

Optimization of Methodology for Determination of Inorganic Chromium Species in Sewage Sludge Samples by HPLC-ICP-MS/MS

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This study developed a methodology for extracting and speciating Cr^{III} and Cr^{VI} in sewage sludge by high performance liquid chromatography, inductively coupled plasma tandem mass spectrometry (HPLC-ICP-MS/MS). The optimal procedure was solid-liquid extraction in a water bath with stirring at 50 °C for 16 h and 50 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA) extraction phase at pH 9.5. In the validated method, the recovery in the fortification experiment was greater than 70% and the relative standard deviation was less than 7%. Speciation analyses showed that all Cr^{VI} was spontaneously reduced to Cr^{III} in the sludge sample. The limits of detection and quantification were 0.060 and 0.20 µg L⁻¹, respectively, and the analyses of Cr^{III} by means of ICP-MS/MS were linear from 0.20 to 50 µg L⁻¹. The extraction efficiency of Cr specie in this validated method improved from 4.09 to 53.3% compared to the EPA 1311 method. The methodology was successfully applied to quantify Cr^{III} in a sludge sample with a lower concentration than that established by European legislation 80568/4225/91.

Keywords: sewage sludge, chromium, extraction, reduction, inorganic chromium species

Introduction

Sewage sludge is a solid material resulting from effluent treatment at sewage treatment plants.¹ This material has been used in farming as a fertilizer or soil amendment due to the high content of macro and micronutrients and particularly for organic matter.² However, sludge may also have organic and inorganic contaminants in its chemical composition.¹

Inorganic contaminants are generally toxic metals such as As, Cd, Pb, Hg and Cr. In addition, Cr may be found in the environment in oxidation (III) and (VI) states.^{1,3} Cr^{III} in low concentrations is not toxic to human beings or the environment, and is additionally important for human metabolism.⁴ On the other hand, Cr^{VI} is highly toxic and is generally related to health problems such as carcinogenesis and mutagenicity.⁵

Therefore, environmental laws are very strict in monitoring Cr^{VI} levels in food and environmental matrices.⁶ Resolution No. 375 of the National Environment Council (CONAMA)⁷ in Brazil currently establishes 1000 mg kg⁻¹ as the limiting concentration of total Cr in the sewage sludge. However, the maximum permitted concentrations

of each Cr species have been set by few countries such as Hungary and Greece, which determine 1 and 10 mg kg⁻¹, respectively, as the limiting Cr^{VI} concentration in sewage sludge.^{8,9}

In order to reach these limits, several extraction methodologies have been developed for this metal to determine Cr^{VI} in matrices such as water,¹⁰ soils,^{11,12} and foods of plant, animal and industrial origin.¹³⁻¹⁵ In determining Cr^{III} and Cr^{VI} in environmental samples, it is common to separate the chromium species in the extraction stage.⁶ Thus, methodologies involving precipitation or cartridges containing adsorbents have been used for separating Cr^{III} and Cr^{VI}, then the extracts obtained with each chemical species are analyzed by instrumental techniques such as atomic absorption spectrometry (AAS), graphite furnace atomic absorption spectrometry (GFAAS) and inductively coupled plasma mass spectrometry (ICP-MS).¹⁶⁻¹⁹ However, there are no extraction methodologies for sewage sludge samples which enable the simultaneous quantification of Cr^{III} and Cr^{VI}.

In the past, the US Environmental Protection Agency (US EPA) has defined a methodology known as the toxicity characteristic leaching procedure (TCLP) to determine the mobility of toxic contaminants such as Cr in liquid and solid waste through the EPA protocol 1311.²⁰

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In this method, it is common to quantify Cr in extracts by inductively coupled plasma optical emission spectrometry (ICP OES).^{21,22}

However, it is necessary to use separation techniques such as high performance liquid chromatography (HPLC), followed by a detection technique such as inductively coupled plasma tandem mass spectrometer (ICP-MS/MS)¹³ for simultaneous analysis of each Cr species at trace levels. In addition, the use of the ICP-MS/MS as a detector enables greater selectivity during the analysis, because it is possible to filter the target analyte with the second quadrupole (MS/MS) and eliminate the interfering ions.²³

Therefore, the objective of this study was to optimize and validate a methodology based on solid-liquid extraction and HPLC-ICP-MS/MS analysis to determine Cr^{III} and Cr^{VI} in sewage sludge samples.

Experimental

Reagents and solutions

HCl (36%, v/v), glacial CH₃COOH, and pure HNO₃ (65%, v/v) acids and Na₂SO₄, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA.2Na), NaOH, NaH₂PO₄, Cr(NO₃)₃ and K₂CrO₄ reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) with purity greater than 99% (m/m). The NH₄OH (30%, v/v) was obtained from Neon (Suzano, SP, Brazil).

The standard Cr^{III} and Cr^{VI} solutions at the concentration of 1000 mg L⁻¹ were prepared in ultrapure water (Millipore, Burlington, MA, USA) from high purity analytical standards of Cr(NO₃)₃ and K₂CrO₄, respectively. These solutions were diluted to prepare the working solutions.

Instrumentation

In the optimization stage of Cr^{III} and Cr^{VI}, extraction conditions, Scilogex vortex (USA), Unique ultrasonic bath (Brazil), stirring water bath (Nova Ética, Brazil), heating plate (Tecnal, Brazil), Quimis pH meter (Brazil) and Kindly centrifuge (Brazil) were used. A CEM (USA) Mars 6 microwave oven was also used during the work.

Atomic absorption spectrometry analyses

An atomic absorption spectrophotometer (AAS 240 FS, Varian, Australia) was used to determine the Cr content in the sludge sample during the extraction method optimization step. Analyses were performed in flame mode using air-acetylene (acetylene 2.8 AA).

HPLC-ICP-MS/MS analyses

A high performance liquid chromatograph (HPLC 1260 Infinity, Agilent Technologies, Japan) coupled to an inductively coupled plasma-tandem mass spectrometer (ICP-MS/MS 8800, Agilent Technologies, Japan) was used for Cr speciation. The system was equipped with a G1311B quaternary pump, G1316A column housing and G1329B auto-sampler.

Speciation of Cr^{III} and Cr^{VI} was performed on an anion exchange chromatographic column of polymethacrylate resin (30 × 4.6 mm, 10 μm) (model G3268, Agilent Technologies, Japan). The target-analytes were eluted in the isocratic mode at a flow rate of 1.3 mL min⁻¹ with a mobile phase consisting of 5 mmol L⁻¹ EDTA, 15 mmol L⁻¹ Na₂SO₄ and 5 mmol L⁻¹ NaH₂PO₄ with pH 7 adjusted with 1.0 mol L⁻¹ NaOH solution. The analyses were performed at 30 °C and the injection volume was 100 μL.

The ICP-MS/MS operating conditions were set for 1550 W radio frequency, 15 L min⁻¹ plasma argon gas flow, 1.1 L min⁻¹ charger gas rate, and 0.9 L min⁻¹ auxiliary gas, while the sampling depth was 8 mm and the temperature was set at 2 °C in the nebulization chamber. Nickel cone skimmer and sampler were used. The collision gas was 99.999% pure helium and 4.3 mL min⁻¹ flow. The quadrupoles were adjusted to monitor the mass *m/z* 52 attributed to the most abundant isotope of Cr (83.8%).

Chromium complexation in standard solution

Cr^{III} is poorly soluble in water at pH 4 to 12 and does not elute in the anion exchange chromatographic column.^{24,25} Thus, a complexation step was necessary before HPLC-ICP-MS/MS analysis to convert the Cr^{III} in the standard solution to [Cr-EDTA]⁻. The methodology consisted of adding 0.1 mL of 10 mg L⁻¹ Cr^{III} standard solution and 4.9 mL EDTA solution (15 mmol L⁻¹) with pH adjusted to 7 using 1.0 mol L⁻¹ NaOH solution. The system was subsequently heated at 60 °C for 30 min and dilutions were performed using the mobile phase.²⁶ These standard solutions were used to adjust the analysis conditions by means of HPLC-ICP-MS/MS.

Sewage sludge samples

The sewage sludge samples were obtained from the sewage treatment plant of Montes Claros, Minas Gerais, Brazil. After collection, sewage sludge samples were screened in 2-mm sieves and stored in a refrigerator at 4 °C until sample preparation. The sewage sludge characteristics

are shown in Table S1 (Supplementary Information (SI) section).²⁷

Extraction method optimization

Sludge sample fortification

The extraction of Cr^{III} and Cr^{VI} from the sludge sample was optimized from fortification and recovery experiments. Hence, samples of 0.200 g in triplicate were fortified with 0.1 mL of 250 mg L⁻¹ Cr^{III} solution and allowed to stand for 24 h. The same procedure was performed by fortifying sludge samples only with Cr^{VI}.

Microwave assisted solid-liquid extraction

Extraction of Cr species was initially based on the work by Guidotti *et al.*¹¹ Following this methodology, 0.200 g of fortified sludge sample and 5 mL of an extractor phase were added to a Teflon tube. This tube was introduced into the microwave oven and the power of the apparatus ranged from 1030 to 1800 W in 25 min to reach 100 °C. The system remained at this temperature for 5 min and then cooled for 15 min. The supernatant was filtered on qualitative filter paper, the final volume was adjusted to 25.00 mL with ultrapure water, and finally analyzed by means of AAS. Four extractor phases were evaluated based on a previous study,⁶ as can be observed in Table 1.

Table 1. Parameters evaluated in the optimization of Cr^{III} and Cr^{VI} extraction in sewage sludge samples

Solid-liquid extraction	Parameter	Level
Microwave-assisted extraction	extractor phase	HCl in pH = 1
		EDTA at 50 mmol L ⁻¹ in pH = 7 (adjusted with NaOH)
		EDTA at 50 mmol L ⁻¹ in pH = 9.5 (adjusted with NH ₄ OH)
		NH ₄ OH in pH = 10
Conventional	homogenization	plate (1 h)
		vortex (5 min)
	time / h	ultrasonic bath (30 min)
		vortex/ultrasonic bath (5/30 min)
		shaking water bath at 100 rpm (24 h)
temperature / °C	1, 6, 16 and 24	
	30, 40 and 50	

EDTA: ethylenediaminetetraacetic acid.

Conventional solid-liquid extraction

A new experimental procedure based on conventional solid-liquid extraction was optimized to increase the recovery percentage of Cr^{III} and Cr^{VI} in the fortified sludge samples.

Therefore, 0.200 g of fortified sludge sample and 5 mL of 50 mmol L⁻¹ EDTA solution at pH 9.5 were used. The flask containing the sample and the extraction phase was shaken by different homogenization modes, time and temperature, which are detailed in Table 1. The supernatant was filtered on qualitative filter paper at the end of each extraction, then the final volume was adjusted to 25.00 mL with ultrapure water and the analyses were performed by AAS.

The recovery data for each parameter evaluated in Table 1 were submitted to analysis of variance (ANOVA) at a 5% significance *F*-test and the means were compared by the Duncan test using the RStudio statistical software program.²⁸

Cr speciation by HPLC-ICP-MS/MS

Triplicate sludge samples were fortified with standard solution simultaneously containing Cr^{III} and Cr^{VI} and allowed to stand for 24 h. Then, these samples were subjected to solid-liquid extraction under optimal conditions. The obtained extracts were analyzed by HPLC-ICP-MS/MS to study Cr speciation.

An analytical curve was prepared to determine recovery percentages of each Cr species by diluting the standard Cr^{III} and Cr^{VI} solution in sludge extract (matrix calibration) to minimize problems associated with the effect of matrix. Each prepared standard solution was heated at 60 °C for 30 min prior to HPLC-ICP-MS/MS analysis so that Cr^{III} was complexed by EDTA in the matrix extract.

Extraction method validation

Five validation parameters were evaluated to ensure the reliability of the optimized method results. The limits of detection (LOD), quantification (LOQ), linearity, precision and accuracy were evaluated as recommended by IUPAC.²⁹

Comparison between methods

The total Cr concentration in a non-fortified sewage sludge sample was obtained using the EPA 3051A method.³⁰ In the same sample, the optimized method and the EPA 1311 reference method²⁰ were performed in order to compare the Cr recovery efficiency by both methods. More details about these methods can be observed in Figure 1.

Application of the method in real sample

A sludge sample was submitted to the optimized and validated method to determine the Cr concentration. However, we chose to quantify the species of this metal

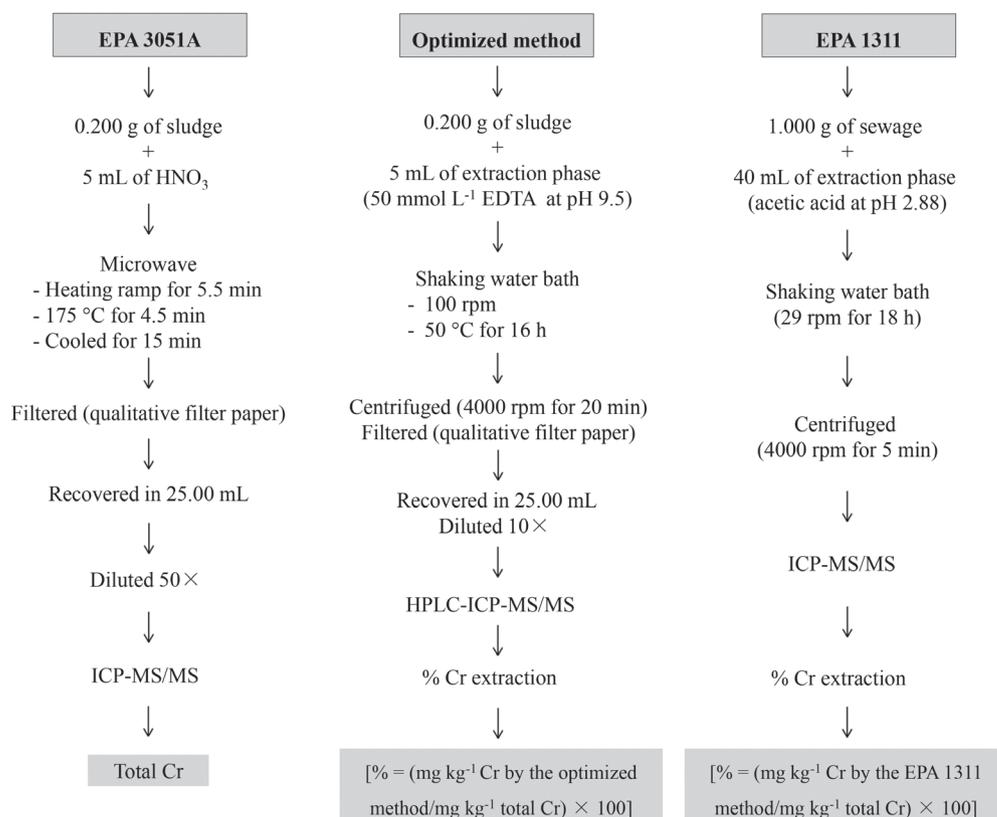


Figure 1. Representation of methodologies used to determine Cr in a sewage sludge sample: EPA 3051A (total Cr), optimized method (Cr available) and EPA 1311 reference method (Cr available).

through the standard addition method due to the problems associated with the matrix effect and the lack of a Cr-free (matrix blank) sludge sample. In this procedure, the sludge sample was divided into three aliquots in which one was not fortified, the second was fortified with 0.1 mL of 25 mg L⁻¹ Cr solution, and the third fortified with 0.1 mL of Cr solution at 125 mg L⁻¹. They were then subjected to extraction by the optimized method. The obtained extracts were diluted 10 times before the analyses. The Cr concentration in the sludge sample was obtained by extrapolation of the analytical curve.

Results and Discussion

Extraction method optimization

An AAS was preferably used during the optimization of Cr extraction in sludge samples. This was necessary because the analyses in this equipment represented approximately 95% savings in gas consumption compared to HPLC-ICP-MS/MS quantifications. However, it is not possible to distinguish Cr^{III} from Cr^{VI} in instrumental analysis by atomic absorption, so the sludge samples were separately fortified with each species in order to evaluate their recovery individually.

The first optimized solid-liquid extraction was the microwave-assisted extraction phase consisting of HCl solution at pH 1. However, the obtained recovery was unsatisfactory, being close to 10% for Cr^{III} (Figure 2).

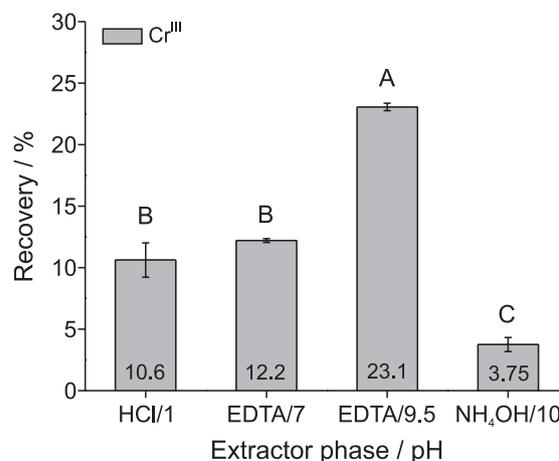


Figure 2. Cr^{III} recovery percentage in sludge samples using four microwave extraction phases.

Thus, new extraction phases were evaluated as shown in Table 1. Among the four solutions analyzed, the extraction phase constituted by the pH 9.5 EDTA solution showed the highest Cr^{III} recovery percentage, in addition to being

statistically different from the others. This solution has also been used in previous studies to extract Cr species from soils^{11,31} and dietary supplements.²⁴

Although the change in the extraction phase represented advances in microwave-Cr^{III} recovery, Cr^{VI} was not extracted under these conditions. Therefore, an alternative was to subject the sample to conventional solid-liquid extraction using the best extraction phase. The first parameter optimized in this method was the homogenization of the sludge-phase extraction system. We evaluated five levels in this parameter, as shown in Table 1. These types of homogenization were used since they are the most cited in the literature^{12,14,32} for extraction of Cr species.

The recovery percentages obtained for each homogenization type are shown in Figure 3.

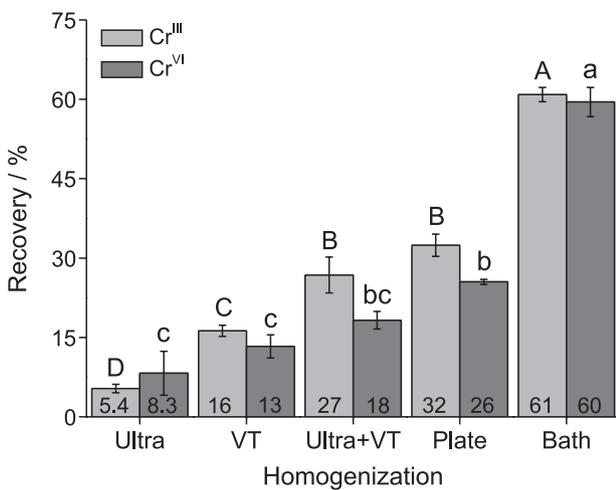


Figure 3. Cr^{III} and Cr^{VI} recovery percentages using five sample homogenization types as extraction phase made up by EDTA solution in pH 9.5. Observation: Ultra: ultrasonic bath; VT: vortex; Bath: shaking water bath.

It was observed that homogenization using a water bath for 24 h resulted in a higher recovery percentage for both Cr^{III} and Cr^{VI} in the sludge sample. This method significantly differed from other homogenization types, so it was chosen for the next steps in this study. This homogenization method was also used to determine Cr^{VI} in soils and sediments.^{33,34}

The extraction time was the second variable studied for solid-liquid extraction. Four levels were evaluated, as shown in Table 1. The recovery percentages obtained at each time are shown in Figure 4.

As can be observed, the 16-h and 24-h extraction times did not statistically differ. Therefore, the 16-h time was selected as the best time for the Cr extraction from the sewage sludge samples. A previous work¹² also used alkaline EDTA solution with this extraction time and obtained recovery percentages below 42.9% Cr in organic soils.

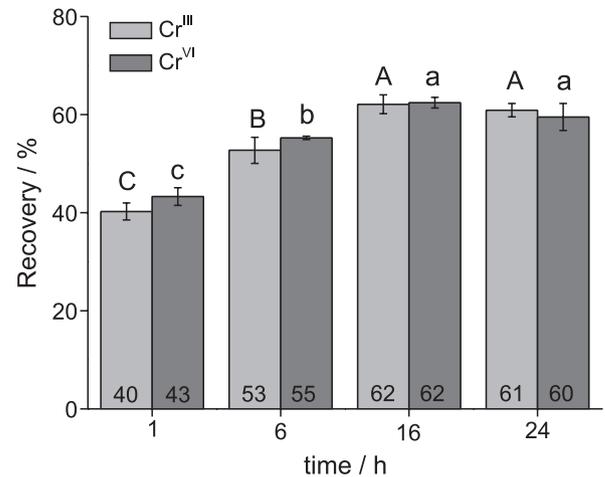


Figure 4. Cr^{III} and Cr^{VI} recovery percentages in sludge samples using four extraction times in water-bath with stirring.

Three extraction temperatures were evaluated in the last optimization parameter, as shown in Table 1. The recovery percentages obtained at each temperature are shown in Figure 5.

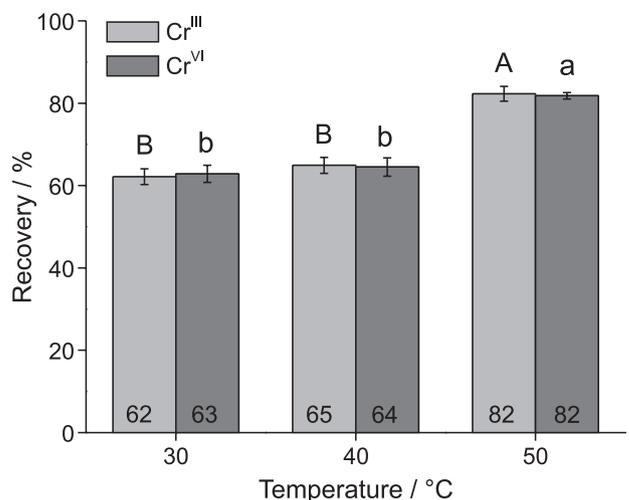


Figure 5. Cr recovery percentages in sludge samples using three temperatures for water-bath extraction in 16 h.

It could be seen that the heating at 50 °C significantly differed in this parameter from the other evaluated temperatures and showed an increase of about 20% in the Cr recovery percentage. Therefore, this temperature was adopted for Cr extraction from sewage sludge samples. Temperatures between 40 and 70 °C have already been used for solid-liquid Cr^{VI} extraction from sediment and weld smoke samples, with extraction percentages between 14 and 106% having been obtained.³⁵⁻³⁷

After optimizing these four extraction parameters, it was possible to observe that the Cr^{III} recovery percentage was four times higher than the methodology proposed by Guidotti *et al.*¹¹

Cr^{III} and Cr^{VI} speciation by HPLC-ICP-MS/MS

Cr^{III} and Cr^{VI} speciation were studied using a standard solution containing both Cr species which was prepared in the mobile phase and analyzed by HPLC-ICP-MS/MS. The obtained chromatogram is shown in Figure 6a.

As can be seen, the analyses were performed in a total time of 3.0 min and the retention times of Cr^{III} and Cr^{VI} were 1.0 and 2.2 min, respectively. On the other hand, an analysis of the extract obtained from both Cr^{III} and Cr^{VI} fortified sludge sample resulted in a 1.0-min signal chromatogram assigned to Cr^{III} (Figure 6b).

Two sewage sludge samples were fortified in order to investigate the absence of Cr^{VI} signal in the extract chromatogram; one with only Cr^{III} and one with only Cr^{VI}. Both samples were extracted under the optimized conditions and the obtained chromatograms are shown in Figure 7.

It can be observed that both chromatograms showed only the 1-min signal for Cr^{III}. These results indicated that all Cr^{VI} was reduced to Cr^{III} in the sewage sludge sample or during the extraction process. Thus, the results obtained for Cr^{VI} in the optimization step of the extraction method shown in Figures 3, 4 and 5 refer to the detected concentrations of Cr^{III}.

New experiments were performed in order to show which stage the Cr^{VI} reduction occurred in. Therefore, the standard solution containing Cr^{III} and Cr^{VI} at a concentration of 50 µg L⁻¹ was subjected to the same conditions as the optimized method, but without the sewage sludge sample. The obtained extract was analyzed by HPLC-ICP-MS/MS, and the chromatogram is shown in Figure 8.

In this chromatogram it can be observed that the two signals attributed to Cr^{III} and Cr^{VI} were detected in 1 and 2.2 min, respectively. This study revealed that optimized

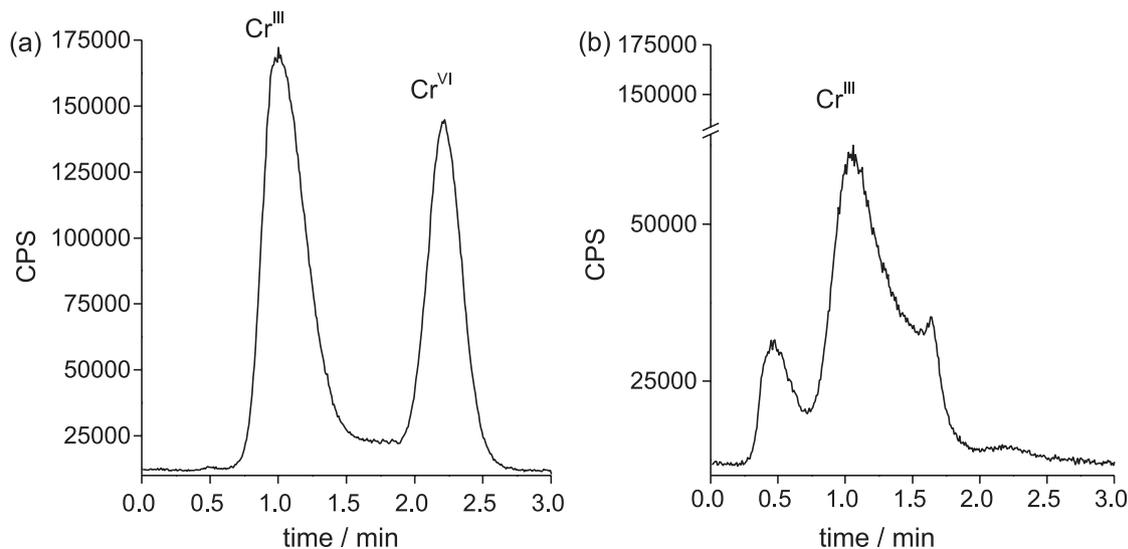


Figure 6. (a) Standard solution chromatograms with Cr^{III} and Cr^{VI} at 50 µg L⁻¹ prepared in the mobile phase, and (b) sewage sludge obtained in optimized extraction conditions.

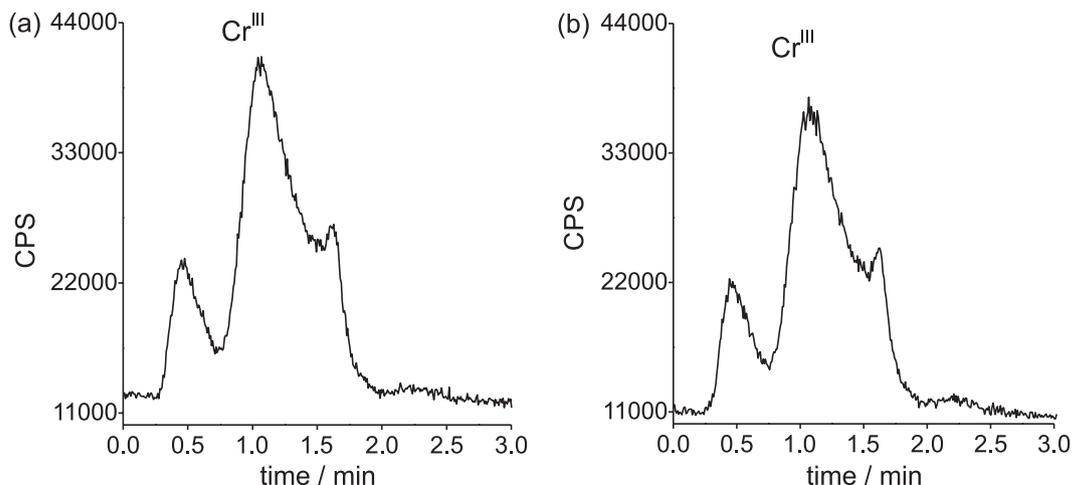


Figure 7. Chromatograms of the extracts obtained from sludge samples fortified with Cr species at 6.25 mg kg⁻¹: (a) Cr^{III} and (b) Cr^{VI}.

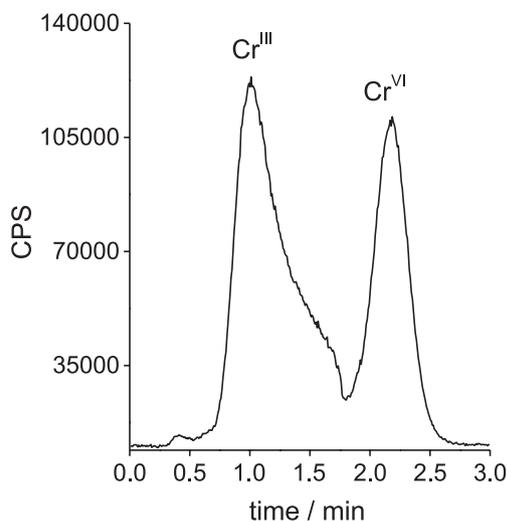


Figure 8. Chromatogram of the standard solution containing Cr^{III} and Cr^{VI} at 50 µg L⁻¹, which was submitted to the optimized extraction method without sewage sludge.

extraction conditions did not contribute to reduce Cr^{VI} into Cr^{III}. In addition, the chosen extraction phase promotes the stabilization of Cr species, as a pH around 10 makes it difficult to reduce Cr^{VI}, and EDTA prevents the oxidation or precipitation of Cr^{III}.^{3,6,38}

On the other hand, the sewage sludge presented a chemical composition which allows this type of reaction (Table S1, SI section), because the reduction of Cr^{VI} in environmental matrices may occur in the presence of Fe^{II} ions, sulfide and organic matter. In addition, the humic and fulvic acids which make up organic matter may also contribute to this reduction reaction.³⁹

Extraction method validation

The method selectivity was evaluated by analyses of (i) mobile phase, (ii) extractor phase and (iii) non-fortified sludge extract (blank) by HPLC-ICP-MS/MS. Based on the chromatograms we concluded that the signal observed at 0.5 min refers to a matrix component, while the signal at 1.7 min is a baseline elevation. It is worth mentioning that no signal was observed at the retention time of 2.2 min during the sewage sludge extract analysis (Figure 7). This result indicates that ⁵²Cr^{VI} did not show interference in polyatomic ions, for example ClOH⁺ (*m/z* = 52), which can be removed by triple quadrupole collision cell.

Bearing in mind that all Cr in the sludge was in the Cr^{III} form, we began the validation step by only using this chemical species. In addition, we chose to directly analyze the extracts by means of ICP-MS/MS, as it was not necessary to separate the Cr species before the quantification step. The results obtained in validating the optimized method are shown in Table 2.

The LOD and the LOQ were obtained by multiplying the standard deviation of the method blank (extractor phase) by three and ten times, respectively, which was analyzed by ICP-MS/MS in ten repetitions. This strategy was necessary due to the absence of a Cr-free sludge sample. As shown in Table 2, the obtained values were lower than those observed in detection and quantification studies of Cr in sewage sludge by ICP OES, which were 6.00 and 11.6 µg L⁻¹ for LOD and LOQ, respectively.^{40,41}

Instrumental linearity was evaluated using standard Cr^{III} solutions prepared in the extractor phase at concentrations of 0.2; 1.0; 10; 20; 30; 40 and 50 µg L⁻¹ in two repetitions. As can be seen in Figure S1 (SI section), the calibration curve was linear in the studied range and presented coefficient of determination (*r*²) greater than 0.99.

The precision and accuracy of the method were evaluated using Cr^{III} fortification/recovery assays at 10 and 50 µg L⁻¹ concentrations, with seven replicates at each level. Cr recovery was determined by subtracting the sludge blank contribution in order to avoid overestimating the results. The optimized extraction method was accurate because recovery percentages were within the acceptable range of 70 to 120% as recommended by IUPAC.²⁹ The accuracy was considered adequate, as relative standard deviation values were about three to seven times lower than a maximum value of 20%.

After validating the methodology, we then compared the extraction efficiency of the optimized and validated method with the reference method recommended by the EPA.²⁰

Comparison between methods

A sludge sample was submitted to the optimized extraction method and only Cr^{III} was identified in the analyses by HPLC-ICP-MS/MS. Thus, 53.3% of the metal in the sample was extracted under the optimum conditions. On the other hand, the amount of Cr extracted was only

Table 2. Results of the Cr^{III} extraction method validation in sewage sludge by means of ICP-MS/MS

Calibration data				Recovery ± RSD / %	
LOD / (µg L ⁻¹)	LOQ / (µg L ⁻¹)	Range / (µg L ⁻¹)	<i>r</i> ²	10 µg L ⁻¹	50 µg L ⁻¹
0.060	0.20	0.20-50	0.9990	73.8 ± 3	74.4 ± 7

LOD: limit of detection; LOQ: limit of quantification; *r*²: coefficient of determination; RSD: relative standard deviation.

4.09% when the same sample was submitted to extraction through the EPA 1311 method.²⁰ This means that the optimized method had about 13 times higher extraction efficiency than the reference method.

It is noteworthy that the bioavailable Cr extraction was less than 60% in both methods. It is likely that Cr^{III} is strongly adsorbed on sewage sludge components.

Method applied in real sample

The optimized and validated method was applied to a sewage sludge sample and showed a Cr^{III} concentration of 20.5 mg kg⁻¹. This concentration was less than the acceptable maximum limit of Cr^{III} in sewage sludge, which is 500 mg kg⁻¹ according to legislation 80568/4225/91 harmonized with the European Directive.⁹ Previous work⁴⁰ also quantified Cr^{III} in sludge samples at lower concentrations than those established by this regulatory organ, with concentrations ranging from 13.5 to 350.7 mg kg⁻¹.

Conclusions

We have optimized a method for inorganic chromium species in sewage sludge samples. The method was validated through the main figures of merit and it was accurate, precise, linear and presented lower limits of detection and quantification than those mentioned in the literature. Only Cr^{III} species was detected in the analysis of sewage sludge extracts by HPLC-ICP-MS/MS, as all Cr^{VI} was reduced by the components of the sludge matrix. This method was more efficient than the reference in the bioavailable Cr extraction because it increased the extraction by 13 times compared to the EPA 1311 method. The Cr^{III} concentration quantified in the sludge sample was below the maximum limit acceptable by law 80568/4225/91 harmonized with the European Directive.

Supplementary Information

Supplementary data are freely available at <http://jbcs.sbq.org.br> as PDF file.

Acknowledgments

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