Analytical Chemistry Research 5 (2015) 9–13

Contents lists available at ScienceDirect

Analytical Chemistry Research

journal homepage: www.elsevier.com/locate/ancr

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

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ARTICLE INFO

Article history:
Received 25 February 2015
Revised 22 May 2015
Accepted 29 May 2015
Available online 3 June 2015

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

Keywords:
Hydrogen sulphide
Sulphur
High performance liquid chromatography
Gas chromatography
Chlorobium limicola
Green sulphur bacteria

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.

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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http://dx.doi.org/10.1016/j.ancr.2015.05.002
2214-1812/

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