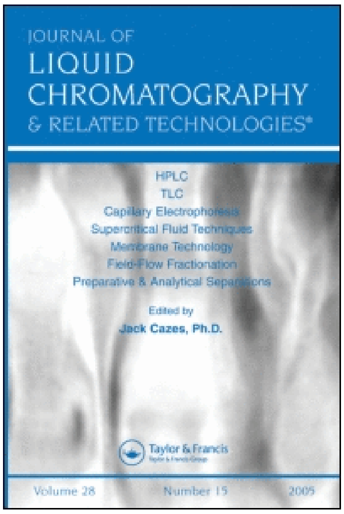

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CHROMIUM SPECIATION IN BACTERIAL CULTURE MEDIUM BY COMBINING STRONG ANION EXCHANGE LIQUID CHROMATOGRAPHY WITH INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY. APPLICATION TO THE REDUCTION OF CR(VI) BY SULFATE-REDUCING BACTERIA

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ABSTRACT

Because of its toxicity, Cr(VI) contamination constitutes a major environmental problem. Reduction of Cr(VI) to Cr(III) by sulfate-reducing bacteria is accordingly a promising biotechnology. Developing this will require research to select the most efficient

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bacteria and to study the precise mechanism of reduction involved. We have, therefore, adapted an analytical method that combines high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS), allowing the speciation of Cr(VI) and Cr(III) in complex medium. This method permits simultaneous determination of both species with high sensitivity and specificity. Applied to the Cr(VI) reduction by the sulfate-reducing bacteria *Desulfomicrobium norvegicum*, it has allowed a preliminary conclusion that the reduction is biphasic and occurs before replication of the bacteria.

INTRODUCTION

Chromium is one of the most widely used metals in industry and is responsible for widespread pollution (1,2). Although it can exist in every oxidation state from −2 to +6, chromium is present in the environment as two major valence states, Cr(VI) and Cr(III). The hexavalent chromium Cr(VI) is very soluble in water. This species is known to be carcinogenic, mutagenic, cytotoxic, and a strong oxidizing agent (3). Conversely, the trivalent form Cr(III) is much less toxic and tends to form insoluble and stable hydroxides at physiological pH (4,5).

Because of its toxicity, pollution by chromium constitutes a major environmental problem. Studies have, therefore, been conducted for cleaning up waste waters and spoiled soils, particularly by using microorganisms that can reduce Cr(VI) to Cr(III). This technique appears efficient, environmentally friendly, and cheaper than more usual processes, such as treatment with lime (6–10). The efficacy of these biotechnological processes involving sulfate-reducing bacteria (SRB) to reduce Cr(VI) is often monitored by measuring the Cr(VI) concentration, using the classical 1,5 diphenylcarbazide colorimetric method, the Cr(III) concentration being calculated by subtraction of Cr(VI) from total chromium (11,12). However, to improve these current biotechnologies, research has been done to select the most efficient bacteria and to understand the biochemical mechanisms implicated in the reduction of chromium (13,14). Chromium speciation by using a sensitive and specific analytical method appeared necessary to adequately investigate the behaviour of Cr(VI) during its reduction by SRB.

In this work we, therefore, adapted a chromium speciation technique based on the methodology described by Byrdy et al. (15) This involved the combining of high-performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS). The chromium species were separated by

HPLC using an ion-exchange column, and they were detected on-line by linking the outlet of the column with the inlet of the ICP-MS apparatus. In addition, we applied this analytical technique to monitor the behaviour of chromium during reduction of Cr(VI) in a complex medium by *Desulfomicrobium norvegicum*, a SRB which has been reported to have a strong chromate-reductase activity and to be able to grow at high concentrations of chromium, up to 500 μM, which can be lethal for other bacteria (13).

EXPERIMENTAL

Standard Chromium Solutions

Standard chromium solutions were made from Cr(VI) and Cr(III) stock solutions prepared as follows: for Cr(VI), the stock solution (20 mM) was obtained from $K_2Cr_2O_7$ (99.9% min, Prolabo, Fontenay S/Bois, France), dried in a 100°C oven for 1 h, and dissolved in Starkey medium, a sterile bacterial growth medium (SY). This solution, which was adjusted at pH 7.2, contained 37 mM NH_4Cl, 8.1 mM $MgSO_4 \cdot 7H_2O$, 28.1 mM Na_2SO_4, 2.8 mM K_2HPO_4, yeast extract (1 g/l), 7.8 mM sodium lactate as carbon source and electron donor, and trace elements solution (1 mL/l) bringing 18 μM $FeCl_2 \cdot 4H_2O$ in the medium (16). For Cr(III), the stock solution (20 mM) was obtained by dissolving $CrCl_3 \cdot H_2O$ (99% min, Sigma-Aldrich Chemie, Steinheim, Germany) in SY. The standard solutions of Cr(VI) and Cr(III) were obtained by diluting the stock solutions in SY to concentration in the range 1 μM–100 μM. In addition, a standard solution containing both 25 μM Cr(VI) and 25 μM Cr(III) was prepared by mixing the standard solutions appropriately.

Standard and assay solutions containing Cr(III) were next treated as follows: the pH was lowered to 4.2 with concentrated HNO_3, then solutions were loaded according to the Cr(III) concentration with EDTA disodium salt (Prolabo, Fontenay S/Bois, France) to reach a final molar ratio of 100 : 1. The solutions were then heated for 1 h at 40°C. To provide blank solution, SY medium was processed in an identical manner by adding EDTA to a final concentration of 10 mM. This corresponded to the concentration used for the standard solution at the higher concentration of Cr(III).

HPLC–ICP-MS Analysis

The chromatographic system consisted of a Hewlett Packard HPLC system series 1100 with a quaternary gradient pump, a Rheodyne model 7725 syringe-loading injector with a 20 μL injection loop connected with a Zorbax SAX

4-Pack guard column (4.6 × 12.5 mm i.d., Rockland Technologies, USA), and a SynChropack Q300 strong anion-exchange analytical column (100 × 4.6 mm i.d., Micra Scientific, Northbrook, USA). The outlet of the latter was linked with the inlet of the Barbington-type nebulizer of the ICP-MS instrument by means of a capillary Teflon tubing (500 × 0.015 mm i.d.). The ICP-MS instrument was a model HP 4500 (Yokogawa Analytical Systems, Tokyo, Japan). The ICP-MS measurement conditions, such as sampling position and ion lenses, were optimized daily using a standard built-in software procedure for injection of a tuning solution (Hewlett Packard, Palo Alto, USA) containing Li, Y, Ce and Tl (10 ppb of each).

Analyses were conducted at 20°C, the temperature of the laboratory being maintained by an air conditioner, as follows: the chromatographic system was conditioned by using an in-line filtered mobile phase consisting of 17.5 mM $(NH_4)_2SO_4$ dissolved in Milli-Q water (18 MΩ cm) and adjusted to pH 9.0 with NH_4OH. By pumping the mobile phase at a flow-rate of $1.5\,mL \times min^{-1}$, stabilisation was reached after about 60 minutes. Next, a sample volume (10 µL)

Table 1. Operating Conditions for HPLC and ICP-MS Instruments

HPLC:	
Column	Zorbax SAX 4-Pack guard column (4.6 × 12.5 mm i.d., Rockland Technologies) SynChropack Q300 strong anion-exchange column (100 × 4.6 mm i.d., Micra Scientific)
Mobile phase	$(NH_4)_2SO_4$ 17.5 mM, pH 9.0
Flow rate	$1.5\,mL \times min^{-1}$
Injection volume	10 µl
Temperature	20°C maintained by air conditionner
ICP-MS:	
Forward RF power	1.3 kW
Reflected RF power	2 W
Plasma Ar flow	$15.0\,l \times min^{-1}$
Auxiliary Ar flow	$0.9\,l \times min^{-1}$
Nebulizer Ar flow	$1.0\,l \times min^{-1}$
Spray chamber	Single pass
Nebulizer	Barbington
Data acquisition mode	Time resolved analysis
Integration time	1.0 s
Isotopes monitored	^{52}Cr, ^{53}Cr
Total time analysis	11 min

of solution to be analysed was injected onto the column and eluted at the same flow-rate by using the same mobile phase. Detection was performed by monitoring, continuously, the two chromium isotopes ^{52}Cr and ^{53}Cr in the eluate. Quantification was performed in the peak area mode, and the chromatographic results were processed using Chromatosoftware (Yokogawa Analytical Systems, Tokyo, Japan). The operating conditions are summarized in Table 1.

Monitoring of Cr(VI) Reduction by *De. norvegicum*

A preculture of *De. norvegicum* was centrifuged at 16,100 × g for 5 min. Bacteria were then suspended in the SY medium preheated to 37°C. An aliquot of the suspension (0.2 mg of total protein (17)) was introduced into 50 mL of SY loaded with Cr(VI) (25 μM, final concentration), from which a volume of 2 mL had been previously taken as sample for control. Culture was next performed at 37°C and samples were taken at time 0, 2, and 6 h to monitor the bio-reduction of Cr(VI). Analyses were performed in triplicate as described above.

RESULTS AND DISCUSSION

HPLC–ICP-MS Analysis

Preliminary analyses of Cr(VI) and Cr(III) standard solutions allowed us to determine the mass to use for single/ion monitoring. At m/z 52, in accordance with Byrdy et al. (15), we observed a high background of about 24,000 counts lowering the signal-to-background ratio. Moreover, as chromium species are dissolved in SY, a complex medium containing organic compounds, the polyatomic ion $^{40}Ar^{12}C^{+}$ may form, and interference problems may thus ensue. By contrast, at m/z 53 we observed a background of only about 9600 counts. Therefore, although at m/z 52, chromium is 83.78% abundant, while at m/z 53 it is 9.55% abundant, this later m/z value was used in this study.

The analyses of Cr(VI), Cr(III), and Cr(VI) mixed with Cr(III), standard solutions performed under the conditions of detection previously described, are reported in Figure 1. For Cr(VI), the chromatogram (Figure 1A) shows a single peak with a retention time of about 9.7 minutes. The retention observed is due to the anionic form of Cr(VI) species at the pH value of the mobile phase used (pH 9.0). For Cr(III), the chromatogram (Figure 1B) also shows a single peak, but with a retention time of about 1.4 minutes. This result clearly demonstrates that chelation by EDTA permits, at low pH (see Experimental section), the formation of one anionic complex of Cr(III), and makes it soluble at pH 9.0, allowing the retention mechanism onto the anionic-exchange column used. Moreover, the

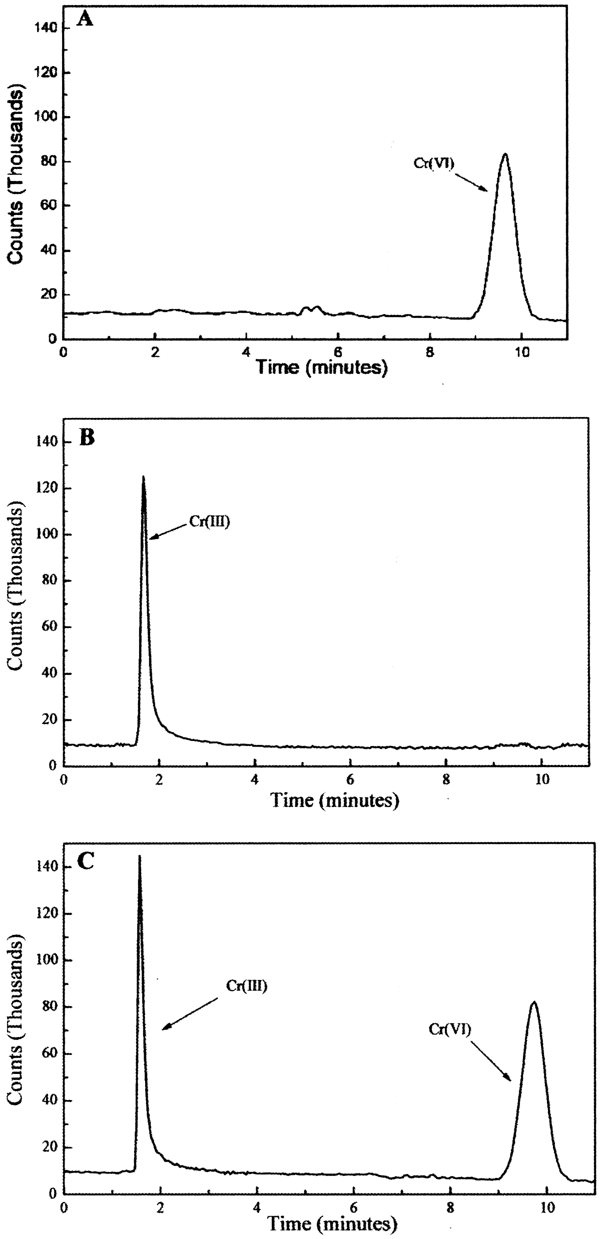

Figure 1. Chromatograms of the standard solutions of 25 μM Cr(VI) (A), 25 μM Cr(III) (B) and a mixture of 25 μM Cr(VI) and 25 μM Cr(III) (C).

chelation precludes the possible oxidation of Cr(III), as previously reported (15). As shown in Figure 1C, analysis of the mixture of Cr(VI) and Cr(III) supplies a chromatogram with two peaks, with retention times corresponding to those of Cr(VI) and Cr(III) alone. Thus, it is clear that the presence of these two species in the same solution induces no analytical interference.

Replicate experiments ($n = 5$) with Cr(VI) and Cr(III) solutions allowed us to check the repeatability of the qualitative analysis. The mean retention time ± SD for Cr(VI) was 9.67 ± 0.05 minutes and that of Cr(III) was 1.41 ± 0.05 minutes, indicating a great separation stability. Indeed, the relative standard deviation of the peak retention time is always less than 0.6% for Cr(VI) and 3.6% for Cr(III).

To evaluate the quantitative aspects of the method, 0, 1, 5, 10, 25, 50, and 100 µM Cr(VI) and Cr(III) standard solutions were processed. The highest concentration (100 µM) used for these experiments was chosen to avoid a contamination of the ICP-MS components. In addition, blank solutions analysed after chromium standard solutions gave chromatograms free from chromium (data not shown), proving that no accumulation of chromium occurs into the chromatographic system.

In these conditions, the plots of peak area versus Cr(VI) or Cr(III) concentration yield calibration curves that could be fitted to a linear response with $R^2 = 0.998$ for Cr(VI) and $R^2 = 0.991$ for Cr(III). As reported by Byrdy et al., relative detection limit was calculated using 3 times the standard deviation of the blank divided by the slope of the log-log representation of the calibration curve (15). For both Cr(VI) and Cr(III), detection limit was found equal to 1 µM, corresponding to an absolute detection limit of 0.52 ng, which is about twice of that determined by Byrdy et al. This difference is due to the high noise caused by the release of chromium from the stainless steel components of our HPLC instrument. Finally, as for the qualitative analysis, the repeatability was determined from replicate experiments ($n = 5$) with chromium standard solutions of 1 µM and 25 µM. The relative standard deviation calculated was 8.2% for 1 µM and 6.3% for 25 µM. The difference between these values appears to be due to the difficulty in accurately integrating the chromatographic peak when concentration is low. Although these relative standard deviation values fall in the usual range, they are higher than those reported by Byrdy et al. (15) This discrepancy can be explained by the fact that in our experimental conditions, chromium species are dissolved in the complex medium SY instead of Milli-Q water.

Monitoring of Cr(VI) Reduction by *De. norvegicum*

As mentioned above (see Experimental section), prior to seeding the culture medium SY loaded with 25 µM Cr(VI), a sample was taken to serve as control. As shown in Figure 2, its analysis exhibits two species, Cr(VI) and Cr(III). This

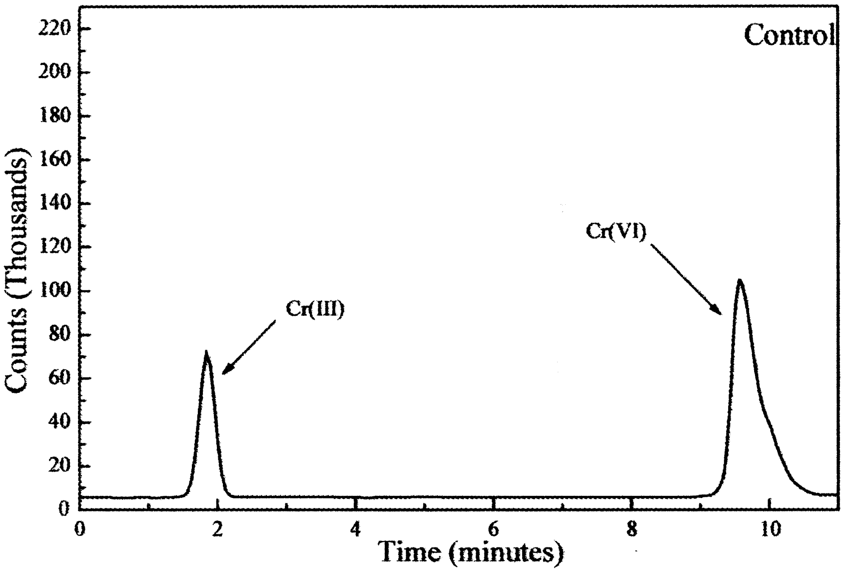

Figure 2. Chromatogram with single-ion monitoring at *m/z* 53 of the culture medium SY loaded with 25 μM Cr(VI) and treated by EDTA as described in the text.

result indicates partial reduction of Cr(VI) in absence of *De. norvegicum* from the medium. This phenomenom is not surprising, as SY contains 18 μM $FeCl_2 \cdot 4H_2O$ and the capacity of this salt to reduce Cr(VI) in Cr(III) is well-known. Consequently, this chemical reduction of chromium needs to be taken into account when studying bioreduction with SRB.

Results of Cr(VI) monitoring reduction are illustrated in Figure 3. No significant differences are observed between control (Figure 2) and inoculated SY at time t = 0 h (Figure 3A). This indicates no further chemical reduction induced by the seeding. By contrast, at time t = 2 h and t = 6 h (Figures 3B and 3C) the Cr(VI) peak diminishes while the Cr(III) peak increases, reflecting the bio-reduction of chromium by *De. norvegicum.* 91% of the Cr(VI) is reduced in 6 hours, 79% during the 2 first hours, and the remaining 12% during the next 4 hours. This result indicates a change in the kinetics during the bio-reduction process and suggests a biphasic phenomenon. Moreover, as the generation time of *De. norvegicum* is longer than that required in these experiments to reduce the chromium, our results demonstrate that quantity of bacteria (0.2 mg/L total protein) seeded is able to reduce the main quantity of Cr(VI) used in this study (18). Finally, for each time (t = 2 h and t = 6 h) the sum of Cr(VI) and Cr(III) concentrations corresponds to the initial Cr(VI) concentration (25 μM) of the

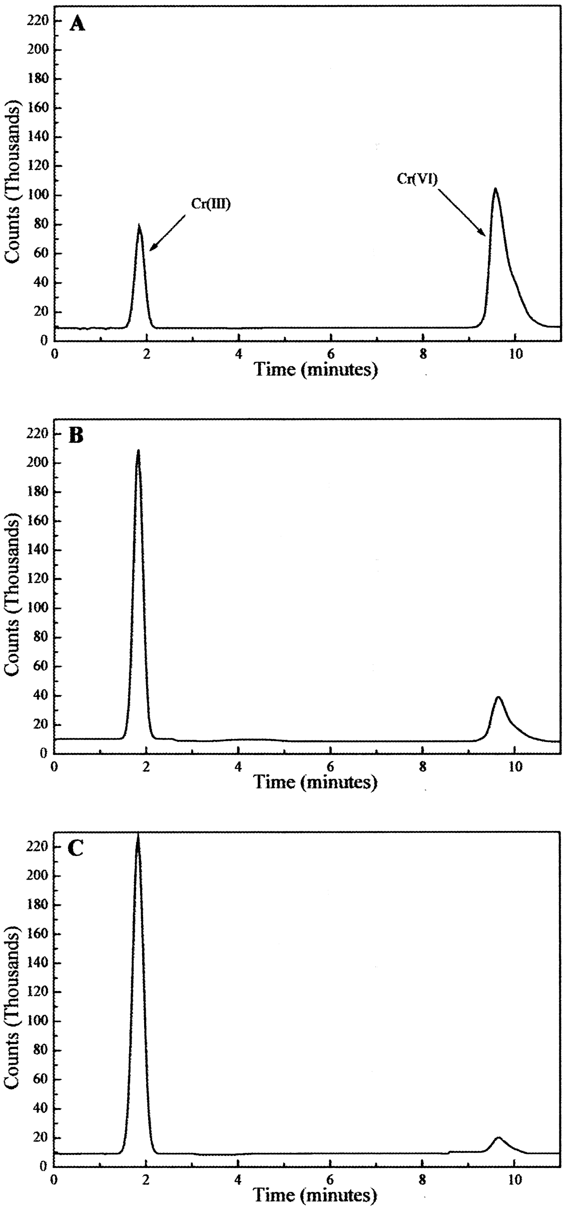

Figure 3. Chromatograms with single-ion monitoring at m/z 53 of the Cr(VI) reduction by *De. norvegicum* seeded in SY medium containing 25 μM Cr(VI). At t = 0 h (A), at t = 2 h (B) and at t = 6 h (C).

culture medium SY. Thus, under our experimental conditions, all of the chromium is shared between Cr(VI) and Cr(III).

CONCLUSION

Combination of HPLC and ICP-MS for chromium speciation appears a useful tool, which allows the monitoring of chemical and biological processes implicated in the bioreduction of Cr(VI) by SRB. This technique has the advantage of measuring the different species present in the sample, simultaneously, and permits accurate analyses in complex medium.

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