Rapid Capillary Electrophoresis Analysis of Glutathione and Glutathione Disulfide in Roots and Shoots of Plants Exposed to Copper

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ABSTRACT:
Introduction – Glutathione and glutathione disulfide can be determined by capillary zone electrophoresis; however, the frequent use of acidic precipitation of protein from samples prior to analysis generates an acidic matrix of strength and pH that may cause changes in the method sensitivity, comigration of species or changes in the equilibria that relate both species in cells or fluids.
Objective – To optimise electrophoretic conditions for glutathione and glutathione disulfide determination, and to improve pre-analytical treatment for better visualization of the signals of both peptides in an acidic matrix.
Methodology – The method consisted of direct photometric detection at 185 nm and 300 mM borate at pH 7.6 as background electrolyte. The variables under study were voltage applied, injection time, capillary length and electrolyte pH. Seedlings were hydroponically grown and the peptides were extracted with metaphosphoric acid.
Results – The resulting acidic matrix was previously treated with the same background electrolyte to prevent comigration and to improve signal resolution. The optimised method showed good reproducibility and linearity, with correlation coefficients above 0.999 and detection limits below 3 μM, and determination of both analytes in less than 3 min. Analyte recovery in the process was in the 88–104% range. The concentration range found in hydroponically grown tomato plants, irrespective of copper level, was 45–100 nmol/g fresh weight for glutathione and below 56 nmol/g fresh weight for glutathione disulfide.
Conclusion – The results obtained here support the applicability of the method to the fast and simultaneous determination of glutathione and glutathione disulfide in tissue of shoots and roots of plants grown under either normal or stressful conditions.

Keywords: glutathione; glutathione disulfide; capillary electrophoresis; copper; phytotoxicity

Introduction

Glutathione (GSH) constitutes an important source of nonprotein thiols both in animal and in plant cells and it has the crucial function of cell defence. This tripeptide is part of the anti-oxidant ascorbate–glutathione cycle that helps to prevent or minimise damage caused by reactive oxygen species. This function involves oxidation of the thiol group to form mainly glutathione disulfide (GSSG) (Noctor *et al*., 2002). Attempts have been made to relate changes in the levels of both peptides present in tissues or fluids to stressful situations resulting from various environmental conditions such as heavy metals, ozone and luminic radiation, among others (Cobbett and Goldsborough, 2002; Foyer *et al*., 1995).

In view of the growing interest in the analysis of GSH, GSSG and homologous peptides in various matrices, several methods have been proposed (Camera and Picardo, 2002). Among these, methods based on liquid chromatography (Davey *et al*., 2003; Rellán-Álvarez *et al*., 2006) and enzymatic determination (Richie *et al*., 1996) are the most frequently reported, although methods based on capillary electrophoresis (CE) have also been proposed (Carru *et al*., 2002; Maeso *et al*., 2005). Examining reported methods based on capillary zone electrophoresis, similarities include the use of a background electrolyte (BGE) borate buffer in the 0.05–0.3 M concentration range, with pH values near its pK_a (Camera and Picardo, 2002), and the observation that BGE concentration and pH, together with capillary length and voltage applied, have the strongest effect on the time required for analyte separation. Detection following CE has been carried out with different systems, the most sensitive of which are based on laser-induced fluorescence (Musenga *et al*., 2007), mass spectrometry (Ohnesorge *et al*., 2005) and photometric detection (Maeso *et al*., 2005).

In order to prevent alteration of the equilibrium between GSH and GSSG in the sample, pre-analytical sample treatment separates small peptides from any kind of protein which may interfere in the determination, particularly from those enzymes that use these peptides as a substrate. Acidic chemical precipitating agents most commonly used include trichloroacetic, metaphosphoric, phosphoric, perchloric and sulfosalicylic acids (Camera and Picardo, 2002). The extraction step thus generates an acidic matrix with a pH that may result in low method sensitivity for EC-based methods and cause comigration of species or changes in the equilibria relating both species in cells or fluids, possibly generating less representative results of actual levels in living organisms. It has previously been proposed that adjustment of the sample pH

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before CE results in better resolution of the signals of both peptides (Mendoza *et al*., 2004). However, the use of alkali for neutralization may result in the formation of a colloidal precipitate in the injection vial which may increase analytical time if precipitate separation is necessary, and may cause capillary obstruction and produce wrong signals if the colloidal precipitate is not removed. Other reported EC methods that have low detection limits and short analytical time are incompatible with strongly acidic matrices such as those produced using metaphosphoric acid (MPA), which is frequently used in studies to determine the effect of different environmental factors on the levels of peptides such as glutathione and their relationship with plant stress. The purpose of this study was to optimise electrophoretic conditions for simultaneous determination of GSH and GSSG in a short period of time, and to improve pre-analytical treatment for better visualization of the signals of both peptides derived from shoots and roots of tomato plants in an MPA matrix. The optimised method was then applied to the determination of GSH and GSSG in tomato plants exposed to phytotoxic levels of copper.

Experimental

Chemicals and reagents. The BGE used in CE was prepared from H_3BO_3 and metaphosphoric acid (MPA). The nutrient solution was prepared with the following salts: $Ca(NO_3)\cdot 4H_2O$, NH_4NO_3, $Mg(NO_3)_2\cdot 6H_2O$, K_2SO_4, KNO_3, NaCl, Fe-EDTA, K_2HPO_4, H_3BO_3, $MnSO_4\cdot 4H_2O$, $ZnSO_4\cdot 7H_2O$, $CuSO_4\cdot 5H_2O$ and $Na_2MoO_4\cdot 2H_2O$. Plant material was digested with HNO_3 and H_2O_2. All of the above-mentioned reagents were PA grade and were supplied by Merck (Darmstadt, Germany). GSH and GSSG (purity >99%) used as standards were supplied by Sigma (St. Louis, MO, USA).

Standard solution and background electrolyte. The BGE consisted of a 300 mM borate solution daily prepared from a 0.5 M sodium borate stock solution. Prior to filtration through a 0.22 μm cellulose membrane the BGE pH was adjusted as required in this study by adding 0.5 M NaOH. GSH and GSSG stock solutions (500 μM) were prepared in water. Optimization of the assay conditions was initially conducted using solutions of both analytes in pure water (Milli-Q, Millipore, Bedford, MA, USA). For subsequent optimization and analytical application the standards were prepared in 2 and 5% aqueous MPA since MPA was used to precipitate proteins in the sample preparation stage.

CE instrumentation. Analysis was carried out using a Quanta 4000E (Waters Associates, Milford, MA, USA) capillary electrophoresis system with direct photometric detection at 185 nm. A positive power supply was used, varying the voltage from 10 to 25 kV. Sample or standard injection was hydrostatically performed, with 30, 45 and 60 s times. Fused silica capillary tubes 40 and 60 cm long and 75 μm i.d. were used. Daily conditioning was carried out by running 0.1 M KOH for 2 min, water for 5 min and BGE for 5 min. Working temperature was 25°C. Data were processed using Millenium data analysis software (Waters associates).

Plant growth, collection and preparation of sample. Similar-looking, 10-day old tomato (*Lycopersicon esculentum* L.) seedlings, obtained from sand-grown seeds, were transferred to nutrient solution (1 L) containing N, 7.4 mM (8:1 $NO_3^- - NH_4^+$); K, 1.2 mM; Ca 2.2 mM; Cl, 0.06 mM; Mg, 0.3 mM; Na, 0.06 mM, Fe (as Fe-EDTA) 19.5 μM; B, 15 μM; Mn, 5.4 μM; P, 20 μM; Zn, 1.4 μM; Mo, 0.5 μM; and one of three concentrations of Cu (as $CuSO_4$) 0.4, 3 or 12 μM. The pH of the nutrient solution was adjusted to 6.0 using 0.1 M NaOH (Mendoza *et al*., 2004). Growth chamber conditions involved irradiation at 400 μE/m/m/s, 16:8 h photoperiod, temperature of 24/20°C and 50% relative humidity. Four repetitions were performed per treatment. The nutrient solution was renewed every other day for 28 days, at which point 1 g each of shoots and roots were harvested from each repetition for GSH and GSSG analysis. The samples were snap frozen and stored in liquid nitrogen until analysis. The remaining plant material was separated into shoots and roots, weighed and oven-dried for 48 h at 60°C.

In order to carry out peptide extraction, the sample was ground with a mortar previously cooled with liquid nitrogen, and 2% MPA (2 mL) was added with swirling to form a homogeneous suspension. The suspension was centrifuged at 8800*g* for 10 min at 4°C. The supernatant was filtered through a 0.45 μm cellulose nitrate membrane. The resulting solution was immediately stored at −80°C. Results of oxidised and reduced glutathione concentration in shoots and roots were expressed as nmol/g fresh weight (FW).

Plant metal absorption from the nutrient solution was quantified by flame atomic absorption spectroscopy (Perkin Elmer) following acid digestion of the plant material in a microwave oven (Milestone), using the following schedule: step 1, 125 W for 1 min; step 2, 0 W for 2 min; step 3, 250 W for 5 min; step 4, 400 W for 6 min; step 5, 600 W for 6 min; and step 7, venting for 5 min. Thus, about 300 mg of shoots tissue were digested with 65% HNO_3 (4 mL) and 30% H_2O_2 (2 mL). The same procedure was applied to digest 100 mg of roots. The cooled digests were diluted with water to a final volume of 20 mL, filtered and stored in polyethylene bottles until analysis.

Statistical analysis. One-way ANOVA was applied, comparing the mean values of the different treatments by Duncan's multiple range test to a level of 5% (Statgraphics statistical software, version 4.0 plus, Statistical Graphics Corporation).

Results and Discussion

Initial conditions for the analysis of solution of GSH and GSSG in water included a 60 cm capillary, 300 mM borate electrolyte at pH 7.8 and 25 kV voltage with positive polarity of the power supply (Serru *et al*., 2001). Under these conditions, an 81 μA current was observed in the capillary with GSH and GSSG both eluting before 7 min [Fig. 1(a)]. In an attempt to shorten migration time, the capillary length, applied voltage and/or the pH were each adjusted [Fig. 1(a–d)]. With silica capillaries, an increase in pH increases the electrosmotic flow because of dissociation of SiOH to SiO^- functional groups on the capillary inner wall, which carries an increase in surface charge and thus in Z potential (Baker, 1995). On the other hand, an increase in pH may favour the presence of negatively charged glutathione species, which improves the method sensitivity. Assessment of the results shown in Fig. 1 indicated use of a pH of 7.6, a lower value than the electrolyte pK_a, a 40 cm capillary and a voltage of 20 kV. These conditions generated a current of 85 μA [Fig. 1(d)]. The effect of sample injection time on the performance and resolution of the signals of both analytes was assessed, considering 30, 45 and 60 s of hydrostatic sample injection. The highest resolution was obtained with 30 s; thus this was the injection time for further tests.

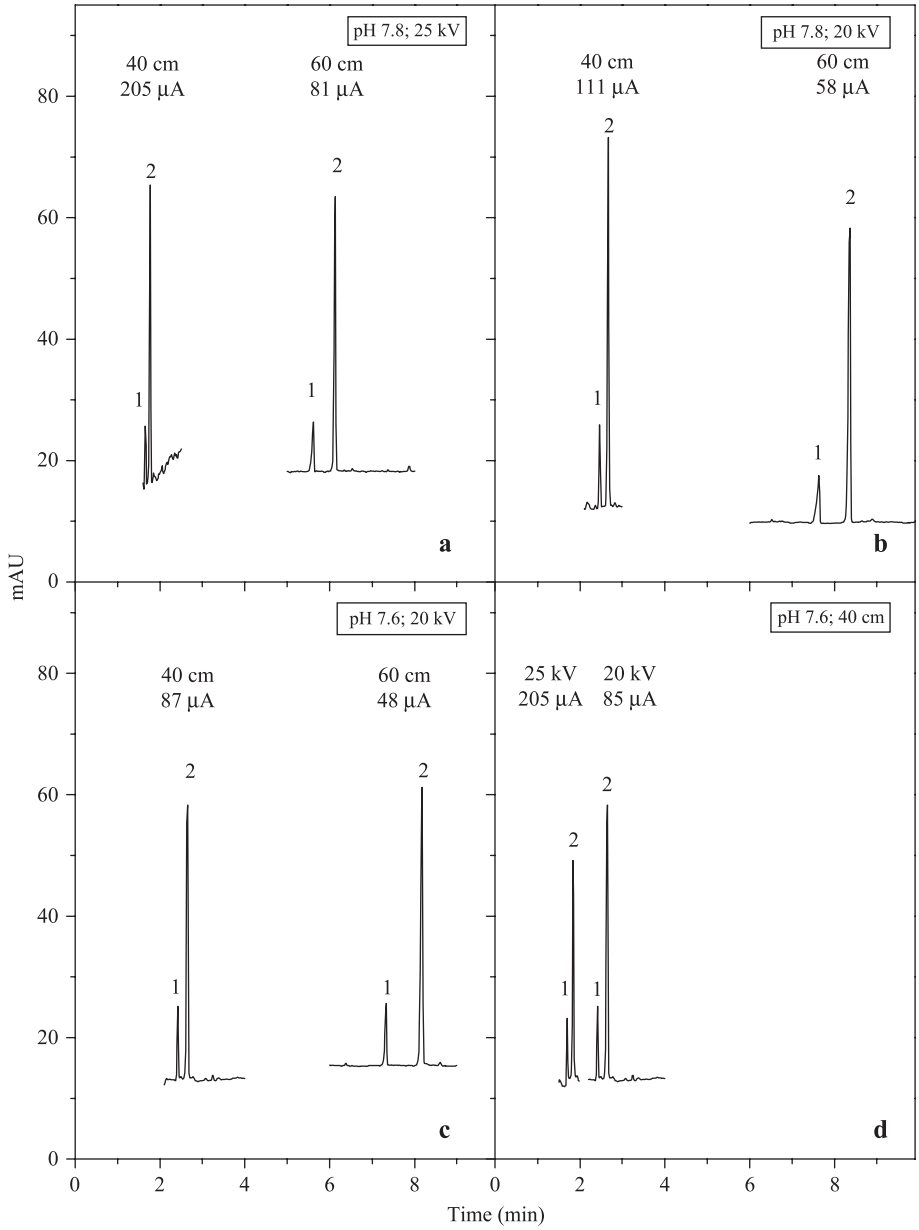

Figure 1. Effect of capillary length, applied voltage, and electrolyte pH on generated current and migration time of GSH (peak 1) and GSSG (peak 2) at a concentration of 40 µM of each standard.

Since this optimization used standard GSH and GSSG solutions prepared with purified water, it was necessary to adjust the conditions to the acidic (MPA) sample matrix. Therefore standards of GSH and GSSG were prepared using 2% (Herrero-Martinez *et al.*, 2000) and 5% (Rellán-Álvarez *et al.*, 2006) aqueous MPA, Electropherograms recorded for these solutions initially showed no sharply defined baseline and no signal for either peptide (Fig. 2). In order to visualise the signals, it was necessary to increase the pH to values near the BGE pH. To this end, the standards were diluted with the same BGE as used in the measurement (300 mM borate, pH 7.6) to reach a final concentration of 10 µM for both analytes, a value within the probable range of the sample analytes. For the solution containing 2% MPA, 1:5 dilution was the most appropriate for good resolution of both signals. This resulted in an increase in the pH from 2.2 to 6.6 and a decrease in the current from 124 to 114 µA. The solution containing 5% MPA was best visualised with 1:10 dilution, reaching a pH of 6.4 and a current of 117 µA. However, the latter resulted in the nearly complete loss of GSH signal, which was verified with standard addition (Fig. 2).

Finally, in order to quantify GSH and GSSG in the extracts of shoots and roots, a calibration curve with six concentration points was established from the standards prepared in 2% MPA and diluted at a 1:5 ratio with BGE, the final concentration range of the standards being 2–80 and 5–80 µM for GSSG and GSH, respectively (Table 1). The detection limit (LOD) was calculated as the analyte concentration providing a signal equal to the blank signal plus three times the blank standard deviation. Measurement reproducibility was established in relation to the area under the curve and migration time, recording both parameters under the experimental conditions mentioned above five times in a day (intraday) and for five consecutive days (interday) (Table 2).

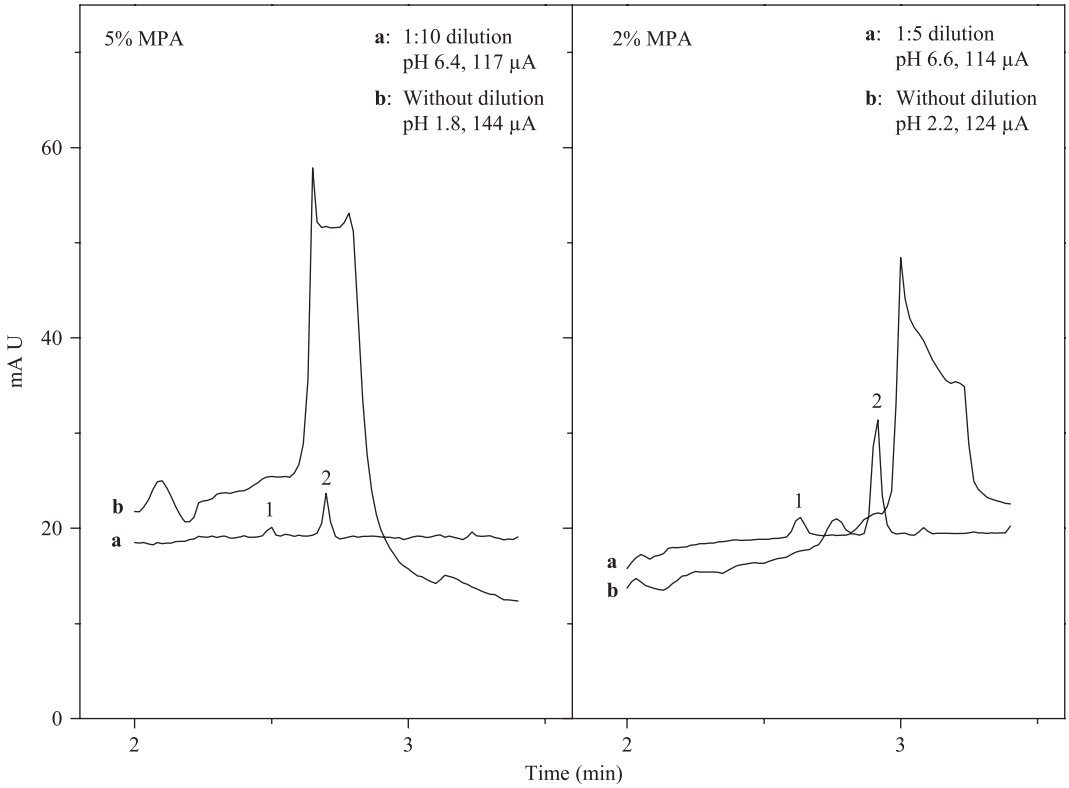

Figure 2. Effect of dilution of standard prepared in 2 and 5% MPA. Final concentration of both standards of 10 µM. (1) GSH, (2) GSSG.

Table 1. Calibration curve and limit of detection obtained for the method

Analyte	Calibration curve[a]	r[b]	SE[c]	LOD (µM)[d]
GSH	$A = 371.1\,C - 911.3$	0.9995	340.2	2.75
GSSG	$A = 1186\,C - 1541$	0.9999	370.6	0.937

[a] A = area, C = concentration (µM).
[b] r = Correlation coefficient.
[c] SE = estimated standard error of intercept.
[d] LOD = limit of detection.

Table 2. Intraday and interday repeatability of optimised method

	GSH		GSSG	
	Area	Time (min)	Area	Time (min)
Intraday				
Mean[a]	26,176	2.59	113,814	2.88
SD[b]	728.8	0.01	1415.4	0.003
RSD(%)[c]	2.78	0.21	1.24	0.10
Interday				
Mean	26,386	2.58	114,674	2.88
SD	971.8	0.01	1653.1	0.005
RSD(%)	3.68	0.35	1.44	0.16

[a] $n = 5$.
[b] Standard deviation.
[c] Relative standard deviation.

In order to calculate the recovery percentage, a control sample was spiked with 20 µL (100 µM) of both standards and extracted following the above described procedure. The resulting analyte concentration was compared with that obtained with a non-spiked sample. This procedure was carried out on three consecutive days and results are shown in Table 3. In order to verify GSSG and GSH signals, samples and standards (200 µL) were run with addition of 2-mercaptoethanol (5 µL) so as to reduce GSSG (Senft *et al*., 2000).

In most of the studies where capillary electrophoresis has been used for glutathione determination, separation times range from 3 to 7 min (Herrero-Martinez *et al*., 2000; Maeso *et al*., 2005; Rellán-Álvarez *et al*., 2006). Although, both Carru *et al*. (2002) and Lochman *et al*. (2003) reported times below 2 min for simultaneous analysis of reduced and oxidised glutathione analysis, using a borate-based BGE in conjunction with 20 and 30 cm capillaries, they provided little information concerning electrophoresis conditions used and generated current. In the present study it was not possible to reduce the capillary length below 40 cm without significantly increasing the generated current which resulted in an increase in capillary temperature and thus affecting sample stability.

The method validation showed acceptable sensitivity, with detection limits below 3 µM for both peptides. Similarly, linearity was good, with a correlation coefficient above 0.999 and a linear range up to 80 µM GSH or GSSG. Limit of detection values are comparable to those reported by Herrero-Martinez *et al*. (2000)

Table 3. Recovery of GSH and GSSG in a sample of tomato leaves with added standard

	GSH	GSSG
Initial concentration (μM)	6.63 ± 0.21[a]	6.59 ± 0.79
Expected concentration (μM)	51.5 ± 0.2	51.4 ± 0.7
Determined concentration (μM)	53.8 ± 4.3	45.5 ± 1.7
Recovery (%)	104 ± 8.1	88.5 ± 2.9

[a] Mean ($n = 3$) ± SD.

using CE with a diode array detector, or those reported by Carru *et al*. (2002) using photometric detection. Values are also similar to those found through chromatographic techniques (Rellán-Álvarez *et al*., 2006), although not as low as those found through CE coupled to the use of more sensitive detectors such as laser-induced fluorescence (Musenga *et al*., 2007) and mass spectrometry (Ohnesorge *et al*., 2005). The method reproducibility, both intra- and interday, was good, with RSD values below 4% for the area under the curve and below 1% for migration time. Compared with GSSG, recovery was greater and closer to 100% for GSH, showing absence of oxidation of the added GSH.

Dry shoot and root yields in plants treated with 3 μM Cu (Table 4) showed no significant differences with control plants (0.4 μM Cu). However, plants treated with 12 μM showed a yield decrease of 77 and 71%, respectively, with slight shoot chlorosis and root darkening. It has been demonstrated that plants exert a control over the levels of some elements in shoots such that they can make up for deficit or excess of elements that might affect their development (Marchner, 1995). Considering the results of this study, it could be stated that Cu homeostasis in tomato plants keeps Cu concentration in shoots in a range within the values found in the control plants (20 μg/g) and those observed with the highest metal doses (55 μg/g). A different situation was observed in roots, where Cu concentration correlated positively with the metal concentration applied to the nutrient solution ($r = 0.92$, $n = 12$). Considering that physiological processes in roots including Cu participation are limited (Marchner, 1995), this correlation may be accounted for by passive absorption of the metal resulting from a mass effect. This greater metal absorption in turn acts to the detriment of the plant, as demonstrated by significant decrease observed in plant growth (Table 4).

The optimised method was applied to the analysis of both peptides from tomato plants grown in the nutrient solution.

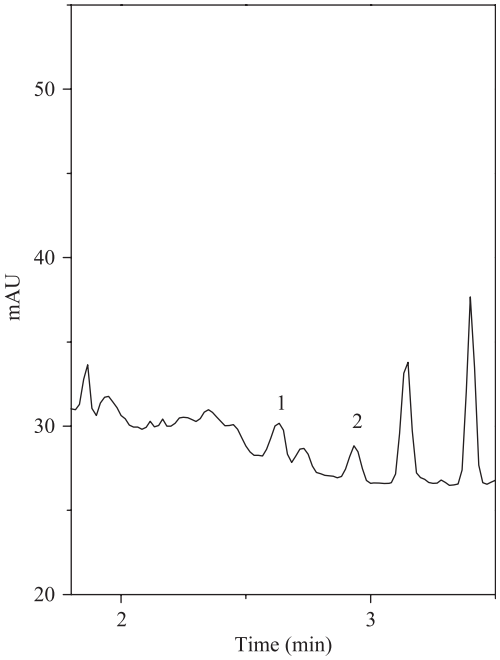

Figure 3. Electropherogram obtained with shoots of control plants treated and analysed with the optimised method. (1) GSH and (2) GSSG.

An electropherogram obtained from the shoots is presented in Fig. 3. Copper treatments showed larger effects on GSH and GSSG levels in roots than in shoots (Table 4). In roots the highest glutathione levels were for the reduced form GSH in control plants, falling by about 20 and 55% with application of 3 and 12 μM Cu, respectively. GSSG in roots was detected only in plants treated with 12 μM Cu. In shoots, GSH concentration did not change with copper treatment, while GSSG concentration increased slightly.

The GSH and GSSG concentrations determined in plants in this study showed similar values comparable to those described in the literature for studies carried out in the same type of matrix with spectrophotometric or chromatographic methods (Drazkiewicz *et al*., 2003). The highest concentrations were obtained for GSH in roots, which is in agreement with results obtained by Wang *et al*. (2004) for Cu and Shanker *et al*. (2004) for Cr. The decrease in GHS in roots with increasing Cu concentrations could indicate greater utilization of this peptide in roots as a precursor of

Table 4. Dry mass, Cu, GSH and GSSG concentration in shoots and roots of plant exposed to copper

Cu treatment (μM)	Dry mass (g/pot)	Cu (μg/g)	GSH (nmol/g)	GSSG (nmol/g)
		Shoots		
0.4	3.89 ± 0.36 A[a]	20.3 ± 0.7 B	55.8 ± 6.3 A	38.6 ± 7.7 B
3	3.28 ± 0.31 A	51.9 ± 0.7 A	52.8 ± 8.5 A	52.7 ± 4.7 A
12	0.86 ± 0.07 B	55.6 ± 4.6 A	65.0 ± 8.4 A	55.4 ± 5.5 A
		Roots		
0.4	0.80 ± 0.08 a	409 ± 66 c	98.3 ± 3.7 a	n.d.
3	0.74 ± 0.12 a	684 ± 20 b	77.6 ± 3.6 b	n.d.
12	0.23 ± 0.01 b	990 ± 18 a	44.9 ± 2.8 c	20.1 ± 1.4

[a] Mean ± SD ($n = 4$). Different letters in each column represent significant differences of mean ($p < 0.05$) in shoots (capital letter) and roots (small letters) of plant; n.d. = not detected.

metal-complexing biomolecules, such as phytochelatins. This effect, in turn, appears to be independent of the metal damage in roots since in the prolonged treatment there was a significant decrease in root biomass.

It has been reported that, under normal conditions, the glutathione pool in shoots is found to be mostly reduced (Noctor *et al*., 2002), so that findings where GSH concentration is higher than GSSG concentration would indicate that sample treatment previous to analysis has not greatly affected the levels of both peptides. In this sense, our results confirm this tendency and are coincident with the results of several authors (Herrero-Martinez *et al*., 2000; Rellán-Álvarez *et al*., 2006). Likewise, the levels found in tomato roots showed the same orders of magnitude as those reported by Rellán-Álvarez *et al*. (2006) in the roots of *Beta vulgaris*, and those found by Zaharieva and Abadía (2003) in the same species. Additionally, the highest GSH concentration was obtained in roots, in agreement with the results of Shanker *et al*., (2004) and Wang *et al*. (2004).

Results obtained here support the applicability of the method to the determination of GSH and GSSG in tissue of shoots and roots of tomato grown either under normal or stressful plant conditions. It should be pointed out that the optimised method, considering pre-analytical operations and CE separation, requires an estimated time of 30 min for complete sample analysis and may be set up in a routine laboratory equipped with a low-cost CE instrument.

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