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Determination of reduced sulfur compounds by high-performance liquid chromatography in hydrothermal seawater and body fluids from *Riftia pachyptila*

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This paper describes a method for the determination of reduced sulfur compounds in hydrothermal seawater and body fluids from the hydrothermal tube worm *Riftia pachyptila*. Sulfur is a key component of the hydrothermal ecosystem based on chemosynthesis. Sulfur compounds were derivatized at pH 8 (4.3 for sulfide in biological fluid) with a fluorescent reagent, monobromobimane, and separated by reverse-phase HPLC. The eluted compounds were detected by spectrofluorimetry. This method allowed the quantitative analysis of sulfide, sulfite, thiosulfate, cysteine and glutathione in seawater, vascular blood and coelomic fluids from *R. pachyptila*. The detection limits were in the 0.1 μmol l^{-1} range with a precision lower than 10%. The method has been applied to hydrothermal seawater. The organisms are distributed along a gradient of sulfide (produced by the vent) and thiosulfate. Analysis of biological fluid was performed with a new sample treatment allowing the analysis of total sulfide (free and bound to haemoglobin) with results comparable to published methods.

Keywords: *Sulfide; sulfite; thiosulfate; cysteine; glutathione; hydrothermal seawater;* Riftia pachyptila, *biological fluids*

Deep sea hydrothermal vents are characterised by a set of unique environmental parameters. The chemical interactions between the magma source and the surrounding seawater produce strongly reducing hot waters enriched in methane and hydrogen sulfide, among others, and depleted in magnesium and oxygen.[1–3] In the absence of light, the hydrothermal food chain is based on chemoautotrophic bacteria which derive the energy necessary for the fixation of inorganic carbon from the reduced sulfur compounds present in the vent fluid and the oxygen present in deep-sea water. Moreover, the most abundant invertebrate species, *e.g.*, tubeworms, mussels and clams,[4] are actually symbiotic associations with such chemoautotrophic bacteria.

The hydrothermal vent vestimentiferan *Riftia pachyptila* probably represents the most achieved and studied example of this kind of symbiosis.[4,5] This worm is devoid of digestive tract and houses in an internal organ, the trophosome, abundant sulfur oxidising bacteria which are completely dependent on their host for their supply of inorganic compounds. Therefore, the host has evolved a specific mechanism to ensure proper acquisition and transport of H_2S, O_2 and CO_2. Acquisition of O_2 and CO_2 from the water occurs mainly through a diffusion process across the branchial plume, while sulfide apparently requires an ionic transport of HS^-[6,7] yet to be characterised. Transport of O_2 and H_2S is achieved bound to the proteins on different sites[8–10] of the circulating haemoglobin,[8] while CO_2 is mainly transported as bicarbonate dissolved in the body fluids.[11] On the other hand, the metabolic activity of the bacteria produces sulfate and protons which are either accumulated or excreted by the host. Evidence of active proton excretion exists[6,7] but nothing is known about the fate of sulfate.

Knowledge of the concentrations of the different sulfur compounds in the environment and in the biological fluids are a first and necessary step towards the elucidation of these processes. This work presents the adaptation of an analytical method published by Vetter *et al.*[12] and Fahey *et al.*[13] to analyse different sulfur components by reverse-phase HPLC after derivatization with monobromobimane. The study has been focused on the following compounds: HS^- (sulfide, or H_2S), SO_3^{2-} (sulfite), $S_2O_3^{2-}$ (thiosulfate), methanethiol, cysteine and glutathione. Polythionates and polysulfides have not been studied yet. The development was performed on the following matrices: hydrothermal seawater, vascular blood and coelomic fluid from the hydrothermal vent worm *R. pachyptila*.

Experimental

To measure sulfur compounds in biological and seawater samples, the protocol should allow the fixation of the different sulfur components to stop their reaction during the preparation and should also separate as many sulfur compounds as possible during a single assay. The principle of the method is based on the use of monobromobimane (mBBr), a fluorescent agent, which reacts selectively with thiols and reduced sulfur compounds to produce highly fluorescent derivatives as illustrated in reaction (1). These fluorescent derivatives can then be separated by reverse-phase HPLC.

$$RSH + mBBr \rightarrow mBSR + HBr \quad (1)$$

The sulfur compounds detected through such a method are therefore: sulfite, cysteine, thiosulfate, glutathione, methanethiol and sulfide. Sulfate cannot be analysed by this method but can be quantified by ionic chromatography (work under progress).

Solutions

The stock standard solutions (SO_3^{2-}, $S_2O_3^{2-}$, methanethiol, cysteine and glutathione) were prepared at a concentration of 20 mmol l^{-1}. Degassed water or seawater was prepared daily by ultrasonication under vacuum during 5 min. Sodium thiosulfate pentahydrate (Merck, Darmstadt, Germany; *pro analysi*) and anhydrous sodium sulfite (Merck) solutions were prepared in degassed water, cysteine (Aldrich, Steinheim, Germany; 97%) and glutathione (Aldrich, 98%) in a v/v mixture 90 + 10 water–acetonitrile (Panreac, Barcelona, Spain: HPLC gradient). The stock sulfide solution (about 50 mmol l^{-1}) was prepared by

dissolving rinsed $Na_2S \cdot 9\ H_2O$ crystals (Prolabo, Paris, France; rectapur) in degassed H_2O. This solution was periodically standardised following the procedure described in Grasshoff.[14] Stock standard solutions were stable for ten days under He at +4 °C; −18 °C for cysteine. Working standard solutions were prepared every day in degassed water or degassed seawater. The thiolyte reagent was obtained from Calbiochem (Los Angeles, CA, USA) and prepared at 20 mmol l^{-1} in acetonitrile (Panreac, HPLC gradient). This solution was kept frozen and was stable for one month.[12] The mobile phase was obtained with acetonitrile (Panreac, HPLC gradient) and ultrapure water (18 MΩ). The solvents were buffered with (per litre) a mixture of 4.916 g sodium perchlorate monohydrate (Panreac 99.5%) and 2.5 ml acetic acid (Panreac, HPLC gradient) and kept at constant temperature in a water bath during the analysis. The buffer solutions were prepared in 50 ml ultrapure water as follows: pH 8, 0.596 g of HEPES (Sigma, St. Louis, MO, USA; H-7523), 0.093 g of EDTA (Sigma E-5134) and sodium hydroxide 0.1 mol l^{-1} (Merck); and pH 4.27, 14 ml acetic acid 0.2 mol l^{-1} (Panreac, HPLC gradient), 6 ml anhydrous sodium acetate 0.2 mol l^{-1} (Sigma S-2889). The methane sulfonic acid (Sigma) was prepared at 25 mmol l^{-1} in water, this solution was kept for one month at ambient temperature in darkness. The 2-pyridyl disulfide solution (PDS; Aldrich 99%) was prepared at 20 mmol l^{-1} in acetonitrile and was stable for one month at +4 °C. The water used in all experiments was ion exchanged UHQ (18 MΩ, ELGA, Villeurbann, France).

Apparatus

The HPLC system comprised a Spectra-Physics Analytical Spectrasystem P2000 pump (San Jose, CA, USA) Spectra-Physics Spectrasystem AS3000 autosampler (injected volume 10 µl), a guard column (Upchurch C135B; CIL), a reverse-phase column of Spherisorb (250 × 4.6 mm; CIL S5 ODS2-250A). The detector (spectrofluorimeter; Jasco FP 920) was connected to an integrator (Spectra-Physics Chromjet SP 4400). The excitation and emission wavelengths were, respectively, 390 and 480 nm. The centrifuge was a Jouan A14.

Procedures

The different analytical procedures obtained after adaptation of the method to the analysis of seawater, biological fluids and sulfide in biological fluids are summarised in Table 1 for the elution gradients and in Fig. 1 for the sample treatment. All gradients were linear. A matrix effect was observed during the work on biological fluids causing an incomplete separation of the sulfide and blank peak. This poor resolution was overcome by modifying the elution gradient.

Table 1 Elution gradients used for the analysis of reduced sulfur compounds in seawater and biological fluids. The flow rate was 1 ml min^{-1}

Sample	Time/min	Acetonitrile (%)	Water (%)
Seawater (A)	0	10	90
	10	30	70
	18	75	25
Biological fluid (B)	0	10	90
	10	30	70
	19	55	45
	21	40	60

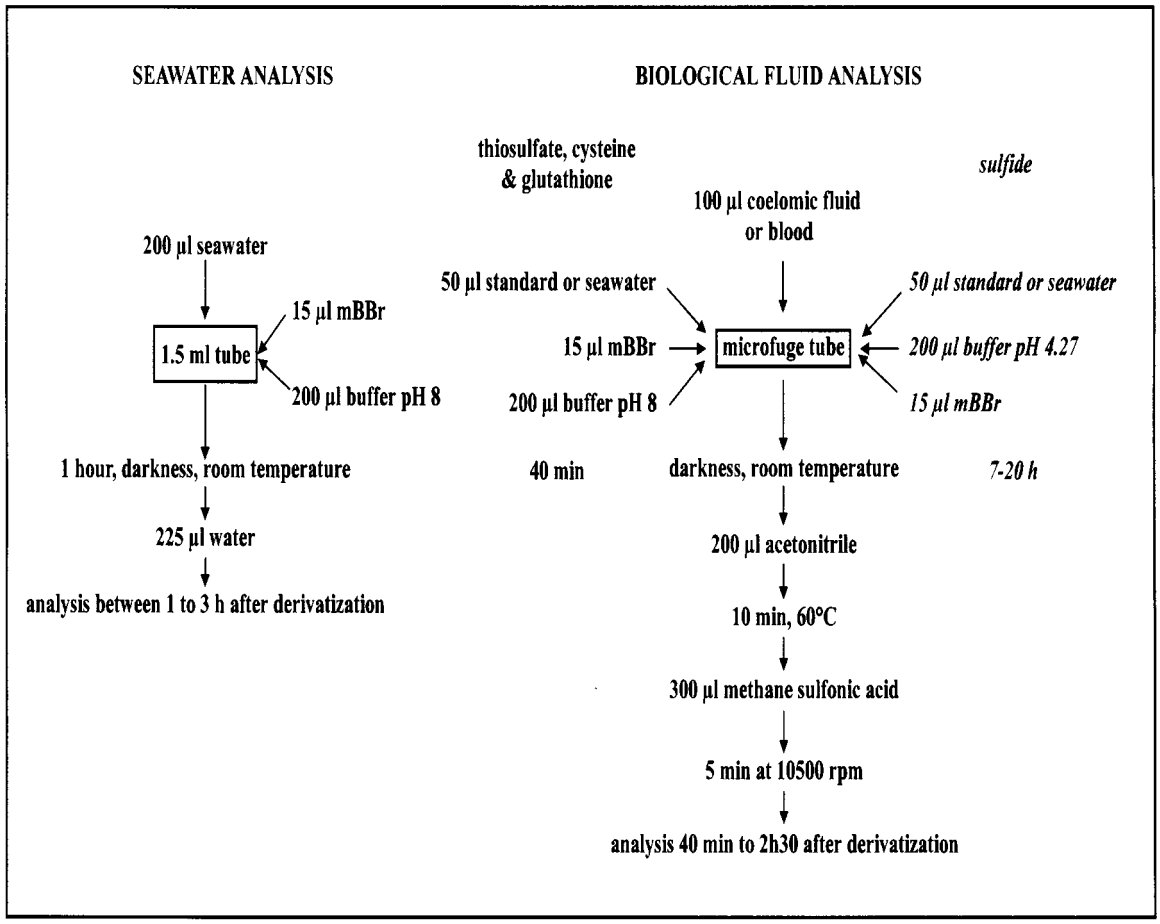

Fig. 1 Flow chart of analytical procedures for seawater and biological fluids analysis.

The volume of reagent added was tested (10, 15 and 25 μl) on a mixture of 1 mmol l^{-1} of reduced sulfur compounds. An addition of 15 μl of the 20 mmol l^{-1} thiolyte reagent (mBBr) was in sufficient excess to ensure the complete derivatization of 1 mmol l^{-1} of reduced sulfur compounds. Larger volumes increased the reaction rate but also the cost of an analysis. The derivatization reaction is completed after 1 h for seawater (in 1.5 ml glass flask), 40 min for biological fluids (in 1.5 ml microfuge tube) in darkness. The buffering of the solution at pH 8 where the RS^- form is predominant was necessary to have a faster reaction rate. The analysis of sulfide in biological fluid was the exception with a pH of 4.7–4.8 leading to a slower reaction rate (6 h). The adducts formed were stable for at least 20 h. The acetonitrile precipitated the proteins present in the biological fluid while denaturation of the enzymes was obtained at 60 °C during 10 min. The methanesulfonic acid stabilised the mBBr adducts and the precipitated proteins were removed by centrifugation. Each analysis should include a reagent blank to identify peaks arising from the reagents. A second blank was also prepared by reacting the reduced sulfur compounds and thiols present in the sample with PDS prior to derivatization. This reagent forms stable derivatives that will no longer react with bimane. This blank indicated any fluorescent non-sulfur component that may give a chromatographic peak unrelated to the bimane derivatization.[12]

Sulfide analysis

The haemoglobin of *Riftia pachyptila* functions as an oxygen and sulfide carrier. Sulfide is transported reversibly bound to the haemoglobin and is stabilised against spontaneous oxidation.[8,15,16] Sulfide binding is strongly dependent on the pH as demonstrated by Childress *et al.*[17] with a sulfide binding maximum at pH 8 and a decrease of the bonding for higher and lower pH. Working at pH 8 implies analysing only the unbound sulfide, the sulfide added during the calibration being possibly bound to the remaining free sites giving erroneous results.

To analyse total sulfide, it is therefore necessary to preliminarily release the fraction bound to the haemoglobin by decreasing the pH (see Fig. 2). The maximum peak area corresponding to the maximum amount of released sulfide was obtained for a pH between 4.5 and 5. The working buffer was chosen at 4.3 leading to a pH measured in the sample of 4.8. This decrease in pH in the reaction mixture caused a decrease in the derivatization rate. At pH 4.3, the plateau was attained for a reaction time of 6 h and was stable up to 20 h.

Sampling and sample treatment

Water sampling was done during the HOT 96 French American cruise (9° and 13° N East Pacific Rise, February–March 1996) on the N/O Nadir hosting the submersible Nautile. Water sampling was made at several points including the smokers, the animal communities and the surrounding cold seawater. A new device conceived by Caprais and Sarradin[18] based on evacuated Teflon coated bottles was used for the first time to collect fluid samples (150 ml) in the vicinity of the animals. Titanium syringes (750 ml)[1,19] were used for the sampling of hot vent fluid.

Water samples were derivatized (200 μl water, 15 μl mBBr, 200 μl buffer pH 8) on board and stored at −20 °C until analysis.

Biological samples used in this study came from various cruises (HERO 91 and HERO 92) on the East Pacific Rise (9 and 13° N). Biological fluids were sampled on board from animals recovered from the bottom by the submersible, kept frozen (−80 °C) immediately after sampling, derivatized and analysed later in a shore-based laboratory.

Results and discussion

Performance of the method

The working range was 1 mmol l^{-1} total sulfur compounds. The linearity for each compound has been verified up to 800 μmol l^{-1} on two ranges: 0–150 and 150–800 μmol l^{-1} by plotting calibration curves for each compound (duplicate injections of 0, 50, 100, 150, 200, 400 and 800 μmol l^{-1} in water) and following the method presented by Huber[20] (at ± 5%). Performances of the method are presented in Table 2. The chromatograms obtained are shown in Fig. 3.

Quantitative analysis

Daily calibration in seawater was performed using two standard mixtures (0, 50, 100, 150 μmol l^{-1}): a standard mixture containing sulfite, cysteine, thiosulfate and glutathione on the one hand, a standard solution of sulfide on the other hand (sulfide is very unstable and is rapidly oxidised into thiosulfate and sulfite). This method using linear fitted calibration curves gave equivalent results within the ± reproducibility range and is less time and sample consuming than the standard addition method for the analysis of seawater and biological fluids. The

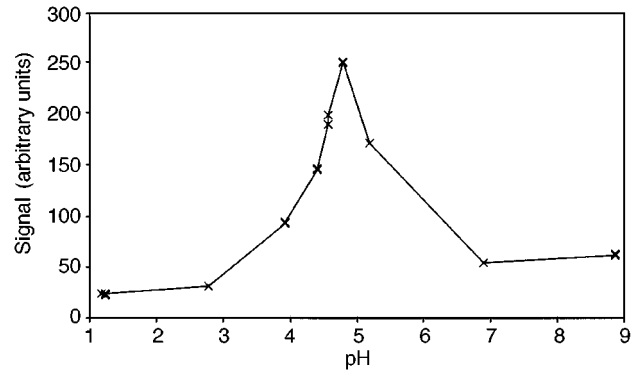

Fig. 2 Influence of the pH on the sulfide released from the haemoglobin in coelomic fluid. The *y* axis represents the area of the sulfide peak obtained.

Table 2 Performance of the method in seawater and in coelomic fluid

	Seawater					Coelomic fluid			
Parameter	SO_32-	Cys	$S_2O_3^{2-}$	Glu	HS^-	Cys	$S_2O_3^{2-}$	Glu	HS^-
D.L./μmol l^{-1}*	0.03	0.7	0.03	0.01	0.4	†	†	†	†
Repeatability (%)‡	2.5	0.20	1.1	0.2	1.1	1.2	0.5	2.9	0.5
Reproducibility (%)§	4.6	9.1	3.5	3.3	4.7	8.1	4.4	7.2	5.0
R_t/min	3.3	5.2	5.6	6.7	14.9	5.0	5.8	8.3	19.7

* D.L., detection limit, IUPAC norm (3 × standard deviation of 20 blanks × slope of the standard curve). † Sulfur compounds were present in the working matrix; the determination of the detection limit was therefore not possible. ‡ Repeatability, five injections of the same standard (100 μmol l^{-1}) by the same operator in the same conditions. § Reproducibility, injections of five identical standards (100 μmol l^{-1}) by the same operator in the same conditions.

quantitative analysis of sulfide in biological fluids requires the standard additions method.

Sulfite in biological fluids

Fig. 4 shows a typical curve obtained after a standard addition procedure of sulfite in coelomic fluid. The peak areas increase only after an addition of 50 to 100 μmol l^{-1}. Sulfites are unstable sulfur compounds and can react with the oxygen present in the biological fluids at ≈ 2 mmol l^{-1} in haemoglobin-containing air saturated coelomic fluid; 3.5 mmol l^{-1} in blood.

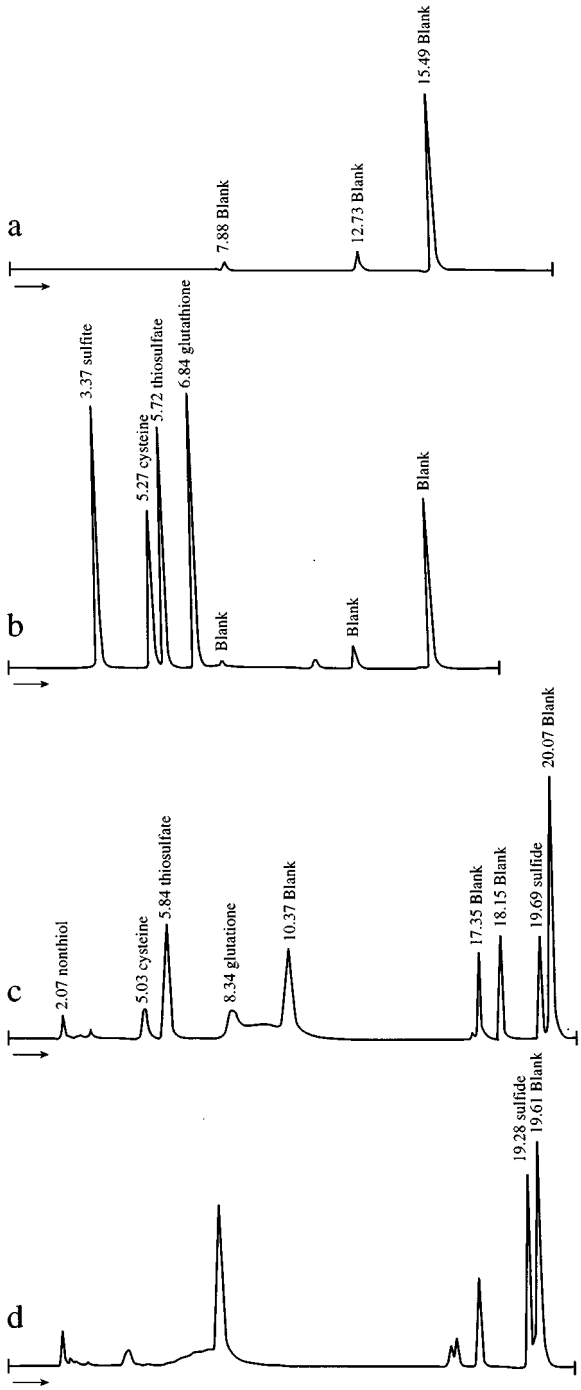

Fig. 3 (a) Chromatogram of a reagent blank, elution gradient A, 1 ml min^{-1}; (b) chromatogram of a 100 μmol l^{-1} standard mixture (sulfite, cysteine, thiosulfate, glutathione) in seawater, elution gradient A, 1 ml min^{-1}; (c) chromatogram of coelomic fluid spiked with a 50 μmol l^{-1} standard mixture (cysteine, thiosulfate, glutathione), elution gradient B, 1 ml min^{-1}; (d) Chromatogram of coelomic fluid spiked with 50 μmol l^{-1} sulfide, elution gradient B, 1 ml min^{-1}.

Storage after derivatization

After centrifugation, the samples were frozen at −18 °C until analysis. A study on frozen samples spiked with 50 μmol l^{-1} of cysteine, thiosulfate and glutathione and 25 μmol l^{-1} of sulfide does not result in any loss of analyte within the ± reproducibility range after two months. This result is also stated in Vetter *et al.*[12] who found no significant loss after up to 20 months.

Applications

Seawater

Seawater sampled during the HOT96 cruise around the vent organisms was analysed. The results obtained are presented in Table 3 and highlight the different concentration ranges observed for each biological community. The organisms are distributed along a gradient of sulfur compounds: sulfides are produced by the hydrothermal vent and the oxidised compounds appear during the dilution of the hydrothermal fluid by the surrounding seawater. Traces of cysteine and glutathione were observed in some samples among the organisms (0.1–1.3 μmol l^{-1}). These results will need to be compared with the concentrations in the biological fluids to find if the distribution of the organisms along the chemical (sulfide) gradient is a function of the sulfur compounds concentrations.

Biological fluids

The method was applied to several samples of *Riftia pachyptila* (blood and coelomic fluid) sampled during HERO 91 and HERO 92 on the East Pacific Rise (9 and 13° N). These values are indicative as the conservation method (frozen at −80 °C just after sampling, derivatised and analysed on shore) had not been established. However, the ranges obtained are identical to those

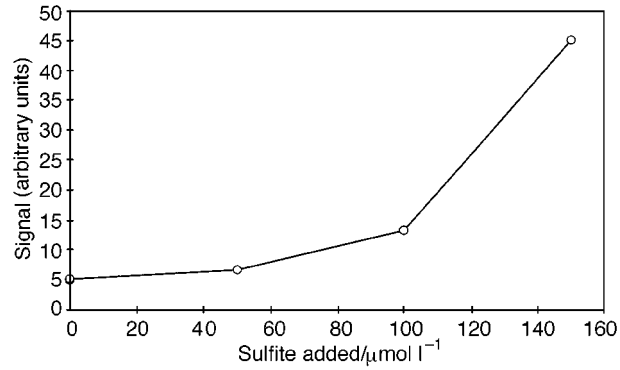

Fig. 4 Standard additions of sulfite in coelomic liquid.

Table 3 Analysis of sulfur compounds in water samples from the East Pacific Rise, 9 and 13° N, HOT 96 cruise

Sample location	Sulfite/ μmol l^{-1}	Thiosulfate/ μmol l^{-1}	Sulfide/ μmol l^{-1}
Vent	1.6 ± 1	26 ± 1	160 ± 30
Among *Alvinella* spp.	2 ± 2	14 ± 7	66 ± 105
Among *Riftia pachyptila*	1 ± 1.6	7 ± 9	24 ± 37
Among mussels	0.2 ± 0.3	1.6 ± 2	1.3 ± 1.2
Seawater	> D.L.†	0.2 ± 0.3	> D.L.

* Analysed by colorimetry.[21] † D.L., detection limit.

Table 4 Concentrations of sulfur compounds in biological fluids of *Riftia pachyptila*

Sample		Sulfide/ μmol l^{-1}	Thiosulfate/ μmol l^{-1}	Cysteine/ μmol l^{-1}	Glutathione/ μmol l^{-1}
Coelomic fluid	Sample 6.6	32	8	< D.L.*	< D.L.
	Sample 2.4	114	48	2	1
Blood	Sample 17.2	370	16	7	< D.L.
	Sample 17.4	338	124	5	< D.L.
Coelomic liquid	Childress *et al.*[15]	89 ± 87	13 ± 17	na†	na
Blood		714 ± 332	24 ± 34	na	na

* D.L., detection limit. † na, not analysed.

found by Childress *et al*.[15] (see Table 4) and reflect the ability of the worm's haemoglobin to bind and transport high sulfide concentrations to its endosymbiont.

Conclusion

The method has been applied to the quantitative analysis of sulfur compounds in seawater, blood and coelomic liquid from *R. pachyptila*. The detection limits were in the 0.1 μmol l^{-1} range with a repeatability and reproducibility (precision) lower than 10%. The new procedure allowed the analysis of the total sulfide concentrations in the biological fluids (free and bound to the haemoglobin) with results comparable to the few presented in the literature. The method has been applied to hydrothermal seawater and biological fluids. This work is a first step toward the study of the S cycle in the hydrothermal ecosystem. The following step will be the simultaneous sampling of organisms and surrounding seawater to determine the interactions of fluid and organisms. The method will later be applied to other biological organisms present in the hydrothermal ecosystem such as Alvinellids, mussels and crabs.

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