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Short communication

Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high-performance liquid chromatography methods[1]

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Abstract

Depending on the sulfur species, picomoles of different inorganic sulfur compounds can be detected and separated by HPLC in one arrangement in a sample volume less than 50 μl. The combination of fluorescence labelling of reduced inorganic sulfur compounds such as sulfide (S^{2-}), sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$) with monobromobimane followed by an extraction of elemental sulfur (S^0) by chloroform treatment enables the detection of all mentioned sulfur compounds as well as sulfate (remaining aqueous phase) in the same sample. While the derivatized sulfur compounds could be detected by their fluorescence emission at 480 nm, elemental sulfur is identified by its UV absorption at 263 nm. Sulfate in the remaining aqueous phase is detected by HPLC with indirect UV detection at 254 nm. Detection ranges for the different sulfur compounds examined are as follows: sulfide (5 μ*M* to 1.5 m*M*), sulfite (5 μ*M* to 1.0 m*M*), thiosulfate (1 μ*M* to 1.5 m*M*), elemental sulfur (2 μ*M* to 32 m*M*) and sulfate (5 μ*M* to >1 m*M*).

Keywords: Sulfur compounds; Inorganic anions

1. Introduction

The sulfur cycle in nature fundamentally consists of different reduced and oxidized inorganic sulfur compounds occurring in different oxidation states from −2 (sulfide) to +6 (sulfate). Especially phototrophic bacteria are able to use sulfur compounds as electron donors for an anoxygenic photosynthesis in which the sulfur moiety is oxidized [9,10,12]. Determination of oxidized sulfur compounds in small quantities turned out to be very difficult. In particular, the detection of non-polar elemental sulfur, which often occurs in sulfidic water samples, is not easy in polar aqueous phases [13]. Because of the relatively high sulfate content (~0.2%) in sea water, it is difficult to determine the amount of sulfate produced by bacterial oxidation of reduced sulfur compounds using common analytical methods (e.g., turbidometric determination as described by Dodgson [4]).

The classical detection methods for different sulfur components require either the use of highly toxic components such as cyanide for the detection of thiosulfate [14] and elemental sulfur [1], or large sample volumes (e.g., for sulfate [2,3], see also Ref. [15]). Sufficient amounts of different sulfur inter-

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[1] Dedicated to Prof. Dr. Hans Trüper on the occasion of his 60th birthday.

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mediates are not always available from natural environments for sulfur analysis because of rapid bacterial and chemical sulfur transformations. Thus, it is necessary to use more modern and highly sensitive methods. Chromatographic separation and detection methods were developed for several sulfur components. However, not all of the sulfur oxidation states could be detected in one sample arrangement at the same time. Fahey and Newton [5], Fahey et al. [6,7] and Reed et al. [11] have shown that it is possible to detect low molecular thiols in complex mixtures after derivatization of the samples with monobromobimane using fluorescence detection. By using the monobromobimane derivatization procedure, Vetter et al. [15] succeeded in detecting only the reduced sulfur compounds S^{2-} (sulfide), SO_3^{2-} (sulfite) and $S_2O_3^{2-}$ (thiosulfate) by HPLC in marine environments. The detection of sulfate in the same sample was only possible by the application of radioactive ^{35}S isotopes. Elemental sulfur was never detected by this combination of radiolabeled sulfur and the bimane HPLC method. Since elemental sulfur does not react with monobromobimane, this sulfur compound is only detectable by its absorption at 263 nm, as reported by Beffa et al. [2].

A combination of several HPLC methods presented in this study now offers the possibility to determine traces of sulfur compounds in very small samples using a relatively low amount of material and time. By these methods, all sulfur compounds – from sulfide beyond elemental sulfur up to sulfate – could be detected in the same sample without using radiolabeled sulfur.

2. Experimental

2.1. Chemicals

Monobromobimane (3,7-dimethyl-4-bromomethyl-6-methyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione) was purchased from Sigma (Deisenhofen, Germany) (B 4380). Acetonitrile and methanol were obtained as HPLC-grade from Merck (Darmstadt, Germany). Concentrated acetic acid was diluted to 0.25%, adjusted to pH 4.0 by adding 1 *M* NaOH and filtrated through a 0.45-μm PTFE filter (Sartorius; 1180650 N).

Boric acid, phthalic acid (Merck each) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mmol/l; EDTA 5 mmol/l) were adjusted to pH 8.0 by adding 1 *M* HCl and filtrated as described above.

Chloroform was purchased from Fluka (Neu-Ulm, Germany).

Elemental sulfur (Riedel-de Haen, Seelze, Germany, No. 13803), sodium thiosulfate (Merck, No. 6512), sodium sulfite (Merck, No. 6657) and sodium sulfide (Merck, No. 6638), analytical-reagent quality each, served as standard compounds for calibration.

2.2. Sampling of Baltic Sea water for sulfur determination

Samples of Baltic Sea water (50 μl) were taken with an Eppendorf micropipette from the sampling sites near Hiddensee island (shallow coastal area, Germany) and directly added to 210 μl of the derivatization mixture (see Section 2.3).

After stopping the derivatization process (see Section 2.3), the samples were frozen in liquid nitrogen in a Dewar vessel and stored at −20°C until used for sulfur analysis.

2.3. Monobromobimane derivatization and chloroform extraction

Derivatization of the samples was performed in Eppendorf caps as described by Newton et al. [8]. A 50-μl volume of the sample was added to 210 μl of the previously prepared derivatization mixture, composed as follows: 50 μl of HEPES buffer (50 mmol/l, 5 mmol/l EDTA; pH 8.0), 50 μl acetonitrile and 10 μl monobromobimane (48 mmol/l in acetonitrile). Derivatization was performed in the dark and was stopped after 30 min by adding 100 μl of methanesulfonic acid (65 mmol/l).

Elemental sulfur was extracted from the monobromobimane treated samples with chloroform. For this procedure, 50 μl chloroform (pure) were added to the same volume of the derivatized monobromobimane sample and mixed by shaking, until no yellow color was visible in the aqueous phase anymore. The aqueous phase was removed with an Eppendorf micropipette and served for sulfate detection by HPLC with indirect UV detection (see

Section 2.6). The remaining chloroform phase was used for elemental sulfur detection by reversed-phase HPLC (see Section 2.5).

2.4. HPLC separation

HPLC separations of low-molecular-mass thiols in the derivatized samples were performed with a Merck–Hitachi system, consisting of an L-6210 intelligent pump, connected to an F-1050 fluorescence detector with excitation at 380 nm and emission at 480 nm. Separations were performed on a reversed-phase-column LiChrospher 60, RP select B (125-4; 5 μm), heated up to 35°C in an L-7350 LaChrom column-oven. Flow-rate was 1 ml/min.

The eluent system was: (A) acetic acid (0.25%, pH 4); (B) methanol (100%, v/v) (LiChrosolv, Merck).

The elution protocol was as follows:

0–7 min	88% A, 12% B isocratic
7–15 min	12–30% B linear gradient
15–19 min	30% B isocratic
19–23 min	30–50% B linear gradient
23–30 min	50–100% B linear gradient
30–33 min	100% B isocratic
33–33.1 min	100 – 12% B linear gradient
33.1–36 min	12% B isocratic (column regeneration)

Calibrations were performed with standard solutions of sodium thiosulfate (1.5 m*M*), sodium sulfite (1 m*M*) and sodium sulfide (130 m*M*, pH 7.4). Calibration curves were obtained by plotting the integrated peak area against increasing concentrations of thiosulfate (0–1.5 m*M*), sulfite (0–1 m*M*) and sulfide (0–1.5 m*M*).

The retention times were 2.38 min for sulfite, 5.08 min for thiosulfate and 24.55 min for sulfide.

2.5. Detection of elemental sulfur in the chloroform phase

The hydrophobic elemental sulfur was detected by reversed-phase HPLC and UV absorption at 263 nm as described by Beffa et al. [2].

Analysis of elemental sulfur (S°) was carried out with a Merck–Hitachi HPLC system using a LiChrospher 100, RP 18 column (125-4; 5 μm) and an isocratic solvent of methanol–water (20:1). Flow-rate was 1 ml/min and detection was at 263 nm in a Merck–Hitachi-L-4250 UV–Vis detector. The column was heated up to 35°C in an L-7350 LaChrom column-oven (Merck). Injection volume into the Rheodyne valve totalled 50 μl (volume of the sample-loop: 25 μl).

A calibration curve was obtained by plotting the integrated peak area against increasing sulfur concentrations (0–32 m*M*) in chloroform. The standard solution was prepared by dissolving commercial elemental sulfur (Riedel-de Haen) in pure chloroform to a concentration of 32 mmol/l. The retention time for S° under these conditions was 4.82 min.

2.6. Determination of sulfate in the polar phase after chloroform extraction

Sulfate was detected by HPLC with indirect UV detection in the polar phase after chloroform extraction of the bimane-derivatized sample. A 50-μl volume of the polar aqueous phase was injected using a Rheodyne valve (sample loop volume: 25 μl). Separation of anions was performed on a Polyspher IC AN-1 anion-exchange column (Merck). Indirect UV was measured at 254 nm in a Merck–Hitachi-L-4250 UV–Vis detector. An L-6210 intelligent pump connected to the UV–Vis detector was used. The flow-rate for the isocratic eluent [1.5 m*M* phthalic acid, 1.38 m*M* tris(hydroxymethyl) aminomethane, 300 m*M* boric acid, pH 4.0] was 1.3 ml/min.

Calibration was carried out several times ($n=3$) with dilutions of a $MgSO_4$ standard (312 μ*M* SO_4^{2-}) in the range of 0 to 312 μ*M* sulfate. The retention time for SO_4^{2-} under these conditions was 16.42 min.

3. Results and discussion

Calibration of the HPLC system for the determination of different sulfur compounds in one sample arrangement was performed with standard solutions

of commercial sulfur substances. Contamination of the purchased substances by other sulfur compounds were taken into account when the concentration of sulfur components to be analyzed were calculated. Figs. 1–3 show representative chromatograms obtained by the analysis of water samples from the Baltic Sea using the separate HPLC methods and the combined method for detection of different sulfur compounds.

Fig. 4 simply shows the combined detection methods for different sulfur compounds using only 50 μl of sample.

Table 1 summarizes the retention times, correlation coefficients for the calibrations and detection

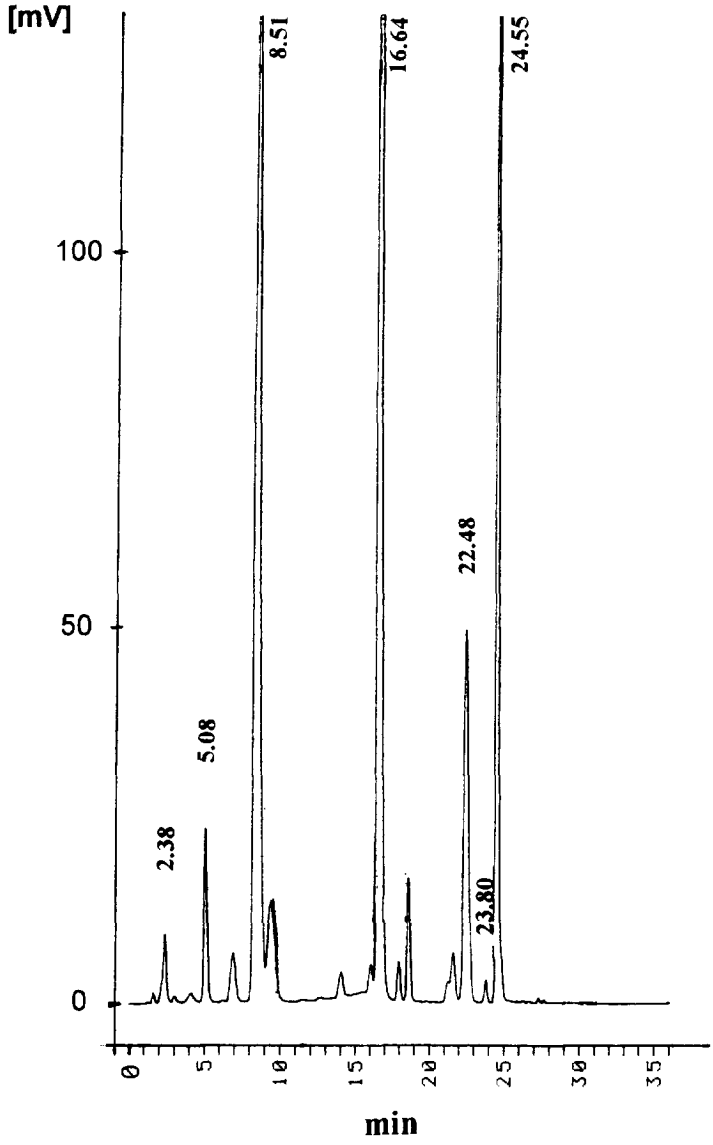

Fig. 1. Typical chromatogram of a water sample (50 μl) from the Baltic Sea obtained by fluorescence detection after monobromobimane derivatization. The retention times at 8.51 min, 9.42 min, 16.64 min and 22.48 min refer to non-thiol peaks (for reference see Ref. [3]). Retention times at 2.38 min (sulfite, 9.1 μ*M*), 5.08 min (thiosulfate, 23.3 μ*M*), 23.80 min (polysulfide, quantification not possible) and 24.55 min (sulfide, 190 μ*M*) refer to sulfur compounds found in the Baltic Sea water sample.

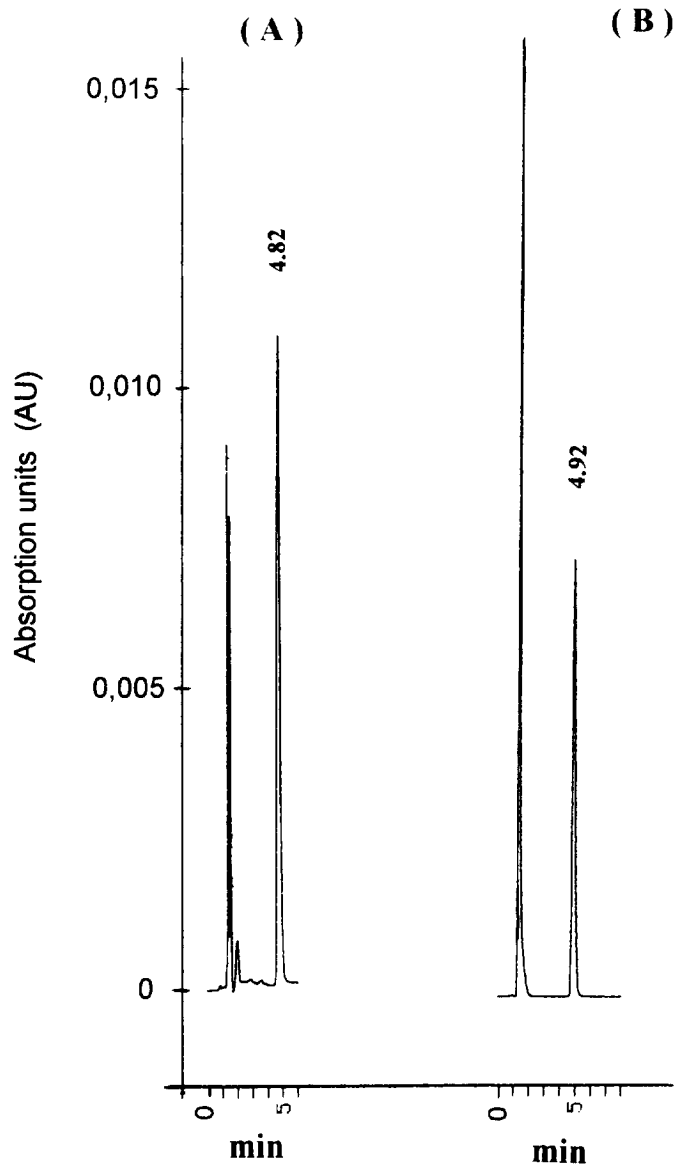

Fig. 2. Typical chromatograms of an elemental sulfur containing standard (A) and an elemental sulfur containing sample (50 μl) from the Baltic Sea (B) obtained by UV detection at 263 nm using the hydrophobic phase of a chloroform extraction of the previously bimane-derivatized sample. The peaks at 4.82 min (A) and 4.92 min (B) refer to elemental sulfur (780 μ*M* or 633 μ*M*, respectively) in the samples.

limits for different inorganic sulfur compounds. No significant differences were observed in the calibration curves with and without chloroform treatment of a sulfate standard solution.

The chloroform treatment of the derivatized bimane samples had no influence on the detection of elemental sulfur or sulfate within the same sample. Neither the application of a given sulfate concentration as standard before or after chloroform extraction nor the bimane derivatization led to significant variations in the concentrations of the substances to be detected. Also, the detection of elemen-

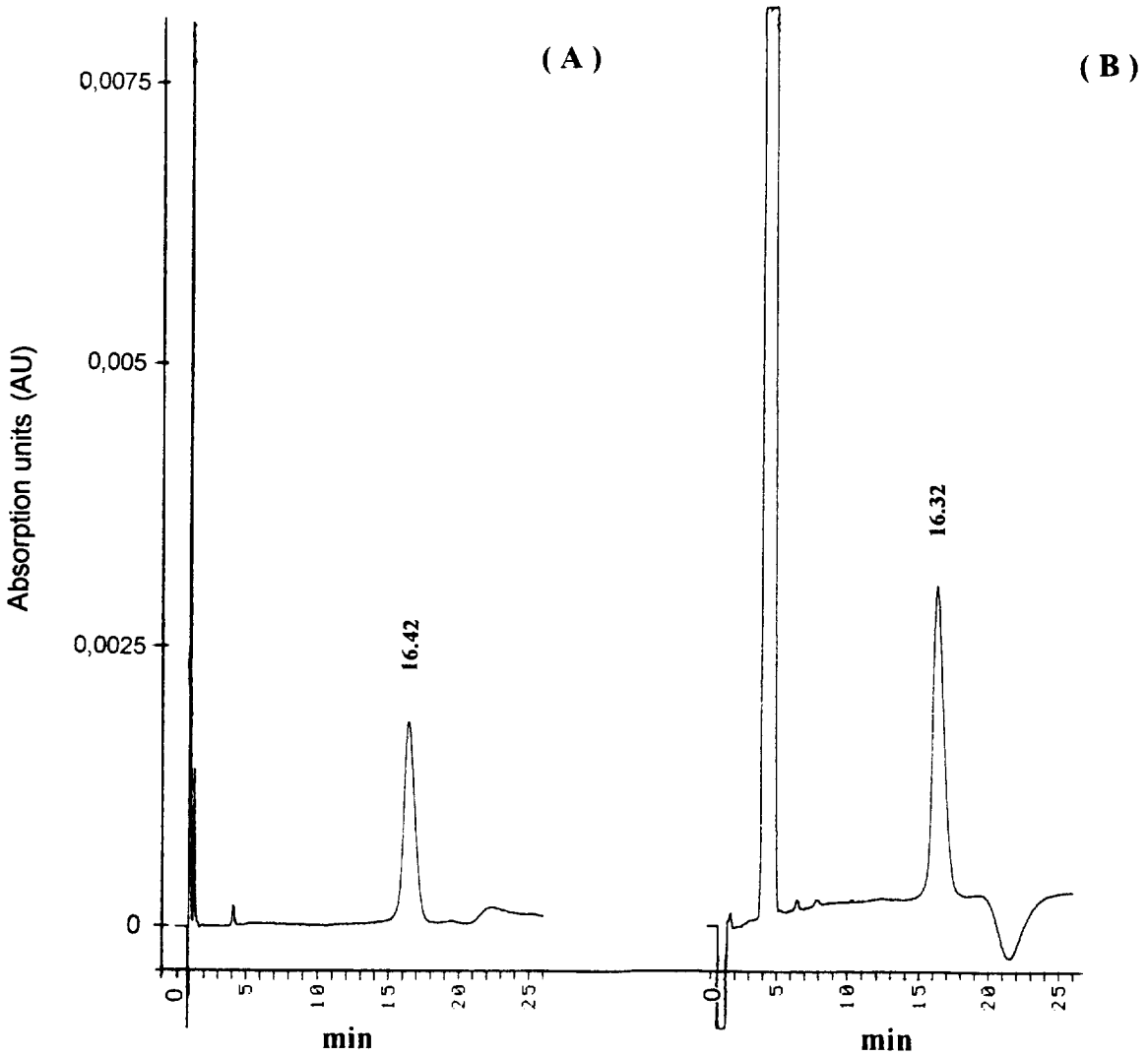

Fig. 3. Typical chromatograms of an aqueous sulfate containing standard (A) and of a sulfate containing sample (50 μl) from the Baltic Sea (B) after chloroform extraction of the previously monobromobimane-derivatized sample obtained by indirect UV detection at 254 nm. The measurement of (B) was performed with the polar aqueous phase. The peaks at 16.42 min (A) and 16.32 min (B) refer to sulfate (312 μ*M* or 391 μ*M*, respectively) in the samples.

tal sulfur was not disturbed by the previous bimane treatment.

The combination of several methods for the detection of inorganic sulfur compound traces in small sample volumes (<50 μl) by using HPLC offers an important advantage for microbiological studies concerning sulfur metabolism and the analysis of pore water in marine sediments. The described procedure now enables a precise and quick detection of reactive intermediate sulfur compounds such as sulfite or sulfide, formed by bacteria during turnover of sulfide to sulfate or vice versa, before theses intermediates interfere with each other or before they are chemically oxidized. It is not necessary to analyze the chloroform or bimane treated samples immediately, because storage at −20°C for several

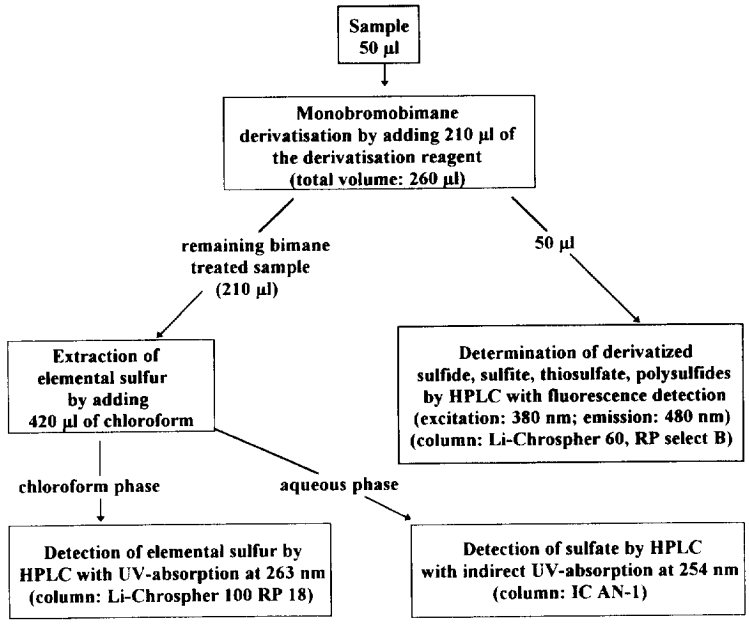

Fig. 4. Simple scheme for preparation, separation and detection of different inorganic sulfur compounds in small sample volumes.

months caused no significant loss of the sulfur compounds in concentrations or any chemical oxidations.

4. Conclusions

The combination of the different HPLC methods presented here is useful for the simultaneous determination of oxidized and reduced sulfur compounds in a very small sample volume. The chloroform treatment of bimane derivatized samples had no effect on the determination of elemental sulfur and sulfate in the non-polar or polar phases, respectively.

Acknowledgments

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Table 1
Calibration data for different inorganic sulfur compounds performed with commercial sulfur compounds (analytical-reagent quality) using standards of sodium sulfite (1 m*M*), sodium thiosulfate (1.5 m*M*), sodium sulfide (130 m*M*, pH 7.4), sodium sulfate (312 µ*M*) and elemental sulfur (32 m*M*)

Sulfur compounds	Retention time	Correlation coefficient	Range tested	
			Lower limit	Upper limit
Thiosulfate	5.08[a]	0.99998	1 µ*M*	1.5 m*M*
Sulfite	2.91[a]	0.99974	5 µ*M*	1.0 m*M*
Sulfide	24.55[a]	0.99998	5 µ*M*	1.5 m*M*
Elemental sulfur	4.92[b]	0.9997	2 µ*M*	32 m*M*
Sulfate (without chloroform extraction)	16.42[c]	0.995	5 µ*M*	>1 m*M*
Sulfate (after chloroform extraction)	16.32[c]	0.9938	5 µ*M*	>1 m*M*

Columns used:[a] LiChrospher 60, RP select B; [b] LiChrospher 100, RP 18; [c] Polyspher ICAN-1.

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