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CAPILLARY ZONE ELECTROPHORESIS FOR DETERMINATION OF REDUCED AND OXIDISED ASCORBATE AND GLUTATHIONE IN ROOTS AND LEAF SEGMENTS OF *Zea mays* PLANTS EXPOSED TO Cd AND Cu

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Abstract. The concentration of non-enzymatic antioxidants such as ascorbate and glutathione in tissues is one of the major plant responses to biotic and many abiotic stresses, including metals. Therefore, it is crucial to develop the most effective methods for simultaneous quantitative analysis of these antioxidants. Capillary zone electrophoresis allows relatively fast and effective analysis. The aim of the paper was to apply and optimise the capillary electrophoresis conditions for simultaneous determination of glutathione, glutathione disulphide, ascorbate, and dehydroascorbate in small plant tissue samples exposed to copper and cadmium. The method ensures good linearity and reproducibility, with correlation coefficients 0.988 for ascorbate and 0.999 for glutathione and glutathione disulphide, and with detection limits approximately 2.50, 0.65 and 0.50 ppm, respectively. Cu stress was found to increase the ascorbate concentration and glutathione content in leaves, while Cd increased glutathione in the oldest leaf segments and root.

Key words: non-enzymatic antioxidants, heavy metal, stress, plant

INTRODUCTION

Glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine), ascorbate (L-AA), and α-tocopherol can serve as non-enzymatic defence mechanisms in prevention and limitation of cell damage under oxidative stress. The conversion of the reduced form of L-AA to dehydroascorbic acid (L-DHA) as well as GSH to glutathione disulphide (GSSG) is commonly known as a reliable index of oxidative stress. The ascorbate-glutathione cycle is important in maintenance of cellular homeostasis and plays a crucial role in removal of reactive oxygen species (ROS). Additionally, ascorbate is involved in pho-

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toprotection, electron transport, and regulation of photosynthesis, while GSH takes part in intracellular binding of heavy metals or detoxification thereof by phytochelatin (PC) synthesis [Drążkiewicz et al. 2003a]. Numerous physiological functions of L-AA and GSH, as well as the significance of the ratio of oxidised and reduced forms of both compounds in the evaluation of oxidative stress, is of increasing concern in simultaneous determination of GSH, GSSG, L-AA, and L-DHA in stressed plant tissues.

Different methods for measurement of GSH and L-AA in biological samples have been developed, including enzymatic and high-performance liquid chromatography (HPLC) equipped with a UV spectrophotometric detector, electrochemical detection, or fluorescence spectroscopy [Nováková et al. 2008, Monostori et al. 2009]. However, most of them are not capable of simultaneous determination of ascorbate and glutathione and their oxidised forms. Furthermore, many of these methods have various limitations. For example, spectrophotometric or enzymatic procedures are time- and material-consuming and are not specific and selective enough, while HPLC needs large amounts of buffer and requires purification of plant samples from some organic impurities. Recently, high-performance capillary electrophoresis (HPCE) has been demonstrated to be a useful separation technique that offers high efficiency, fast separations, predictable selectivity, and automation. Particularly, the HPCE method is useful for analysing small plant samples with the weight below 200 mg of fresh matter.

Capillary zone electrophoresis (CZE) with photometric detection has been successfully applied in simultaneous determination of L-ascorbic acid, glutathione, and their oxidised forms in plant tissues [Davey et al. 1997, Herrero-Martinez et al. 2000]. The procedure of Herrero-Martinez et al. [2000] entails reduction of L-DHA by DL-homocysteine, but this method requests large sample aliquots; therefore, it eliminates analysis of small parts of plant tissue. Previously, Davey et al. [1997] described a capillary zone electrophoresis (CZE) procedure for direct determination of GSH, GSSG, L-AA, and L-DHA. However, in this method, quantitation of L-DHA was not achieved. Previously, Kacem et al. [1986] pointed out that direct determination of L-DHA with UV detection is difficult due to the extremely low UV maximum wavelengths absorption (185 nm). Additionally, direct UV L-DHA detection in complex matrices (e.g. plant samples) is complicated by appearance of other organic molecules in a sample assay with similar UV maximum absorption as L-DHA. Usually, both HPLC [Gökmen et al. 2000] and HPCE [Herrero-Martinez et al. 2000] systems, define measurement of DHA concentration as the difference between the total L-AA and initial L-AA (before L-DHA reduction). Previously, several reduction agents such as homocysteine, l-cysteine, glutathione, and meracaptoethanol [Deutsch 2000] were tested. However, dithiothreitol (DTT) is the most common reducing agent used for simultaneous determination of L-DHA and L-AA in the HPLC method.

The aim of the paper is to show the possibility of simultaneous determination of reduced and oxidised forms of glutatione and ascorbate using the HPCE method, in differently aged segments of plants exposed to heavy metal stress.

EXPERIMENTAL

Chemicals and materials. L-AA, GSH, and GSSG used as standards were purchased from Sigma-Aldrich. The BGE and extraction buffer were prepared from H_3BO_4, ACN (acetonitrile) (purity > 99%), MPA (metaphosphoric acid) (Sigma-Aldrich), and sodium hydroxide (1M NaOH, 0.1 M NaOH) (Agilent Technologies). DTT (dithiothreitol 1M) was obtained from AA Biotechnology. Before injection into the capillary, all solutions were filtered through 0.20 µm pore size nylon filters (Chemland, Poland). Separation was performed a using 50-µm i.d. extended light capillary (Perlan Technologies).The Hoagland nutrient solution contained the following salts (in mM): 1 $NH_4H_2PO_4$, 6 KNO_3, 4 $Ca(NO_3)_2$, 2 $MgSO_4$; microelements (in µM): 46 H_3BO_3, 9 $MnCl_2$, 0.76 $ZnSO_4$, 0.32 $CuSO_4$, 0.11 H_2MoO_4, and 85 µM Fe as Fe(III)citrate. Cadmium and copper were added as $Cd(NO_3)_2 \cdot 4\ H_2O$ and $CuSO_4 \cdot 5\ H_2O$, respectively. All solutions were prepared with deionized water (MiliQ deionizer, Millipore).

Standard curves and background electrolyte. The background electrolyte (BGE) (pH 9.0) consisting of a 200 mM borate solution and 20% of ACN (acetonitrile) was prepared daily from a 400 mM borate stock solution [Davey et al. 1997]. The L-AA, GSSG, and GSH stock solutions were prepared in ice-cooled 3% (w/v) MPA solutions at the concentration of 1000 ppm, and stored at 4ºC for use on the same day.

CE instrumentation and separation conditions. Separations were performed on an Agilent 7100 capillary electrophoresis system equipped with a diode array spectrophotometric detector (190–600 nm). A fused-silica 50-µm i.d. capillary with varying effective lengths of 56, 72, and 104 cm (48.5, 80.5, 112.5 cm total length respectively) were used. The applied voltage was set from 24 to 30 kV. The samples were injected by pressure (50 mbar) for 4.0 s, and then the BGE was injected for 4.0 s with 50-mbar pressure. Prior to the first use, new capillaries were rinsed with 0.1 mM NaOH under pressure (approximately with 1 bar) for 10 min, followed by water (10 min) and BGE (10 min); next, voltage of + 25 kV was applied for 45 min. Before each injection, the capillary was preconditioned for 10 minutes by flushing with the run electrolyte. Varying working temperature was used, increasing by 4ºC between 20 and 32ºC. In order to choose the maximum wavelength absorbance, spectral analyses between 190–600 nm were performed using the L-AA, GSH, and GSSG standard. Data analysis was performed on the ChemStation data analysis software (3D-CE ChemStation, Agilent Technologies).

Plant culture and treatments. Young seedlings of maize (*Zea mays* L. cv. Reduta) were used in the experiment. Maize seeds germinated for 2 days on wet filter paper. The seedlings were transferred to plastic pots with 1.5 L of aerated Hoagland nutrient (9 seedlings per pot). After adaptation for 2 days, the nutrient solution was changed and treated with 100 µM Cu and 50 µM Cd. Control (without Cu and Cd) and heavy metal treated plants were grown at a 16/8 h day/night cycle and at 24/17ºC, respectively, relative humidity 60–70% and photosynthetic photon flux density of 150 µmol m^{-2} s^{-1}. The nutrient solution was replaced after 4 days. The plants were analysed after 8 days. Third leaves were collected and divided into three sections of equal length from the youngest (basal), through the middle (mature) to the oldest part of leaf (apical). Additionally, roots were used for further analyses after being washed thoroughly with distilled water.

Sample preparation and DHA reduction. In all the cases, the analyses were performed on the same day after collecting the plant tissues. After harvest, the plant samples obtained (aliquots of 50–200 mg leaf parts and 200–300 mg of roots) were immediately frozen in liquid nitrogen. Subsequently, the plant tissues were homogenized in 0.6 ml 3% (w/v) MPA and centrifuged at 20000 rcf at 4ºC for 5 min. The supernatant was filtered through 0.20 μm nylon filters and the clarified assay was divided into two parts. One part of the samples was supplemented with an adequate aliquot of 3% MPA and immediately injected, and L-AA, GSSG, and GSH were determined. The other part of the samples was supplemented with DTT to final a concentration of 50 mM. The mixture was kept in the dark for 30–45 min to convert any L-DHA to LAA and then it was injected. The amount of L-DHA was calculated by subtracting the initial L-AA from the total L-AA obtained after L-DHA reduction.

Statistical analysis. The two-way ANOVA was used to explore the heavy metal × leaf segments interaction and its influence on the L-AA, L-DHA, GSH, and GSSG content. Differences between the factors were determined with Fisher's Least Significant Difference (LSD) at the 0.05 probability level. Data analysis was carried out with Statistic 6.0 (StatSoft, Inc. 2006).

RESULTS AND DISCUSSION

Conditions for CZE and L-DHA reduction. Previously, several various background electrolytes based on ammonium acetate [Yang et al. 2002], phosphate [Herrer-Martinez et. al 2000, Žunić and Spasić 2008], or borate buffer [Muscari et al. 1998, Carru et al. 2003] were tested to determine some non-enzymatic antioxidants using HPCE. In the present experiment, BGE containing 200 mM borate and 20% ACN was used to verify the electroosmotic flow [Davey et al. 1997]. As expected, the migration times increased with the voltage decrease (fig. 1). The varied voltage tested indicated that 30 kV yielded efficient resolutions with run time below 28 min, without negative Joule heating effects. Another factor greatly affecting separation is the capillary temperature, which influences buffer viscosity, injection volumes, and migration times. As known from the literature, high temperature increases the electroosmotic flow (EOF) and shortens migration time [Frazier et al. 2000]. Previously, various temperatures were used: 18ºC for GSH determination [Žunić and Spasić 2008], 23ºC for analyses of GSH, GSSG, L-AA and L-DHA [Herrero-Martinez et al. 1998], 25ºC for L-AA [Davey et al. 1996], 28ºC for measurement of GSH and GSSG in heart tissues [Muscari et al. 1998], and 40ºC for L-AA and uric acid detection in human plasma [Zinellu et al 2005]. The results obtained (fig. 2) indicate that lower temperature negatively affects the resolution between L-AA and GSSG, prolongs migration time, and decreases current. The estimated optimum for temperature separation that yielded efficient resolution, high efficiency, and a relatively short time run, without a risk of degradation of buffer or compounds, was 28ºC.

A property of the compounds of interest is low maximum wavelengths absorption. Although the maximum absorption of L-AA ranged between 244–265 nm depending on the buffer compositions, the GSH and GSSG were characterised by very low UV ab-

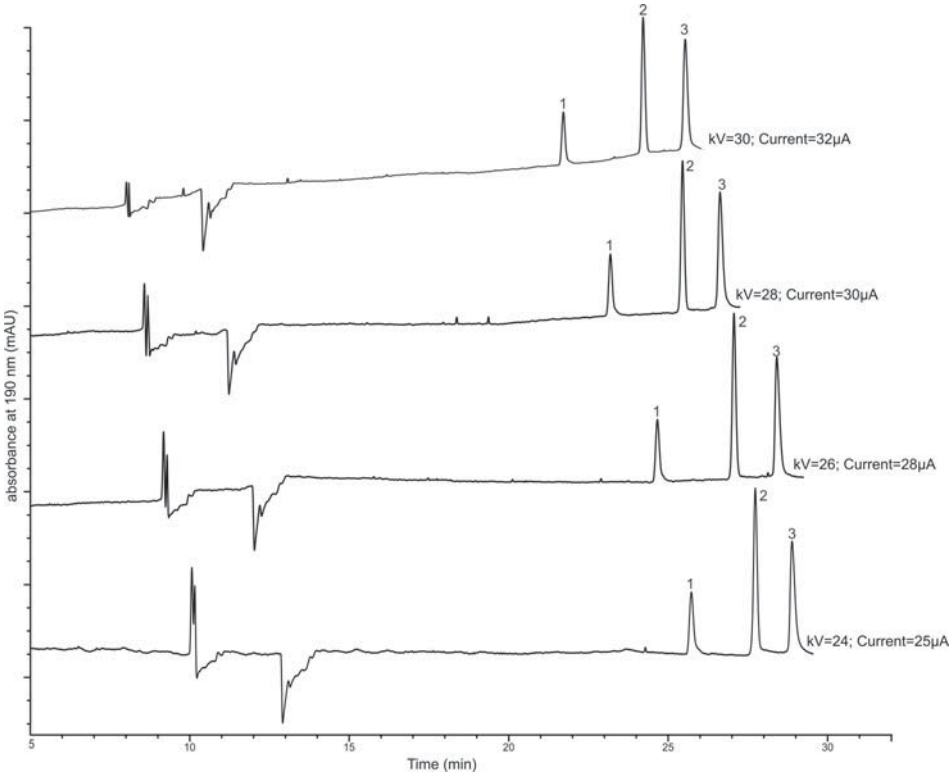


Fig. 1. Effect of voltage on the L-AA (1), GSSG (2), GSH (3) separations at a concentration of 50 ppm of each standard. All other conditions as in Fig. 4

sorptivity in the range of 185–200 nm. The wavelengths applied in the electrophoresis capillary method were most often 254 nm for L-AA [Galiana-Balaguer et al. 2001, Zinellu et al. 2004] and 200 nm for GSH and GSSG [Muscari et al. 1998, Herrero-Martinez et al. 2000, Carru et al. 2003]. However, the properties of the detector limited the possibility of setting the wavelength, especially at extremely low UV wavelength. In order to choose the best wavelength, all the spectra between 190–600 nm were collected during the analysis. The highest absorbance in the standard as well in the plant sample mixture was found to be 268 nm for L-AA and 190 nm for GSSG and GSH (fig. 3).

Three lengths of the capillary were tested (48.5, 80.5, 112.5 cm total length) (data not shown). To resolve several peaks present in the plant sample matrix, it was necessary to use a capillary of 80.5 cm total length (72 cm effective length), which yielded efficient resolution and a relatively short run time. Varied voltage, temperature and capillary length generated current ranges of 25–32 μA (fig. 1), 22–35 μA (fig. 2), and 16–48 μA respectively. The current values obtained were lower than those observed by Carru et al. [2003] and Mendoza [2009]. However, the authors mentioned used shorter capillaries, which increased the current level to a greater extent.

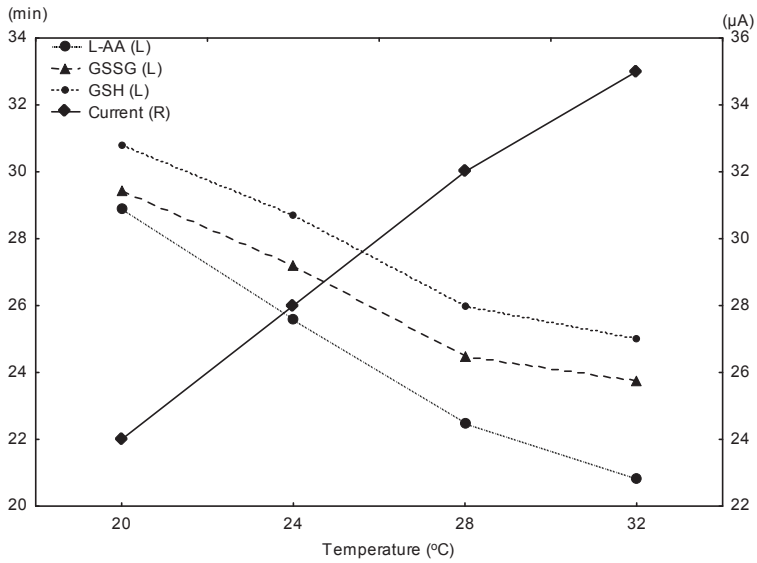


Fig. 2. Influence of capillary temperature on migration times and current. All other conditions as in Fig. 4

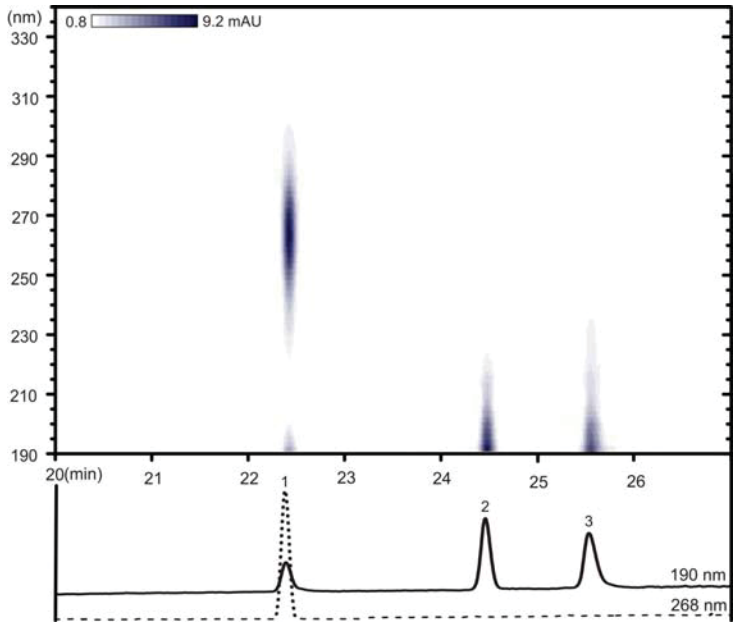


Fig. 3. DAD isoabsorbance plot of the L-AA, GSSG, GSH standard and electropherogram at UV 190 and 268 nm. Conditions as in Fig. 4

Finally, the following conditions were used for the analysis of L-AA, L-DHA, GSH, and GSSG in the plants samples: 200 mM borate pH 9.0 containing 20% (v/v) ACN, 30 kV voltage with positive polarity of the power supply, 28ºC capillary temperature, and a simultaneously monitored signal at 190 and 268 nm. In this system, applied voltage generated a current of around 32 μA while the L-AA, GSSG, and GSH peaks appeared at 22.3, 24.5, and 25.3 respectively (tab. 1).

Table 1. Precision of the uncorrected area, corrected area, and migration times of 50 ppm L-AA, GSSG and GSH

	L-AA			GSSG			GSH		
	Area	CorrArea	time (min)	Area	CorrArea	time (min)	Area	CorrArea	time (min)
Mean*	40.60	0.0303	22.27	60.08	0.0409	24.46	56.30	0.0370	25.33
SD**	0.681	0.00055	0.169	1.25	0.00065	0.31	1.90	0.00185	0.48
RSD(%)***	1.68	1.99	0.698	2.08	1.74	1.15	3.37	5.39	1.68

* n = 6
** Standard deviation
*** Relative standard deviation

The reduction of L-DHA to L-AA was performed by using the DTT reagent. Gökmen et al. [2000] have indicated that the concentration of DTT and reaction time greatly affects the efficiency of L-DHA reduction. According to these authors, 0.1 mg of DTT per 1 ml of samples is enough to complete L-DHA reduction; however, in higher amounts, it decreases the reaction time and prevents oxidation of L-AA. In the present study, 50 mM DTT for 45 min in the dark was used. However, as mentioned above, to reduce the consumption of chemicals, a lower amount of DTT could be used, depending on the expected L-DHA. The results of L-DHA reduction by DTT in the plant samples is illustrated in the electrochromatograms (fig. 4).

Reproducibility and linearity of the HPCE method. Using the HPCE method, linearity calibration curves were received for all the compounds over the range of 5–250 ppm. The correlation coefficients of the linear regression equation were 0.988, 0.999, and 0.999 for L-AA, GSSG, and GSH, respectively (tab. 2). As low concentrations as 2.5 ppm of L-AA, 0.5 ppm of GSSG, and 0.65 ppm of GSH, which correspond approximately to 7.5 μg L-AA·g^{-1}, 1.5 μg GSSG·g^{-1}, and 2.0 μg·GSH·g^{-1}, respectively, could be detected. The limit of detection was set at approximately three times the baseline noise. According to Ali et al. [2006], reproducibility is a major problem in capillary zone electrophoresis. In the present study, the relative standard deviation (RSD) and the mean value of the peak area, normalised peak area, and migration time were obtained by injecting 50 ppm of each compound six times. The results obtained indicated that the RSD value for uncorrected peak areas ranged from 1.7–3.4% and between 1.7–5.4% for

the normalised peak area. For all the compounds, the RSD of migration time was below 1.7%. The RSD values obtained were similar to the RSD values reported by Havel et al. [1999] and Carru et al. [2003] for GSH, GSSG and slightly higher than those obtained by Herrero-Martinez et al. [1998] for L-AA in plants.

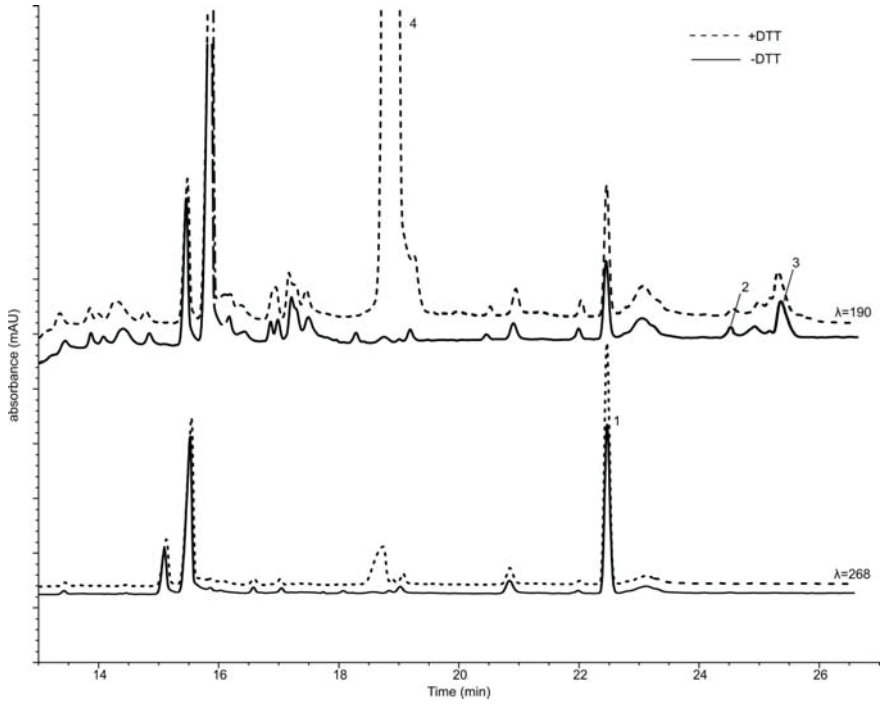


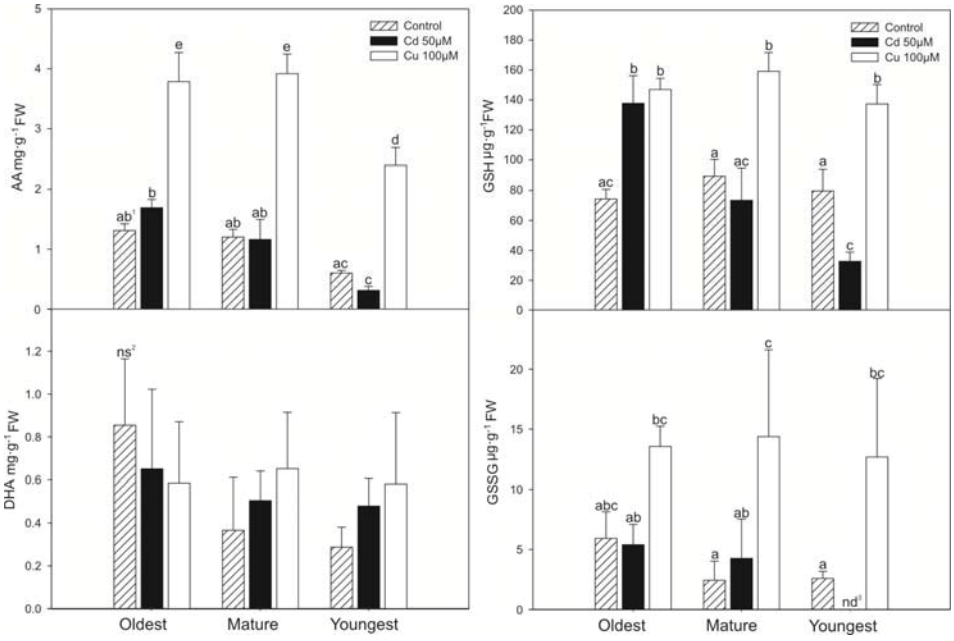
Fig. 4. Electropherogram of maize leaf monitored at 190 and 268 nm with and without DTT, (1-L-AA, 2-GSSG, 3-GSH, 4-DTT). Conditions: buffer, 200 mmol borate, 20% ACN (v/v), pH 9.0, kV = 30, capillary 80.5 cm total length (72 cm effective length) × 50 μm i.d. extended light path, temperature 28°C

Table 2. Calibration curve, limit detection, and efficiency obtained for the method

	L-AA	GSSG	GSH
Calibration curve	CA* = 0.00156Amt** – 0.01052	CA = 0.00070Amt + 0.00035	CA = 0.00069Amt + 0.00061
Correlation coefficient	0.988	0.999	0.999
LOD*** (ppm)	2.5	0.50	0.65
Efficiencies	176×10^3	194×10^3	119×10^3

* CA = Corrected area
** Amt = Amount (ppm)
*** Limit of detection

Concentration of L-AA, L-DHA, GSH, and GSSG in Cd and Cu stressed plant tissues. The method was used for measuring the L-AA, GSH, and their oxidised forms in the roots and differently aged segments of plants exposed to cadmium and copper. Typical electropherograms are shown in fig. 4. Cadmium and excess Cu did not exert any significant effect on the GSH and GSSG level in the roots (tab. 3); however, a tendency toward an increase in both these forms is visible. A similar effect was observed in the case of total ascorbate; however, in this case, this was a result of the significant increase in its oxidised or reduced forms after Cu or Cd action, respectively. The concentration of reduced and oxidised forms of glutathione and especially ascorbate was much higher in the leaves than in the roots of the control plants and exhibited similar or higher values than those described in the literature for monocotyledonous plants [Córdoba-Pedregosa et al. 2003, Rellan-Alvarez et al. 2006, Tie et al. 2012]. However, there are some differences in the case of dicotyledons and/or stressed plants [Mendoza et al. 2008]. Furthermore, the content of the reduced forms was higher than that of the oxidised ones, especially in the case of GSH, which is in accordance with the results of other authors [Drążkiewicz et al. 2003a, Córdoba-Pedregosa et al. 2003, Chao and Kao 2010].



[1] Means with the different letter within the same parameter are significantly different according to the Fisher's test ($p < 0.05$) (Two-way ANOVA leaf section × heavy metal treatments)
[2] ns – not significant
[3] nd – not detected

Fig. 5. Amount of AA, DHA, GSH, and GSSG in leaf segments of maize plants under Cu and Cd treatments. Bar and whisker plots give the mean (bar) and standard error (whiskers).

Table 3. Amount of L-AA, DHA, GSH, and GSSG in the roots of maize plants under different cadmium and copper treatments

	L-AA μg·g^{-1} FW	DHA μg·g^{-1} FW	GSH μg·g^{-1} FW	GSSG μg·g^{-1} FW
Control	169.4 a*	173.9 a	26.97 a	1.613 a
Cd 50 μM	324.2 b	107.7 a	35.83 a	2.061 a
Cu 100 μM	159.2 a	443.3 b	37.25 a	4.668 a

* Means with the different letter within the same parameter are significantly different according to the Fisher's test ($p < 0.05$)

Previously, Maksymiec and Baszyński [1996] and Krupa and Moniak [1998] indicated that the plant stress response seems to be mostly age-dependent. Along with the leaf ageing, the AA and GSH showed a tendency to increase; however, this effect was not significant. A similar effect was seen in the case of the oxidised forms of the substances. This is in accordance with the data obtained previously for GSH [Drążkiewicz et al. 2003a], but in the case of ascorbate, there are no investigations concerning similar objects.

Excess Cu increased the concentration of AA about 3–4 fold in comparison to the control, with a decreasing tendency during leaf segment ageing. The DHA content did not change significantly after Cd or Cu treatment, suggesting that direct AA oxidation did not occur. Excess Cu at similar concentrations usually exerted a stimulatory effect on AA accumulation, and despite its oxidative properties, Cu did not immediately convert AA to its oxidative form [Drążkiewicz et al. 2003a]. In contrast, there was no increase in the AA concentration after Cd action and this is in line with the tendency of the Cd-induced decrease in the AA concentration in the leaves [Wu et al. 2004]. GSH and GSSG concentration increased in the whole leaves after the Cu treatment. The Cd effects were weaker and involved a 75% increase in GSH only in the oldest segments. These results confirm that heavy metals such as Cu and Cd initiate phytochelatin synthesis from the GSH substrate, but Cd has a higher stimulatory effect on this process [Drążkiewicz et al. 2003b].

CONCLUSION

The determination of non-enzymatic antioxidants such as L-AA, L-DHA, GSH, and GSSG in plant samples is a difficult task due to their instability and difficulty in detecting. The described method allows simultaneous measurements of the above-mentioned compounds in plant samples using the HPCE technique. The study has confirmed that this HPCE system is valuable in terms of low consumption of chemicals, its rate, and easiness. Furthermore, plant sample preparation does not require any complicated procedures. Hence, this method ensures high sensitivity, stability, and reproducibility. Finally, the validated method can be a useful tool for analysis of two principal compounds

important in ROS homeostasis in a low volume of various plant samples, including small parts of tissues and young seedlings.

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WYKORZYSTANIE ELEKTROFOREZY KAPILARNEJ DO POMIARU ZAWARTOŚCI ZREDUKOWANEJ I UTLENIONEJ FORMY GLUTATIONU ORAZ KWASU ASKORBINOWEGO W KORZENIACH I SEGMENTACH LIŚCI *Zea mays* STRESOWANYCH Cd I Cu

Streszczenie. Zawartość nieenzymatycznych antyoksydantów takich jak kwas askorbinowy oraz glutation w roślinach jest jedną z głównych form odpowiedzi roślin na biotyczne i abiotyczne czynniki stresowe, w tym na metale ciężkie. Dlatego też niezbędne jest rozwijanie efektywnych metod jednoczesnego ilościowego oznaczania tych antyoksydantów. Elektroforeza kapilarna pozwala na takie relatywnie szybkie i efektywne analizy. Celem pracy było zastosowanie oraz optymalizacja metody jednoczesnego pomiaru zawartości glutationu, disiarczku glutationu, kwasu askorbinowego oraz kwasu dehydro-

askorbinowego w małych próbkach roślinnych z użyciem elektroforezy kapilarnej. Opracowana metoda zapewnia dobrą liniowość i powtarzalność ze współczynnikami korelacji na poziomie 0,988 dla kwasu askorbinowego oraz 0,999 dla glutationu i disiarczku glutationu, z limitem detekcji odpowiednio 2,50; 0,65 oraz 0,50 ppm. Stwierdzono, że stres indukowany nadmiarem Cu powodował wzrost zawartości kwasu askorbinowego i glutationu w liściach, podczas gdy po zastosowaniu Cd obserwowano wzrost zawartości glutationu w starszej części liścia i korzeniu.

Słowa kluczowe: nieenzymatyczne atyoksydanty, metale ciężkie, stres, roślina

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