SIMULTANEOUS DETERMINATION OF GLUTATHIONE AND GLUTATHIONE DISULFIDE IN AN ACID EXTRACT OF PLANT SHOOT AND ROOT BY CAPILLARY ELECTROPHORESIS

JORGE MENDOZA, TATIANA GARRIDO, RAÚL RIVEROS, CECILIA GONZÁLEZ AND JOSÉ PARADA*

Universidad de Chile, Facultad de Ciencias Químicas y Farmacéuticas, Departamento de Química Inorgánica y Analítica, Casilla 233, Santiago, Chile

ABSTRACT

This study describes the fast and simultaneous determination of glutathione and glutathione disulfide by Capillary Zone Electrophoresis in plant extracts of shoot and root of tomato plants. Frequent use of acidic precipitation of protein 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. In this study, the resulting acidic matrix was previously treated with the same background electrolyte to prevent comigration and to improve signal resolution. Optimization of some parameters of the technique allowed the determination of both analytes in less than three minutes. The optimized method showed good reproducibility and linearity, with correlation coefficients above 0.999 and detection limits below 3 µM for both peptides. Analyte recovery in the process was in the 88-104% range. The concentration found in tomato plants hydroponically grown in the absence of stress factors was in the 51-100 nmol g^{-1} range, fresh weight for GSH and 5-32 nmol g^{-1} range, fresh weight for GSSG.

Keywords: Glutathione; Capillary Electrophoresis; Metaphosphoric acid; Acidic precipitation; Glutathione disulfide

INTRODUCTION

Glutathione constitutes an important source of nonprotein thiols both in animal and in plant cells and it has the crucial function of cell defense and antioxidizing protection. This tripeptide is part of the ascorbate-glutathione cycle that helps to prevent or minimize damage caused by reactive oxygen species. This function involves oxidation of the thiol group to form mainly glutathione disulfide (GSSG) [1, 2]. 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, luminic radiation, among others [3-5]. In this respect, the GSH/GSSG ratio has been utilized rather than the individual levels of each peptide as an indicator of oxidative status in plants and animals [2].

In view of the growing interest in the analysis of GSH, GSSG, and homologous peptides in various matrices, several methods have been proposed [6, 7]. Among these, methods based on liquid chromatography [8, 9] and enzymatic determination [10] are the most highly demanded, although methods based on Capillary Electrophoresis (CE) have also been proposed [11-13]. Several aspects make capillary electrophoresis a highly adequate technique to obtain simple and fast methods for glutathione determination, such as good reproducibility, simplicity of procedure, short analytical time, low injection volume, and low cost [14, 15]. If we go through proposed methods based on capillary zone electrophoresis (CZE), a similarity is observed in the process of analyte separation, where the background analyte is usually constituted by borate in the 0.05-0.3 M concentration range, with pH values near its pK_a [16, 17], BGE concentration and pH, together with capillary length and voltage applied having the strongest effect on the time required for analyte separation. On the other hand, detection using CE has been carried out with different systems, the most sensitive of which are based on laser-induced fluorescence [18], mass spectrometry [19], and, less sensitive even though more widely used, systems based on photometric detection [11].

There are several studies dealing with glutathione determination in plant shoot [5, 8], with concentration values ranging from a few nmol g^{-1} to several hundreds of nmol g^{-1}. On the contrary, root has received less attention, even though it is the fundamental organ for nutrient absorption and also the main way of xenobiotic uptake by the plant. In this sense, the presence of GSH and GSSG has been reported in the root of *Beta vulgaris* [8, 20], *Vigna radiata* L. [21] and *Raphanus sativus* L. [22], whereas only GSH has been found in the species *Cicer arietinum* L. [23] and *Arabidopsis thaliana* [24, 25].

Pre-analytical sample treatment is intended to separate small peptides from any kind of protein which may interfere in the determination and particularly from those enzymes that use these peptides as a substrate. If these were not deactivated they would alter equilibrium between GSH and GSSG in the sample. As chemical precipitating agents, acids are the most commonly used ones, among them trichloroacetic, metaphosphoric, phosphoric, perchloric, and sulfosalycylic acid [7]. The extraction step thus generates an acidic matrix of an acid strength and pH which may affect the method sensitivity, cause comigration of species or changes in the equilibria relating both species in cells or fluids, possibly generating less representative results of the actual levels in living organisms. We have previously[26] proposed adjustment of the sample pH before CZE for better resolution of the signals of both peptides. However, the use of alkali for neutralization may imply the formation of a colloidal precipitate in the injection vial which may produce wrong signals or capillary obstruction if the colloid has not been previously visualized; additionally, it may increase analytical time in case precipitate separation is necessary. For these reasons, the purpose of this study was to optimize electrophoretic conditions for simultaneous determination of reduced and oxidized glutathione in a short time, and to improve pre-analytical treatment for better visualization of the signals of both peptides in an acidic matrix obtained with MPA from shoot and root of tomato plants. Since extracts obtained with MPA are 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.

EXPERIMENTAL

Standard solution and electrolyte background

The background electrolyte consisted of a 300 mM borate solution daily prepared from a 0.5 M sodium borate stock solution, adjusting the pH to the values under study by using 0.5 M NaOH and filtering the resulting solution through a 0.22 µm cellulose membrane. Highly pure (Sigma, St. Louis, MO, USA) GSH and GSSG were used as standards, and 500-µM stock solutions of both analytes were prepared. Preliminary tests to establish the best conditions were carried out with standards prepared in water purified in a Milli-Q system (Millipore, Bedford, MA, USA). Considering that GSH and GSSG extraction from the plant matrix would be performed with metaphosphoric acid (MPA), stock solutions of the standards containing 2 and 5% MPA were prepared. Six concentration points in the 5-80-µM range for GSH and in the 2-80-µM range for GSSG were considered for the calibration curves. Analyte electrophoretic mobility was calculated from the experimentally obtained parameters using water migration time as a reference[14].

Instrumentation

Analysis was carried out in a Quanta 4000 (Waters associates, Milford, MA, USA) capillary electrophoresis system using 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-sec times. Fused silica capillary tubes 40- and 60- cm long and 75-µm id were used, conditioned with 2 M KOH for 30 min the first time they were used. Additional 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 processing was carried out with Millenium data analysis software (Waters associates).

Plant growth, collection and preparation of sample

Tomato (*lycopersicon esculentum* L.) plantules were obtained from seeds germinated in sand at room temperature.The plants were grown in a plant-growth chamber. They were irradiated with artificial light at 400 µEinstein $m^{-2}s^{-1}$, with a 16-hour photoperiod, at day/night temperature of 24/20 °C and 50% relative humidity. The plants were grown in pots containing 1-L nutritive solution of definite composition and pH adjusted to 6.0 [26]; solution replacement for fresh solution was done every other day. Six repetitions, grown for 28 days, were utilized. After this period, the sample was collected for GSH and

e-mail: jmendoza@ciq.uchile.cl

GSSG analysis by selecting 1 g of shoots and roots from each repetition. These samples were immediately frozen and kept in liquid nitrogen until analysis. The remaining plant material was separated into shoot and roots, weighed and stove-dried for 48 h at 60°C.

In order to carry out peptide extraction, the sample was ground with a mortar previously cooled in liquid nitrogen, 2 mL 2% MPA was added with vigorous stirring to form a homogeneous suspension. The suspension was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was extracted with a syringe and filtered through a 0.45-µm cellulose nitrate membrane. The solutions thus obtained were immediately stored at -80°C.

The results of oxidized and reduced glutathione concentration in shoot and root were expressed as nmol g^{-1} fresh weight (fr wt.), then subjected to one-way variance analysis and the mean values were compared by Duncan's test to a level of 5%.

RESULTS AND DISCUSSION

Trying to apply the best instrumental conditions for GSH and GSSG analysis and based on the already reported methodology [27, 28], work was started with a 60-cm, 300-mM borate electrolyte at pH 7.8 and 25 kV [27] with positive polarity of the power supply; under these conditions, a 81-µA current was observed in the capillary with signals appearing before 7 minutes for both peptides and a difference of 0.5 min between them (Figure 1a). In order to shorten migration time, the capillary was shortened to 40 cm, keeping a voltage of 25 kV and both signals appeared in less than 2- min run, with a time difference of 0.1 min between them. However, a significant current rise occurred, in some cases above 200 µA. In order to decrease the current, voltage was decreased to 20 kV, keeping the electrolyte pH, and thus the current was observed to fall to 111 µA, with an increase in migration time of about 1 min (Figure 1b). At this voltage and pH, current difference between the 40- and 60-cm capillaries was about 50 µA, unlike the value observed at a voltage of 25 kV, where the difference was 120 µA. The effect of decreasing the BGE pH from 7.8 to 7.6 was mainly observed as a decrease in the current and an increase in signal resolution (Figures 1b and 1c). By considering a 40-cm capillary and BGE pH at 7.6, voltage decrease from 25 to 20 kV showed a response similar to the previous cases, where current fell about 120 µA, increasing signal resolution but also increasing migration time of both analytes (Figure 1d). In all the cases in study, at the same concentration of GSH and GSSH, a more intense signal was always observed for GSSG.

The method optimized in this study was based on methods proposed for GSH and GSSG analysis in samples of animal fluids or tissues [6, 7]. In most studies, borate has been used as a run electrolyte, which favors the presence of both peptides in their dissociated state, the same as at physiological pH. Considering that one of the purposes of this study was to decrease analytical time, the parameters capillary length, voltage applied and electrolyte pH were adjusted so that analyte migration time and the current generated in the process would be minimized. An increase in the voltage applied implied an increase in electroosmotic flow (EOF) and thus a decrease in migration time. The voltages used in this study ranged from 20 to 25 mV (Figure 1). In turn, since a decrease in the capillary length implies an increase in electric field and thus an increase in EOF, the application of this principle, regardless of the increase in run voltage, also produced a decrease in migration time. However, increasing either parameter implied an increase in the capillary current. This undesired effect may lead to heat generation within the capillary, producing wide peaks, nonreproducible migration times, sample decomposition or denaturation and in some cases, electrolyte boiling, which may cause cuts in the electrophoresis system [14]. Thus, conditions were chosen where current was not above 120 µA. In GSH and GSSG determination by CE using borate as the main BGE component current values ranging from 27 [11] to 150 µA [29] have been reported without loss of efficiency and keeping analyte stability

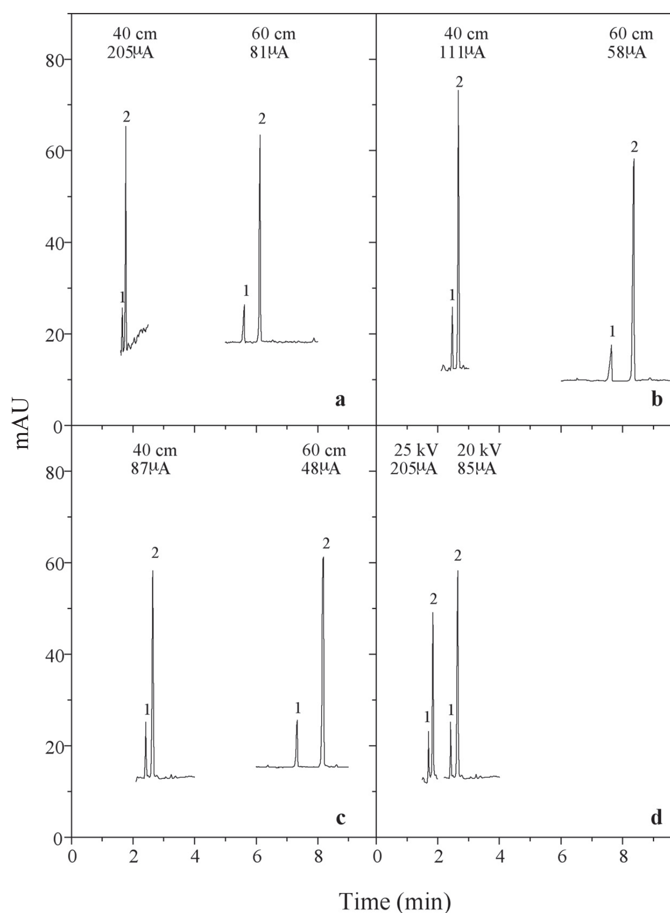

Figure 1: Effect of capillary length, applied voltage, and electrolyte pH on generated current and migration time of GSH (peak 1) and GSSG (peak 2) at 40-µM concentration of each standard. In figures a, b and c, pH and voltage were kept constant and the capillary length was varied. In figure d, pH and capillary length were kept constant and voltage was varied. a) pH 7.8, 25 kV b) pH 7.8, 20 kV c) pH 7.6, 20 kV and d) pH 7.6 and capillary length of 40 cm.

Another parameter affecting GSH and GSSG analysis is BGE pH. On the one hand, in silica capillaries, an increase in pH increases EOF because of dissociation of the SiOH to SiO^- functional groups on the capillary inner wall, which carries an increase in surface charge and thus in Z potential [14]. On the other hand, an increase in pH may favor the presence of negatively charged glutathione species, which improves the method sensitivity. For this study, pH 7.6, a lower value than the electrolyte pK_a, was chosen because at higher pH values the decrease in the capillary length caused an important current increase in the capillary. With the selected pH, considering a 40-cm capillary and a voltage of 20 kV, the generated current was 85 µA. Similar conditions for GSH and GSSG determination were found by Carru et al. [30] when working with 300 mM and 7.8 borate.

The above information was obtained using standard GSH and GSSG solutions prepared with purified water. However, it was necessary to adjust the conditions since the sample matrix corresponded to an acidic matrix. Based on information describing GSH and GSSG extraction using 2%[5] and 5%[8] MPA, standards of both peptides were prepared containing the above described MPA percentages. Electropherograms recorded for these solutions did not show a sharply defined baseline and no signal for either peptide (Figure 2). In order to visualize the signals, it was necessary to raise the solution 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, pH 7.6 borate) 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 implied increasing the pH from 2.2 to 6.6 and decreasing the current from 124 to 114 µA. For the solution containing 5%

MPA, the best visualization was attained with 1:10 dilution, reaching a pH of 6.4 and a current of 117 µA. However, this resulted in the nearly complete loss of GSH signal, which was verified with standard addition (Figure 2).

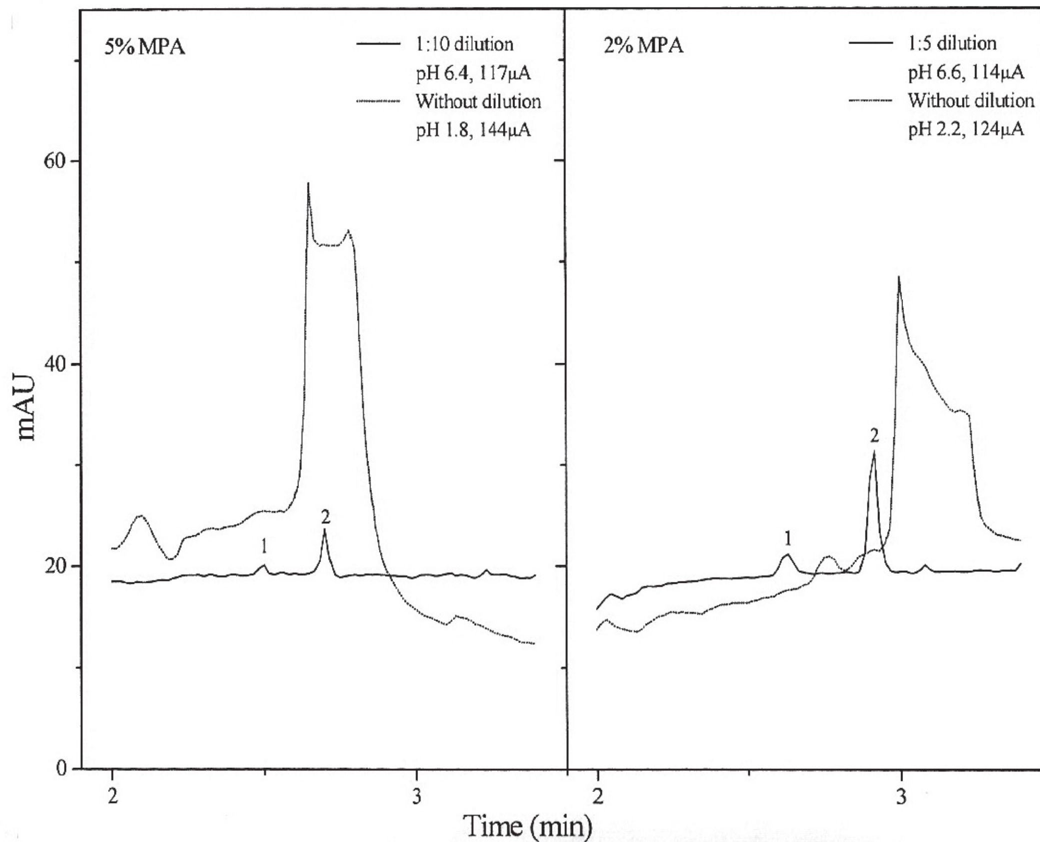

Figure 2: Effect of dilution of standard prepared in 2 and 5% MPA. Final concentration of both standards of 10 µM, GSH (1), GSSG (2).

The effect of sample injection time on the performance and resolution of the signals of both analytes was assessed, considering 30, 45, and 60 seconds of hydrostatic sample injection (Table 1). Good correlation was observed between area under the curve and injection time, with correlation coefficients of 0.998 and 1 for GSH and GSSG, respectively. Increasing injection time kept the time difference between both signals in about 0.2 min, which implied a decrease in resolution (Table 1).

Table 1.- Area, migration time and mobility of GSH and GSSG; and resolution for each injection time

	GSH			GSSG			
Injection time (s)	Area	Migration time (min)	Mobility ($cm*s^{-1}*V^{-1}$)	Area	Migration time (min)	Mobility ($cm*s^{-1}*V^{-1}$)	Resolution
30	36290	2.42	-0.1194	115195	2.65	-0.0852	3.44
45	55719	2.36	-0.1332	167515	2.57	-0.0941	2.38
60	71167	2.23	-0.1502	220040	2.42	-0.1075	1.44

Finally, in order to quantify GSH and GSSG in the extracts of shoot and root, a calibration curve 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. The parameters obtained for both curves are shown in Table 2. The detection limit (LOD) was calculated as the concentration of the analyte providing a signal equal to the blank signal plus three times the blank standard deviation. Measurement reproducibility was established in relation to area under the curve and migration time, recording both parameters under the above mentioned experimental conditions five times in a day (intraday) and for five consecutive days (interday) (Table 3).

Table 2.- 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 3: Intraday and interday repeatability of optimized method

	GSH		GSSG	
	Area	Time (min)	Area	Time (min)
Intraday				
Mean [a]	26176	2.59	113814	2.88
SD[b]	728.8	0.01	1415.4	0.003
RSD(%) [c]	2.78	0.21	1.24	0.10
Interday				
Mean	26386	2.58	114674	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 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 nonspiked sample. This procedure was carried out on three consecutive days and results are shown in Table 4. In order to verify GSSG and GSH signals, control samples and standards were run with addition of 2-mercaptoethanol so as to reduce GSSG.

Table 4.- Recovery of GSH and GSSG in a sample of tomato leaves with added standard

	GSH	GSSG
Initial concentration (μM)	6.63[a] ±0.21[b]	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),
b. ± SD

In most of the studies where capillary electrophoresis has been used for glutathione determination, the required separation time ranges from 3 to 7 min [5, 8, 11]. Studies where time is below this range are less frequent. In this respect, both Carru et al. [12] and Lochman et al. [31] report times below 2 min for simultaneous analysis of reduced and oxidized glutathione analysis, using a BGE constituted by borate and 20- and 30-cm capillaries. However, they provide little information concerning electrophoresis conditions used and generated current. In the present study it was not possible to reduce the capillary length further without significantly affecting the generated current; otherwise, capillary temperature would have increased 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. Limit of detection values are comparable to those reported by Herrero et al. [5] using CE with a diode array detector or those reported by Carru et al. [12] using photometric detection. Values are also similar to those found through chromatographic techniques [8], although not as low as those found through CE coupled to the use of more sensitive detectors such as laser-induced fluorescence [18] and mass spectrometry [19]. EC methods have been described based on other modalities such as sample stacking [32] or MECK [13] with lower detection limits than those found in the present study, but they have not been successfully applied to highly acid matrices such as those formed by MPA. 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. Recovery was greater and closer to 100% for GSH, compared with GSSG, showing absence of oxidation of the added GSH.

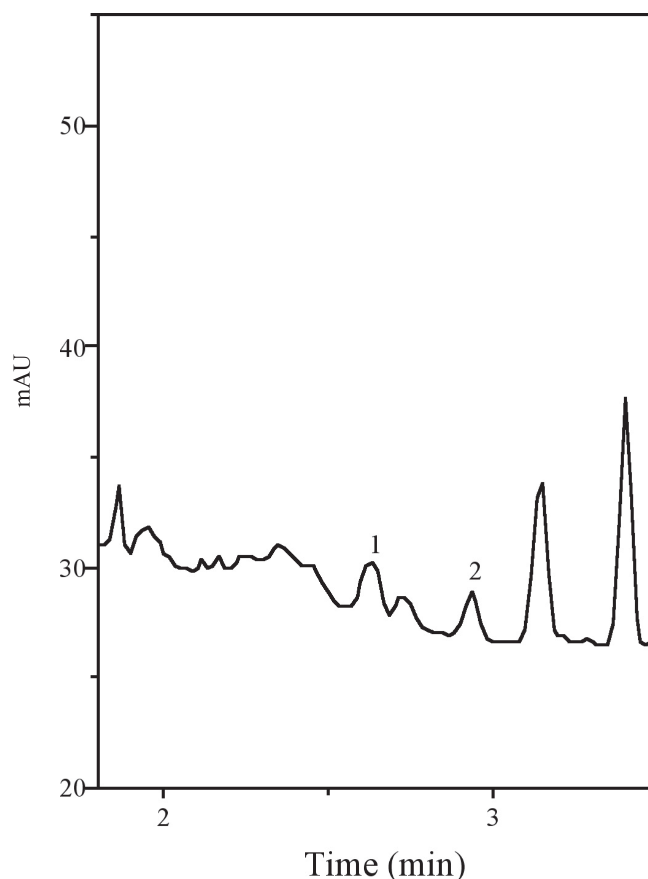

Figure 3: Electropherogram obtained with shoot of plant treated and analyzed with the optimized method. (1) GSH and (2) GSSG.

Analysis of both peptides in tomato plants grown in nutritive solution was started under the previously established conditions. The samples were short-term thawed, diluted with electrolyte, and immediately analyzed by CZE. An electropherogram obtained with shoot of plant is presented in Figure 3. Both peptides showed significant differences ($p<0.05$) between the concentrations in each plant organ. In the case of GSH, the concentration in root was higher (99.7

±4.5 nmol g^{-1} fr wt.) than that found in shoot (51.8 ±2.9 nmol g^{-1} fr wt.). On the contrary, GSSG concentration was higher in shoot (31.9 ±2.4 nmol g^{-1} fr wt.) than in root (5.1 ±0.6 nmol g^{-1} fr wt.), with GSH/GSSG ratio values of 1.6 and 19.5 for root and shoot, respectively. Such values are similar to those reported in the literature for both peptides in the same kind of matrix but obtained by spectrophotometric or chromatographic methods [33].

It has been reported that, under normal conditions, the glutathione pool in shoot is mostly found reduced [1] 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 [5, 8]. Likewise, the levels found in tomato root showed the same orders of magnitude as those reported by Rellan et al. [8] in the root of *Beta vulgaris*, which they report as 92.1 and 46.1 nmol g^{-1} fr wt. for GSH and GSSG, respectively, and those found by Zaharieva and Abadía who report 30 and 10 nmol g^{-1} fr wt., respectively, in the same species [20]. Additionally, the highest GSH concentration was obtained in root, in agreement with the results of Shanker et al. [21] and Wang et al. [34].

The results obtained here ensure the applicability of the method to the determination of GSH and GSSG in tissue of shoot and root of tomato grown either under normal conditions or under conditions of stress for the plant. It should be pointed out that the optimized method, considering pre-analytical operations such as 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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