

Validation of an Electrothermal Atomization Atomic Absorption Spectrometry Method for Quantification of Total Chromium and Chromium(VI) in Wild Mushrooms and Underlying Soils

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An ETAAS method was validated to quantify total Cr and Cr^{VI} in mushrooms and the underlying soils. The method includes a sample pretreatment for total Cr dissolution using a wet acid digestion procedure and a selective alkaline extraction for Cr^{VI}. The limits of detection were, expressed in $\mu\text{g/L}$, 0.15 and 0.17 for total Cr and Cr^{VI}, respectively. The linearity ranges under the optimized conditions were 0.15–25.0 and 0.17–20.0 $\mu\text{g/L}$ for total Cr and Cr^{VI}, respectively. The limits of quantification were, expressed in $\mu\text{g/g}$ of dry weight, 0.0163 and 0.0085 for total and hexavalent chromium, respectively. The precision of the instrumental method for total Cr and Cr^{VI} was lower than 1.6%, and for the analytical method, it was lower than 10%. The accuracy of the method for Cr^{VI} quantification was evaluated by the standard additions method, with the recoveries being higher than 90% for all of the added concentrations. For total Cr, certified reference materials (lichen CRM 482 and soil sample NCS ZC73001) were used. An interference study was also carried out in a mushroom simulated matrix, and it was verified that the deviations of the expected values were lower than 4.0% for both total Cr and Cr^{VI}. The validated method was applied to the evaluation of total Cr and Cr^{VI} in 34 wild mushrooms and 34 respective underlying soil samples collected in two different regions of Portugal (Beira Interior and Trás-os-Montes), with different locations regarded as noncontaminated or contaminated areas. The species were identified by a mycologist and subdivided into 10 genera and 15 species: *Amanita* (*rubescens*, *muscaria*, and *ponderosa*), *Boletus* (*regius*), *Lactarius* (*deliciosus*, *vellereus*, and *piperatus*), *Suillus* (*granulatus* and *luteus*), *Tricholoma* (*acerbum*), *Agaricus* (*sylvicola*), *Volvariella* (*gloiocephala*), *Lecopaxillus* (*giganteus*), *Macrolepiota* (*procera*), and *Psilocybe* (*fascicularis*). The mean values found for total Cr were 1.14 and 1.11 $\mu\text{g/g}$ of dry weight, and for Cr^{VI}, the mean values were 0.103 and 0.143 $\mu\text{g/g}$ of dry weight for cap and stalk, respectively. For soils, the mean concentrations found were, for total Cr, 84.0 $\mu\text{g/g}$ and, for Cr^{VI}, 0.483 $\mu\text{g/g}$. The bioconcentration factors (BCFs) based on dry weight for cap and stalk were determined, and the values found, for both total Cr and Cr^{VI}, were always <1 , although for hexavalent chromium, the BCFs were 10 times higher than for total chromium.

KEYWORDS: Mushroom; soil; total Cr; Cr^{VI}; atomic absorption spectrometry

INTRODUCTION

Heavy metals are considered to be among the most pollutant agents in all of the environmental compartments. In the soils, heavy metals are derived from natural components or geological sources, as well as from anthropogenic activities. Apart from the occupational exposure, the human exposure to toxic metals

is mainly through the diet, and the vegetable fraction is an important contribution for metal intake.

Mushrooms constitute part of the human diet because they provide carbohydrates, proteins, vitamins, and minerals (1). Moreover, mushrooms are considered a delicacy, are part of the traditional cuisine culture of some countries, and are generally consumed worldwide. Considering their relative position in the food chain, the occurrence of high metal contents in mushrooms is considered important because they may constitute a possible toxicological hazard (2). In fact, the mushrooms grown in polluted soils can uptake the metals and

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concentrate them in the edible parts. Several studies were already carried out to evaluate the contents of toxic metals in mushrooms either to tentatively characterize bioindicators of pollution through the bioconcentration factors or to evaluate the contribution to metal intake through the consumption of contaminated mushrooms. Although the main factors contributing for the accumulation of metals by mushrooms are poorly identified, the metals uptake is determined by several conditions, namely, environmental (metal contents in soil, water, and air, pH, and soil composition) and genetic properties (ecology, species, and morphological portion) (2–4).

Most of the studies on the accumulation of toxic metals in mushrooms published thus far focus on mercury, lead, cadmium, and arsenic (3–5). Referring to chromium, we only had access to three papers where the contents of total chromium in mushrooms are reported (1, 2, 5). In a review on trace element concentrations in edible mushrooms (3), while there is reference to a great number of studies on cadmium, lead, and mercury, the author only mentions four studies where total chromium was determined. To our knowledge, there are no studies on the determination of Cr^{VI} content in mushrooms.

Chromium is an extensively used metal for several industrial purposes, in the form of both tri- and hexavalent, depending upon their final use. Cr^{VI} is released in the environment as a result of coal-fired, power production, electroplating, timber treatment, pulp production, mineral ore, and petroleum refining (6, 7). This chromium species is very toxic, exhibiting genotoxic and carcinogenic actions both in animals and humans, while Cr^{III} is considered a bioelement with important metabolic functions (6).

Besides the different biological and toxicological properties of the chromium species, their mobilization from soils and uptake by vegetables are also different, depending upon several factors, namely, microbial activity (8) and organic contents in soil (9). While Cr^{III} is strongly retained in soil particles, Cr^{VI} is very weakly adsorbed and is readily available for plant uptake (6).

The purpose of this investigation was to validate a method to quantify total Cr and Cr^{VI} in mushrooms by electrothermal atomization atomic absorption spectrometry (ETAAS) and its subsequent application to measure the levels of total chromium and Cr^{VI} in mushrooms and underlying soils, collected in two regions of Portugal (Beira Interior and Trás-os-Montes). The results were then interpreted in relation to some factors, such as metal contents in soil from both regions, the different portions of mushrooms (cap and stalk that were analyzed), and the ecology of the mushrooms (mycorrhizal and saprophyte).

MATERIALS AND METHODS

Reagents and Materials. (a) All of the solutions were prepared with doubly deionized water, and the chemicals used, HF, HCl, HNO₃, NaOH, NH₄NO₃, and CaCl₂, were of Suprapure grade (Merck).

(b) Total chromium standards were prepared daily from a 1000 mg/L solution of Cr^{III} nitrate (Spectrosol, BDH) in HNO₃ (0.2%, v/v). An aqueous stock solution of Cr^{VI} at 1000 µg/mL was prepared by dissolving 0.2829 g of potassium dichromate (Riedel-de-Haen, Germany) in 100 mL of double deionized water; other diluted standard solutions were prepared daily from this stock solution. An aqueous stock solution of Cr^{III} at 1000 µg/mL was prepared by dissolving 0.5124 g of chromium chloride hexahydrate (Fluka, Germany) in 100 mL of double deionized water; other diluted standard solutions were prepared from this stock solution.

(c) **Chemical Modifiers.** A total of 1 g/L of Mg(NO₃)₂ solution and 2 g/L of Mg(NO₃)₂ plus 3 g/L of Pd(NO₃)₂ solution, Suprapur grade from Merck, were prepared in 15% (v/v) Suprapure nitric acid.

(d) **Certified Materials.** Soil NCS ZC73001 (Promochem, Germany) and lichen CRM 482, Community Bureau of Reference, BCR (Promochem, Germany).

(e) **Decontamination of Material.** To avoid contamination of the samples, all PTFE materials (Teflon vessels, pipettes, micropipette tips, and auto-sampler cups) were immersed in freshly prepared 15% v/v proanalysis HNO₃ (Merck) during 24 h, then rinsed thoroughly with doubly deionized water, and dried in a dust-free area before use.

Apparatus. (a) **Water Purification System.** Seralpur PRO 90 CN and Seradest LFM 20.

(b) **Balance.** All analytical weighings were performed with a Mettler Toledo balance AB265-S model.

(c) **Stove.** Heraeus stove D-6450 model.

(d) **Oscillating Agitator.** GFL 3006.

(e) **pH Meter.** WTW pH 330/SET-2, Best. NR 100 788, Germany.

(f) **Centrifuge 5810 R.** Eppendorf model.

(g) **Spectrometer.** Metal quantifications were carried out in a Perkin-Elmer HGA-850 furnace installed in a model AAnalyst 300 spectrometer with a deuterium arc background correction, equipped with an AS-800 autosampler and a hp deskjet 920C. The analyses were performed using Perkin-Elmer HGA tubes with an integrated platform.

Analytical Conditions. The spectrometer settings and furnace programs used with pyrolytic graphite-coated tubes were as follows: ashing temperature, 1600 °C (20 s for ramp time and 20 s for hold time); atomization temperature, 2500 °C (30 s for ramp time and 30 s for hold time); atomization temperature, 2500 °C; chemical modifier, Pd(NO₃)₂ at 3 g/L plus Mg(NO₃)₂ at 2 g/L; background correction, deuterium arc; HGA tubes with integrated platform; gas stop flow, atomization step. All data were taken at 357.9 nm. The slit width was 0.7 nm, and argon was used as the purge gas, with an internal flow rate of 300 mL/min. Readings on the spectrometer were taken by using the peak area mode (integrated absorbance) for total Cr and Cr^{VI}.

The autosampler was programmed to pipet sequentially the modifier (10 µL) followed by the standard solution/acid digest/alkaline extract sample solution (15 µL) and to dispense them together onto the platform.

Sampling. The mushrooms were collected by hand or with a plastic knife, were slightly cleaned, being the residual soil particles manually removed using paper, a brush, or a plastic knife, and were subdivided into the cap and stalk parts. A total of 34 mushrooms pertaining to 10 genera and 15 species, were collected during the autumn of 2005 and spring of 2006 in the wild, growing from different sites in the Trás-os-Montes and Beira Interior regions (northwest Portugal). In both regions, mushrooms were collected in different locations regarded as noncontaminated or contaminated according to the nearby industries, tobacco plantation, eucaliptus plantation, and railways. The identification and edibility of the mushrooms was performed by expert mycologists. In Beira Interior, 15 samples were collected, 3 saprophytes [*Agaricus silvicola* (1) and *Volvariella gloiocephala* (2)] and 12 mycorrhizal [*Amanita rubescens* (1), *Amanita ponderosa* (8), and *Boletus regius* (3)]. In Trás-os Montes, 19 samples were collected, which were 6 saprophytes [*Psilocybe fascicularis* (1), *Leucopaxillus giganteus* (1), *Macrolepiota procer* (4)] and 13 mycorrhizal [*Amanita muscaria* (2), *Lactarius deliciosus* (4), *Lactarius piperatus* (1), *Lactarius vellereus* (1), *Suillus granulatus* (1), *Suillus luteus* (1) and *Tricholoma acerbum* (4)].

The underlying soil samples were collected with a plastic spade, and the roots, small stones, gravel, leaves, sticks, and other external materials were removed.

Sample Preparation. The mushrooms were grouped according to the genus and species. The cap and stalk of all of them were separated. Dependent upon the size of the mushrooms, the samples for analysis were constituted by one or several caps or stalks (2–10 specimens of the same genus). These parts were cut into small portions with a plastic knife previously rinsed with 15% HNO₃ and doubly deionized water, placed in polyvinyl chloride (PVC) decontaminated tubes, and dried in a stove at 30–35 °C for 3 days. One representative portion of each soil sample (about 10 g) was dried under the same conditions. All of the dried samples were reduced to powder in an agate mortar.

Soil pH. Measurement of the soil pH was performed in suspensions prepared with 1 g of soil in 2.5 mL of 0.01 M CaCl₂ solution, after

standing for 30 min (10). The mean measured results for soil pH were 5.17 ± 0.26 , ranging from 3.88 to 6.66.

Total Chromium. Approximately 0.25 g of the dried powdered parts of the mushrooms or the soil samples were accurately weighed and transferred to a Teflon container. A total of 1 mL of HF and 3 mL of HNO_3 were added to the samples, and the container was covered loosely with its cap and heated at 105 °C overnight to enable the volatilization of silicates. After the volume of the digest was reduced, the remaining residue was added with 1 mL of HNO_3 and 1 mL of HCl and the Teflon container was closed for digestion during 17 h in a stove thermostatically controlled at 105 °C to completely dissolve the sample. The cool digested solution was transferred to a decontaminated tube and diluted to 8 mL with doubly deionized water. Blank reagent acid samples were processed in parallel with the samples and submitted to the same acid pretreatment.

Chromium(VI). For the selective extraction of Cr^{VI} , a procedure previously adopted by our group was applied (11). Briefly, 0.2 g of dried powdered cap and stalk mushrooms and soil samples was accurately weighed and placed into a 10 mL polypropylene tube; then 2.5 mL of 0.01 mol/L NaOH solution were added; the cap was fitted; and the tubes were shaken horizontally in an oscillating agitator for 7 h at 300 oscillations/min, at room temperature, to selectively extract the Cr^{VI} . After this period, 0.5 mL of 1 mol/L NH_4NO_3 solution was added and the sample was shaken briefly and centrifuged for 30 min at 12 500 rpm. The Cr^{VI} was measured in the supernatant at the same instrumental conditions as for total chromium. Alkaline Cr^{VI} standard solutions and blank reagents were prepared daily under the same conditions as the mushroom samples and soils.

Method Validation. For the evaluation of the instrumental precision, absorbance signals were determined in the same acid-digested samples/alkaline extracts 20 times for total Cr and Cr^{VI} , respectively, under the established instrumental conditions. For the analytical method, readings of 20 different acid-digested aliquots/alkaline extracts of the same sample were performed for total Cr and Cr^{VI} , respectively.

Linearity was observed in the working ranges (in $\mu\text{g/L}$) from 0.15 to 25.0 and 0.17–20.0 for total Cr and Cr^{VI} , respectively. To calculate the detection limit of the instrumental method, 20 determinations were carried out on 0.2% HNO_3 solution and alkaline solution and the value was calculated as $3s/m$, where "s" is the standard deviation of the blank 0.2% HNO_3 /alkaline solution measurements and "m" is the slope in the calibration curve. The limit of quantification was calculated as $10s/m$.

To verify the stability of hexavalent chromium during the extraction time of the procedure, we extracted a mushroom sample and a soil sample during 1, 3, 5, 7, and 17 h. In parallel, a standard solution was agitated with the same alkaline solution. The Cr^{VI} was quantified in the respective alkaline extracts. It was found that Cr^{VI} was stable up to 17 h and that 7 h of contact time period was enough to quantitatively extract Cr^{VI} from the samples. After we established the best time of extraction without alteration of the oxidation state of the Cr species (7 h), cap and stalk mushroom samples as well as soil samples with high, medium, and low Cr^{VI} contents were submitted to this extraction time. The obtained results constituted a further confirmation of the precision of the method described above.

Because reference materials for Cr^{VI} are not available for a mushroom matrix, the method of standards addition was applied for accuracy evaluation. Four different concentrations (between 0.25 and 2.5 $\mu\text{g/L}$) of standard solutions of Cr^{VI} were added to different aliquots of mushroom samples (six replicates for each concentration) and submitted to the alkaline extraction procedure, and the respective analyte recoveries were calculated.

The analytical method adopted for total Cr quantification in mushrooms and soils was also validated by analyzing the certified reference material, lichen CRM 482 and soil sample NCS ZC73001. For this purpose, 11 aliquots of lichen and 10 aliquots of soil sample were submitted to the acid pretreatment under the established conditions, and the respective concentrations were calculated.

To study the putative interferences of the principal mushroom constituents on the measurements of the total Cr and Cr^{VI} , a simulated matrix was prepared, containing the principal organic and inorganic species, as shown in Table 1 (12). This simulating solution was

Table 1. Principal Inorganic and Organic Constituents of Mushrooms^a Used To Prepare the Simulated Matrix Used in the Interference Studies

inorganic constituent	concentration ($\mu\text{g/g}$)	organic constituent	concentration ($\mu\text{g/g}$)
potassium	3200	carbohydrate	4000
phosphorus	800	fat	5000
chloride	690	protein	18 000
magnesium	90	total sugars	2000
calcium	60		
sodium	50		
iron	6		
zinc	4		
manganese	1		
selenium	0.09		

^a From ref 11.

subdivided into four aliquots, and to each one, the minimum and maximum concentrations of total Cr or Cr^{VI} found in the analyzed mushroom samples were added (0.03–13.8 and 0.0085–2.1 $\mu\text{g/g}$ for total Cr and Cr^{VI} , respectively). To evaluate the interferences on total Cr, four concentrations of total Cr were added to the simulated matrix enriched with Cr^{VI} (0.0085 and 2.1 $\mu\text{g/g}$). To evaluate the interferences on Cr^{VI} , four concentrations of Cr^{VI} were added to the simulated matrix enriched with total Cr (0.03–13.8 $\mu\text{g/g}$). All of the spiked simulating matrices were submitted to the overall acid digestion or to the alkaline extraction, and the absorbance signals were measured under the validated analytical conditions for total Cr and Cr^{VI} .

The pH values of soil suspensions ranged from 3.88 to 6.66; to verify the putative influence of the different values of pH on the selectivity of Cr^{VI} alkaline extraction, soils with these extreme pH values were fortified with different concentrations of Cr^{III} (0.5–5.0 $\mu\text{g/L}$) and the Cr^{VI} extraction procedure was applied. To verify the selectivity of the Cr^{VI} alkaline extraction procedure, soil samples with different Cr^{VI} contents (low, 0.069 $\mu\text{g/g}$; medium, 0.48 $\mu\text{g/g}$; high, 1.78 $\mu\text{g/g}$) were added with four concentrations of Cr^{III} (0.5–5.0 $\mu\text{g/L}$). After Cr^{VI} was submitted to the alkaline extraction, it was quantified in the supernatant.

The results obtained showed no significant differences between the concentrations of Cr^{VI} naturally present in the soil samples and the concentrations found after adding different levels of trivalent chromium. It was concluded that the Cr^{III} present was not extracted in the alkaline solution.

Application of the Validated Method. The validated methods were applied to quantify total Cr and Cr^{VI} in the collected mushroom and soil samples as described above. In Table 2, the mushroom species analyzed, the region where they were collected, and the number of each species analyzed are presented.

Ecologically, the samples analyzed were grouped in the genera saprophytes (9 samples) and mycorrhizal (25 samples), with the species of these two genera being subdivided as mentioned: *Amanita* (10): *rubescens* (1), *muscaria* (1), *ponderosa* (8); *Boletus* (3): *regius* (3); *Lactarius* (6): *deliciosus* (4), *vellereus* (1), *piperatus* (1); *Suillus* (2): *granulatus* (1), *luteus* (1); *Tricholoma* (4): *acerbum* (4); *Agaricus* (1): *sylvicola* (1); *Volvariella* (2): *gloiocephala* (2); *Lecopaxillus* (1); *giganteus* (1); *Macropleiota* (4): *procera* (4); *Psilocybe* (1); *fascicularis* (1).

A total of 10 of the analyzed mushrooms are edible species; 4 are not edible; and 1 is poisonous, as mentioned in Table 2.

Statistical Analysis. The comparison of the obtained results between the two regions and between genera for the several metals was performed. The values were log-transformed to approximate normal distribution. Statistical significant differences were determined by the paired *t* test. A *p* value of ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A wet acid digestion procedure in a closed vessel, including a preliminary decomposition step with HF and HNO_3 and a subsequent residue dissolution with HCl and HNO_3 , was

Table 2. Species, Ecology, Edibility, and Sample Number (*n*) of Analyzed Mushrooms of the Beira Interior and Trás-os-Montes Regions of Portugal

mushroom species	mushroom ecology	edibility	Beira Interior (<i>n</i>)	Trás-os-Montes (<i>n</i>)
<i>Agaricus sylvicola</i>	saprophyte	edible	1	
<i>Amanita ponderosa</i>	mycorrhizal	edible	8	
<i>Amanita rubescens</i>	mycorrhizal	edible	1	
<i>Boletus regius</i>	mycorrhizal	edible	3	
<i>Volvariella gloiceps</i>	saprophyte	edible	2	
<i>Amanita muscaria</i>	mycorrhizal	not edible		1
<i>Lactarius deliciosus</i>	mycorrhizal	edible		4
<i>Lactarius piperatus</i>	mycorrhizal	not edible		1
<i>Lactarius vellereus</i>	mycorrhizal	not edible		1
<i>Lecopaxillus giganteus</i>	saprophyte	edible		1
<i>Macrolepiota procera</i>	saprophyte	edible		4
<i>Psilocybe fascicularis</i>	saprophyte	poisonous		1
<i>Suillus granulatus</i>	mycorrhizal	edible		1
<i>Suillus luteus</i>	mycorrhizal	edible		1
<i>Tricholoma acerbum</i>	mycorrhizal	not edible		4

Table 3. Performance of the Method

	precision (CV%)		linearity ($\mu\text{g/L}$)	detection limit		quantification limit	
	instrumental procedure	overall procedure		($\mu\text{g/L}$)	($\mu\text{g/g}$)	($\mu\text{g/L}$)	($\mu\text{g/g}$)
total Cr	1.3	10.0	0.15–25	0.15	0.0048	0.51	0.0163
Cr ^{VI}	1.6	6.2	0.17–20	0.17	0.0026	0.57	0.0085

Table 4. Statistical Results (Mean Values, $\mu\text{g/g}$ of Dry Weight \pm SD) for the Recoveries Obtained by the Standard Additions Method (*n* = 6, for Each Added Concentration, $\mu\text{g/L}$)

	C_1^a ($\mu\text{g/g}$)	C_2^b ($\mu\text{g/L}$)	C_3^c ($\mu\text{g/g}$)	recovery (%)
Cr ^{VI}	0.009 45 \pm 0.001 26	0.25	0.013 00 \pm 0.000 15	94 \pm 4
		0.5	0.016 57 \pm 0.000 23	95 \pm 3
		1.0	0.023 85 \pm 0.000 30	96 \pm 2
		2.5	0.0440 \pm 0.000 75	92 \pm 2

^a C_1 = initial concentration ($\mu\text{g/g}$) of Cr^{VI} in the whole mushroom sample. ^b C_2 = concentration of the standard metal solution ($\mu\text{g/L}$) added to the whole mushroom sample prior to the application of the overall procedure. ^c C_3 = final concentrations ($\mu\text{g/g}$) found in spiked whole mushroom samples.

effective for the simplification of the matrices (mushrooms and soils) to obtain the necessary dissolution of total chromium for ETAAS determination. The alkaline extraction adopted for the quantification of Cr^{VI} enabled the selective separation of this species from both matrices, as verified by the validation procedure.

The parameters determined to evaluate the performance of the method both for total Cr and Cr^{VI} in mushroom samples are summarized in **Table 3**. The precision, evaluated for both the instrumental and analytical procedures, was $\leq 10\%$ for the total Cr and Cr^{VI}. The limits of detection were 0.15 and 0.17 $\mu\text{g/L}$, and the linearity was 0.15–25.0 and 0.17–20.0 for total Cr and Cr^{VI}, respectively. On the basis of 0.25 or 0.20 g of dried sample in a final volume of 8 or 3 mL, the limits of quantification were 0.0163 and 0.0085 $\mu\text{g/g}$ for total Cr and Cr^{VI}, respectively. These values show that the method is very sensitive, enabling the quantification of very low levels of Cr^{VI}, which, if present, must be at the lowest possible levels given that it is a very toxic species.

The accuracy of the method for the quantification of Cr^{VI} was evaluated by the standard additions method, and the results are presented in **Table 4**. The recoveries were higher than 92% for all of the added concentrations.

The certified reference materials, lichen CRM 482 and soil sample NCS ZC73001, were used to perform the accuracy

Table 5. Statistical Results (Mean Values, $\mu\text{g/g}$ of Dry Weight \pm SD) of Certified Reference Materials

	certified material	total Cr	
		certified value ($\mu\text{g/g}$)	measured value ($\mu\text{g/g}$)
	lichen CRM 482 (<i>n</i> = 11)	4.12 \pm 0.15	3.86 \pm 0.18
	soil sample NCS ZC73001 (<i>n</i> = 10)	58 \pm 2	57.5 \pm 1.1

studies for total chromium in mushroom and soil samples, respectively. The results showed that there were no contamination or losses during the pretreatment procedure for the analyzed metal, with the concentrations obtained being in good agreement with the certified values (see **Table 5**). For the lichen CRM 482 reference material, the certified value of total chromium was $4.12 \pm 0.15 \mu\text{g/g}$ and the value found after applying the implemented method was $3.86 \pm 0.18 \mu\text{g/g}$ (deviation of 6.3% considering the mean value). For the soil sample NCS ZC73001 reference material, the certified value for total chromium was $58 \pm 2 \mu\text{g/g}$ and the found value was $57.5 \pm 1.1 \mu\text{g/g}$ (deviation of 3.2% considering the mean value).

The results of the interference study conducted in the simulated matrix spiked with the minimum and maximum concentrations of total Cr or Cr^{VI} found in the mushroom samples are summarized in **Table 6**. As it can be observed, the deviations from the expected values are always lower than 4.0% for both total Cr and Cr^{VI} and all of the added metal concentrations. From this study, we can conclude that there was no noticeable interference of the principal mushroom constituents on the contents of total Cr and Cr^{VI}.

To confirm the repeatability of the alkaline extraction procedure of Cr^{VI}, eight aliquots of the same cap and stalk of mushroom samples containing three different levels of Cr^{VI} (<0.0085 , 0.103, and 0.580 $\mu\text{g/g}$ in caps and <0.0085 , 0.143, and 0.81 $\mu\text{g/g}$ in stalks) were submitted to the alkaline extraction, the metal was quantified, and the coefficient of variation of the results was determined. As is shown in **Table 7**, the coefficient of variation was lower than 11.4% for all of

Table 6. Deviations (%) from Expected Values for Total Cr and Cr^{VI} Obtained in the Interference Study (*n* = 5 for Each Added Concentration)

interference species	concentration added ($\mu\text{g L}^{-1}$)		concentration found ^a ($\mu\text{g L}^{-1}$)		deviation from expected values (%)	
	total Cr	Cr ^{VI}	total Cr	Cr ^{VI}	total Cr	Cr ^{VI}
total Cr _{min} ^b	1			0.95 ± 0.04		1.6
	2.5			2.40 ± 0.08		2.22
	5			4.85 ± 0.15		2.0
	10			9.60 ± 0.30		2.1
	1			0.93 ± 0.03		1.9
total Cr _{max} ^c	2.5			2.37 ± 0.08		2.2
	5			4.75 ± 0.20		2.0
	10			9.80 ± 0.10		2.1
	2.5		2.38 ± 0.08		2.5	
Cr ^{VI} _{min} ^d	5		4.60 ± 0.10		1.5	
	10		9.20 ± 0.20		1.4	
	20		19.40 ± 0.50		1.6	
	2.5		2.38 ± 0.12		3.4	
	5		4.75 ± 0.25		4.0	
Cr ^{VI} _{max} ^e	10		9.40 ± 0.50		3.7	
	20		19.60 ± 0.20		0.5	

^a Mean values ($\mu\text{g/L}$) ± SD. ^b Minimum concentration of total Cr found in the analyzed mushrooms samples added to the interference matrix (0.03 $\mu\text{g/g}$). ^c Maximum concentration of total Cr found in the analyzed mushrooms samples added to the interference matrix (13.8 $\mu\text{g/g}$). ^d Minimum concentration of Cr^{VI} found in the analyzed mushrooms samples added to the interference matrix (0.0085 $\mu\text{g/g}$). ^e Maximum concentration of Cr^{VI} found in the analyzed mushrooms samples added to the interference matrix (2.1 $\mu\text{g/g}$).

Table 7. Repeatability of the Alkaline Extraction Procedure of Cr^{VI}

	low concentration	median concentration	high concentration
cap sample ($\mu\text{g/g}$)	<0.0085	0.103	0.580
coefficient of variation (%)	11.4	6.2	9.8
stalk sample ($\mu\text{g/g}$)	<0.0085	0.143	0.81
coefficient of variation (%)	7.8	9.0	6.2
soil sample ($\mu\text{g/g}$)	0.086	0.427	1.78
coefficient of variation (%)	4.5	8.5	7.0

the assayed samples. The obtained data show that even at very low contents of Cr^{VI} present in the mushroom samples, the results of its quantification are precise and reproducible.

A similar study was performed in soils containing three different levels of Cr^{VI} (0.086, 0.427, and 1.78 $\mu\text{g/g}$). The coefficient of variation was lower than 8.5%, showing that the extraction procedure is also precise when applied to soils with different contents of Cr^{VI} (see **Table 7**).

The contents of total Cr and Cr^{VI} (expressed as mean values in $\mu\text{g/g}$ of dry weight ± standard deviation) in the mushrooms grouped in the genera saprophytes and mycorrhizal and in soil samples collected in two different regions of the interior of Portugal (Trás-os-Montes and Beira Interior) are summarized in **Table 8**.

Total Cr contents found in the total mushroom cap samples (*n* = 34) ranged from 0.02 to 13.84 $\mu\text{g/g}$ of dry weight. When the mushroom caps are grouped in mycorrhizal and saprophyte, no differences were found between these two groups (1.49 ± 1.41 versus 0.20 ± 0.16 $\mu\text{g/g}$ of dry weight). Referring to the levels of total Cr in the mushroom caps collected in the two regions, there was no statistical difference in the contents. In the stalk of the total mushroom samples (*n* = 34), total Cr ranged from 0.04 to 6.50 $\mu\text{g/g}$ of dry weight. Again, mycorrhizal stalks presented higher levels of total Cr than saprophytes, although the statistical difference had no significance (1.30 ± 0.73 versus

0.59 ± 0.56 $\mu\text{g/g}$ of dry weight, in mycorrhizal and saprophytes, respectively, *p* = 0.1395).

When the total chromium values in the caps and stalks in the total mushrooms of Trás-os-Montes were compared, a significantly higher content was found in the stalks (1.02 ± 1.24 and 1.33 ± 0.90 $\mu\text{g/g}$, for caps and stalks, respectively, *p* = 0.0389). Considering all of the saprophyte samples, there was no difference in the total chromium contents between caps and stalks.

Other authors have already quantified total Cr in mushrooms, with maximum and minimum referred values of 0.2 and 2.0 $\mu\text{g/g}$ (5), 0.8 and 7.52 $\mu\text{g/g}$ (2), and 0.0 and 56.0 $\mu\text{g/g}$ (13). In a recent study, in which only two edible species were analyzed, the chromium contents were 16.2 and 32.1 $\mu\text{g/g}$ (1). In a review by Kalac and Svoboda (3) that reports the contents of several metals in mushrooms, only four papers are mentioned for chromium evaluation, with the values being between 0.1 and 2 $\mu\text{g/g}$. In both our study and those published by others, a great variability in chromium contents was found. This is not surprising, because several factors can contribute to the uptake of chromium by the mushrooms: soil contamination, pH, organic soil contents, and the ecology of the mushrooms.

Referring to the Cr^{VI} contents, they ranged from <0.0085 to 0.580 $\mu\text{g/g}$ of dry weight in the caps of all mushroom samples (*n* = 34). There were no differences in the Cr^{VI} contents between mycorrhizal and saprophyte genera or between regions. In the stalks, Cr^{VI} contents ranged from <0.0085 and 0.81 $\mu\text{g/g}$ of dry weight and there were no significant differences between mycorrhizal and saprophytes. Interestingly, a significant difference (*p* < 0.05) was observed in the contents of Cr^{VI} in the stalks from Beira Interior and Trás-os-Montes regions (0.075 ± 0.042 versus 0.197 ± 0.103 $\mu\text{g/g}$ of dry weight, respectively, with the median value being 0.048 and 0.143 $\mu\text{g/g}$). This can be justified by the significantly lower levels of total Cr in the soils of the Beira Interior region when compared to Trás-os-Montes (30.4 ± 15.6 versus 126.3 ± 53.8 $\mu\text{g/g}$ of dry weight, respectively, with the median value being 71.8 and 15.3 $\mu\text{g/g}$), as well as the lower levels of Cr^{VI} in the first region, although not statistically significant (0.307 ± 0.080 versus 0.623 ± 0.284 $\mu\text{g/g}$ of dry weight, respectively, *p* = 0.0554). Also, there were no differences between the levels of Cr^{VI} in the caps and stalks

Table 8. Contents (Mean Values, $\mu\text{g/g}$ of Dry Weight \pm SD) of Total Chromium and Hexavalent Chromium in Mushroom and Soil Samples^a

	cap		stalk		soil	
	Cr	Cr ^{VI}	Cr	Cr ^{VI}	Cr	Cr ^{VI}
total samples	<i>n</i> = 34 1.14 \pm 1.03 (0.02–13.84) Med ^b = 0.30	<i>n</i> = 34 0.103 \pm 0.045 (<0.0085–0.580) Med = 0.058	<i>n</i> = 34 1.11 \pm 0.55 (0.04–6.50) Med = 0.41	<i>n</i> = 34 0.143 \pm 0.061 (<0.0085–0.81) Med = 0.088	<i>n</i> = 34 84.0 \pm 34.5 (5.8–343.0) Med = 71.8	<i>n</i> = 34 0.483 \pm 0.170 (0.069–1.785) Med = 0.286
Beira Interior samples	<i>n</i> = 15 1.30 \pm 1.94 (0.09–13.84) Med = 0.27	<i>n</i> = 15 0.058 \pm 0.023 (0.0196–0.179) Med = 0.049	<i>n</i> = 15 0.83 \pm 0.66 (0.13–4.57) Med = 0.35	<i>n</i> = 15 0.075 \pm 0.042 c ^c (0.009–0.304) Med = 0.048	<i>n</i> = 15 30.4 \pm 15.6 e (5.8–87.5) Med = 15.3	<i>n</i> = 15 0.307 \pm 0.080 (0.069–0.679) Med = 0.262
Trás-os-Montes samples	<i>n</i> = 19 1.02 \pm 1.24 d (0.02–11.54) Med = 0.34	<i>n</i> = 19 0.139 \pm 0.078 a (<0.0085–0.580) Med = 0.065	<i>n</i> = 19 1.33 \pm 0.90 d (0.04–6.50) Med = 0.53	<i>n</i> = 19 0.197 \pm 0.103 a,c (<0.0085–0.812) Med = 0.143	<i>n</i> = 19 126.3 \pm 53.8 e (6.8–343.0) Med = 71.8	<i>n</i> = 19 0.623 \pm 0.284 (0.086–1.785) Med = 0.342
mycorrhizal samples	<i>n</i> = 25 1.49 \pm 1.41 (0.08–13.84) Med = 0.45	<i>n</i> = 25 0.103 \pm 0.045 (0.019–0.497) Med = 0.060	<i>n</i> = 25 1.30 \pm 0.73 (0.11–6.50) Med = 0.53	<i>n</i> = 25 0.130 \pm 0.059 (<0.0085–0.471) Med = 0.082	<i>n</i> = 25 95.7 \pm 44.7 (5.8–343.0) Med = 71.8	<i>n</i> = 25 0.562 \pm 0.219 (0.086–1.785) Med = 0.286
saprophyte samples	<i>n</i> = 9 0.20 \pm 0.16 (0.02–0.70) Med = 0.15	<i>n</i> = 9 0.105 \pm 0.138 b (<0.0085–0.580) Med = 0.056	<i>n</i> = 9 0.59 \pm 0.56 (0.04–2.31) Med = 0.27	<i>n</i> = 9 0.178 \pm 0.195 b (<0.0085–0.813) Med = 0.096	<i>n</i> = 9 51.7 \pm 35.4 (6.1–144.0) Med = 15.3	<i>n</i> = 9 0.266 \pm 0.134 (0.069–0.638) Med = 0.257

^a Minimum and maximum values are indicated in parentheses. ^b Med = median value. ^c Significantly different by the paired *t* test at 95% confidence level: d and e for total Cr and a–c for Cr^{VI}.

of the total mushrooms (*n* = 34) (0.143 \pm 0.061 versus 0.103 \pm 0.045 $\mu\text{g/g}$ of dry weight, respectively, with the median value being 0.088 and 0.058 $\mu\text{g/g}$).

Total chromium in the total soil samples (*n* = 34) ranged from 5.8 to 343 $\mu\text{g/g}$ of dry weight, with the mean pH value of soil samples being 5.17 \pm 0.24. For the soil samples collected in the two regions, Beira Interior and Trás-os-Montes, a significant difference (*p* < 0.05) was observed for total Cr contents (30.4 \pm 15.6 versus 126.3 \pm 53.8 $\mu\text{g/g}$ of dry weight, respectively, with the median value being 15.3 and 71.8 $\mu\text{g/g}$), with mean pH values of 4.95 \pm 0.10 and 5.46 \pm 0.35, respectively, in the two regions. This finding is in accordance with the characteristics of Trás-os-Montes soils, which are predominantly serpentinic, resultant from ultrabasic rocks. Serpentinic soils have high contents of chromium that can range between 634 and 125 000 $\mu\text{g/g}$ (14). Generally, we found a negative correlation between the stalk and cap BCFs and the pH values of the respective soils, for both total chromium and hexavalent chromium. This is in accordance with the findings of other authors in an experiment performed on mineral contents in onions (15). In fact, the pH of the soils influences the metal contents of the vegetables, with the lowering of pH values normally favoring the uptake of the metals.

There were no significant differences between the total Cr contents of soil samples subjacent to mycorrhizal and saprophyte genera (5.8–343 versus 6.1–144 $\mu\text{g/g}$ of dry weight, respectively), and the pH values were 5.14 \pm 0.26 and 5.25 \pm 0.28, respectively.

Referring to Cr^{VI} contents in the total soil samples, there were neither significant differences between Beira Interior and Trás-os-Montes regions (0.069–0.679 versus 0.086–1.785 $\mu\text{g/g}$ of dry weight) nor between mycorrhizal and saprophyte genera (0.086–1.785 versus 0.069–0.638 $\mu\text{g/g}$ of dry weight). For total samples (*n* = 34), the levels ranged from 0.069 to 1.785 $\mu\text{g/g}$ of dry weight.

To evaluate the chromium bioconcentration capacity of the mushrooms (16), total Cr and Cr^{VI} BCFs were calculated as the quotient between the concentration in cap or stalk of each mushroom and the respective underlying soil (on the basis of

dry weight). The geometric means and median values of all of the obtained BCFs were also calculated (Table 9).

Considering all of the analyzed samples (*n* = 34), the values of BCF for total Cr were 0.008 \pm 0.031 (median value of 0.006) and 0.012 \pm 0.022 (median value of 0.009) for cap and stalk, respectively. For Cr^{VI}, BCF values were 0.142 \pm 0.119 and 0.126 \pm 0.178 (median values of 0.171 and 0.231), for cap and stalk, respectively. Considering the two regions and cap and stalk conjunctly, the BCF geometric means for total Cr and Cr^{VI} ranged from 0.004 to 0.024 and from 0.093 to 0.187, respectively.

The BCF values can be indicative of the tendency of vegetable species to accumulate toxins present in the soil. For example, for mercury, some mushroom species are bioaccumulators, with very high BCFs, attaining mean values of 73 and 38 for caps and stalks, respectively, in some species (17). A similar study made for copper and zinc showed the capacity of some mushroom species to bioconcentrate these elements, with very high BCFs (maximum values of 22 and 19 were found for copper and zinc, respectively) (15). In the present study, the geometric mean and median values found, for both total Cr and Cr^{VI}, were always <1, and we can conclude that chromium does not bioconcentrate in the analyzed mushroom species, which, following the adopted criteria, can be considered bioexclusors (14). However, it must be highlighted that Cr^{VI} was more extensively concentrated by the mushrooms than was total Cr, showing BCFs 1 order of magnitude higher. Interestingly, the uptake of Cr^{VI} in the animal cells is much higher than the uptake of trivalent chromium. Given the high toxicity of Cr^{VI}, its presence in food is a matter of concern.

The cap/stalk ratios of the contents of total chromium and hexavalent chromium for all of the mushroom samples were also calculated, and the values found were of the same order of magnitude (0.99 \pm 0.45 and 0.97 \pm 0.22 for total and hexavalent chromium, respectively) (see Table 9).

The cap/stalk ratios for total Cr in Beira Interior and Trás-os-Montes regions were 1.17 \pm 1.02 and 0.86 \pm 0.36, respectively, and, for Cr^{VI}, were 1.26 \pm 0.39 and 0.74 \pm 0.24, respectively. For mycorrhizal and saprophyte samples, the cap/

Table 9. Bioconcentration Factors (BCF Values;^a Geometric Mean \pm SD) and Cap/Stalk Quotient (Arithmetic Mean \pm SD) Based on Dry Weight with the Concentration ($\mu\text{g/g}$) and pH Values of Underlying Soils

	Cr				Cr^{VI}			
	BCF cap	BCF stalk	cap/stalk	soil ($\mu\text{g/g}$)	BCF cap	BCF stalk	cap/stalk	soil ($\mu\text{g/g}$)
all samples (n = 34)	0.008 \pm 0.031 (0.001–0.377)	0.012 \pm 0.022 (0.001–0.299)	0.99 \pm 0.45 (0.02–7.00)	84.0 \pm 34.5 pH =	0.142 \pm 0.119 (0.000–1.696)	0.126 \pm 0.178 (0.000–2.367)	0.97 \pm 0.22 (0.00–2.32)	0.483 \pm 0.170 pH =
Beira Interior sample (n = 15)	Med = 0.006 Med = 0.020 (0.001–0.38)	Med = 0.009 Med = 0.030 (0.001–0.299)	Med = 0.73 Med = 0.65 (0.02–7.00)	5.17 \pm 0.24 4.95 \pm 0.10 pH =	Med = 0.171 (0.030–0.990)	Med = 0.231 (0.010–1.390)	Med = 0.87 1.26 \pm 0.39 (0.18–2.32)	5.17 \pm 0.24 0.307 \pm 0.080 pH =
Trás-os-Montes samples (n = 19)	0.004 \pm 0.004 (0.001–0.034)	0.007 \pm 0.010 (0.001–0.066)	0.86 \pm 0.36 (0.05–3.33)	126.3 \pm 53.8 pH =	0.115 \pm 0.186 (0.000–1.696)	0.093 \pm 0.256 (0.000–2.380)	0.74 \pm 0.24 (0.00–2.08)	0.623 \pm 0.284 pH =
mychorizial sample (n = 25)	0.009 \pm 0.042 Med = 0.004 (0.01–0.377)	0.012 \pm 0.025 Med = 0.006 (0.001–0.299)	1.16 \pm 0.61 Med = 0.75 (0.02–7.00)	95.7 \pm 44.7 5.46 \pm 0.35 pH =	0.167 \pm 0.097 (0.027–1.000)	0.115 \pm 0.116 (0.000–1.310)	1.13 \pm 0.27 (0.00–2.32)	0.562 \pm 0.219 5.46 \pm 0.35 pH =
saprophyte sample (n = 9)	0.005 \pm 0.008 (0.001–0.028)	0.012 \pm 0.052 (0.001–0.169)	0.54 \pm 0.23 (0.18–0.95)	51.7 \pm 35.4 pH =	0.091 \pm 0.370 (0.000–1.696)	0.162 \pm 0.532 (0.000–2.380)	0.52 \pm 0.22 (0.00–0.91)	0.266 \pm 0.14 pH =
	Med = 0.005	Med = 0.01	Med = 0.57	5.25 \pm 0.28	Med = 0.115	Med = 0.380	Med = 0.65	5.25 \pm 0.28

^a BCF = concentration of the dry mushroom sample (cap/stalk)/concentration of the dry soil sample. ^b Med = median value.

stalk ratios for total Cr were 1.16 ± 0.61 and 0.54 ± 0.23 , respectively, and, for Cr^{VI} , were 1.13 ± 0.27 and 0.52 ± 0.22 , respectively. Although the values are apparently different, there is no statistical significance between the ratios of mycorrhizal and saprophytic samples. The results obtained showed that the cap/stalk ratios were >1 for the Beira Interior region and for mycorrhizal samples, for total and hexavalent chromium, respectively.

For all mushroom samples analyzed, $n = 34$, the percentage of Cr^{VI} relative to total Cr was, in mean values, 9.0 and 12.9%, for cap and stalk, respectively. For the total underlying soils, $n = 34$, the percentage found was, in mean value, 0.57%.

The percentage of total chromium in the caps and stalks relative to the total Cr in the underlying soils was, in mean value, 1.3% for both. This value is in agreement with that referred by others, which found 1% as the uptake extension of total chromium by the plants (8). For the Cr^{VI} , the percentages found were, in mean value, 21.3 and 29.6%, respectively.

The high contents of total Cr present in the soils with a low percentage of Cr^{VI} (0.57%) and the higher percentages of this species both in caps and stalks (9 and 12.9%) allow us to conclude that mushrooms accumulate Cr^{VI} in a greater extension, and this is in agreement with the greater bioavailability of Cr^{VI} in the soils. Thus, it can be concluded that mushrooms are good bioindicators of Cr^{VI} soil pollution.

LITERATURE CITED

- Jonnalagadda, S. B.; Pienaar, D. H.; Haripersad, K. Elemental distribution in selected *Agaricus* and *Rhizina* mushrooms in South Africa. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* **2006**, *41*, 507–514.
- Michelot, D.; Siobud, E.; Doré, J.-C.; Viel, C.; Poirier, F. Update on metal content profiles in mushrooms—Toxicological implications and tentative approach to the mechanisms of bioaccumulation. *Toxicon* **1998**, *12*, 1997–2012.
- Kalac, P.; Svoboda, L. A review of trace element concentrations in edible mushrooms. *Food Chem.* **2000**, *69*, 273–281.
- Gadd, G. M. Interaction of fungi with toxic metals. *New Phytol.* **1993**, *124*, 25–60.
- Barcan, V. Sh.; Kovnatsky, E. F.; Smetannikova, M. S. Absorption of heavy metals in wild berries and edible mushrooms in an area affected by smelter emissions. *Water, Air, Soil Pollut.* **1998**, *103*, 173–195.
- Bolan, N. S.; Adriano, D. C.; Natesan, R.; Koo, B.-J. Effects of organic amendments on the reduction and phytoavailability of chromate in mineral soil. *J. Environ. Qual.* **2003**, *32*, 120–128.
- Singh, V. K.; Tiwari, P. N. Removal and recovery of chromium(VI) from industrial waste water. *J. Chem. Technol. Biotechnol.* **1997**, *69*, 376–382.
- Desjardin, V.; Bayard, R.; Huck, N.; Manceau, A.; Gourdon, R. Effect of microbial activity on the mobility of chromium in soils. *Waste Manage.* **2002**, *22*, 195–200.
- Banks, M. K.; Schwab, A. P.; Henderson, C. Leaching and reduction of chromium in soil as affected by soil organic content and plants. *Chemosphere* **2006**, *62*, 255–264.
- Eckert, D.; Sims J. T. Recommended soil pH and lime requirement tests. In *Recommended Soil Testing Procedures for the Northeastern United States*; Sims, J. T., Wolf, A., Eds.; Northeast Regional Bulletin 493, Agricultural Experiment Station, University of Delaware: Newark, DE; pp 11–16.
- Furtmann, K.; Seifert, D. On the routine determination of chromium(VI) in soils. *Fresenius' J. Anal. Chem.* **1990**, *338*, 73–74.
- Holland, B.; Welch, A. A.; Unwin, I. D.; Buss, D. H.; Paul, A. A.; Southgate, D. A. T. *The Composition of Foods*, 5th revised and extended ed.; Royal Society of Chemistry: Cambridge, U.K. 1998.
- Michelot, D.; Poirier, F.; Melendez-Howell, L. M. Metal content profiles in mushrooms collected in primary forests of Latin America. *Arch. Environ. Contam. Toxicol.* **1999**, *36*, 256–263.
- Shanker, A. K.; Cervantes, C.; Loza-Taveras, H.; Avudainayagam, S. Chromium toxicity in plants. *Environ. Int.* **2005**, *31*, 739–753.
- Ariyama, K.; Nishida, T.; Noda, T.; Kadokura, M.; Yasui, A. Effects of fertilization, crop year, variety, and provenance factors on mineral concentrations in onions. *J. Agric. Food Chem.* **2006**, *54*, 3341–3350.
- Alonso, J.; Garcia, M. A.; Pérez-Lopéz, M.; Melgar, M. J. The concentration and biocaccumulation factors of copper and zinc in edible mushrooms. *Arch. Environ. Contam. Toxicol.* **2003**, *44*, 180–188.
- Falandysz, J.; Jedrusiak, A.; Lipka, K.; Kannan, K.; Kawano, M.; Gucia, M.; Brzostowski, A.; Dadej, M. Mercury in wild mushrooms and underlying soil substrate from Koszalin, North-central Poland. *Chemosphere* **2004**, *54*, 461–466.

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