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Chemometrics applied to the analysis of induced phytochelatins in *Hordeum vulgare* plants stressed with various toxic non-essential metals and metalloids

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ABSTRACT

Hordeum vulgare plants were stressed with Hg, Cd and As and their phytotoxicity was evaluated in terms of growth inhibition and total metal uptake by the plant. The synthesised phytochelatins ($(\gamma$-Glu-Cys$)_n$-Gly, $n=2$–5; PCs) were determined by HPLC with amperometric detection at a glassy carbon electrode. The results indicate that *H. vulgare* is a good phytostabilisation plant due to its capacity to accumulate heavy metals in roots. Cd and Hg are the most uptake toxic elements, being Cd the most potent inducer of PCs. The data obtained on the different PCs and related peptides induced by each heavy metal were used to perform a Principal Component Analysis (PCA) of the results as a function of the contaminating toxic element or its concentration level. The nature of the stressor element could be predicted from the pattern of PCs and related peptides identified by PCA. PCs were the most strongly induced peptides under Cd and Hg stress, whereas As only tended to synthesise small thiols such as glutathione and γ-glutamylcysteine, both precursors of PCs synthesis. This finding indicates that PCs are induced at different rates depending on the metal stressor used.

1. Introduction

Plants respond to heavy metal stress by inducing SH-containing peptides such as phytochelatins (PCs) [1,2]. Phytochelatins are small, cysteine-rich peptides capable of binding heavy metal ions via thiolate coordination. PCs consist of L-glutamic acid, L-cysteine and a carboxy-terminal glycine. The general structure of these peptides is $(\gamma$-Glu-Cys$)_n$-Gly, where n has been reported as being as high as 11, but is generally in the range of 2–5 [3]. Phytochelatins (PCs), which possess a high antioxidant capacity, are thought to be the most important mechanism of detoxification in plants subject to metal stress, and are involved in the accumulation, chelation, sequestration and metabolism of metal ions [4,5]. The PC pathway consists of two parts: metal-activated synthesis of peptides and transport of metal-PC complexes into the vacuole. Glutathione (γ-Glu-Cys-Gly; GSH) is known to be the precursor of phytochelatins, and phytochelatin synthase the enzyme responsible. When plants are exposed to heavy metals in the environment, PCs can reduce the free metal concentration in the cytosol by binding and transporting the metal to specific compartments, mainly the vacuole, prior to biotransformation into organic compounds or chemical reduction of the element [5].

Several metals have been proved to be inducers of PCs in plants. Cadmium is known to be the most potent PC inducer and is also the most extensively studied metal. A large number of papers have been published on the analysis of PCs in Cd-stressed plants. For example, Bräutigam et al. [6] studied Cd-induced peptides in *Chlamydomonas reinhardtii*, observing several PCs and related peptides by MS detection prior oxidation of the thiols, while Najmanova et al. [7] used RP-HPLC-ED to analyse the abundance of PC species in tissues of *Linum usitatissimum* grown in the presence of Cd(II). They determined PCs content and proved the translocation of Cd–PC complexes from roots to shoots. Mohamed et al. [8] used HPLC with post-column derivatisation to determine PCs in Cd-treated *Brassica juncea*, and demonstrated that GSH and PC biosynthesis increased in both roots and shoots. Many more articles have been published describing the response to Cd stress in plants; however, time-consuming or expensive methodologies were used. Another metal which has been extensively studied is mercury, which is known to be one of the most hazardous pollutants in the environment due to its bioaccumulation and biomagnification in diverse ecosystems. Consequently, decontamination of Hg-polluted sites is a universal goal. Several publications were found which report the content of PCs in Hg-stressed plants.

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Chen et al. [9] analysed the generation of PCs in *Brassica chinesis* exposed to different Hg species, and also determined Hg–PC complexes. Iglesia-Turiño et al. [10] analysed PCs in *Brassica napus*, but only found PC_2. In previous studies by our research group [11], *Hordeum vulgare* plants were stressed with Hg or Hg and Cd simultaneously, and PCs from $n=2$ to 5 were detected and quantified. PCs in *Asparagus acutifolius* and *Retama sphaerocarpa* grown in the Almadén mining district, one of the most Hg-contaminated sites in the world, were determined in order to evaluate plant response to Hg pollution (unpublished results).

Arsenic is another contaminant which is widely distributed in the environment. Although it is considered a metalloid rather than a metal, both inorganic species, As(V) and As(III), are highly toxic to plants. For this reason, some authors include arsenic in the term "heavy metal", although strictly speaking it is not a metal [12,13]. As(V) is a phosphate analogue, and it can therefore compete with phosphate in the cytoplasm, replacing it in ATP to form the unstable complex ADP-As, which leads to the disruption of energy flows in cells. Meanwhile, As(III) is highly toxic to plants because it reacts with sulphydryl groups in enzymes and tissue proteins, leading to inhibition of cellular function and death. Although arsenate [As(V)] is the predominant species in aerobic soils, it is thought that once inside the cytoplasm, As(V) is readily reduced to As(III) by GSH, making it the predominant As species in roots and shoots [14,15]. As(III) is then complexed with PCs and subsequently stored in the vacuoles. In a study of PCs induced by As stress, Sneller et al. [16] analysed the short- and long-term toxicity of arsenate in *Silene vulgaris* plants. They reported that PCs accumulation is linearly related to toxicity and can therefore be used as a biomarker for As toxicity; they also proved that PC_2 is the predominant PC in the presence of arsenite, in agreement with Schmöger et al. [17].

Several studies have compared the cellular damage induced in plants according to the metal stressor used. Nevertheless, very few have compared PC content in plant extracts as a function of the metal stressor. Thus, PCs induced by Cd and Hg have been determined by Rellán-Álvarez et al. [18], Ortega-Villasante et al. [19] and Sobrino-Plata et al. [20] in *Zea mays* extracts and in *Medicago sativa*, respectively, demonstrating that Cd is a more potent inducer of PC synthesis than Hg, as inferred from the higher proportions of thiols found at high levels of Cd contamination. However, these authors did not always detect PCs, or if detected they were unable to distinguish between different length PCs and could not determine which PC was the most induced by each metal. In another study comparing PCs induced by Cd and Pb in *Phaeodactylum tricornutum* [21], it was observed that when cells were exposed to Pb, the most abundant PC was PC_2, but PC_4 was the most abundant when Cd was added to the cultures. Nevertheless, the higher capacity of Cd to activate PC synthesis remained unproven.

Other studies have been conducted using different toxic elements; for example, Grill et al. [1] analysed the induction of PCs by Cd(II), Bi (III), As(V), Cu(II), Pb(II), Zn(II) and Ag(I) in *Schizosaccharomyces pombe* cells and showed that Cd is the most potent inducer of PCs. In another multi-metal study conducted by Maitani et al. [22], several metalloids were analysed in addition to metals. Ag(I), As(III), As(V), Cd(II), Cu(II), Ga(III), Hg(II), In(III), Ni(II), Pb(II), Pd(II), Se(IV) and Zn(II) were tested as PC inducers in *Rubia tinctorum* roots. However, this study used a time-consuming HPLC methodology with post-column derivatisation. Moreover, the authors did not describe the PC pattern for each metal or whether there were predominant PCs for each metal or not.

In the present study, barley (*H. vulgare*) was selected as the study plant because it is easy and fast to germinate and is readily available since it is a major cereal grain which is widely cultivated. Hydroponic culture (a method of growing plants using only nutrient solution) is considered a viable research method for phytoremediation studies prior to evaluating the behaviour of plants in greenhouse and/or field soils. Plants were stressed hydroponically with Hg(II), Cd(II) or As(III), and an analysis of the induced PCs was performed using an optimised HPLC methodology with amperometric detection as a sensitive, cheap and fast technique. The results obtained on the different PCs induced by each toxic element were used to apply a more advanced data processing technique in order to identify a pattern in the behaviour observed depending on the contaminating metal and/or its concentration. To this end, chemometrics was applied for the first time in phytoremediation studies. Principal Component Analysis (PCA) was applied to the PC quantification data obtained, with good results. Thus, the aims of this study were (i) to induce PCs in *Hordeum vulgare* plants stressed by different concentrations of several toxic metals and metalloids, (ii) to evaluate heavy metal phytotoxicity in plants, expressed as growth inhibition and total content of these toxic elements in roots and shoots, (iii) to quantify the PCs formed by applying HPLC with amperometric detection using an external calibration curve, and (iv) to apply chemometric tools to the data obtained, in order to evaluate the possible correlation between thiol concentrations in plants and the heavy metal supplied to the plants and/or its concentration level. The results obtained may contribute to improving current knowledge on heavy metal stress response in plants.

2. Material and methods

2.1. Chemicals

L-cysteine, γ-Glu-Cys (80% purity as trifluoroacetate salt) and Cys-Gly (85% purity) were provided by Sigma-Aldrich (St. Louis, MO, USA). Phytochelatins $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n=2$–5), as trifluoroacetate salts, were provided by DiverDrugs S.L. (Barcelona, Spain) with a purity ranging from 86.2% to 99.0%. Glutathione (GSH) and As_2O_6 were obtained from Merck (Darmstadt, Germany). $HgCl_2$ was purchased from Probus (Badalona, Spain). $Cd(NO_3)_2 \cdot 4H_2O$ was obtained from Panreac (Barcelona, Spain).

$Ca(NO_3)_2$, $Fe(NO_3)_3 \cdot 9H_2O$ and $CuSO_4 \cdot 5H_2O$ from Probus, KNO_3, $MnSO_4 \cdot H_2O$ and $ZnCl_2$ from Merck and $Mg(NO_3)_2 \cdot 6H_2O$, KH_2PO_4, H_3BO_3 and $Mo_7O_{24}(NH_4)_6$ from Panreac, were used to prepare the Hoagland solution (nutrient solution).

For plant extract preparation, cleaning the column and preparation of all solutions, ultrapure filtered water obtained from USF Purelab Plus (Hamburg, Germany) was used.

2.2. HPLC instrumentation

An Agilent (Santa Clara, CA, USA) 1100 chromatographic system was used, equipped with a quaternary pump, a Rheodyne 7725i 20 μL loop manual injector (Rohnert Park, CA, USA), a vacuum degasser and a handheld control module. An Ascentis C18 5 μm particle size analytical column measuring 25 cm × 4.6 mm was provided by Supelco (Bellefonte, PA, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in ultrapure filtered water, pH = 2.00, and 0.1% TFA in acetonitrile. Gradient separation was achieved at ambient temperature with a gradient profile as described in Dago et al. [23]. The flow rate was 1.2 mL min^{-1}.

The electrochemical detector (ED) was a CC-5C flow cell BAS (West Lafayette, IN, USA), with a three electrode system and a 0.005 in. gasket, connected to an Autolab PGSTAT 12 (Eco Chemie, Utrecht, the Netherlands). GPES software version 4.9.007 (Eco Chemie) was used for potentiostatic control and data acquisition.

The working electrode was a glassy carbon electrode BAS, the surface of which was polished daily using a suspension of 0.3 μm

alumina particles from Metrohm (Herisau, Switzerland), rinsed thoroughly with ethanol and sonicated for 5 min in ethanol and 5 min in ultrapure filtered water. The optimised potential for the working electrode was 1.2 V. A stainless steel auxiliary electrode and an Ag/AgCl (NaCl 3 mol L^{-1}) reference electrode were also used.

2.3. Plant material

Barley (*H. vulgare* cv. Graphic) seedlings were cultivated hydroponically using Hoagland solution adjusted to pH 5.5–6.5. The nutrient solution contained 268 mg L^{-1} of N, 235 mg L^{-1} of K, 200 mg L^{-1} of Ca, 31 mg L^{-1} of P, 0.30 mg L^{-1} of S and 48.6 mg L^{-1} of Mg as macronutrients; 0.5 mg L^{-1} of B, 2.50 mg L^{-1} of Fe, 0.5 mg L^{-1} of Mn, 0.05 mg L^{-1} of Zn, 0.02 mg L^{-1} of Cu and 0.01 mg L^{-1} of Mo as micronutrients. Seeds were placed on top of a mesh situated over a plastic container filled with nutrient solution so that the seeds were slightly in contact with the nutrient solution. Four days after germination, the nutrient solutions were changed for Hoagland solutions to which four different concentrations of Hg(II), Cd(II) or As(III) had been added: 0 (control), 20, 50, and 500 μmol L^{-1}. Three pots per treatment with 20 seeds per pot were considered. Barley shoots and roots were collected after 14 days of treatment and fresh weight was obtained from shoots and roots separately. Plants were cleaned with 0.1 mol L^{-1} EDTA solution, frozen at once with liquid nitrogen to disrupt cell walls and stored at −80 °C. Subsequently, samples were ground separately in liquid nitrogen.

2.4. Sample preparation for HPLC

Water extraction was selected as the most convenient method for analysis of PCs and their complexes. For this purpose, 100 mg of sample fresh weight was mixed with 500 μL of ultrapure filtered water at 1500 rpm for 1 h in an Eppendorf MixMate (Hamburg, Germany). For preconcentration and clean-up of the samples, DSC-18 solid phase extraction cartridges were used in a Visiprep SPE Vacuum Manifold, both supplied by Supelco. For evaporation under a stream of nitrogen, a Visidry drying attachment was used. Prior to the clean-up procedure, samples were filtrated through 0.45 μm nylon filter discs by Osmonics (Minnetonka, MN, USA) and pH was adjusted to 2.00 with TFA. SPE cartridges were then conditioned and equilibrated before adding the sample. Finally, the compounds of interest were eluted with methanol leaving behind any impurities not removed in the washing step. After that, the eluent was evaporated and then reconstituted in ultrapure filtered water. Many of the irrelevant signals that appear at low retention times were eliminated through this clean-up procedure, and clear chromatograms were obtained.

2.5. Data treatment

Chromatograms were baseline-corrected and converted into data matrices by means of home-made programs implemented in MATLAB® (version 7.9.0.529 (R2009b), MathWorks, Natick, MA).

Principal component analysis (PCA) of the data was performed to obtain qualitative results for pattern recognition. PCA is a linear feature extraction method that consists of projecting the *m*-dimensional data set (in our case, *m* being the number of PCs or related peptides) in a dimension smaller than *m*. The uncorrelated and orthogonal coordinates of this reduced space are the eigenvectors (principal components) of the covariance or correlation matrix of the data set. These new variables are more descriptive because they are chosen to describe the maximum amount of variance in the data matrix. The eigenvalue of a principal component is directly related to the percentage of "information" contained in the corresponding component, so that only the most relevant components are preserved. In spite of the limitations due to its linearity, PCA is a very useful classification technique which is widely used in the field of analytical chemistry. PLS_Toolbox 3.5 software was used for the PCA [24], and a detailed description of this method is given elsewhere [25].

The distribution of the samples on the principal components, known as the scores plots, is a powerful strategy for classifying samples according to the data measured. These graphs reveal patterns and other features that may be correlated to sample characteristics. An analysis of the distribution of variables (the loadings plot) provided information about their correlations and possible relationships with phytochelatin concentrations. Additionally, a simultaneous study of scores and loadings (biplots) was used to explore the relationships between samples and variables.

2.6. Analysis of total metal content

Approximately 100 mg of fresh sample was digested in glass reactors with 2 mL of HNO_3. Digestion was conducted at 90 °C for 1 h, after which the digested extracts were cooled and diluted with ultrapure water. The samples were then transferred to plastic or glass tubes, depending on the metal analysed (glass tubes in the case of Hg and plastic ones with the other metals), and diluted with 1% HNO_3. All samples were analysed using an ICP-OES Perkin-Elmer Optima 3200 RL (Waltham, MA, USA) for a first approximation of metal concentrations. Then, following prior dilution where necessary, samples were analysed using an ICP-MS Perkin-Elmer Elan-6000, and 2% rhodium was added as an internal standard. Triplicate analyses of each sample were measured and a blank was then subtracted from the final concentration.

3. Results and discussion

3.1. Effect of Hg, Cd and As on growth inhibition

Plants exposed to different levels of Hg, Cd or As suffered clear symptoms of phytotoxicity after 18 days of treatment, as reflected in the general visible trend towards decreased plant size with increasing heavy metal concentration (Fig. 1). This reduction was particularly evident at the highest concentration. Shoot and root fresh weight (milligrams per seedling) was determined for all levels (Fig. 2). An analysis of growth inhibition revealed a strong correlation between the heavy metal supplied to the plant and

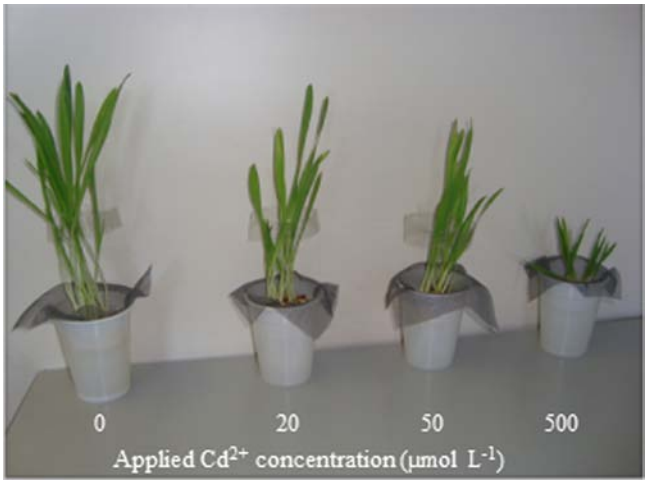

Fig. 1. Seedlings of *Hordeum vulgare* grown hydroponically in the absence and presence of 20, 50 and 500 μmol L^{-1} of Cd(II) for 14 days.

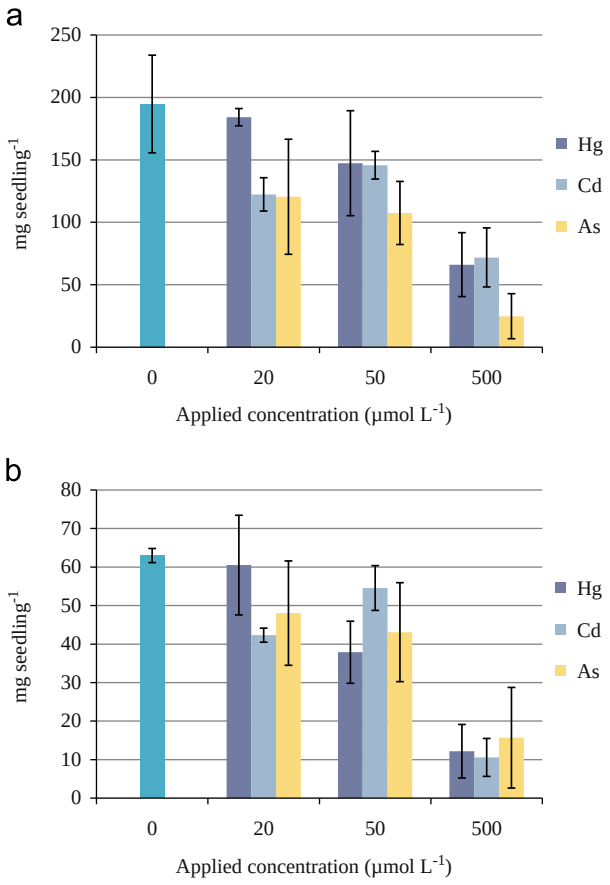

Fig. 2. Effect of Hg, Cd and As on growth of *Hordeum vulgare* seedlings treated with these elements at the indicated concentrations (0, 20, 50 and 500 μmol L^{-1}) for 14 days. Plant fresh weight biomass (milligrams per seedling) of both shoots (a) and roots (b) is represented. The bar in the zero point represents the no-contamination level.

plant biomass. All the toxic elements assayed caused a marked reduction in the fresh weight of shoots and roots, indicating the plants' capacity to uptake heavy metals into their tissues. Regarding the results shown in Fig. 2, plant growth at the first concentration level (20 μmol L^{-1}) was inhibited in a very irregular manner. The relative growth inhibition observed in roots treated with Hg, Cd and As was 4%, 33% and 24%, respectively. In shoots of plants stressed with 20 μmol L^{-1} of the different heavy metals, a similar decrease in biomass was observed. At 50 μmol L^{-1} of each toxic element, two different groups were observed in roots: Hg and As caused a relative growth inhibition of between 30% and 40%, whereas Cd caused an inhibition of 13%. All heavy metals provoked a reduction in shoot biomass of plants treated with 50 μmol L^{-1}, which decreased by 25% for Hg and Cd, and 45% for As. Furthermore, at the highest concentration, all heavy metals caused a very marked inhibition of growth of between 75% and 85% in roots and between 65% and 90% in shoots, thus evidencing heavy metal toxicity.

3.2. Heavy metal accumulation in shoots and roots

To investigate the heavy metal uptake efficiency of barley, which was grown under Hg, Cd or As stress, the total heavy metal content in roots and shoots was compared (Table 1). The metal levels in shoots and roots of seedlings grown in 20, 50 and 500 μmol L^{-1} solutions clearly increased in a dose-dependent manner. Furthermore, the concentration of heavy metals in roots was higher than in shoots.

Heavy metal uptake in shoots and roots was highest for cadmium, with values of 120 ± 21 and 2970 ± 570 μg Cd g^{-1} of fresh weight in shoots and roots, respectively, for a 500 μmol L^{-1} Cd-solution. Moreover, although the biomass of plants stressed with 20 and 50 μmol L^{-1} of Cd was almost equal, the concentration of metal increased at the higher dose and the largest increase was observed at the most contaminated level. In shoots, the Cd uptake concentration was similar to that of As, and much higher than those of Hg. Conversely, roots accumulated similar concentrations of Cd and Hg, whereas uptake of As was lower.

Although the concentration of heavy metals in roots is always higher than that in shoots, some of them are much more mobile than others and can be transferred more easily to the aerial part of plants. Comparing the shoots and roots of plants stressed with three different toxic elements, Hg and Cd would appear to be less mobile because only small quantities of metal were translocated from roots to shoots and, of these, Hg was the least mobile. Conversely, As would seem to be much more mobile in view of the amount absorbed by roots and the concentration of As in shoots. The metal translocation efficiency of any given plant species is indicated by the translocation factor (TF), which is a ratio of the metal concentration in shoots to roots [26]. In the present study, the observed TF values were always smaller than 1, indicating that *H. vulgare* can be classified in the "shoot metal excluder" group which, due to a tendency to store heavy metals in root tissues, is suitable for phytostabilisation of metal-contaminated soils. This accumulation of toxic elements in roots may be a strategy for protecting the aerial parts of plants from the toxicity of these heavy metals [10,18].

3.3. Induced phytochelatins content

As an example, chromatograms of roots stressed with 50 μmol L^{-1} of Hg, Cd or As are shown in Fig. 3, together with the control level and the standards. The chromatographic peaks were identified by comparing the retention times with those obtained by injection of standards. Quantification was performed by external calibration curves of all the studied thiols and the figures of merit are summarised in Table 2. Tables 3 and 4 present the results obtained from shoots and roots, respectively. As it can be seen, an increase in the heavy metal concentration induced an increase in thiol synthesis. This phenomenon was evidenced by an increase in small thiol or PC concentrations, depending on the stressor element. It can be seen that Cd was by far the most potent PC inducer assayed, with different PCs synthesised and with higher concentrations in both shoots and roots. At the control level, with no heavy metal supplied, GSH and γ-Glu-Cys were detected in both shoots and roots, although PC_2 was also quantifiable in roots. However, this was not unusual, since Rauser [3] detected the presence of GSH, PC_2 and PC_3 in plants grown under normal conditions without heavy metal stress.

Generally, thiol concentrations were higher in roots than in shoots, with a maximum value of 573 ± 14 nmol PC_2 g^{-1} fresh weight in roots stressed with 20 μmol L^{-1} of Cd. For all the heavy metals studied, the concentration of thiols in both shoots and roots increased according to the concentration supplied to the plants. The length of the PC chain depended on the level of metal concentration as well as the metal supplied.

In Hg-stressed roots, increasing concentrations of Hg induced the synthesis of PC_2, although a marked decrease in PC_2 content was observed at the most contaminated level when GSH concentrations increased. In media with very high levels of Hg contamination, PC synthesis was inhibited and only its substrate, GSH, was observed. PC content in Hg-stressed shoots was very low, and only PC_3 was observed at the 20 μmol L^{-1} level and PC_5 at 500 μmol L^{-1}.

Table 1
Heavy metal concentrations (microgram metal per gram fresh weight) in shoots and roots of *Hordeum vulgare* seedlings grown in the absence and presence of Hg, Cd and As (20, 50 and 500 μmol L^{-1} for 14 days). Data are mean ± standard deviation of three replicates.

	Shoots				Roots			
	0	20	50	500	0	20	50	500
Hg	< 0.21	< 0.21	0.36 ± 0.04	30 ± 2	< 0.21	17.0 ± 0.5	36 ± 3	1740 ± 110
Cd	0.09 ± 0.01	8.8 ± 0.2	14.3 ± 0.5	120 ± 21	0.8 ± 0.2	82 ± 5	126 ± 4	2970 ± 570
As	0.4 ± 0.1	2.6 ± 0.1	4.2 ± 0.2	105 ± 2	22.4 ± 0.4	37 ± 6	62 ± 5	207 ± 2

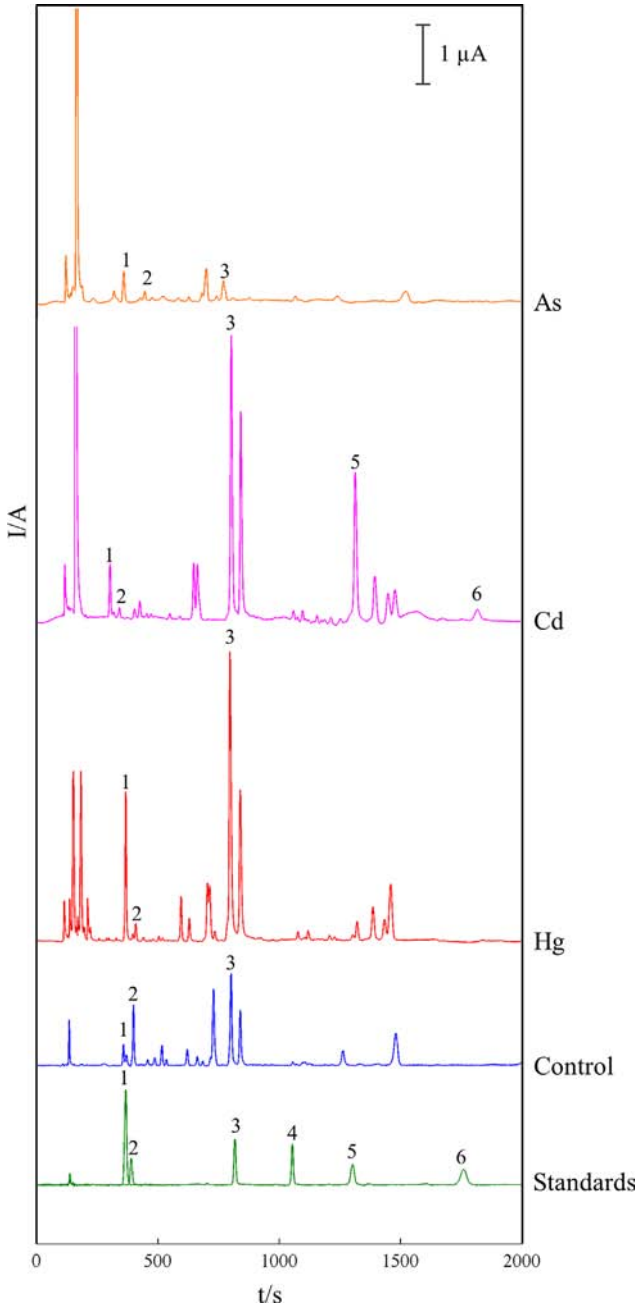

Fig. 3. Chromatograms of barley roots stressed with 50 μmol L^{-1} of Hg, Cd and As. It also shows a chromatogram of no-contamination level (control) and a chromatogram of standards (50 μmol L^{-1}). Peaks correspond to: 1. GSH, 2. γ-Glu-Cys, 3. PC_2, 4. PC_3, 5. PC_4 and 6. PC_5.

Cadmium is more likely to induce the synthesis of longer-chain PCs in both shoots and roots. In addition to GSH and γ-Glu-Cys, PC_3, PC_4 and PC_5 could also be observed in shoots at all levels, increasing their proportion as metal content increased. In roots, PC_2 was the most abundant thiol although PC_4 and, at some levels, PC_3 and PC_5, could also be observed. Moreover, the amount of PCs synthesised in Cd experiments was higher by far than in the other ones, indicating that from among the metals studied, Cd is the most potent inducer of PCs. At high Cd levels, a decrease in PC synthesis was observed, similar to that noted with Hg.

In As-stressed plants, longer-chain PCs were practically not synthesised, and the concentration of GSH and γ-Glu-Cys increased as metal levels rose. With As, the only PC synthesised in shoots was PC_3, whereas only PC_2 was detected in roots.

To better compare the formation of thiols with the different heavy metals, the total γ-Glu-Cys units are represented in front of the supplied element concentration in both shoots and roots (Fig. 4). In shoots (Fig. 4a), an increase in heavy metal concentration produced an increase in the formation of γ-Glu-Cys units in almost all cases. Cd was confirmed as being the most potent

Table 2
Figures of merit for thiol determination by HPLC with amperometric detection at a glassy carbon electrode.

Compound	Calibration function[a]	Detection limit[b] (μmol L^{-1})	Quantification limit[c] (μmol L^{-1})
GSH	$A=0.315c-8\times10^{-8}$, $r^2=0.999$	0.87	2.92
γ-Glu-Cys	$A=0.158c-1\times10^{-7}$, $r^2=0.999$	1.26	4.20
PC_2	$A=0.213c-4\times10^{-7}$, $r^2=0.993$	3.20	10.66
PC_3	$A=0.237c-6\times10^{-7}$, $r^2=0.994$	2.88	9.60
PC_4	$A=0.209c-6\times10^{-7}$, $r^2=0.987$	4.62	15.42
PC_5	$A=0.334c-6\times10^{-7}$, $r^2=0.998$	1.66	5.55

[a] A is the peak area (ampere per second) and c the injected concentration (moles per litre).
[b] Calculated as three times the standard deviation of the y-intercept and divided by the slope.
[c] Calculated as ten times the standard deviation of the y-intercept and divided by the slope.

Table 3
Thiol concentration (nanomoles per gram fresh weight) in shoots of *Hordeum vulgare* stressed with different heavy metals. (n.d. non detectable and n.q. non quantifiable).

	Thiol concentration (nmol g^{-1} fresh weight)					
	GSH	γ-Glu-Cys	PC_2	PC_3	PC_4	PC_5
No metal	21 ± 4	22 ± 3	n.q.	n.q.	n.d.	n.d.
20 μmol L^{-1} Hg	16 ± 3	14 ± 6	n.q.	30 ± 5	n.d.	n.d.
50 μmol L^{-1} Hg	8 ± 2	n.q.	n.d.	n.d.	n.d.	n.d.
500 μmol L^{-1} Hg	6 ± 1	n.q.	n.q.	n.d.	n.q.	15 ± 1
20 μmol L^{-1} Cd	n.q.	16 ± 2	n.d.	71 ± 12	n.d.	n.d.
50 μmol L^{-1} Cd	11.3 ± 0.7	24 ± 4	n.d.	125 ± 6	31 ± 8	21 ± 3
500 μmol L^{-1} Cd	18 ± 4	22 ± 3	n.q.	171 ± 3	67 ± 3	50 ± 1
20 μmol L^{-1} As	8 ± 2	40 ± 2	n.q.	n.d.	n.d.	n.d.
50 μmol L^{-1} As	21 ± 2	35 ± 6	n.q.	67 ± 10	n.q.	n.d.
500 μmol L^{-1} As	183 ± 26	89 ± 16	n.q.	44 ± 5	n.q.	n.d.

Table 4
Thiol concentration (nanomoles per gram fresh weight) in roots of *Hordeum vulgare* stressed with different heavy metals. (n.d. non detectable and n.q. non quantifiable).

	Thiol concentration (nmol g^{-1} fresh weight)					
	GSH	γ-Glu-Cys	PC_2	PC_3	PC_4	PC_5
No metal	14 ± 5	78 ± 22	112 ± 18	n.d.	n.q.	n.d.
20 μmol L^{-1} Hg	56 ± 10	n.q.	227 ± 6	n.d.	n.q.	n.d.
50 μmol L^{-1} Hg	80 ± 8	15 ± 2	256 ± 42	n.d.	n.q.	n.d.
500 μmol L^{-1} Hg	251 ± 27	18 ± 1	194 ± 38	n.d.	n.d.	n.d.
20 μmol L^{-1} Cd	48 ± 4	61 ± 5	573 ± 14	n.d.	222 ± 15	n.q.
50 μmol L^{-1} Cd	31 ± 3	11 ± 2	388 ± 32	n.d.	294 ± 6	20 ± 4
500 μmol L^{-1} Cd	65 ± 11	32 ± 8	177 ± 52	76 ± 14	79 ± 14	n.q.
20 μmol L^{-1} As	22 ± 3	n.d.	36 ± 10	n.d.	n.d.	n.d.
50 μmol L^{-1} As	33 ± 10	34 ± 14	68 ± 5	n.q.	n.q.	n.d.
500 μmol L^{-1} As	81 ± 22	133 ± 33	n.q.	n.d.	n.q.	n.d.

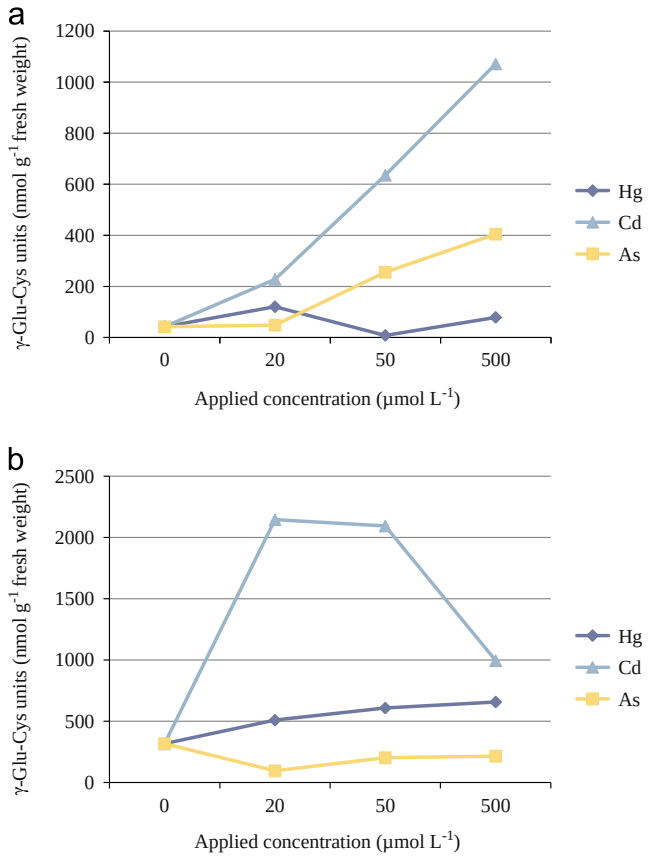

Fig. 4. Total γ-Glu-Cys units (nanomoles per gram fresh weight) synthesised in shoots (a) and roots (b) of *Hordeum vulgare* stressed with different concentrations (0, 20, 50 and 500 μmol L^{-1}) of Hg, Cd and As.

inducer of thiol from among the toxic elements studied. An increase in the units was also observed with As, whereas Hg did not show a visible increase. The presence of thiols in roots (Fig. 4b) was higher than in shoots. Moreover, Cd was also the element that induced most thiols, but its behaviour did not correlate with an increase in heavy metal concentrations. At the 50 μmol L^{-1} level, the amount of γ-Glu-Cys units appeared to stabilise and began to decrease, probably due to the toxicity of this metal, which as mentioned earlier affects the synthesis of PCs. Hg showed a linear behaviour with an increase in metal concentration, although with a small slope of the curve. On the other hand, As did not induce a clear increase in thiol units with increased heavy metal concentrations.

3.4. Principal Component Analysis

The biosynthesis of PCs proceeds by stepwise addition of dipeptidyl units to GSH or to the previously formed PC. Our finding that different heavy metal ions induce different chain length PCs suggests that the nature of the ion involved in induction and complexation reactions could affect the capacity of the previously formed PC to act as a γ-Glu-Cys acceptor, as reported by Morelli and Scarano [21]. However, in some cases the amount of synthesised PC does not depend on the stressor element; rather, it is the combination of all the thiols synthesised which yields the pattern that permits identification of the pollutant. Therefore, a Principal Component Analysis (PCA) was performed with the data obtained from PC quantification.

Two different groups of PCA studies were carried out in order to obtain the correlation between the synthesised PCs and (i) the concentration of heavy metal and (ii) the nature of the stressor element. In all PCA studies, the multivariate data (variables) are the concentrations of the different thiols (GSH, γ-Glu-Cys, PC_2, PC_3, PC_4 and PC_5).

The first block of PCA studies was applied to the different heavy metals supplied, and they were performed for shoots and roots separately. Thus, in the first study, six different PCA were performed with different data matrices, the dimensions of which were 4 (objects) × 6 (variables). Note that in this nomenclature, the objects were the four different levels of concentration (0, 20, 50 and 500 μmol L^{-1}), while the variables were the six thiols under study. Six different PCA were performed using the same data arranged in different matrices obtained from the three different metals applied to both shoots and roots.

In the second block of PCA studies, the dimensions of the corresponding data matrices were also 4 (objects) × 6 (variables). In this case, the objects were the three different heavy metals supplied to the plants (Hg, Cd and As) plus the control level (no metal), while the variables were the six thiols studied. This type of data arrangement was applied to six different PCA applications using data on the three different concentrations of metals (20, 50 and 500 μmol L^{-1}) in both roots and shoots.

Before performing all the PCA analyses, data were autoscaled to provide similar weights for all thiols in the PCA model.

The results obtained for the first group of PCA studies indicated a good correlation between the amount of thiols and the concentration of heavy metal supplied to the plant. Nevertheless, the scores plot makes it possible to group the analysed samples according to the different toxic element content in the media only on the basis of thiol content. Moreover, the loadings plot indicates the correlation between the thiol concentrations. As an example, PCA scores and loadings plots of barley shoots stressed with different concentrations of Hg are shown in Fig. 5. The evolution of the magnitude of eigenvalues as a function of the number of components suggests that, in this example, three principal components are significant. The percentages of variance retained by principal components 1, 2 and 3 were 64.64%, 21.18% and 12.71%, respectively; more than 98% of significant information is thus captured by the model. The map of scores (Fig. 5a) shows that the independent replicate samples were always in close positions. This finding is consistent with a satisfactory repeatability of the entire method, from cultivation to analytical measurements. Four characteristic patterns were observed, which are represented by the different levels of metal. As regards the loading plot in this example (Fig. 5b), the distribution of variables on principal components 1 and 2 shows that γ-Glu-Cys and GSH are clustered

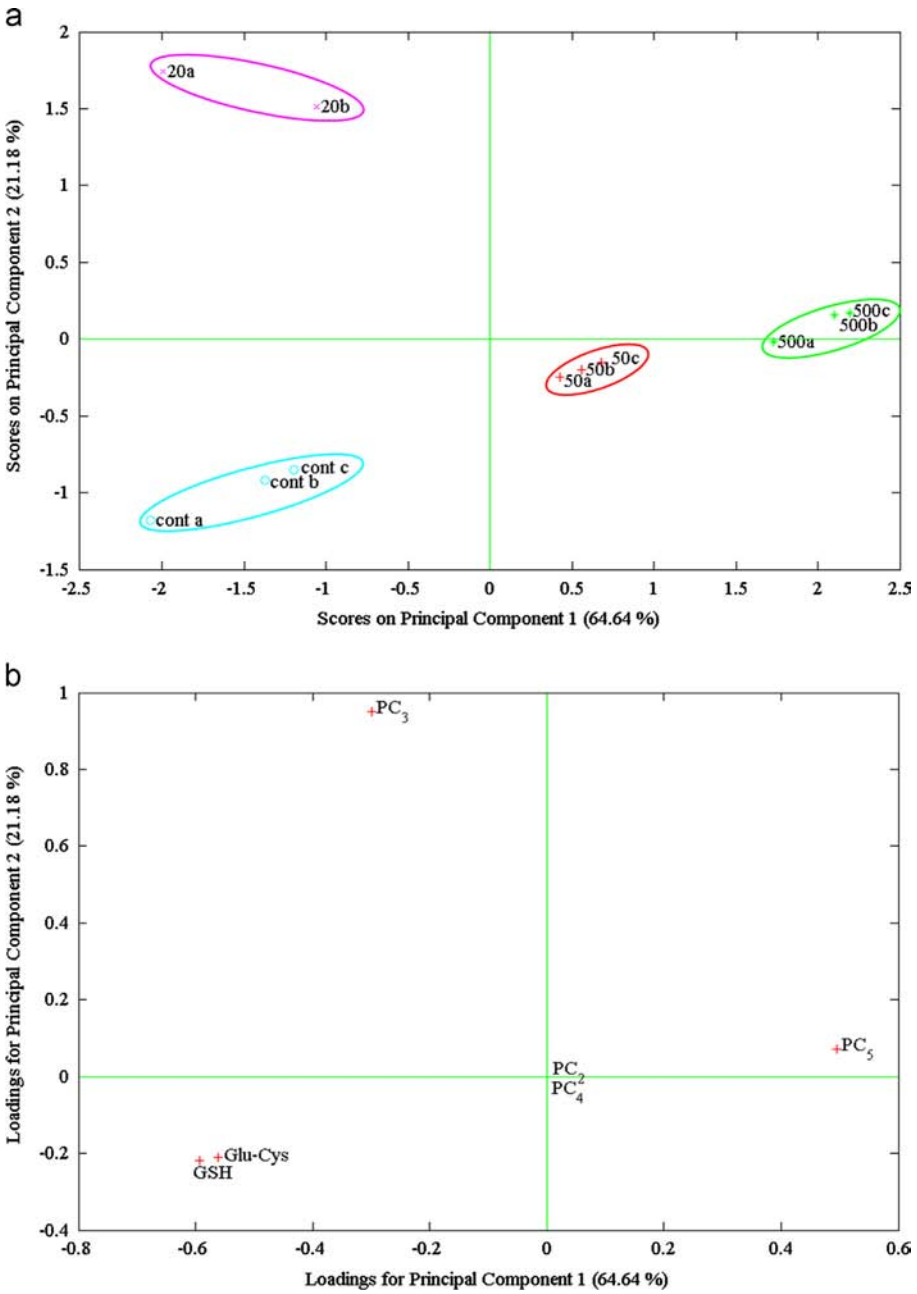

Fig. 5. PCA characterisation of barley shoots stressed with different levels of Hg using thiols concentration as analytical data. (a) Plot of scores of principal component 1 and 3 where *cont* is the control level and 20, 50 and 500 the Hg concentration in micromoles per litre and a, b and c the three independent replicates of each level. (b) Plot of loadings of principal components 1 and 3 where Glu-Cys, GSH, PC_2, PC_3, PC_4 and PC_5 are the studied thiols.

to the left. This finding confirms that these two peptides were reasonably correlated and their behaviour was similar. However, in this example, longer-chain PCs did not correlate with each other, although this did occur in other cases. PC_2 and PC_4 appear at the centre, which means that none of the components explains its variance because they are not quantifiable in any of the samples.

A greater wealth of information can be extracted from the simultaneous analysis of scores and loadings (Fig. 5). Principal component 1 confirms that γ-Glu-Cys and GSH were the most representative thiols for control and 20 μmol L^{-1} levels. Moreover, PC_3 was also representative of the 20 μmol L^{-1} level. According to this principal component, PC_5 was more abundant at the 500 μmol L^{-1} level. Principal component 2 indicates that PC_3 was more abundant at the 20 μmol L^{-1} level than in control, whereas in contrast, γ-Glu-Cys and GSH were the predominant thiols at control level. The 50 μmol L^{-1} level showed a good correlation with PC_5 for principal component 2. The highest concentration (500 μmol L^{-1}) showed a good correlation with PC_5 as regards principal components 1 and 2, but γ-Glu-Cys and GSH were not representative of this level, as inferred by their opposite positions in the loadings plot.

These results indicate that smaller thiols are almost always important at low metal concentration levels, whereas longer-chain PCs are more characteristic at high metal concentrations.

The second group of PCA analyses was conducted in order to discriminate the four cases of heavy metal pollutants considered in this study (no contamination, Hg, Cd and As). The results obtained for shoots and roots with all levels considered permitted discrimination of four different clusters in the principal component scores plot. As an example, Fig. 6 shows the scores and loadings

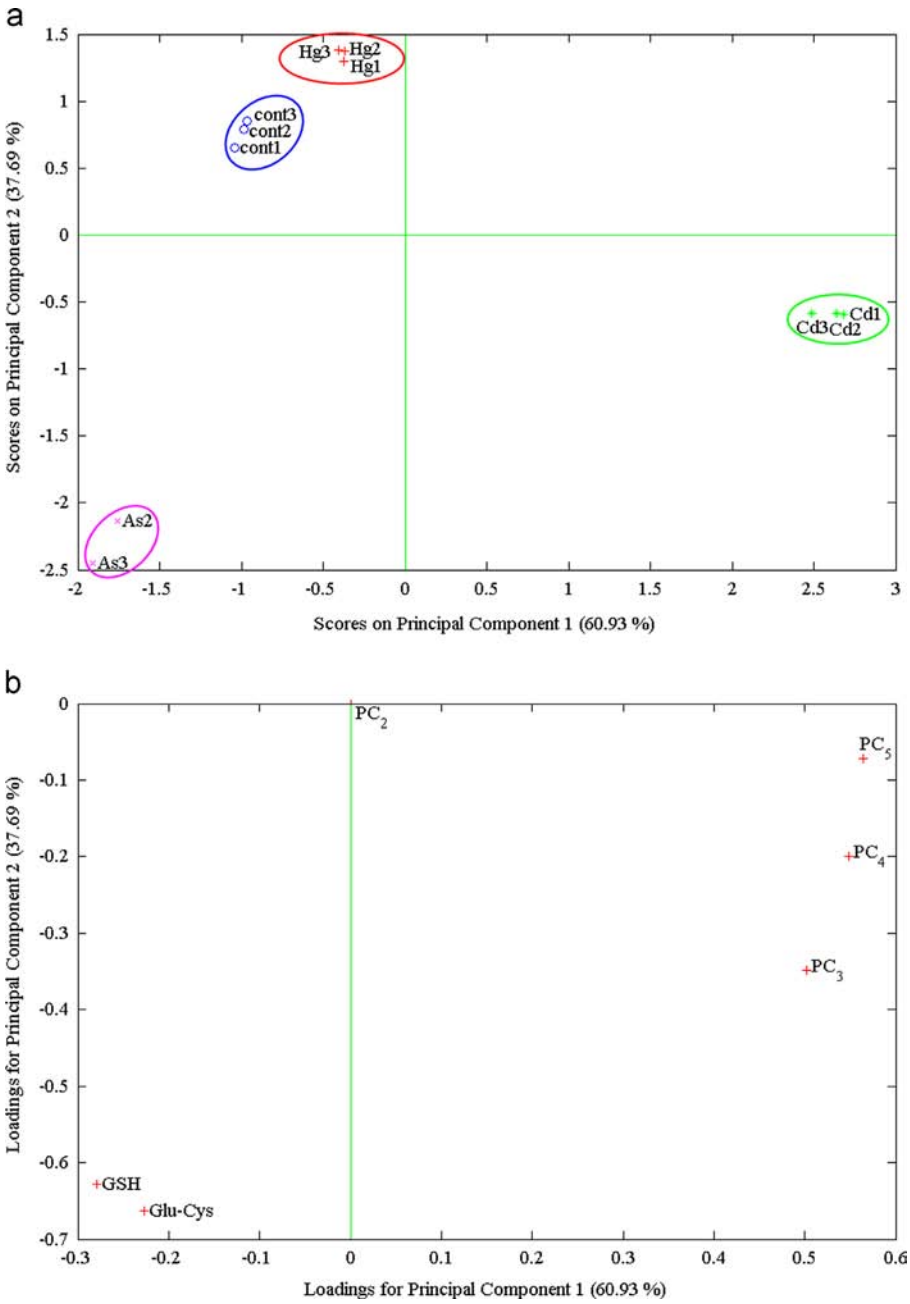

Fig. 6. PCA characterisation of barley shoots stressed with 500 μmol L^{-1} of the different metals studied using thiols concentration as analytical data. (a) Plot of scores of principal component 1 and 2 where cont is the control level (with no-contamination), Hg, Cd and As are the stressing elements and 1, 2 and 3 are the three independent replicates of each heavy metal. (b) Plot of loadings of principal components 1 and 2 where Glu-Cys, GSH, PC_2, PC_3, PC_4 and PC_5 are the studied thiols.

plots for shoots, with 500 μmol L^{-1} of different toxic elements, as a function of the first two principal components. In this case, three principal components explained more than 99% of the variance. Thus 60.93% was captured by principal component 1, 37.69% by principal component 2 and 1.05% by principal component 3. The scores plot (Fig. 6a) shows that samples stressed with the same heavy metal were always close together, which seems to indicate that a pattern of contamination according to the stressor element could be identified depending on the amount and type of thiols synthesised. In the loadings plot (Fig. 6b), the six thiols are represented on the plane between principal components 1 and 2. From this plot, it can be seen that GSH and γ-Glu-Cys are on the left side of the plot, suggesting that they were reasonably correlated and their behaviour was similar. The same is the case for PC_3, PC_4 and PC_5, which appear on the right side. It can also be stated that these two groups of thiols were inversely correlated with each other because they appear in opposite positions of the plot. However, PC_2 does not show any correlation with the other PCs. As regards principal component 1, it was possible to distinguish between samples containing more GSH and γ-Glu-Cys and samples with PCs. Samples with small thiols should be placed on the left side of the plot, whereas samples where long-chain thiols are more representative should be placed on the right side. Nevertheless, principal component 2 did not distinguish between GSH and γ-Glu-Cys but did differentiate between PCs.

When extracting information from scores and loadings plots together (Fig. 6), for principal component 1, non-contaminated samples or those stressed by As and Hg were more correlated with GSH and γ-Glu-Cys, whereas those stressed by Cd were more related to PC_3, PC_4 and PC_5. Taking into account principal component 2, As

samples correlated most strongly with GSH, γ-Glu-Cys and PC_3, which appear at the bottom of the plot, whereas those stressed by Hg were more related to PC_5, which appears at the top.

Thus, the predominant thiols in plants stressed with high concentrations of As were GSH and γ-Glu-Cys, indicating that As is not a very good inducer of PCs and tends to synthesise small thiols, although some PCs, such as PC_3, may be formed and explained in the PCA model by analysis of scores and loadings of principal component 2. PCs are completely related to Cd contamination and PC_5 was the most abundant thiol in Hg-contaminated samples. On the other hand, the characteristic thiols appearing at control level were GSH and γ-Glu-Cys, but these occupied a higher position in the scores plot because of their low content compared with those of As contamination.

To conclude, *H. vulgare* seems to be a good phytostabilisation plant due to its capacity to accumulate heavy metals in its roots. Cd and Hg were the most uptake metals. The synthesis of PCs was evaluated in Hg, Cd and As stressed plants, and the results indicate that Cd is the most potent inducer of PCs. Using the pattern of PCs and peptides observed by PCA, it is possible to predict the nature of the metal stressor. As was more likely to synthesise small thiols, such as GSH and γ-Glu-Cys, whereas Cd and Hg tended to synthesise PCs.

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