Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 998 (2003) 31–40
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of dissolved thiols using solid-phase extraction and liquid chromatographic determination of fluorescently derivatized thiolic compounds

Degui Tang*, Martin M. Shafer, Kou Vang, Dawn A. Karner, David E. Armstrong
Environmental Chemistry and Technology Program, University of Wisconsin-Madison, 660 North Park Street, Madison, WI 53706-1484, USA

Received 10 July 2002; received in revised form 11 March 2003; accepted 7 April 2003

Abstract

A method employing solid-phase extraction coupled with HPLC separation of thiol-monobromobimane (mBBr) derivatives was developed and optimized to quantify dissolved thiols at concentrations as low as 0.1 n*M* for glutathione (GSH) and γ-glutamylcysteine (γEC) in natural waters. The reducing reagent, tri-*n*-butylphosphine (TBP), is needed for complete derivatization. At the optimal addition of TBP ([TBP]/[mBBr]=0.4–1.6), no interference from copper was observed. The thiol fluorescence signal was totally suppressed if the mole ratio of TBP to mBBr was 2.6 or greater. Consistent recovery of thiols standards in a NaCl solution (0.5 *M*) was obtained using the Waters HLB reversed-phase resin, and blank levels of GSH and γEC were extremely low (less than 0.03 n*M*). The detection limits for GSH, γEC and phytochelatin-2 (PC-2) were 0.03, 0.03, and 0.06 n*M*, respectively.

Keywords: Derivatization, LC; Monobromobimane; Glutathione; Thiols

1. Introduction

Fahey and Newton [1] developed a method for thiol determination based upon fluorescence detection of HPLC separated thiol-monobromobimane (mBBr) derivatives. This method has been adopted for thiol determination in anoxic environments [2,3], and in marine microorganisms [4,5]. However, for detection of dissolved thiols at the sub-n*M* levels typical of surface waters, sensitivity and method blanks are major obstacles. With few exceptions [6], preconcentration is needed to increase the sensitivity of the method. Direct immobilization of thiols using a thiol–disulfide exchange resin [7] or extraction of the products from the thiol–disulfide interchange reaction onto a C_{18} cartridge [8] has been proposed. However, the efficiency of the thiol–disulfide interchange reaction in environmental samples is likely to be poor or variable due to the presence of compounds which bind to thiols, making them inaccessible to the interchange reaction [9]. The C_{18} cartridge has been used to extract large sulfhydryl-enriched

*Corresponding author. Tel.: +1-608-262-2123; fax: +1-608-262-0454.
E-mail address: deguitang@facstaff.wisc.edu (D. Tang).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00639-3

peptides, i.e., phytochelatins [10], but its usage for small peptides, e.g., glutathione, has not been reported. Stripping voltammetry has been used to detect dissolved thiols [11], but quantification of specific thiol species is difficult. A recent study showed improved electrochemical detection of thiols through coupling with HPLC separation [12], but its application to environmental samples has not been reported.

For the fluorescence measurement of thiols in environmental samples, two parameters are of concern regarding derivatization efficiency [13]: (1) the use of a reducing reagent, and (2) the presence of trace metals. To detect the disulfide form of thiols, the reducing reagent, e.g., tri-*n*-butylphosphine (TBP), is needed. However, it has been shown that TBP can cause large decreases in the fluorescence signals [14]. An extra solvent extraction step (e.g., *n*-hexane) is typically required to remove it from the aqueous phase before derivatization [15]. The presence of trace metals can also impact thiol detection, due to formation of thiol–metal clusters [16], which makes the sulfhydryl group less accessible to the fluoroprobe. The reducing reagent, TBP, has been shown to ameliorate this problem when another fluoroprobe (ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, SBD-F) was used for thiol determination [13]. The possible influences of trace metals on thiol determination when using the mBBr derivatization technique, however, have not been reported.

The recognized importance of thiols in controlling the bioavailability of trace metals [17] and other toxic compounds in natural waters necessitates a sensitive technique for their detection. In this manuscript, we report the development of a solid-phase extraction method to preconcentrate the fluoro-tagged thiols, followed by HPLC analysis of these derivatized compounds. Factors influencing derivatization, such as the appropriate use of both the reducing reagent and fluoroprobe, the presence of trace metals (copper), and reaction temperature, are also addressed. We demonstrate that the enhancement or suppression of the overall thiol fluorescence signals, caused by the reducing reagent (TBP), depends on the relative amounts of TBP and fluoroprobe used.

2. Experimental

2.1. Instrument and chemicals

Glutathione (GSH, >97.0%), L-cysteine (Cys, >99.5%), γ-glutamylcysteine (γ-EC, 99%), tri-*n*-butylphosphine (TBP, 95%), methanesulfonic acid (MSA, >99.0%), trifluoroacetic acid (TFA, >99.5%), monobromobimane (mBBr, >97%), acetonitrile (>99.93%), methanol (>99.9%), and copper chloride (97%) were purchased from Sigma and used without further purification. Phytochelatin-2 (PC-2) standard (95% purity) was synthesized by Biopeptide, Inc. Milli-Q water (18.3 MΩ) was used to prepare aqueous solutions. The HPLC system included a gradient controller (Waters 600), a high-pressure pump (Waters 600E), a temperature-controlled autosampler (Waters 717), and a fluorescence detector (Spectroflow 980, ABI Kratos). The HPLC system is controlled by Waters Millenium software. A reversed-phase C_{18} column (Beckman Ultrasphere ODS, 250×4.6 mm, particle size, 5 μm) with a C_{18} guard column (Beckman All-Guard cartridge, ODS, 7.5×4.6 mm, 5 μm) was used at room temperature for thiol species separation. The fluorescence detector was tuned to 390 nm for excitation and a high-band-pass filter (470 nm) was used for isolation of the emission signals.

The thiol-mBBr derivatives were separated using a binary gradient of mobile phase A (0.08% TFA in aqueous solution) and B (100% acetonitrile). Both solutions were purged continuously by high purity helium before and during analysis. During conditioning of the column and prior to injection, the mobile phase composition was 8% B and 92% A. The percentage of mobile phase B was changed as follows after injection: 8–10% from 1 to 15 min; 10–14% from 15 to 20 min; 14–16% from 20 to 24 min; 16–18% from 24 to 28 min; 18–22% from 28 to 35 min; 22–35% from 35 to 40 min; 35–80% from 40 to 42 min; 80–8% from 42 to 47 min. In all steps, the gradients used were linear except for the last step in which mobile phase B was rapidly cut from 80 to 8%. Separation was carried out at a flow-rate of 1.0 ml/min except that flow was linearly increased to 1.3 ml/min from 20 to 24 min and returned to 1 ml/min from 24 to 28 min, to wash out

most of the reagent peak (mainly caused by TBP if used) before PC-2 elution. After a 15-min period to re-equilibrate the column with the initial mobile phase, the autosampler was set to inject the next sample (150–200 μl).

2.2. Fluorescence derivatization

For routine analysis, all standards were prepared in a 15 m*M* MSA solution, and field samples were stabilized by acidification with MSA to a similar level. The stock of 1 m*M* thiol standards in the MSA (15 m*M*) solution was stored at 4 °C for up to 2 months. Experiments demonstrated that frozen (−20 °C) standards are stable for at least 6 months. The working solution (1 μ*M*) was made from stocks every 2 weeks. For derivatization, 30 μl of TBP (0.5% in acetonitrile) was added to 50 ml of acidified sample or standard, and 10 min was allowed for reduction. The acidified samples or standard solutions were then neutralized by addition of 140 μl of NaOH (6 *M*), and solution pH was subsequently buffered near 8.5 by adding 1 ml of buffer containing boric acid (2 *M*), NaOH (0.8 *M*), and EDTA (10 m*M*). The addition of 120 μl of the mBBr solution (7.7 m*M* in acetonitrile) gave a concentration of 18.5 μ*M* in a 50-ml sample solution before derivatization and a mole ratio of TBP to mBBr of about 0.65. The derivatization reaction was conducted at 60 °C in the dark for 40 min before 800 μl of MSA (6 *M*) was added to terminate the reaction.

2.3. Solid-phase extraction

A Waters HLB reversed-phase cartridge (60 mg) was used to extract the thiol–mBBr derivatives from the derivatized solution. The cartridges were attached to a solid-phase extraction manifold (Supelco Visiprep), and the vacuum was maintained at less than 5 p.s.i. during the entire procedure. The HLB cartridges were conditioned with 1 ml methanol and 2 ml MSA (15 m*M*) sequentially before loading samples. Samples were suctioned through the HLB cartridges at about 2 ml/min, followed by a rinse with 2 ml MSA solution (15 m*M* with 2% methanol). Then 1 ml methanol was used to elute thiol–mBBr derivatives into a 4-ml amber glass vial, followed by evaporation to dryness using N_2. The derivatives were dissolved in a MSA solution (15 m*M*) to achieve the required concentration factor. These procedures were carried out under dim light. Experiments demonstrated that the HLB cartridge can be used up to 10 times without losing extraction efficiency. At the highest concentration level of thiol standards (20 n*M*), no breakthrough was observed with a loading volume of 100 ml.

3. Results and discussion

3.1. Chromatogram and thiol recovery

Compared with the results from direct analysis, all fluorescence signals in the HLB extracted samples are enhanced, including possible interfering peaks. Typical chromatograms are presented in Fig. 1. Chromatographic separation of standards in the NaCl solution (0.5 *M*) yielded extremely low blank signals of GSH and γEC (less than 0.03 n*M*) (chromatogram a), and fluorescence signals increased with thiol concentrations (chromatogram a–c). Chromatograms b and c represent injections of 200 μl of the concentrated solution of thiol–mBBr derivatives, in which thiol standards were originally added to a NaCl solution (0.5 *M*) to make concentrations of 0.5 n*M* and 5.0 n*M*, respectively. Chromatogram d represents an extraction from the growth medium of a marine diatom culture (see below). The solid-phase extraction step increases the sensitivity by at least a factor of 20 for all thiols, compared with that from the direct measurement [4,5]. The enhancement of fluorescence signals after concentration by solid-phase extraction enables the detection of thiols at concentrations that are otherwise undetectable in the direct measurement approach.

The thiol–mBBr fluorescence signals were detectable after solid-phase extraction at thiol levels as low as 0.1 n*M* in NaCl solution (0.5 *M*) for GSH, γEC and PC-2. Thiols at concentrations higher than 10 n*M* in solution can be measured without the solid-phase extraction procedure. The data from five independent experiments showed consistent overall percent recoveries of GSH, γEC and PC-2 from 0.5 *M* NaCl solution over concentrations ranging from

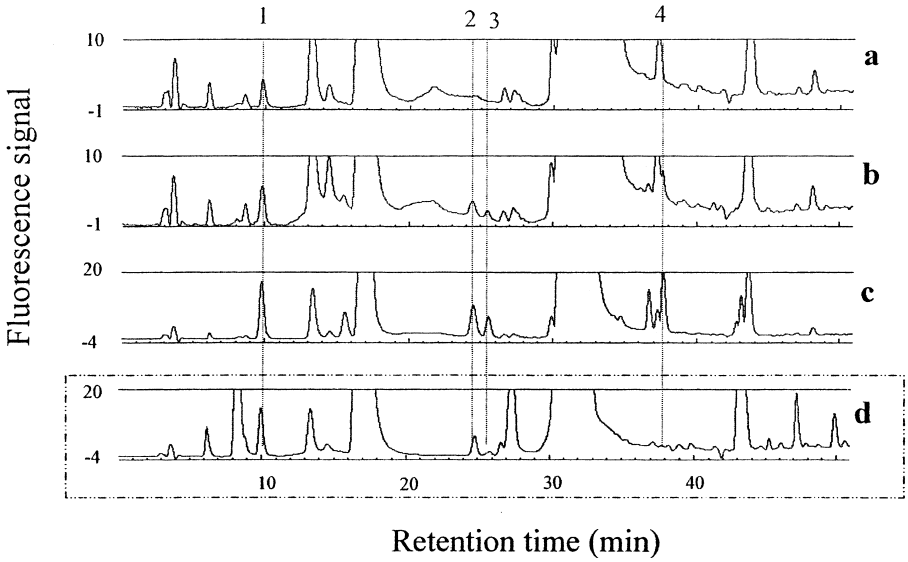

Fig. 1. Chromatograms for (a) 0 n*M*, (b) 0.5 n*M*, (c) 5 n*M* standards, and (d) the growth medium of diatom, *Thalassiosira weissflogii*. The concentration factor is 200 after solid-phase extraction. The vertical lines 1, 2, 3, and 4 represent Cys, GSH, γEC and PC-2 peaks, respectively; the number on the left indicates different scales of signals in chromatograms a and b, compared with chromatogram c and d.

0.3 to 20 n*M* with average values (±standard deviation) of 86.2 (±5.7), 76.4 (±5.1), and 74.3 (±5.0), respectively (Table 1). Although standard deviations (n*M*) increased with concentration, relative standard deviations (%) were largely independent of concentration. For PC-2, variability is largely related to its elution on the shoulder of the large TBP-related reagent peaks eluting from 30 to 35 min. Variability at higher concentrations of γEC may be caused by the close vicinity of the γEC peak to GSH, making baseline definition difficult. The proposed method is suitable for detection of nanomolar and sub-nanomolar levels of GSH and γEC. Additional optimization of the method is needed before quantification of Cys at sub-nanomolar levels can be achieved, due to its low observed recovery (<50%, data not shown) and the interference from a reagent peak (Fig. 1). However, these factors should not limit direct analyses (without pre-concentration) of Cys at concentrations higher than 10 n*M*.

Table 1
Percent recovery (*R*) and standard deviation (SD) for thiol determinations at different concentration levels in 0.5 *M* NaCl solution from five independent experiments[a]

Thiol	GSH		γEC		PC-2	
	R (%)	SD (n*M*)	*R* (%)	SD (n*M*)	*R* (%)	SD (n*M*)
0.1 n*M*	89.3	0.01	78.7	0.01	76.1	0.01
0.3 n*M*	77.3	0.01	68.8	0.02	66.3	0.03
0.5 n*M*	78.8	0.02	69.7	0.05	70.3	0.08
1 n*M*	85.9	0.05	74.3	0.10	70.2	0.15
5 n*M*	87.0	0.29	81.2	0.33	76.5	0.75
10 n*M*	92.1	0.62	80.5	1.19	79.0	1.46
20 n*M*	93.1	0.93	81.9	2.13	81.5	2.90
Average of *R*	86.2		76.4		74.3	
SD of *R*	5.7		5.1		5.0	

[a] Experiments were carried out in November and December of 2002 using one batch of HLB cartridges.

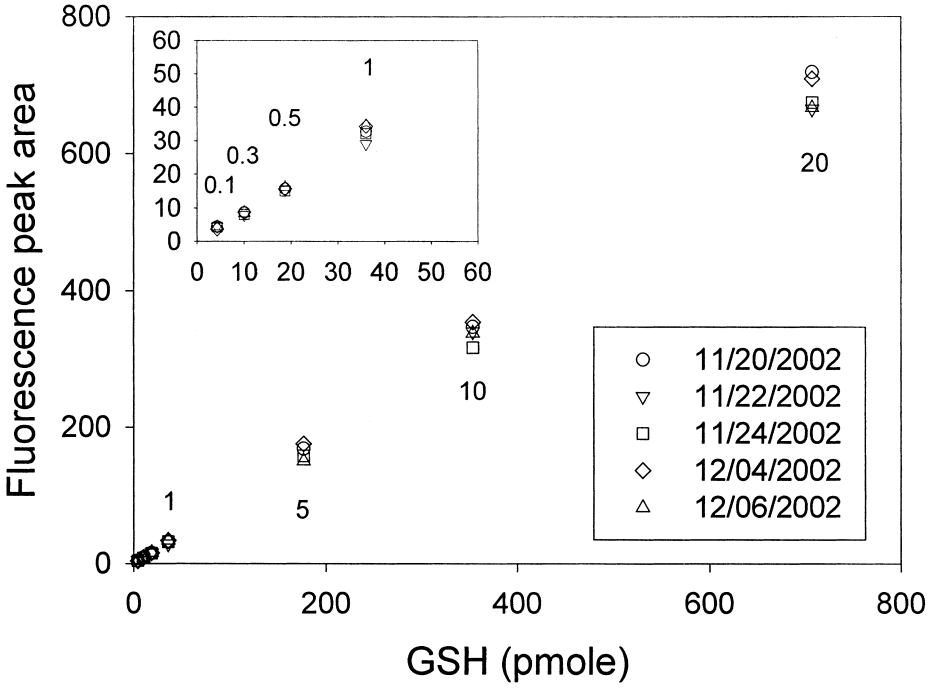

Fig. 2. GSH fluorescence signals increase with the recovery-corrected amount of GSH injected into the HPLC after solid-phase extraction. Numbers above or below the data symbols represent the GSH concentrations in original solutions.

Fluorescence intensity increased with thiol concentrations ranging from 0.1 to 20 n*M* (Fig. 2), as demonstrated from the fluorescence response for GSH plotted against the recovery-corrected GSH concentration for the five experiments described in Table 1. The inter-experimental consistency of the method is shown by the narrow range of the data points for each concentration level. Detection limits, calculated as three times the standard deviation for the lowest thiol concentration (0.1 n*M*), are 0.03, 0.03, and 0.06 n*M* for GSH, γEC and PC-2, respectively.

The precision of thiol recovery was also evaluated on subsamples of an experimental seawater medium ''Aquil'' [18] (without Zn and EDTA) in which a marine diatom *Thalassiosira weissflogii* had been grown for 30 h in the presence of Cu (120 n*M*). Average concentrations of thiols found from six separate extractions and measurements were (average±standard deviation) 2.32±0.29 (n*M*), and 0.72±0.03 (n*M*), for GSH, and γEC, respectively. PC-2 was not detected in these samples. The chromatogram for one subsample is shown in Fig. 1d. These results demonstrate the capability of the proposed method to reproducibly detect sub-nanomolar levels of dissolved GSH and γEC. In the following sections, we discuss some factors which are unrelated to pre-concentration steps, but nonetheless important for successful derivatization. These experiments were conducted at concentrations sufficiently high for direct measurements without pre-concentration using HLB cartridges.

3.2. Derivatization temperature and reaction time

Although the derivatization of thiols by mBBr is a relatively fast process [1], efficiency is temperature-dependent [9]. We found fluorescence intensity to change with reaction time and temperature (Fig. 3). At room temperature, fluorescence signals increased rapidly during the first 120 min and then changed slowly with further reaction time. At 60 °C, the reaction was essentially complete within 80 min for γEC and GSH, and even sooner for Cys and PC-2. The fluorescence signals then decreased with time. When $CuCl_2$ (400 n*M*) was also added to some

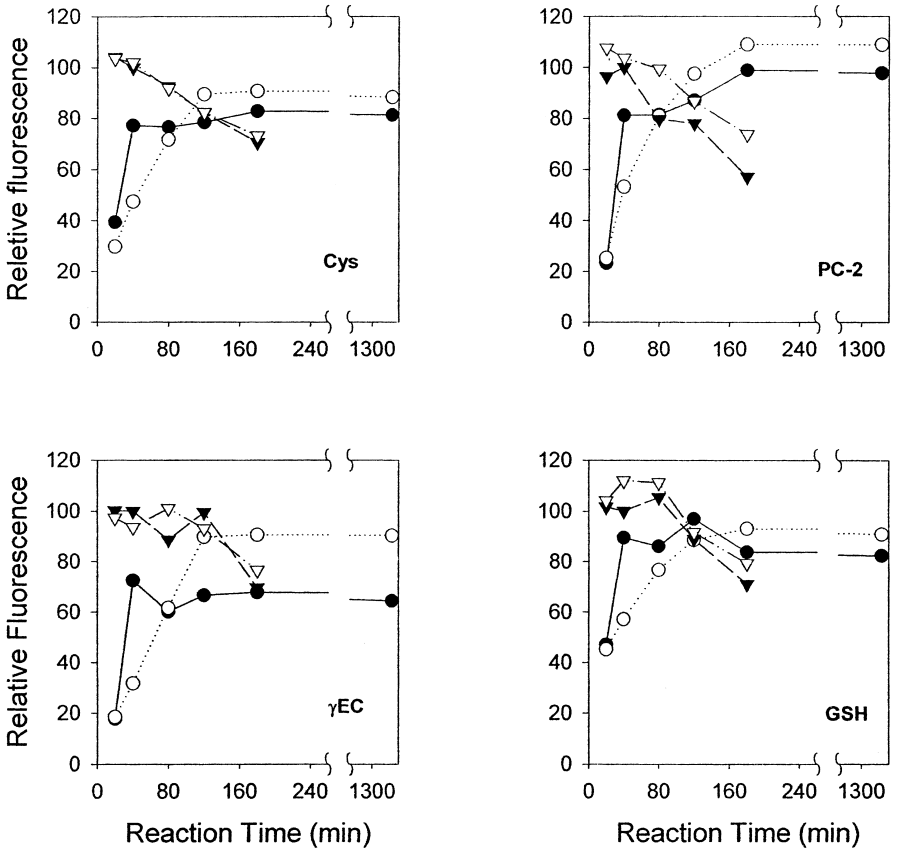

Fig. 3. Relative fluorescence changes of thiols (50 n*M* each) in NaCl solution with reaction time and temperature. The label ''circle'' represents reaction at room temperature without Cu present (''filled circle'') or with addition of 400 n*M* Cu (''open circle''). The label ''triangle'' indicates results obtained at 60 °C without Cu present (''filled triangle'') or with 400 n*M* Cu (''open triangle''). Signals for each thiol were normalized to the signal measured at 60 °C with a 40-min reaction time.

samples at concentrations twice that of the total SH group in solution, fluorescence intensity increased in samples derivatized at room temperature. However, the difference was eliminated in samples derivatized at 60 °C. These results indicate that copper has no influence on thiol-fluorescence when derivatization is performed at 60 °C and that the higher temperature is very important for complete derivatization of naturally occurred thiols (see below). In summary, derivatization is complete within a short time period and fluorescence signals are usually higher when samples are derivatized at 60 °C rather than at room temperature. Therefore, we recommend a reaction temperature at 60 °C and reaction time of 40 min for routine analysis.

3.3. Fluorescence tag

We also investigated the optimum concentration of the fluoroprobe, mBBr. In practice, mBBr should be in large excess of total thiols to insure that the fluorescence signal of each thiol is linearly related to its initial concentration. As shown in Fig. 4, fluorescence intensity increased with the amount of mBBr added. The fluorescence ratio, obtained by normalizing to the response of the lowest mBBr addition, increased dramatically at lower [mBBr]/[thiol] mole ratios for both thiol concentrations (10 and 50 n*M*) studied. The signal began to level off at [mBBr]/[thiol] ratios >200. We conclude that the amount of mBBr added should be at least 200 times more than

the total thiol concentration in samples to obtain adequate intensity and complete recovery. A higher [mBBr]/[thiol] ratio may be beneficial for samples with lower thiol concentrations, especially for the determination of PC-2 (Fig. 4).

3.4. Reducing reagent

Reducing reagents are used to reduce the disulfide bond and release the sulfhydryl group before derivatization in total thiol determinations. However, suppression of the fluorescence signal by reducing reagents has been reported [14,15]. To study the possible influence of the reducing reagent (TBP) on thiol quantification, experiments were conducted using different mole ratios of TBP and mBBr during the derivatization. Fluorescence intensities remained fairly constant (for Cys) or increased slightly (for GSH, γEC and PC-2) with an increase in the [TBP]/[mBBr] ratio up to 2.6 (Fig. 5). The intensities, however, were severely suppressed when the ratio was 5.2 or higher. Thus, careful control of the mole ratio between TBP and mBBr is critical for a successful derivatization. The reported suppression of the fluorescence signals by the reducing reagent [14] may result from the use of excessive reducing reagent. The extra solvent extraction step [15] may not be necessary if an appropriate amount of TBP is used (less than that of mBBr). Interestingly, the blank signal for Cys also decreased in the presence of TBP, a possible benefit for the direct measurement of Cys. The fluorescence signals for samples without TBP addition (points with [TBP]/[mBBr]=0 in Fig. 5) were similar to those with a proper TBP addition. This is expected because reduced forms of thiols were used in this experiment under acidic condition, and acidification prevents oxidation from thiol to its disulfide. However, in environmental samples, due to the presence of oxidative species, a reducing reagent is needed for complete derivatization (see next section).

3.5. Trace metal (Cu)

Some trace metals have a very high affinity for the sulfhydryl group, a factor which could influence the fluorescence detection of thiols [13]. In the experiments shown in Figs. 3 and 5, thiol standards containing 200 n*M* of total thiols were prepared in a NaCl solution (0.5 *M*) acidified with MSA to pH less than 2.0. An excess amount of $CuCl_2$ (400 n*M*) was also added to some samples to determine the effect of Cu on fluorescence. For reactions conducted at room temperature in the presence of reducing reagent (TBP), Cu addition resulted in a higher signal at longer reaction times (Fig. 3.); for reactions at 60 °C,

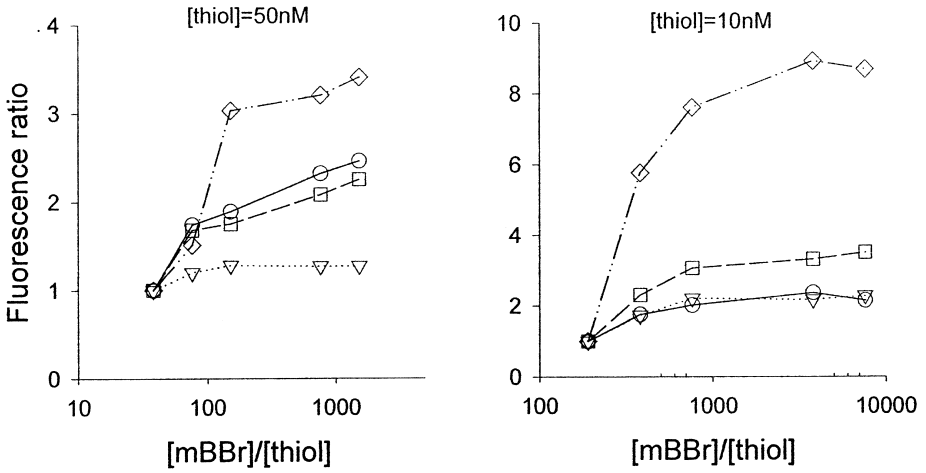

Fig. 4. Thiol fluorescence ratio changes with the mole ratio of mBBr to total thiol in solution. Fluorescence intensity is normalized to the value for the lowest mBBr to thiol ratio. The labels ‘‘circle’’, ‘‘triangle’’, ‘‘square’’, and ‘‘diamond’’ represent Cys, GSH, γEC, and PC-2, respectively.

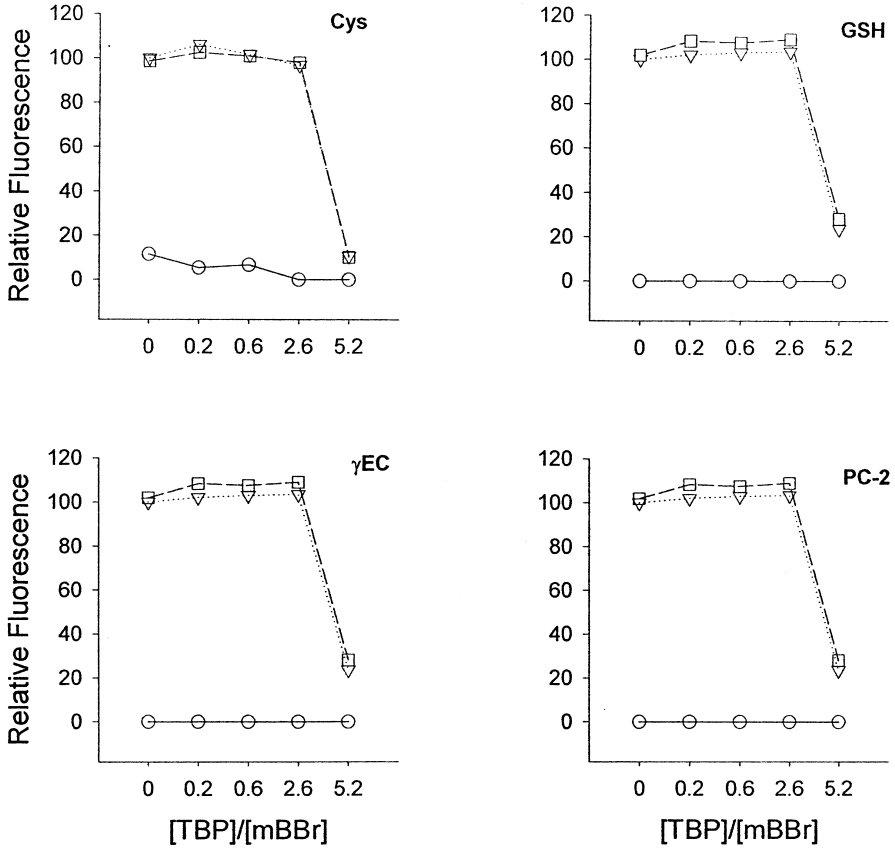

Fig. 5. Relative fluorescence of thiol–mBBr derivatives versus the mole ratio of reducing reagent (TBP) to fluorescence tag (mBBr). The labels represent normalized fluorescence intensity for blanks (''circle''), 50 n*M* thiol solutions (''triangle''), and 50 n*M* thiol plus 400 n*M* Cu solutions (''square''). Signals were normalized to the signal for a sample containing the respective thiol but neither Cu nor TBP.

Cu addition did not influence fluorescence signals, except for the slightly higher signal for PC-2. Furthermore, as shown in Fig. 5, fluorescence was not affected by Cu addition under conditions of no added TBP ([TBP]/[mBBr]=0) or when the [TBP]/[mBBr] ratio was <2.6 in these samples.

In another series of experiments, copper was spiked into a NaCl solution (0.5 *M*), containing 50 n*M* of GSH at neutral pH (without acidification), and the samples were stored for 28 h to mimic the possible reaction between GSH and Cu in natural waters after GSH release from algae. As shown in Fig. 6, the TBP suppression of the thiol-fluorescence signal occurred, at [TBP]/[mBBr]≥2.6 (''open square''), rather than [TBP]/[mBBr]≥5.2 as expected from the results shown in Fig. 5; at low [TBP]/[mBBr] rations (≤0.1), copper addition also decreased the GSH fluorescence signals. At [TBP]/[mBBr] between 0.4 and 1.6, a consistent and strong fluorescence signal of GSH was obtained. These results suggest that the optimal TBP addition level for complete derivatization of Cu–GSH complexes is at [TBP]/[mBBr] between 0.4 and 1.6. The difference in results between experiments may result from slow kinetics of the reaction between GSH and Cu(II) under acidic conditions. In the experiments shown in Figs. 3 and 5, Cu was added to samples at pH<2.0, while the results in Fig. 6 were obtained when Cu was added at neutral pH. This is another reason why natural samples should be acidified to preserve thiols before analysis [19]. At neutral pH, GSH quickly reacts with Cu by either forming Cu(II)–GSH complexes or Cu (I)–GSH complexes after reduction of Cu(II) to Cu(I) [20]. We have

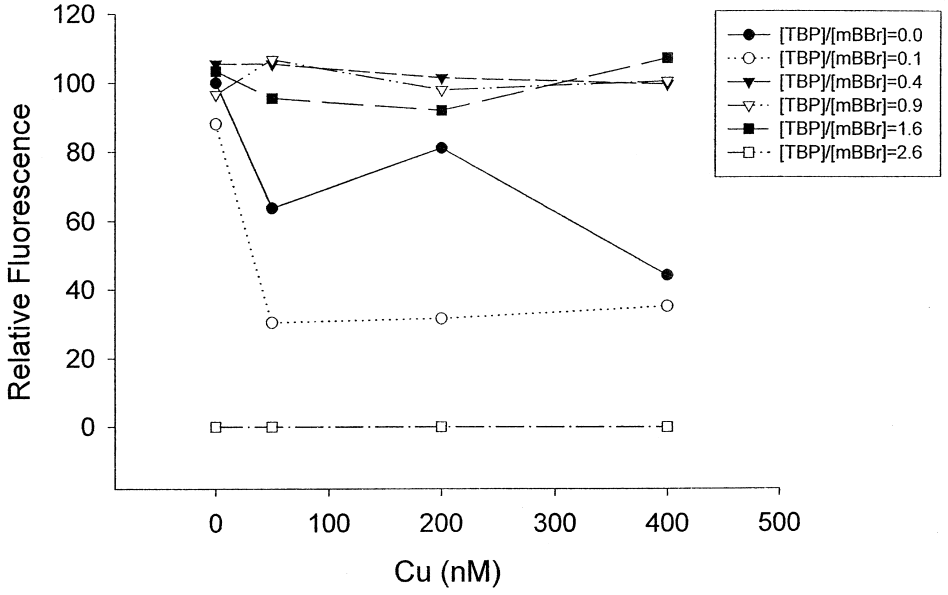

Fig. 6. Relative fluorescence for glutathione (GSH, 50 n*M*) as influenced by Cu addition at neutral pH.

demonstrated that the reducing reagent, TBP, makes the Ag(I)–thiolates accessible to a different fluoroprobe [13]; here we show that for mBBr derivatization, Cu(II)– and Cu(I)–thiol complexes will not suppress the fluorescence signal of GSH at the optimal TBP level. Thus, the ambient Cu in surface waters will not significantly influence thiol determinations, as long as the samples are preserved in acidic condition and the derivatization is conducted at 60 °C for 40 min at the optimal TBP level. Other soft metals such as Ag(I) and Hg(II) are present at levels typically less than 10 p*M* in natural waters, so their effects on thiol quantification should be minimal.

4. Conclusions

This study demonstrates the capability of a new method for detection of low levels of dissolved thiols in aqueous systems using solid-phase extraction coupled with HPLC detection. The method will significantly enhance the ability to investigate the release of dissolved thiols from algae and their role in regulating trace metal bioavailability in surface waters. The proposed method gives consistent recovery and a good linear range for the thiols studied for concentrations ranging from 0.1 to 20 n*M*. The estimated detection limits for GSH, γEC and PC-2 are 0.03, 0.03, and 0.06 n*M*, respectively. Careful control of the derivatization conditions is crucial for successful detection in both the direct and the solid-phase extraction measurements. To achieve optimum fluorescence signals, the reducing reagent, tri-*n*-butylphosphine, should be added at the optimal addition level ([TBP]/[mBBr]=0.4 to 1.6); Cu, which is present at higher concentrations than thiols in most natural waters, does not interfere with the determination of thiols under these derivatization conditions.

Acknowledgements

The work detailed in this manuscript was funded by SERDP, a partnership of US Department of Defense, Department of Energy, and the Environmental Protection Agency. Special thanks go to Bill Krick at the Wisconsin State Laboratory of Hygiene and Jerry Gipp at the Department of Pharmacology, University of Wisconsin-Madison, for providing equipment.

References

[1] R.C. Fahey, G.L. Newton, Methods Enzymol. 143 (1987) 85.
[2] R.D. Vetter, P.A. Matrai, B. Javor, J. Obrien, in: W.J. Cooper (Ed.), Acs Symposium Series: Biogenic Sulfur in the Environment, 1989, p. 243.
[3] C. Gru, P.M. Sarradin, H. Legoff, S. Narcon, J.C. Caprais, F.H. Lallier, Analyst 123 (1998) 1289.
[4] B.A. Ahner, F.M.M. Morel, Limnol. Oceanogr. 40 (1995) 658.
[5] J.W. Rijstenbil, J.A. Wijnholds, Mar. Biol. 127 (1996) 45.
[6] D.G. Tang, C.C. Hung, K.W. Warnken, P.H. Santschi, Limnol. Oceanogr. 45 (2000) 1289.
[7] A.K.M. Kabzinski, Talanta 46 (1998) 335.
[8] A. Vairavamurthy, K. Mopper, Anal. Chim. Acta 236 (1990) 363.
[9] F.E.C. Sneller, L.M. van Heerwaarden, P.L.M. Koevoets, R. Vooijs, H. Schat, J.A.C. Verkleij, J. Agric. Food Chem. 48 (2000) 4014.
[10] J.G. Lee, B.A. Ahner, F.M.M. Morel, Environ. Sci. Technol. 30 (1996) 1814.
[11] Y. Hiraku, M. Murata, S. Kawanishi, Biochim. Biophys. Acta: Gen. Subjects 1570 (2002) 47.
[12] A.C.A. Le Gall, C.M.G. Van Den Berg, Deep-Sea Res. Part I Oceanogr. Res. Papers 45 (1998) 1903.
[13] D.G. Tang, L.S. Wen, P.H. Santschi, Anal. Chim. Acta 408 (2000) 299.
[14] V. Rizzo, L. Montalbetti, M. Valli, T. Bosoni, E. Scoglio, R. Moratti, J. Chromatogr. B 706 (1998) 209.
[15] R.J. Kok, J. Visser, F. Moolenaar, D. deZeeuw, D.K.F. Meijer, J. Chromatogr. B 693 (1997) 181.
[16] R.A. Bell, J.R. Kramer, Environ. Toxicol. Chem. 18 (1999) 9.
[17] M.F.C. Leal, M. Vasconcelos, C.M.G. van den Berg, Limnol. Oceanogr. 44 (1999) 1750.
[18] N.M. Price, G.I. Harrison, J.G. Hering, R.J. Hudson, P.M.V. Nirel, B. Palenik, F.M.M. Morel, Biol. Oceanogr. 6 (1988/1989) 443.
[19] D. Shea, W.A. Maccrehan, Anal. Chem. 60 (1988) 1449.
[20] M.F.C. Leal, C.M.G. Van den Berg, Aquat. Geochem. 4 (1998) 49.