffer mixed solution, or in a 100% lake water solution alone) as 'unknown samples', with each of the reference solutions prepared using the phosphate-buffered $NaNO_3$ solution. The measured thiol concentrations in the ACYS and GLU 'unknowns' generally matched well with the known concentration of thiols in these solutions, with measured values ranging from 86% to 109% of the known values (Table 1). The 1σ uncertainties associated with the measured thiol concentrations are typically 4–6%, with one exception: the '100 nM GLU + 100 nM ACYS' sample has a 1σ uncertainty of ~19%.

We used the UHPLC-MS approach to determine the dissolved thiol concentrations of water samples that were collected from six lakes at the University of Notre Dame Environmental Research Center (UNDERC) in May 2022. As is the case for similar freshwater lakes in North America and Europe (Dugan et al., 2017), chloride concentrations in the UNDERC lakes are below 80 μM (3 ppm) (Solomon et al., 2018), and hence no chloride interference effect occurs in the UHPLC-MS analysis. Significant concentrations of thiols were detected in all six lakes, with concentrations varying from 0.8 to 3.7 μM (Table 2). We analyzed a different sample from one of the six original lakes (Crampton Lake), but sampled in October 2022. This analysis indicated that the thiol concentration from the October Crampton Lake sample was below the detection limit of our analytical approach, indicating that thiol concentrations in lake waters can vary significantly between seasons. In order to test whether some component of the lake sample matrix was

Table 1
Thiol analysis tests.

Thiols *	Solution	n	Expected (nM)	Measured (nM)	Accuracy
200 nM ACYS	100% Buffer	3	200	209 ± 9	105%
400 nM ACYS	100% Buffer	3	400	413 ± 25	103%
200 nM GLU	100% Buffer	3	200	186 ± 11	93%
400 nM GLU	100% Buffer	3	400	345 ± 6	86%
100 nM GLU + 100 nM ACYS	100% Buffer	3	200	217 ± 42	109%
200 nM GLU + 200 nM ACYS	100% Buffer	3	400	417 ± 24	104%
300 nM ACYS	1:9 Lake water/Buffer	1	300	316	105%
500 nM ACYS	1:9 Lake water/Buffer	1	500	432	86%
500 nM ACYS	100% Lake water	1	500	431	86%

* The two model thiols used are N-Acetyl-L-cysteine (ACYS) and glutathione (GLU). The buffer solution contains 1.8 mM Na_2HPO_4, 18.2 mM NaH_2PO_4, 50 mM $NaNO_3$ and ultrapure water. The lake water sample was collected from Crampton Lake at the University of Notre Dame Environmental Research Center (UNDERC) in October 2022 for which our method indicated a thiol concentration below the detection limit of our approach.

Table 2
Thiol concentrations in lake surface water samples from UNDERC (May 2022).

Sample	Total thiols (μM)
Paul Lake	2.2
Morris Lake	1.9
Tenderfoot Lake	0.8
Hummingbird Lake	1.9
Bay Lake	2.8
Crampton Lake	3.7

interfering with the thiol analysis, and to verify our finding of a thiol concentration below the detection limit, we conducted three thiol spike tests in which, in addition to following our approach of adding the buffer solution and qBBr to the October Crampton Lake sample, we also added a known concentration of ACYS to each sample. As shown in Table 1, the presence of natural lake water in the sample did not significantly affect the thiol measurement. The sample which contained 300 nM ACYS yielded a measured thiol concentration of 316 nM, and the measured concentrations of total thiols for the samples in which 500 nM ACYS was added to the 1:9 lake water/buffer solution and to the 100% lake water solution were the same within analytical uncertainty. These tests demonstrate that the lake water matrix does not affect the thiol analyses, and that nM levels of thiols in both the defined solutions and natural samples can be accurately detected using our approach, with typical accuracy of approximately ±10% (Table 1) and an operational detection limit of approximately 100 nM.

Because the lake water matrix did not affect thiol analyses in this study, so our results showed that a dilution step was not necessary for these samples. However, water matrices can vary significantly for between samples taken from different locations, or even between samples taken at the same location but from different seasons and/or water depth. Therefore, the best practice is to start the thiol analysis of a sample with an unknown thiol concentration by diluting the sample using the same chloride-free buffer that is used to make the qBBr reference solution. It is also important to characterize the water samples before the thiol analysis in order to determine if sample pretreatments are required. For example, dissolved chloride interferes with the analytical approach due to chloride substitution for Br on the qBBr molecule, but for aqueous samples with less than approximately 4.6 mM (163 ppm) chloride, this effect is negligible (when using 2 μM qBBr in the analyses). Analyses of samples with higher chloride concentrations are possible, but must involve precise matrix matching between the sample and the thiol-free qBBr standard that is used. One potential approach for analyzing thiol concentrations in high-chloride samples would be to use a portion of each sample to create the qBBr reference solution for that sample after converting the thiols in the sample to sulfide bonds through a thiol-Michael addition reaction. This approach would yield a thiol-free qBBr reference solution that is matrix-matched to the samples exactly (Nair et al., 2014). Beside chloride, sulfide can also react with qBBr (Huynh et al., 2020) and hence would interfere with the thiol analysis using our approach. For samples that contains significant concentrations of sulfide, it is necessary to remove dissolved sulfide with a N_2 purge pretreatment step (Huynh et al., 2020). In addition, there is some evidence that mercury and methylmercury ions bind more strongly to thiols than does qBBr and that they are not removed during the qBBr treatment (Bakour et al., 2023; Huynh et al., 2020). Hence, thiol concentration measurements on samples with significant levels of bound mercury or methylmercury using any qBBr approach would not measure these Hg-bound thiol sites.

This study describes a new analytical approach for measuring the concentration of thiol sites in aqueous samples. Our results demonstrate the ability of the UHPLC-MS approach to analyze thiol concentrations in natural water samples, and our preliminary results of lake water analyses suggest the widespread presence of thiols in natural waters. Because of the high thermodynamic stability of aqueous complexes between chalcophile elements and thiol binding sites (Yu and Fein, 2015; Nell and Fein, 2017), and because total dissolved metal concentrations in pristine surface waters such as these are typically on the nM level (Sander et al., 2013), the μM level of thiols present in these waters suggests that thiol binding controls the aqueous speciation and environmental behavior of chalcophile elements. Our study offers a means for quantifying dissolved thiol concentrations down to the nM level, and can be adapted to measure the concentration of thiol binding sites on environmental surfaces such as mineral, bacterial, soil, sediment, and solid-phase organic matter surfaces as long as qBBr binding onto those surfaces is controlled dominantly by binding with thiol sites. A better understanding of the concentration and role of thiol binding sites in natural environments will yield more accurate models of the fate and transport of chalcophile elements in the environment, and will aid the development of more effective and efficient remediation approaches for waters and soils contaminated by toxic chalcophile elements, such as Hg, Se, and Cd.

CRediT authorship contribution statement

Qiang Yu: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Jeremy B. Fein:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jeremy B. Fein reports financial support was provided by U.S. National Science Foundation.

Data availability

Data will be made available on request.

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