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Analytical protocols for the determination of sulphur compounds characteristic of the metabolism of *Chlorobium limicola*

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In memory of Giulio Izzo (March 18th 1949 – June 1st 2015) passionate ecologist.

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ABSTRACT

Chlorobium limicola belongs to the green sulphur bacteria that has a potential for technological applications such as biogas clean up oxidising hydrogen sulphide to elemental sulphur through photosynthetic process. In the present work, analytical methods are described for the determination of different sulphur species in *C. limicola* cultures – sulphide by GC-FPD, sulphate by ionic HPLC and elemental sulphur by RP HPLC. The latter method eliminates the need for chloroform extraction of water suspensions of elemental sulphur. Data from sulphide and elemental sulphur analyses have been compared with ones coming from more traditional analytical methodologies.

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1. Introduction

Photoautotrophic bacteria from *Chlorobiaceae* family, known as green sulphur bacteria (GSB), are characteristic because of their metabolism. They can actually oxidise hydrogen sulphide (H_2S) to elemental sulphur through a so called anoxygenic photosynthetic process. The complete photochemical reaction is reported in Eq. (1):

$$2nH_2S + nCO_2 + h\nu = 2nS^0 + n(CH_2O) + nH_2O \quad (1)$$

Chlorobium limicola is a GSB that lives in mud, in stagnant waters containing H_2S and in meromictic lakes. It has a high tolerance to sulphide and can easily live in low light environments [1,2]. The cultures of these bacteria are thus maintained with sulphide and may contain, beyond sulphide, elemental sulphur and sulphate ion. The elemental sulphur (S_8) is located outside the cell arranged in so called globules. It may be further oxidised to sulphate ion in conditions of excess of light or sulphide shortage. Depending on culture conditions, yielded elemental sulphur can be divided between two fractions, a first one that is freely suspended in the water medium and a second one clung to the bacterial cell wall [3,4]. Many GSB use and produce sulphur species with intermediate number of oxidation like sulphite and thiosulphate. It is reported in literature that these species do not play a role in *C. limicola* metabolism [2].

C. limicola has been tested in industrial processes aimed at the removal of H_2S from biogas and has proven itself to be very effective [5–7]. In view of future new biotechnological applications of this GSB, it is important to have analytical methods that can reliably determine all sulphur species in the culture. For the analysis of sulphide, various protocols are reported in literature [8]. A colorimetric method has been developed and is described in many different versions [9]. It is the most widespread non-chromatographic protocol. Sulphide can also be determined by GC either as H_2S [10,11] or as derivatized species [12]. Sulphate is generally quantified by ionic HPLC. The quantitative analysis of elemental sulphur in water suspension is the critical step of this analytical chain, because it generally implies extraction and/or derivatization steps that are long and labour intensive [13]. Tetrahydrofuran has been described as a good solvent for direct dissolution of sulphur and has proven itself to be a valuable tool to ease analytical preparation [14], but it has never been used to dissolve sulphur in water suspension.

In the present work, an analytical chain for the determination of sulphur species in *C. limicola* cultures has been set and tested. Sulphate has been determined by ionic HPLC, sulphide as H_2S by GC with FPD detection through a new protocol for sample preparation based on the work by Knöry and Cutter [15]. A new method has been set for the HPLC analysis of elemental sulphur, based on direct dilution of cultures with THF without any preliminary preparative step. The results yielded by the protocols for sulphide

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and elemental sulphur determinations have been compared with ones given by blue methylene and chloroform extraction respectively, and yielded mass balances have been evaluated.

The need to validate chromatographic methods alternative to spectrophotometric ones popular for the determination of sulphide and elemental sulphur has many reasons. Spectrophotometric methods are generally time and labour intensive and make use of toxic reagents and solvents. Furthermore, they generally use various millilitres of specimen thus limiting the actual time span of experiments. The proposed chromatographic methods are easy and fast to implement. They use very little specimen and can substitute UV/Vis methods contributing to delete the usage of toxic reagents and/or solvents. The new HPLC method has also turned out to be more precise and accurate in the quantitative analysis of elemental sulphur in bacterial cultures.

2. Materials and methods

2.1. Bacterial strains and growth media

C. limicola (DSM 248, Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) was grown at room temperature with an incandescence lamp with modified Pfennig Medium II, No. 29 DSMZ [16]. In the medium, $MgSO_4$ was replaced by $MgCl_2$ to avoid analytical interferences. The sulphide was added from a standard solution made with $Na_2S^*9H_2O$. The starting concentration of sulphide species was 4.0 mM. Fed batch cultures were held in Hungate Anaerobic Tubes, 16 × 125 mm, with Butyl Stopper and Cap with 9 mm Opening (15 mL) (GPE Scientific Limited, UK). All operations were performed under anaerobic conditions. Three replicate experiments were carried out. Sampling was carried out, from day 0, at day 1, 2, 4 and 6.

2.2. Chemicals

Reversed phase HPLC eluents were HPLC grade THF (Sigma Aldrich Corporation, Saint Louis, MO, USA) and water (18 MΩ) acidified with 0.1% v/v of formic acid (Baker, Deventer, Holland). THF stabilized with 250 mg/L of butylated hydroxytoluene (BHT) (Sigma Aldrich Corporation, Saint Louis, MO, USA) was used for all other preparations. Elemental sulphur (orthorhombic S_8), phenylethyl salicylate 97%, BHT 99.0% min, $MgCl_2^*6H_2O$, chloroform 99.5%, sulphamic acid ⩾99.3%, $Na_2S^*9H_2O$ 98%, S^{2-} standard solution 1000 μg/mL in 1% NaOH, chloroform ⩾99.5% stabilized with 100–200 ppm of amylenes were from Sigma–Aldrich Corporation (Saint Louis, MO, USA); anhydrous sodium carbonate 99.5% and sodium bicarbonate from Ashland Italia S.p.A (San Giuliano Milanese-MI, Italy); anhydrous sodium sulphate, H_2SO_4 96% and sodium hydroxide, pellets, from Baker (Deventer, Holland); zinc acetate from Ashland (San Giuliano Milanese, Italy); N,N-dimethyl-p-phenylene diamine and $Fe(NH_4)(SO_4)_2$ from Carlo Erba (Milano, Italy).

2.3. Sulphide analysis

Colorimetric analyses of sulphide for protocol comparison have been carried out following literature instructions [17]. From six replicates, we found a RSD of 6% for this determination. Quantitative analysis of sulphide species in solution was carried out by GC with FPD detection. The GC apparatus was from Thermo Fisher Scientific: oven, Trace GC; detector, flame photometric; injector, split–splitless; carrier gas, N_2, constant flow of 1 mL/min. The column used in all runs was a Supel-Q (30 m, 0.53 mm ID, 30 μM film thickness) from Supelco (Bellefonte, PA, USA). 20 μL of sample from bacterial cultures were measured in a 2 mL HPLC vials with PTFE/silicone septum. 580 μL of sulphamic acid solution 0.3 M were then added for a final volume of 600 μL quantitatively hydrolysing all sulphide species to H_2S. The vials were shaken at 40 °C for twenty minutes and then 50 μL of headspace gas were sampled and injected in the GC system. The injection was carried out in split mode, splitting ratio, 1:20. The runs were isothermal, T = 120 °C. In these conditions, H_2S had a retention time of 2.5 ± 0.1 min. Standard solutions for the calibration in the 25–100 μM range were prepared from a commercial solution 1000 μg/mL (31.2 mM) of S^{2-} in NaOH 0.25 M diluting with NaOH 0.25 M and following procedure described for real specimens.

2.4. Elemental sulphur analysis

Extractions with chloroform were performed as follows. 2.5 mL of water suspension containing sulphur were measured in a 25 mL separation funnel and 50 μL of a 20 mM phenylethyl salicylate ethanolic solution were added as internal standard. The suspension was extracted thrice with 2.5 mL portions of chloroform, the reunited extracts were filtered on anhydrous sodium sulphate in a 10 mL volumetric flask and brought to volume with chloroform. The UV profile of the solution was registered between 280 and 320 nm. Quantitation of sulphur and phenylethyl salicylate was carried out with absorbances at 309 and 290 nm solving a system of two equations (sulphur, ε_{290} = 5550 ± 20 M^{-1} cm^{-1}, ε_{309} = 2360 ± 10 M^{-1} cm^{-1}; phenylethyl salicylate, ε_{290} = 2650 ± 50 M^{-1} cm^{-1}, ε_{309} = 5050 ± 50 M^{-1} cm^{-1}). Extraction of standard solutions was performed as described and elemental sulphur was dispersed measuring a known volume of a 20 mM THF solution. All spectrophotometric determinations were performed with an "Evolution 201" instrument from Thermo Scientific.

The HPLC apparatus was made up of the following parts – a binary pump "Perkin Elmer Series 200"; an injection group with a 6 μL loop; an UV/Vis detector "Perkin Elmer Series 200 UV/Vis detector"; an electronic interface "Perkin Elmer NCI 900". The data were acquired on a PC with Turbochrom software, version 4.RP HPLC protocol as follows. The specimens were prepared mixing 590 μL of THF, 60 μL of internal standard solution (2 mM phenylethyl salicylate in stabilized THF), 35 μL of formic acid 1% v/v in water and 315 μL of bacterial culture, for a final volume of 1000 ± 2 μL. The resulting suspension was filtered on 13 mm syringe filters with 0.2 μm PTFE membranes (PALL Corporation) and injected in HPLC without any further treatment. Standard solutions were prepared from THF mother solutions maintaining the same proportions of water, formic acid and THF. The solutions were then eluted with the following method: (i) column: Ascentis C18®, 250 × 4.5 mm, 5 μm particles (Supelco, Bellefonte, CA). The column was kept at 50 °C inside an oven; (ii) eluents: A 0.1% v/v formic acid in water, B THF; (iii) elution: 9 min 65% B isocratic; and (iv) flow: 1000 μL/min. The UV/Vis detector was set at 290 nm.

The peaks were preliminarily assigned by comparing their retention times with those determined by injection of pure standard solutions and then confirmed by co-injections with pure standards. The analytical concentration of sulphur was determined using phenylethyl salicylate as internal standard with Eq. (2):

$$C_{SUL} = C_{PES} * (P_{PES}/P_{SUL}) * (S_{SUL}/S_{PES}), \quad (2)$$

where C are the analytical concentrations in μM, P the HPLC sensitivities at 290 nm in (μV s)/μM and S the chromatographic signals (areas) in μV s. In our HPLC system the relative sensitivity (P_{PES}/P_{SUL}) at 290 nm was 0.60 ± 0.01.

2.5. Sulphate analysis

Sulphate was determined by anionic chromatography. The HPLC apparatus was Model 761 Compact IC from Metrohm (Herisau, Switzerland) made up of the following parts: suppressor

module; a binary pump; an injection group with a 20 μL loop; conductivity detector. Data were acquired by a PC equipped with IC NET 2.2 Metrodata© 2003 software (Metrohm, Herisau, Switzerland). The specimens from bacterial cultures were diluted 1:10 with the eluent and filtered over 0.45 μm cellulose filters (Whatman, Buckinghamshire, UK) before injection. The solutions were then eluted with the following method: (i) column: Metrosep A Supp 4 250/4.0 (size 250 mm × 4.0 mm ID, 9 μm particles); (ii) eluent: 1.98 mM of Na_2CO_3 and 1.87 mM of $NaHCO_3$ (14.5 ± 0.5 μS/cm; pH 8.9); (iii) elution: 20 min; and (iv) flow: 1250 μL/min. A C18 cartridge was placed ahead of the column to remove non polar organic contaminants.

Sulphate calibration was carried out in the 0.3–3.0 mM range, and resulting plot was linear – $r^2 > 0.999$. In these conditions sulphate ion had a retention time of 11.9 min.

3. Results and discussion

3.1. Sulphide analysis

The gas-chromatogram from a real specimen is reported in Fig. 1. Due to the high selectivity of the FPD detector, a single peak, the one from H_2S, is visible. The chromatographic signal from H_2S in gas phase was linearly correlated with the square of concentration of sulphide in solution as reported in literature [11], so a logarithmic plot was drawn for the quantitative analysis, $\log S = \log P + 2\log C$ ($R = 0.99660$). The slope of the calibration line was 2 within the uncertainty interval and thus displayed no deviation from quadratic law, as reported for H_2S analysis with FPD detection [11]. RSD on sulphide signal was 6%, determined on six different standard solutions. LOD was 13 nM of sulphide species in analytical solution, determined as three times higher than the average background noise of the baseline. This value corresponds to 400 nM of sulphide species in the culture, about 0.3% of the starting sulphur in the batch experiments with *C. limicola*. The detection limit is much higher than the one reported in literature for analysis of waters containing sulphur, reported as low as 0.2 pM [15]. Nevertheless, that limit is reached with cryotrapping prior to injection, thus at a cost in terms of equipment that is clearly not worth in the case of *C. limicola* cultures. The GC protocol implemented here was compared with the colorimetric method (CM). The average ratio of sulphide determined with the two methods in six separate trials on real bacterial cultures, GC/CM, was found to be 1.17 with a SD of 0.11 and a RSD of 9%. Given the extent of the uncertainty interval for ratios, it is possible that the two methods actually yield the same results, as the unitary ratio is within about 1.5 SDs from average value determined here. The GC-FPD method is thus not standing out for its ability to produce results more accurate than the one produced by colorimetric method. Rather its main advantage stands in the simplicity of preparation and non toxicity of reagents, where N,N-dimethyl-p-phenylene diamine used in CM method is toxic instead.

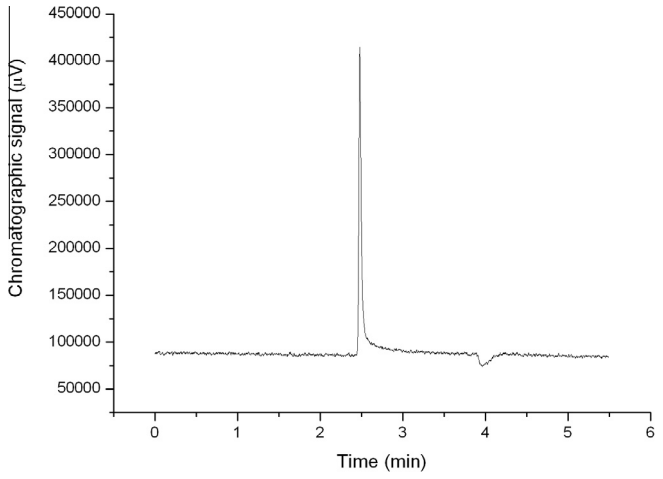

Fig. 1. The GC-FPD chromatogram Day 3 Rep2. The peak of H_2S is clearly visible with a retention time of 2.5 min and is the only peak thanks to the selectivity of flame photometric detector.

3.2. Elemental sulphur analysis

RSD on HPLC signal was 3% for both phenylethyl salicylate and elemental sulphur (six measures each), so RSD for sulphur determination in real specimens was estimated to be 5%. Day by day repeatability of signal, based on six measures repeated for five consecutive days, was 3%. Other parameters of analytical interest are in Table 1. The limit of detection for elemental sulphur (three times higher than the average background noise of the baseline) corresponds in our system to an analytical concentration of 165 nM and a culture concentration of 550 nM. The starting sulphur in culture medium is 4.0 mM as sulphide, that can be theoretically converted to a maximum concentration of 500 μM of elemental sulphur. The limit of revelation is thus about 0.1% of the maximum possible amount of sulphur that can be present in solution/suspension.

A chromatogram of a culture is displayed in Fig. 2. It is possible to see that the three prominent peaks are baseline separated, with $R \gg 2$. The presence of the peak of BHT, the THF stabilizer, does not interfere with the peaks of the analytes. This observation has let us work using stabilized THF that can be manipulated in aerobic conditions thus avoiding the problems that arise working in an anaerobic environment.

THF has been chosen for the preparation of specimens and as eluent for RP HPLC because it is reported in literature to be a good solvent for elemental sulphur [14] and is also one of the three organic solvents of choice for RP HPLC along with acetonitrile and methanol. In order to optimise the method, solubility tests have been carried out. Standard specimens were prepared varying THF/H_2O ratio for a concentration of sulphur of 200 μM and subsequently analysed by HPLC. It is possible to see in Fig. 3 that the specimen preparation method is valid with a minimum THF percentage of 45% in the final mixture. The choice of 65% THF in the method is thus well posed as it guarantees quantitative dissolution of elemental sulphur. It was thus checked if it was possible to raise the amount of sulphur at 65/35 THF/H_2O ratio. Standard specimens were then prepared with various concentrations of sulphur at the stated fixed solvent ratio. It is possible to see in the Fig. 4 that no losses of sulphur were found. The method can thus work even at concentrations much higher than the maximum ones present in this type of cultures.

Table 1
Analytical parameters for the HPLC determination of elemental sulphur (S_8) and phenylethyl salicylate. The limits of detection (LOD) and quantitation (LOQ) were determined as the amounts that give a signal three and ten times higher than the average background noise of baseline respectively. Retention times are all ±0.1 min.

	Calibration range (pmol)	r	LOD (pmol)	LOQ (pmol)	Retention time (min)
Phenylethyl salicylate	120–900	0.99997	0.6	2.0	4.6
Elemental sulphur (S_8)	200–800	0.99997	1.0	3.3	7.3

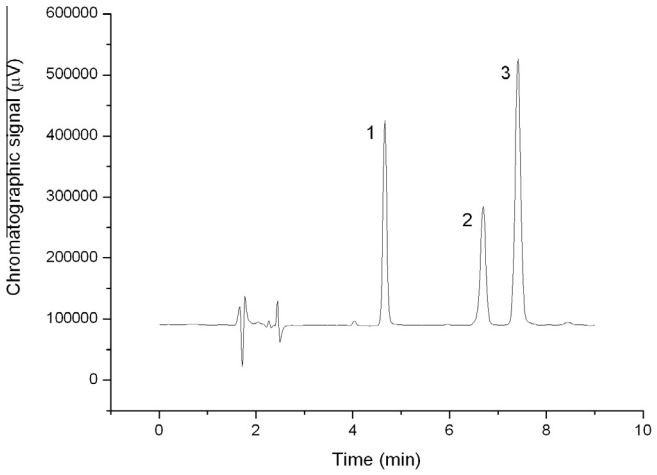

Fig. 2. The HPLC chromatogram of a sample from Rep2 taken on Day 3. It is possible to distinguish the peaks of 1. Phenylethyl salicylate (internal standard) 2. BHT (stabilizer of THF, present in the solvent used for specimen preparation but not in the HPLC eluent) and 3. Orthorhombic elemental sulphur. The peaks are baseline resolved with $R \gg 2$ and no interferences are present.

A comparison has been carried out between HPLC proposed protocol and chloroform extraction method. First of all, phenylethyl salicylate has been tested for its suitability as internal standard in chloroform extraction. The ratio of extraction yields Y_{SUL}/Y_{PES} was found to be 1.03 as an average of six extractions from standard water suspensions of sulphur, SD 0.07. Three separate bacterial cultures (A, B, C) has been then used for comparison trial (three replicates for both extraction and HPLC). Results are reported in Table 2. The values yielded by HPLC are systematically higher than ones from solvent extraction with a ratio comprised between 0.46 and 0.67. This can be attributed to the fact that part of the elemental sulphur is not in water suspension but rather is clung to the bacterial cell wall or is in the form of globules that may well not be available for solvent extraction. The preparation of HPLC specimen requires the direct addition to the bacterial culture of THF that is fully miscible with water instead. This procedure is likely to destroy the globules dissolving more sulphur and making it available for the HPLC determination. As we will further discuss in Section 3.4, the mass balances tell that values from HPLC are closer to the real amounts of elemental sulphur in the cultures,

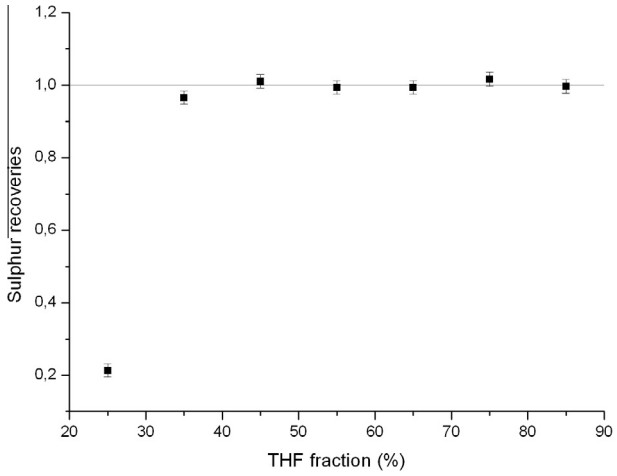

Fig. 3. Recoveries of standard elemental sulphur (300 pmol) in different volumes of THF following addition of water for a final volume of 1 mL. The elemental sulphur remains in solution if the fraction of THF is equal to or higher than 45%. Losses are significant if THF fraction is below 45%.

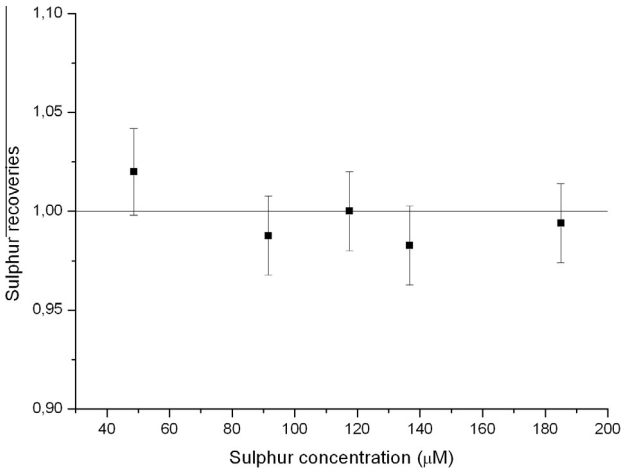

Fig. 4. The graphic shows the recoveries of elemental sulphur at different concentrations in solutions with THF/H_2O 65/35 ratio. Recoveries are quantitative in the whole inspected range.

Table 2
Comparison of the results coming from spectrophotometric protocol and HPLC determinations of elemental sulphur. All results are average of three replicates. *R* refers to the ratio of results from chloroform extraction over ones from HPLC.

Experiment	C_{CHL} (μM)	RSD_{CHL} (%)	C_{HPLC} (μM)	RSD_{HPLC} (%)	*R*
A	165	9	356	10	0.46
B	221	4	331	4	0.67
C	151	14	319	7	0.47

where the values from solvent extraction would give mass balances much lower than unity. This conclusion is further supported by the result discussed above, standard sulphur can be quantitatively and accurately determined by chloroform extraction using phenylethyl salicylate as internal standard.

3.3. Sulphate analysis

LOD was 20 nM of sulphate in solution, determined as three times higher than the average background noise of the baseline. This corresponds to about 0.05% of the total initial sulphur in the medium. RSD was determined with six separate injections of sulphate standard and was found to be 3%.

3.4. Bacterial metabolites and their mass balances

The sulphur species in bacterial cultures were monitored four times during the experiment, one each day from the first one. The amounts of sulphide, sulphate and elemental sulphur determined in bacterial cultures are in Table 3. The concentrations of the different sulphur species vary with the growth stage of the bacterial cultures. In the first 2 days, sulphide has been oxidised to elemental sulphur and no appreciable amount of sulphate is produced. On day 4, maximum concentration of elemental sulphur is attained and sulphate appears. On day 6, finally, sulphide is below detection limit, thus reduced below 0.3% of the initial amount, elemental sulphur has diminished and sulphate has increased. This trend corresponds with typical *C. limicola* metabolism. Where sulphide is freely available its reducing power is exploited to yield elemental sulphur. Once sulphide has exhausted elemental sulphur is oxidised to sulphate.

The overall mass balances of sulphur are reported in Table 4. It is possible to notice that the three analytical systems integrate well and mass balances are close to unity. Deviations from unity may

Table 3
Content of the different sulphur species monitored in bacterial cultures at first, second, fourth and sixth day respectively, given as the average of three replicate determinations. RSD was 7% for elemental sulphur, 4% for sulphate and 9% for sulphide. Starting concentrations were 4000 μM for sulphide, 1170 μM for sulphate and 112 μM for elemental sulphur. BDL stands for below detection limit, starred values are indistinguishable from starting value within experimental uncertainty.

	S^{2-}	SO_4^{2-}	S_8
Day 1	2500	1200	250
Day 2	1300	1200	390
Day 4	110	1600	500
Day 6	BDL	2000	450

Table 4
Mass balances (MB in table) in all replicates of the two batch experiments. It is expressed as the ratio of the sum of the moles of sulphur atoms in all analysed sulphur species over the initial moles of sulphide measured in the bacterial cultures at day 1, day 2, day 4 and day 6 from the beginning of the experiment. ΔMB are the uncertainties calculated from RSD of the single sulphur species following error propagation rules.

	MB_1	ΔMB_1	MB_2	ΔMB_2	MB_4	ΔMB_4	MB_6	ΔMB_6
Rep 1	1.20	0.10	0.85	0.05	0.80	0.05	0.80	0.05
Rep 2	0.80	0.05	0.85	0.05	0.95	0.05	0.90	0.05
Rep 3	1.00	0.10	0.95	0.05	0.90	0.05	1.00	0.05

depend on various factors – small amounts of some other non-monitored sulphur species may form and/or part of the sulphur may be withheld in the biomass. These results confirm that the pursued sulphur species are the significant ones for *C. limicola* metabolism and that the analytical methods are fit for the stated purpose.

4. Conclusions

The methods implemented here have proven themselves fit for the purpose of monitoring *C. limicola* cultures. They are fast and easy to implement, and the orders of magnitude of the LODs are a very small fraction of the initial sulphur in typical *C. limicola* cultures. The HPLC protocol for the determination of elemental sulphur has proven itself much more precise and accurate than chloroform extraction in the analysis of *C. limicola* cultures, that is, RSD is lower and values are closer to the real expected value, as proven by mass balances of sulphur. The specimen preparation for the HPLC method skips the need to perform the liquid–liquid extraction step that is time consuming and uses a lot of chloroform, a toxic and alleged carcinogenic solvent whose usage will be restricted in the future. While THF is also toxic, in HPLC instrumentation solvents are held in a nearly closed circuit and the potential contact is held to a minimum, especially if confronted with exposure to chloroform in a solvent extraction procedure. Exposure is also limited in the specimen preparation that uses limited amounts of solvents and that is prone to further miniaturization in the future as smaller and smaller LC equipment become available. GC method for sulphide detection is not displaying superior analytical performance when compared with colorimetric methods, but statements regarding the use of toxic solvents also apply. More, both HPLC method for elemental sulphur and GC method for sulphide determination use an amount of specimen that is in the order of microlitres, where spectrophotometric methods use amounts in the order of millilitres. This can make a difference in culture managing. As very small amounts are sampled, cultures can be maintained close to integrity for longer times.

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