

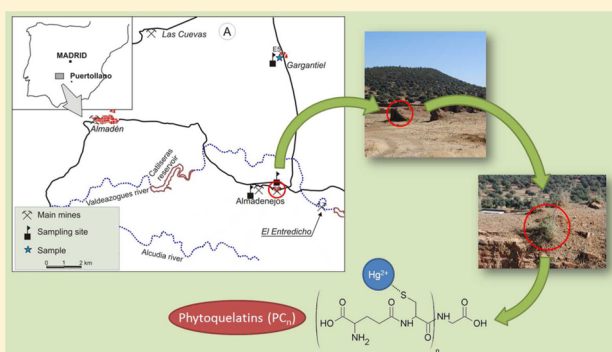
Evaluation of Mercury Stress in Plants from the Almadén Mining District by Analysis of Phytochelatins and Their Hg Complexes

Àngela Dago,[†] Inmaculada González,[†] Cristina Ariño,^{*,†} Alba Martínez-Coronado,[‡] Pablo Higuera,[‡] José Manuel Díaz-Cruz,[†] and Miquel Esteban[†]

[†]Departament de Química Analítica, Facultat de Química, Universitat de Barcelona, Martí i Franquès, 1-11, 08028 Barcelona, Barcelona, Spain

[‡]Instituto de Geología Aplicada, Escuela de Ingeniería Minera e Industrial de Almadén, Universidad de Castilla-La Mancha, Plaza M. Meca 1, 13400 Almadén, Ciudad Real, Spain

ABSTRACT: To evaluate plant response to Hg stress, glutathione, phytochelatins, and their Hg complexes were analyzed using HPLC with amperometric detection in samples of *Asparagus acutifolius* grown in the Almadén mining district (Ciudad Real, Spain), one of the most Hg-contaminated sites in the world. Soils of the Almadén mining district, and specifically from the Almadenejos zone, are highly contaminated, with some zones having values above $4,000 \mu\text{g Hg g}^{-1}$ soil. Although soils have an extremely high concentration of mercury, generally less than 2% is available for plants, as is shown by various soil extractions simulating bioavailability. In plants, Hg concentration increases depending on the content of Hg in soils. In addition, Hg levels in roots are higher than in aerial parts, which is a strategy of plants for protecting their more sensitive aerial parts from the deleterious effects of metal stress. The total content of phytochelatins (PCs) and their complexes are directly related with the amount of mercury in soils. These findings highlight the important role of thiol compounds and their metal complexes in capturing and fixing Hg from soils, giving plants the capacity to deal with the heavy metal toxicity of polluted soils.



1. INTRODUCTION

The development and growth of mining, metallurgy, and industrial activities has increased the concentration of heavy metals in the environment, thus exposing animals and plants to high concentrations of these contaminants. Since plants are the main source of human food, the presence of heavy metals in them is of great interest. Plants absorb metals from soils, water, and air. However, the chief source of metal absorption is soil. Uptake from it depends not only on the total content of the respective metals but also on their accessibility to roots and transfer across the soil-root interface. The total amount of metal in a soil is, in turn, affected by the inherent natural resources of the particular area, in addition to the agricultural and industrial activities.¹

Among the toxic trace metals, mercury (Hg) is one of the most hazardous environmental pollutants.^{2–5} The toxicity and environmental mobility of mercury compounds are closely related to their chemical form. Cinnabar and other mercury sulfides, mercury oxides, metallic mercury, and organo-mercury compounds are the most common mercury forms found near mercury mining sites. Moreover, total concentrations of metals in soils are not a good indicator of phytoavailability, or a good tool for potential risk assessment, due to the varied and complex distribution patterns of metals among various chemical species or solid phases.

The Almadén district (Spain) is known for the largest deposits of mercury in the world yet discovered.^{6,7} Because of this, the Almadén district can be regarded as one of the most Hg-contaminated places on Earth.⁸ The Almadén district covers an area of about 300 km² and comprises many mercury mines that are now inactive, but in their almost 2000 years of activity have produced about a third of the mercury consumed by mankind.⁶ Almadenejos village owes its origin to the discovery and exploitation of Hg mines near the village, including the nearby “Vieja Concepción” and “Nueva Concepción” mines.⁶ Almadenejos is also the site of a historic metallurgical enclosure that housed the furnaces where Hg was extracted from cinnabar ores. This old metallurgical precinct, known as “Cerro de Almadenejos”, can be regarded as one of the most contaminated sites in the whole Almadén district, as seen in its soils, water, and sediments.^{9–11}

Knowledge of the physiological and molecular mechanisms that plants have to degrade or eliminate pollutants in contaminated soils which are troubling for human health is of great interest.¹² Plants’ main mechanism for fighting against

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metal toxicity is phytochelatin synthesis. Phytochelatin (PCs) are small, cysteine-rich peptides that are synthesized in the cytosol in response to heavy metal stress. Metal-PC complexes are formed and sequestered in vacuoles via transport across the tonoplast where their toxicity is less concerning.^{13,14} Thus, PCs are assumed to be involved in the accumulation, detoxification, and metabolism of heavy metal ions in plant cells.¹⁵ The general structure of this set of peptides is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$ to 5).^{16,17} Glutathione serves as the substrate of PC biosynthesis through the transpeptidation of the $\gamma\text{-Glu-Cys}$ moiety of GSH onto a second GSH to form PC_2 or onto a PC molecule to produce an $n+1$ oligomer. For these reasons, the study of PC content in plants is highly relevant, as PCs could be used as indicators of metal pollution.

Of all the studies of the Almadén mining district, none evaluates the heavy metal stress caused in plants by the high Hg concentrations found in soils, sediments, water, or air. However, several researchers have studied mercury distribution in the environment. Higuera et al.⁹ and Martínez-Coronado et al.¹¹ emphasized the high amounts of Hg found in soils and postulate the possible sources of Hg. Ferrara et al.¹⁸ and Higuera et al.¹⁹ analyzed the atmospheric Hg and determined the main emission sources and Hg fluxes over Almadén village. The aquatic environment has also been analyzed by Berzas Nevado et al.,²⁰ Gray et al.,¹⁰ and Rodríguez Martín-Doimeadios et al.,²¹ who determined Hg in waters, sediments, and bivalves. A reconnaissance survey of Hg in sediments, soil, and water and a comparison with other mining districts by Higuera et al.⁸ reached the main conclusion that the Almadén mining district is the most highly Hg-contaminated site in the world. A complete analysis of the Hg concentration in many plants of the Almadén mining district was published by Millán et al.²² and Molina et al.,²³ who pointed out the differing behavior of diverse plants in mercury uptake. Another study determined Hg in plants grown in the laboratory using Almadén soil and compared the results with plants grown in perlite:vermiculite soil spiked with Hg.²⁴ In this article, a decrease in mercury accumulation was observed in Almadén soil, which was attributed to the low bioavailability of mercury in this soil. However, PCs and Hg-PC, as markers for heavy metal stress, were not evaluated in any of these studies. Nevertheless, in the literature there are some studies of the effect of Hg in plants through examination of PC synthesis. In these studies, PCs are mainly determined by HPLC coupled with mass spectrometry,^{25–27} although in some cases fluorescence²⁸ or absorbance detection^{29,30} are used. In our research group, the interactions between Hg and PCs in several *in vitro* experiments were studied with electroanalytical techniques in either static experiments^{31,32} or chromatographic separations^{33,34} showing very low detection limits. Moreover, in previous studies *Hordeum vulgare* plants cultured with mercury or other heavy metals have been considered for the determination of the induced PCs and the Hg-PC complexes formed with HPLC with amperometric detection.^{35,36}

For the present study, wild asparagus (*Asparagus acutifolius*) was selected, because it is a typical evergreen perennial plant that grows naturally in the mining zone of the Almadén district.²³ Several zones with different Hg concentrations were chosen, and aerial and underground parts of plants from these zones were analyzed separately to determine the total concentration of Hg, as well as the concentrations of PCs and their Hg complexes. The correlation with the Hg concentration of the source soil was also looked at, including

both total Hg concentration and the extractable fractions. Therefore, the aims of this study were to examine whether natural plants, grown in the Almadén district, accumulated mercury from the contaminated soil and to analyze these samples with HPLC-amperometric detection, considering the induced PCs and Hg-PC complexes formed in both aerial parts and roots, in order to correlate these species with the mercury found in soils and to find out more about plants' PC synthesis for dealing with metal toxicity.

2. EXPERIMENTAL SECTION

2.1. Plant and Soil Sampling.

Samples of soils and plants were collected in four areas with different Hg content in Almadenejos: (E1) above the ruins of a furnace in the Almadenejos precinct, where the ore was heated to temperatures over 300 °C; (E2) next to the ruins of a furnace, where Hg vapor was condensed in the pottery lines; (E3) at the entrance of the “Nueva Concepción” mine; (E4) the wall limiting the old metallurgical precinct called “Cerco de Almadenejos” in the closest area to the village. Other samples (E5) were collected in Gargantiel, a village 9 km from Almadenejos, which we used as a blank site, as there were low concentrations of mercury in the local soil. The location of the individual samples was recorded with a portable Global Position System (GPS) (Figure 1).

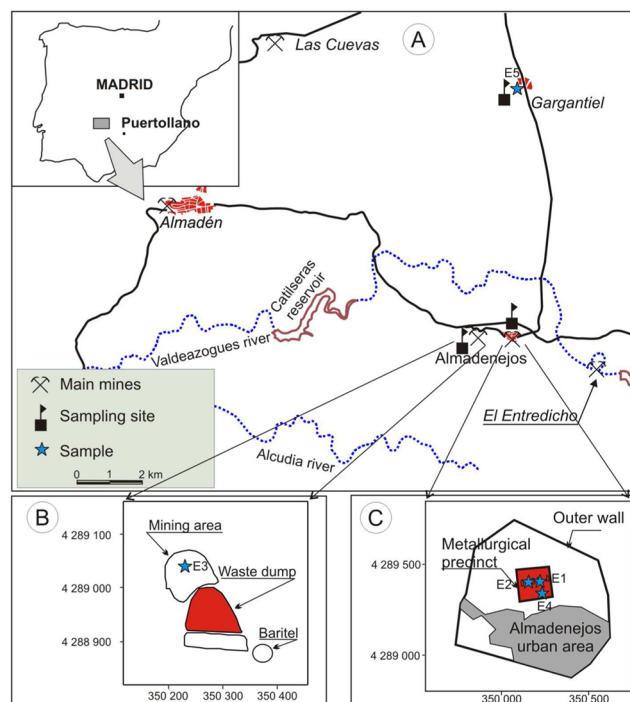


Figure 1. Sketch map of the area. A) General area; B) Nueva Concepción area, Baritel is an old type of mine shaft building; C) Almadenejos area. Numbers in the axis of B and C are UTM VGS 84 coordinates.

We also performed the edaphic characterization of soil samples, including the determinations of the main parameters relevant to mining areas: electrical conductivity (EC), pH, and organic matter (OM) content. EC and pH were measured on soil suspensions: samples (10 g) were homogenized in 25 mL of ultrapure water and subjected to a process of mechanical stirring for 120 min with a Unitronic 320 OR stirring bath to

homogenize and reach an equilibrium.³⁷ Then, pH and EC were determined with a GLP Crison pH meter and a GLP 32 Crison conductivity meter. OM was determined by following the Walkley and Black method.³⁸ Table 1 shows the results of these measurements, emphasizing the physical and chemical similarities of these soils.

Table 1. Edaphic Characterization of Soil Samples from the Different Sampling Sites

sampling site	pH	electrical conductivity ($\mu\text{S cm}^{-1}$)	organic matter (%)	soil texture
E1. Almadenejos precinct (above the furnace ruins)	7.66	214	4.35	loam
E2. Almadenejos precinct (next to the furnace ruins)	7.38	162	4.14	sandy-loam
E3. Mine entrance	7.67	170	6.02	sandy-loam
E4. Almadenejos precinct (wall)	7.36	307	6.67	loamy-sand
E5. Gargantiel	5.72	135	5.61	sandy-loam

2.2. Sample Preparation. Once plant samples had been collected (several portions of aerial parts and roots of different plants from each sampling zone), they were taken to the laboratory, cleaned with ultrapure filtered water, and accurately cut into small pieces. Plants were frozen with liquid nitrogen separating the aerial parts of the plant (stems and leaves) from the underground one (roots). Afterward, samples were ground with liquid nitrogen in a mortar and stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

Soils were ground and homogenized with an agate mortar and pestle and sieved through mesh to obtain a particle size lower than $100\text{ }\mu\text{m}$.

2.3. SPE Procedure and HPLC-ED Analysis for the Determination of PCs and Their Hg Complexes in Plant Samples. Prior to HPLC analysis, water extraction and then a cleanup step in solid phase extraction (SPE) cartridges were performed. For this purpose, 50 mg of fresh sample were mixed with $250\text{ }\mu\text{L}$ of ultrapure filtered water at 1,500 rpm for 1 h in an Eppendorf MixMate (Hamburg, Germany). For the preconcentration and cleanup of the samples, DSC-18 SPE cartridges (Supelco, Bellefonte, PA, USA) were used in a Visiprep SPE Vacuum Manifold (Supelco). For the evaporation under nitrogen stream, a Visidry drying attachment was used. Prior to the cleanup procedure, samples were filtered through $0.45\text{ }\mu\text{m}$ Nylon filter discs (Osmonics, Minnetonka, MN, USA) and pH was adjusted to 2.00 with trifluoroacetic acid (TFA). SPE tube packing was conditioned by rinsing with 2 mL of methanol and 2 mL of 0.1% TFA in water. Then, the sample was accurately transferred to the tube and washed with 1 mL of 0.1% TFA in water. In this step, weakly retained materials were removed. Finally, the relevant compounds were eluted with 2 mL of methanol, leaving behind any impurities not removed in the wash step. After this, the eluent was evaporated and then reconstituted in $100\text{ }\mu\text{L}$ of ultrapure filtered water. This cleanup procedure eliminates many irrelevant signals that appear at low retention times and provides clearer chromatograms. An Agilent 1100 chromatographic system (Agilent, Santa Clara, CA, USA) was used with an Ascentis C18 column (Supelco), particle size $5\text{ }\mu\text{m}$, $25\text{ cm} \times 4.6\text{ mm}$ column. The mobile phase consisted of 0.1% TFA in ultrapure filtered water pH = 2.00

and 0.1% TFA in acetonitrile. Separation was performed with gradient elution, as described in Dago et al.³⁴ The electrochemical detector (ED), consisting of a flow cell (BAS, West Lafayette, IN, USA) and an Autolab PGSTAT-12 (Eco Chemie, Utrecht, The Netherlands), was coupled at the end of the column. The flow cell consisted of a glassy carbon working electrode (BAS) whose surface was polished daily, a stainless steel auxiliary electrode, and an Ag/AgCl ($\text{NaCl } 3\text{ mol L}^{-1}$) reference electrode. The optimized potential for the working electrode was 1.2 V. The complete HPLC-amperometric detection procedure, along with its calibration functions and its limits of detection and quantification, is given in Dago et al.³⁴

2.4. Analysis of Hg Concentration in Plants. To determine Hg concentration in roots and aerial parts, 50 mg of fresh sample were transferred into glass reactors with 2 mL of 65% HNO_3 . Digestion was conducted for 1 h at $90\text{ }^{\circ}\text{C}$. Cooled digested extracts were diluted with 20 mL of ultrapure filtered water. The appropriate volume of these solutions was then diluted with 1% HNO_3 with the addition of KMnO_4 to stabilize Hg(II) and 2% rhodium as internal standard. All samples were analyzed in triplicate by an ICP-MS Elan-6000 (PerkinElmer, Waltham, MA, USA). Hg concentration in procedure blanks and in all reagents were always below the detection limits.

2.5. Analysis of Hg Concentration in Soils. Total Hg concentration in soils was measured with a LUMEX RA-915+ analyzer (Lumex, Saint Petersburg, Russia) with the RP-91C pyrolysis attachment (Lumex). In this equipment, based on Zeeman atomic absorption spectrometry, the sample is vaporized and mercury compounds are fully decomposed by heating to $800\text{ }^{\circ}\text{C}$, whereas organic compounds and carbon particles are catalytically transformed to carbon dioxide and water.^{11,23} Quality control is accomplished by analyzing equipment blanks ($<0.002\text{ mg kg}^{-1}$), duplicate samples (Relative Standard Deviation: 1.51%), and the certified reference material NIST SRM 2710 ($32.6\text{ mg kg}^{-1}\text{ Hg}$), obtaining precision (1.23%) and accuracy (99.32%) for total mercury in solid samples under the conditions of these analyses.

Different extraction procedures were applied to each soil sample in order to determine the extractable fractions of Hg. These methods are summarized as follows:

a) Organic mercury was determined as described in Válega et al.,³⁹ in which a soil sample was first treated with acid leaching ($\text{KBr}/\text{H}_2\text{SO}_4/\text{CuSO}_4$), then by the extraction of the organic mercury halide with toluene and back-extracted with an aqueous sodium thiosulfate solution. Extracted solutions of each soil were analyzed directly by atomic absorption spectrometry (AAS) with thermal decomposition, using an Advanced Mercury Analyzer AMA-254 (Leco, St. Joseph, MI, USA). This simple methodology is based on thermal decomposition of the sample and collection of the mercury vapor on a gold amalgamator.

b) The United States Geological Survey (USGS) extraction method proposed by Hageman⁴⁰ simulates the leachability and the chemical reactions that occur when geological materials are in contact with water. For this purpose, 5 g of soil were mixed with deionized water at a 1:20 ratio for 2 h at 50 rpm in a shaking bath (Selecta, Barcelona, Spain). Prior to the analysis, the extracts were filtered using nitrocellulose syringe filters ($0.45\text{ }\mu\text{m}$ pore size). Hg was determined by Cold Vapor Atomic Fluorescence Spectroscopy (AFS-CV) with Millennium

Table 2. Total Hg and Different Extractable Hg Concentrations in Soils ($\mu\text{g Hg g}^{-1}$ soil) from the Different Sampling Sites in the Almadenejos Precinct and Surrounding Areas^a

sampling site	total Hg	organic Hg	USGS	EPA	AcNH ₄ (pH = 7)	AcNH ₄ (pH = 4.5)	EDTA 0.05 M
E1. Almadenejos precinct (above the furnace)	4830 ± 520	16.1 ± 0.1	5.17 ± 0.05	6.28 ± 0.04	3.84 ± 0.08	87 ± 2	93.66 ± 0.03
E2. Almadenejos precinct (next to the furnace)	4260 ± 467	2.83 ± 0.06	4.36 ± 0.07	7.06 ± 0.01	2.25 ± 0.08	25.999 ± 0.005	76 ± 2
E3. Mine entrance	1995 ± 134	0.84 ± 0.02	1.05 ± 0.02	1.73 ± 0.01	0.4686 ± 0.0005	5.70 ± 0.02	11.2 ± 0.2
E4. Almadenejos precinct (wall)	1340 ± 99	0.11 ± 0.03	0.081 ± 0.004	0.110 ± 0.009	0.229 ± 0.005	0.234 ± 0.003	1.7 ± 0.7
E5. Gargantiel	2 ± 1	<LOD	<LOD	0.0024 ± 0.0009	<LOD	0.0179 ± 0.0009	0.033 ± 0.002

^aValues are the mean of three independent replicates ± standard deviation.

Merlin equipment (PS Analytical, Orpington, UK). With this method, mercury is oxidized with a bromide/bromate mixture and then reduced with Sn(II) chloride. Free mercury atoms in a carrier gas are excited by an ultraviolet light source at a wavelength of 254 nm. By measuring the radiation emitted, the specific element being measured is quantified.

c) The United States Environmental Protection Agency method (EPA 1312), known as the Synthetic Precipitation Leach Procedure (SPLP), determines the mobility of inorganic mercury present in waste materials in acid conditions caused by acidic rain. Five g of soil of each sample was processed with 100 mL of the extracting solution ($\text{H}_2\text{SO}_4\text{:HNO}_3$ in a proportion of 60:40 at a pH adjusted to 4.2), which is considered comparable to that of expected acid rain. The extraction was performed at 25 °C in a bath for 18 h with shake at 30 rpm. The extracts were filtered with borosilicate glass fiber filters (0.7 μm pore size) and analyzed by AFS-CV, as described above.

d) Other extraction procedures were performed by changing only the extracting solution and the conditions of the extractions to 2 h at 25 °C, shaking at 50 rpm. The extractants were 0.05 mol L⁻¹ ethylenediaminetetraacetic acid (EDTA) at pH = 4.5, ammonium acetate at pH = 7, and ammonium acetate at pH = 4.5. All solutions were filtered with 0.45 μm Teflon filters prior to analysis. AFS-CV was used as described above to determine the Hg concentration.

Three independent replicates for each sample were analyzed, and a blank analysis of each extraction procedure was then subtracted.

3. RESULTS AND DISCUSSION

3.1. Hg Determination in Soils. Several extraction methods were carried out in order to determine diverse metal fractions in soils and identify plant-available forms of Hg in soils for correlating with the synthesized PCs. The results for the different Hg fractions in soils are given in Table 2. For total Hg concentration, soils around Almadenejos have between 4,830 and 1,340 $\mu\text{g Hg g}^{-1}$ soil, which is consistent with the studies by Gray et al.¹⁰ and Martínez-Coronado et al.¹¹ The most contaminated soils are those from the furnace ruins (sampling sites E1 and E2), which have up to 1,960 times the Hg concentration of the control soil sampled at Gargantiel (sampling site E5), which has 2 $\mu\text{g Hg g}^{-1}$ soil. As Senesi et al.⁴¹ pointed out, the usual concentrations of nonpolluted soils fall within the range 0.01–0.03 $\mu\text{g Hg g}^{-1}$. Therefore, the soil of Gargantiel cannot be considered uncontaminated. Soils from the mine entrance (sampling site E3) also have a high level of Hg, 1,995 $\mu\text{g Hg g}^{-1}$ soil, more than the zone of the Almadenejos precinct next to the village (sampling site E4), with 1,340 $\mu\text{g Hg g}^{-1}$ soil. Given these results, it is not exaggerated to consider the Almadén mining district one of the

most Hg-contaminated places in the world and the “Cerro de Almadenejos”, the most contaminated place in the Almadén area.

However, despite the very high Hg concentration in these soils, mercury associated with water-soluble phases and exchangeable forms is very low. For all the different extraction methodologies, Hg concentration at the different sites correlates with total Hg concentration, being the furnace ruins the site with the highest level of mercury. The highest amounts of Hg were extracted with EDTA, as Table 2 shows. The carbonate bound fraction, which is determined with ammonium acetate at pH value of 4.5, is also very high. On the contrary, the ammonium acetate extraction at pH = 7 that determined the exchangeable and weakly adsorbed fraction was the least effective extraction reagent. The EPA method, which could be associated with the Hg extractable in acidic conditions caused by acid rain, and the USGS method, which simulates the water leachability of geological materials, provided slightly higher values.

Determinations of total or inorganic mercury do not provide adequate information about its impact on the environment. In particular, organic mercury compounds are generally more toxic than inorganic mercury salts, due to their higher solubility in lipids, which increases the potential for biological uptake and bioconcentration. Organic mercury determined with the methodology described above ranges from 16.1 $\mu\text{g Hg g}^{-1}$ soil to nondetected values. The highest values were found in the furnace ruins, consistent with total Hg concentration. Nevertheless, only a maximum of 0.33% of the total mercury is in their organic forms. In this study, the most effective extractant was EDTA, with a maximum value in the furnace ruins zone (sampling site E1) of only 1.94% of Hg extracted. This highlights the immobility of Hg in this mining zone, where, despite soils' extremely high concentrations of mercury, only small fractions could be solubilized and were therefore available for plants. Thus, most of the mercury in the soils of Almadenejos could be in the form of cinnabar (HgS) particles which are not phytoavailable for plants, as was indicated by Higuera et al.⁹

3.2. Hg Determination in Plants. To determine the total Hg concentration in plants, both aerial parts and roots were analyzed. The results are shown in Table 3. Hg concentration in plants of various origins was consistent with its concentration in related soils. As was foreseeable, plants from above the furnace ruins (zone E1) had more mercury, 76 and 74 $\mu\text{g Hg g}^{-1}$ for roots and aerial parts, respectively, than plants from Gargantiel (zone E5), with values of 0.14 and 0.12 $\mu\text{g Hg g}^{-1}$ in roots and aerial parts, respectively. In general, plants from the highest contaminated zones accumulate more mercury in roots than in aerial parts, although the value of Hg concentration in

Table 3. Concentrations of Total Hg in Plants ($\mu\text{g Hg g}^{-1}$ fresh weight) in Aerial Parts and Roots of *Asparagus acutifolius* from Different Sites of the Almadenejos Precinct and the Surrounding Areas^a

sampling site	Hg concn ($\mu\text{g Hg g}^{-1}$ FW)	
	aerial parts	roots
E1. Almadenejos precinct (above the furnace ruins)	74 \pm 8	76 \pm 43
E2. Almadenejos precinct (next to the furnace ruins)	29 \pm 5	66 \pm 11
E3. Mine entrance	2.9 \pm 0.1	5 \pm 1
E4. Almadenejos precinct (wall)	1.4 \pm 0.1	1.7 \pm 0.7
E5. Gargantiel	0.12 \pm 0.04	0.14 \pm 0.02

^aValues are the mean \pm standard deviation of three replicates.

roots from the E1 zone has a very high associated error which affects the interpretation. In general, the accumulation of more mercury in roots than in aerial parts can be seen as a strategy for protecting the more sensitive aerial parts from the deleterious effects induced by metal stress. Therefore, mercury ions are preferably retained in root tissues, and only small portions are transferred to leaves.^{25,28,29} However, it has to be remarked that the atmospheric Hg pollution in these zones is very high as indicated by Higuera et al.,¹⁹ and, therefore, possibly part of the mercury found in the aerial parts of plants could come from atmospheric deposition phenomena.⁴² In general, the correlation between the total Hg in plants, in both aerial parts and roots, and the Hg obtained by any of the different extraction procedures is close to linearity.

3.3. Analysis of Phytochelatin and Hg-Phytochelatin Complexes. To explore a way of following heavy metal stress response, the variation in thiol composition was analyzed, following the method of Dago et al.³⁴ Six biothiols were determined (GSH, γ -Glu-Cys, and PC₂₋₅), as well as Hg complexes of GSH and PC₂. With this methodology, Hg complexes with other PCs (PC₃, PC₄, PC₅) could be detected, but they could not be individually identified as they were clustered at the same retention time.³⁴ With the aim of analyzing PCs and their Hg complexes, cleaned up and preconcentrated extracts of each sample were injected into the chromatogram, and results of aerial parts and roots were analyzed separately. Figure 2 shows, as an example, a chromatogram of roots of *Asparagus acutifolius* from the Almadenejos precinct (zone E1). Peaks were identified by

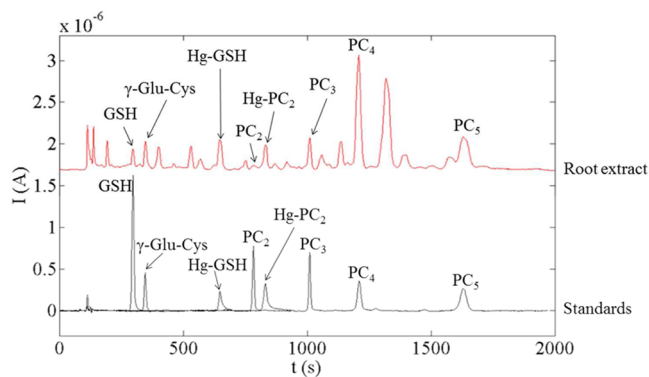


Figure 2. Chromatogram of root extracts of *Asparagus acutifolius* from above the furnace in the Almadenejos precinct zone (zone E1) compared with a chromatogram of standards (5×10^{-5} mol L⁻¹).

comparing the retention times obtained with the injection of standards. External calibration curves for each thiol were used for quantification. Nevertheless, in the case of Hg complexes, no commercial standards were available, and external calibration curves were done by mixing different concentrations of thiols with an excess of Hg. Thus, the concentration of the complex was the same as the concentration of the thiol added, while taking into account that with these injections only the complex peak appears in the chromatogram. This ensures that all the thiol is complexed. Thus, due to its ML stoichiometry, determined in previous studies by Dago et al.,³⁴ the concentration of the complex can be regarded as the same as the concentration of thiol present in the mixture. Figure 3

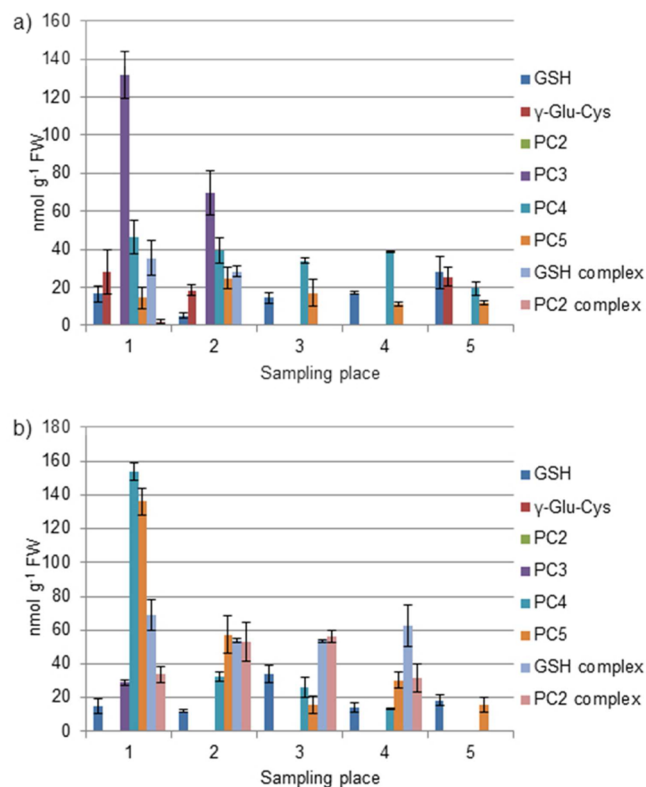


Figure 3. Quantification of thiols and some of its Hg complexes in *Asparagus acutifolius* aerial parts (a) and roots (b), grown in the different sampling places: (1) above a furnace ruins, (2) next to a furnace ruins, (3) in the mine entrance, (4) in the wall of the Almadenejos precinct, (5) Gargantiel.

shows the average and the standard deviation obtained for three replicates of plants from the sampling sites. At first glance, samples with more mercury synthesize more phytochelatin as a response to metal toxicity. It is also noticeable that longer PCs are more abundant in roots than in aerial parts, indicating that PCs, which are mainly synthesized in roots, are not compounds for long-distance root-to-shoot Hg transfer.²⁶ Moreover, longer PCs increase their concentration with increasing metal concentration. However, GSH is more concentrated in aerial parts than in roots, as shown by Sobrino-Plata et al.,³⁰ who pointed out that GSH is synthesized in shoots and then exported to roots, where it could be converted into PCs. Furthermore, GSH generally reduces its concentration with an increase of Hg concentration, because other longer-chain thiols are formed using GSH as the substrate.

Turning to the results from the analyses of aerial parts (Figure 3a), plants from the furnace ruins (sampling sites E1 and E2) generated an extremely large quantity of PC₃ (the maximum concentration of PC₃ is 132 ± 12 nmol g⁻¹ fresh weight in the extract of the aerial parts of E1 zone), as well as a relatively high level of the GSH complex, in comparison with plants from other locations. These findings are consistent with the higher concentration of Hg in soils and also in plants. The concentrations of other thiols did not increase visibly. However, PC₄, γ -Glu-Cys, and the GSH complex also reached their maxima in this extract, with 46 ± 9 , 28 ± 12 , and 35 ± 9 nmol g⁻¹ fresh weight, respectively. Unlike findings for other plant species,^{25,27} PC₂ is not the most abundant thiol in this case, either because it is the precursor molecule for the synthesis of longer-chain PCs or because it can be complexed by mercury. As explained in the literature on GSH,²⁶ GSH concentration diminishes as the Hg concentration in leaves increases, since GSH is the substrate of the PC synthesis that is activated by a rise in metal concentration. A similar explanation could be accepted for PC₂, as longer-chain PCs are formed by the successive addition of a Glu-Cys unit to the GSH or the precursor molecule (PC_{n-1}). Thus, for example, PC₃ is formed by adding a Glu-Cys unit to a molecule of PC₂. Aerial parts of plants from Gargantiel (sampling site E5) had small quantities of small thiols and also of some PCs. However, the presence of small amounts of Hg in plants and soils of this area, which could induce the synthesis of these PCs, is not a strange feature. For instance, Rauser¹⁴ detected the presence of GSH, PC₂, and PC₃ in plants grown under normal conditions, with no heavy metal stress.

Concerning roots (Figure 3b), the extract with the most synthesized PCs came from above the furnace ruins (sampling site E1), and the most abundant thiol compounds in this extract were PC₄ and PC₅, with 154 ± 5 and 136 ± 8 nmol g⁻¹ fresh weight, respectively. In the other root extracts, the concentrations of these thiol compounds diminished more or less in accord with the mercury concentration at the sampling site. In roots, GSH and PC₂ complexes were relatively abundant at all sampling sites, with the exception of Gargantiel (sampling site E5). At sampling site E1, the PC₂ complex was less abundant, perhaps because of the synthesis of longer-chain phytochelatin from PC₂. PC₃, which was the most abundant thiol in aerial parts, was only detected in the extract with the greatest Hg contamination (sampling site E1). The GSH complex shows a big difference between samples from Gargantiel (sampling site E5) and from the wall of the Almadenejos precinct (sampling site E4), with values of 12 ± 2 and 63 ± 12 nmol g⁻¹ fresh weight, respectively. Values then remained more or less constant for the other sampling sites. In root extracts from sampling site E5, very low concentrations of thiols were appreciated, with a maximum of 18 ± 3 nmol g⁻¹ fresh weight of GSH.

To sum up, the concentration of phytochelatin (PCs) and their Hg complexes were consistent with the amount of mercury found in soils; more phytoavailable Hg in soils produces more phytochelatin in plants as a response to metal toxicity. Longer-chain PCs were more abundant in roots than in aerial parts of plants, the opposite of GSH, which was more concentrated in aerial parts than in roots. Knowing that these thiols are mainly synthesized in roots, it is possible that the small GSH molecule could be transferred from roots to aerial parts, while the less mobile longer-chain PCs remain only in roots. When Hg concentration in soils increases, the

concentrations of the longest-chain phytochelatin (PC₃, PC₄, and PC₅) increase, and, in consequence, smaller thiols such as GSH and PC₂ decrease their concentrations because they serve as substrate for the synthesis of longer-chain PCs. Several differences between roots and aerial parts of plants were observed: in aerial parts the main PC synthesized is PC₃, while in roots PC₄ and PC₅ have higher concentrations; and the Hg complexes of GSH and PC₂ are more abundant in roots, as they accumulate more mercury than aerial parts.

With the aim of establishing the Hg extraction procedure that best relates the Hg concentration in soils with the synthesis of PCs, the total SH groups, calculated as Cys-Gly units in both aerial parts and roots, and the concentration of Hg determined in soils and plant tissues were correlated being γ the Hg concentration ($\mu\text{g Hg g}^{-1}$) and x the Cys-Gly units (nmol g⁻¹ fresh weight) in all cases. The results of coefficients of determination are shown in Table 4. As it can be seen, the total

Table 4. Correlations between Total SH Groups (nmol g⁻¹) in Aerial Parts and Roots and the Concentration of Hg ($\mu\text{g g}^{-1}$) in Plant Tissues and Soils^a

	aerial parts	roots
Hg (plant tissues)	0.9584	0.6895
total Hg (soil)	0.8743	0.6988
organic Hg (soil)	0.8296	0.9726
USGS extraction (soil)	0.9504	0.7152
EPA extraction (soil)	0.8288	0.5249
AcNH ₄ (pH = 7) (soil)	0.9981	0.8957
AcNH ₄ (pH = 4.5) (soil)	0.9175	0.9790
EDTA (soil)	0.9637	0.7389

^aFor Hg concentration in soils, different extractions were considered as explained in the text. Values shown correspond to determination coefficients (R^2).

concentration of Hg in roots is not closely related with the synthesis of PCs. Contrarily, the extraction procedure that best relates the total concentration of SH groups in roots with the extractable Hg in soil is ammonium acetate extraction at pH 4.5, giving a R^2 of 0.9790 and of 0.9175, for roots and aerial parts, respectively. The correlation with the Hg concentration in roots is not as good as expected as a consequence of the large deviation of the value of Hg concentration in roots. In order to know which kind of thiols are more related to mercury contamination in soils, the total thiol content has been divided into several groups: GSH, γ -Glu-Cys, PCs (PC₃ + PC₄ + PC₅), the Hg-GSH complex, and the Hg-PC₂ complex. Then, correlation with these groups with the ammonium acetate extraction at pH = 4.5 (the most correlated extraction procedure with total SH groups) were tested. The obtained R^2 coefficients for PCs (PC₃ + PC₄ + PC₅) were 0.8950 and 0.9859 for aerial parts and roots, respectively. The other R^2 coefficients were lower than 0.5 indicating that PCs are the best indicator of Hg effect in plants.

These findings highlight the important role of thiol compounds and their metal complexes in capturing and fixing Hg from soils, giving plants the capacity to deal with the heavy metal toxicity of polluted soils.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +34 93 402 15 45. Fax: +34 93 402 12 33. E-mail: cristina.arino@ub.edu.

Notes

The authors declare no competing financial interest.

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