Routine sample preparation and HPLC analysis for ascorbic acid (vitamin C) determination in wheat plants and *Arabidopsis* leaf tissues

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Running title

Routine HPLC analysis for ascorbic acid

Summary

Plants have developed various mechanisms to protect themselves against oxidative stress. One of the most important non-enzymatic antioxidants is ascorbic acid. There is thus a need for a rapid, sensitive method for the analysis of the reduced and oxidised forms of ascorbic acid in crop plants. In this paper a simple, economic, selective, precise and stable HPLC method is presented for the detection of ascorbate in plant tissue. The sensitivity, the short retention time and the simple isocratic elution mean that the method is suitable for the routine quantification of ascorbate in a high daily sample number. The method has been found to be better than previously reported methods, because of the use of an economical, readily available mobile phase, UV detection and the lack of complicated extraction procedures. The method has been tested on *Arabidopsis* plants with different ascorbate levels and on wheat plants during Cd stress.

Keywords: Ascorbic acid; HPLC analysis; plant tissue; stress; UV detection

Abbreviations: AA: ascorbic acid, reduced form; DHA: dehydroascorbic acid, oxidised form

1. Introduction

It is a well-known fact that the exposure of plants to unfavourable environmental conditions such as drought, high or low temperature, heavy metals, salt stress or pathogen attack increases the production of reactive oxygen species (ROS), thus inducing oxidative stress. Plants have developed various mechanisms to protect themselves against these toxic oxygen intermediates. One of the most important non-enzymatic antioxidants is ascorbic acid (AA). AA appears to be universal in photosynthetic eukaryotes, occurring in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall (33).

In plants at the subcellular level, the presence of AA has been demonstrated in different cell compartments including the chloroplast, cytoplasm, mitochondria and apoplast. Vacuolar concentrations are relatively low at around 0.6-3 mM, while cytoplasmic concentrations are in the range of 20-60 mM (7). About 30 to 40% of the total ascorbate content of plant tissue is in the chloroplast with stromal concentrations as high as 50 mM (10). Values of AA content between 2–20 μmol g^{-1} FW are consistently reported for leaves of higher plants. Leaf global AA concentration detected with about 1.13 μmol g^{-1} FW in *Sorghum*, with 2.3 μmol g^{-1} FW in sunflower (37), with 2.27-4.5 μmol g^{-1} FW in *Arabidopsis* (3), but these values depend on tissue type, plant age, time of day at sampling, and light intensity (13). In certain alpine plants, ascorbate leaf concentration reach 45 μmol g^{-1} FW (35), and the chloroplasts of alpine plants have been reported to contain up to 10 times the amount of ascorbate found in lowland plants (36). This compound occurs in both reduced (AA) and oxidized [dehydroascorbic acid (DHA)] forms; the former relatively unstable in aqueous environments, but generally the reduced form remains available under normal physiological conditions.

AA has the ability to donate electrons and is able to detoxify reactive oxygen species either by direct chemical interaction or through reactions catalysed by ascorbate peroxidase. Changes in ascorbate levels were reported under various environmental stress conditions, such as exposure to UV-B stress (1), high light intensity and drought (36), salinity (22), water and cold stress (19), heavy metal stress (8) and also wounding (16). AA may influence several processes in order to protect plants against oxidative damage (12; 16; 29), for example maintaining the photosynthetic apparatus (5), delaying the senescence of the leaves and protecting chlorophylls and carotenoids (28). It was also demonstrated that AA probably improves the growth of stressed plants through its effect on the contents of proline and soluble sugars, by its antioxidant action and its role in cell division and expansion (9).

Several problems may arise during the analysis of AA and DHA in plant extracts. The compounds have been shown to be degraded, to bind to proteins, and also to be inter-converted by oxidation/reduction during extraction and analysis (35). Numerous analytical methods have been reported in the literature to quantify the amount of AA in biological samples, with various detection limits ranging from μM (27) to nM (12; 23) or even lower (24). High-performance liquid chromatographic (HPLC) procedures have also been developed for AA quantification, but mostly in human and food samples, in fruit and vegetables (11; 34; 30; 26) or only in *Arabidopsis* (6; 18). Only a few recent studies have reported the HPLC determination of ascorbic acid content in plant tissue with low endogenous AA content (14; 31; 32). Several recent studies investigating the effect of abiotic stresses on ascorbic acid content use spectrophotometric methods, and the accurate measurement of these labile compounds in crop plants with a low AA level still remains a challenge both in terms of sample collection and analysis.

There is thus a need for a rapid, sensitive method for AA and DHA analysis in crop plant tissues. The reported methods are very diverse with regard to the eluent. Most of them use phosphate buffer, phosphoric acid, acetate buffer, methanol, acetonitrile or a combination of these, either in isocratic or gradient mode. The present paper recommends a quick, accurate and low-cost sample preparation procedure and an HPLC method with photodiode array (PDA) detection, using isocratic elution, suitable for the routine analysis of AA in various plant leaf tissues, with a high daily sample number and a low detection limit.

2. Materials and methods

2.1. Reagents, chemicals and instrumentation

Ascorbic acid, dithiothreitol (DTT), *meta*-phosphoric acid, trifluoracetic acid (TFA) and TRIS were obtained from the Sigma Chemical Company (St. Louis, MO, USA). HPLC analysis was carried out using an Alliance 2690 system (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector (W996, Waters, Milford, MA, USA). The control of the Waters HPLC components, and the collection and evaluation of the data were managed using Millennium32 software on a Windows-based computer.

2.2. Plant material

Seedlings of Columbia-0 (wild-type) and *vtc2-1* (a vitamin C-deficient mutant) Arabidopsis (*Arabidopsis thaliana* L.) plants grown at 21 °C for four weeks were used for the detection of different ascorbic acid levels.

Two-week-old wheat (*Triticum aestivum* L.) plants grown in hydroponic solution were used for experiments on the storage of plant material. Leaves of wheat plants were ground in liquid nitrogen and extracted either immediately or after 7 days of

storage at -80 °C. For the Cd stress experiments the plants were treated with 1 mM Cd(NO$_3$)$_2$ for a week, after which the leaves and roots were used for analysis.

2.3. Sample preparation

0.5 g leaf material was ground with a pestle in liquid nitrogen in a mortar and extracted with 3 ml of 5% *meta*-phosphoric acid. The homogenate was centrifuged at 10000 g for 10 min at 4 °C and the supernatant was collected for the analysis of AA and DHA.

Total AA was determined after the reduction of DHA to AA using DTT, and the concentration of DHA was estimated from the difference between total AA and native AA. The reaction mixture for total AA contained a 950 µl aliquot of the supernatant and 50 µl of 40 mg ml^{-1} DTT. AA was determined in a similar reaction mixture, except that 50 µl distilled water was added instead of DTT. After incubation for 25 min at room temperature in the dark, 10 µl of the mixture was injected onto the column.

2.4. Chromatography method

Separation was achieved with isocratic elution using 0.1% TFA (pH 2.7). The analysis was carried out by injecting 10 µl of the reaction mixture onto a Hyperprep HS C18 250x4.6 mm column (particle size 8 µm, pore size 100 Å) (ThermoFisher Scientific Inc., Waltham, MA, USA) connected to a 10x4 mm Hypeprep HS BDS C18 100 Å 8 µm drop-in guard column using a flow rate of 1 ml min^{-1}. The column temperature was maintained at 30 °C and the autosampler temperature was kept at 8°C. The run time was 7 min both for the standard and for the samples. AA was detected with PDA at 244 nm (spectrum Figure 1). Under these conditions the AA peak was eluted in around 5.4 min. An external standard calibration curve (Figure 2) was used to quantify the AA in the samples.

2.5. Statistical analysis

The data are presented as the mean values of 5 repetitions. The data were statistically evaluated using the standard deviation and Student's t-test methods.

3. Results

3.1 Sample preparation and reaction mixture

A number of factors have a negative influence on the stability of AA (e.g. light, a rise in temperature, increased pH, and the presence of oxygen or metal ions). It is therefore necessary to reduce the influence of these factors to a minimum (21). Brown Eppendorf tubes were used during sample preparation, the dark brown vials during the HPLC analysis which appeared to protect the AA from natural light. Sample extraction with liquid nitrogen, followed by centrifugation at low temperature (4 °C) seemed to be suitable conditions for sample preparation. Most methods involve sample extraction under acidic conditions, as the precipitation of protein leads to satisfactory AA recovery and stability. To provide an acidic pH, MPA was used for sample preparation. One basic requirement for accurate measurement is the addition of a reducing agent, which was DTT in the present. DTT was also added to the standard solutions. Although the incubation of the reaction mixture was carried out at room temperature, the samples were stored in the autosampler at 8°C up until the time of HPLC analysis, which was performed at 30 °C.

3.2. Method development

The mobile phase was optimized in order to obtain the best separation in the shortest time at the lowest cost. In the first round of method development 1mM

hexadecyltrimethylammoniumbromide (HTMAB: as an ion-pair reagent) and 0.05 %
$NaH_2PO_4.H_2O$:water (3:7) was tried as a mobile phase, but was found to have several
disadvantages; for example, an aqueous solution of HTMAB tends to foam strongly,
while the phosphate buffer often crystallises in the capillary tubes. Therefore, an easier
and safer eluent, 0.1 % TFA (pH 2.7) was chosen. A UV-VIS PDA detector was used
for the detection of reduced AA. To select the wavelength giving maximum absorbance
the spectra of AA was checked (Figure 1) and 244 nm was selected for detection. To
testing linearity, seven working standard solutions containing AA (5, 10, 100, 200, 400,
600 and 800 μM) were prepared. The calibration curve was linear in this range (R^2=
0.9996) and was plotted for every batch (Figure 2). The limit of detection was approx.
1.8 ng. The HPLC chromatograms of AA standard solution (800 μM), *Arabidopsis* leaf
samples and wheat leaf or root samples are shown in Figure 3. To determine the
precision of the AA analysis, an *Arabidopsis* leaf sample was measured six times under
the same conditions and the precision of the method was found to be 98 %. For testing
the recovery, 0, 1.5 or 3 μmol AA was added to *Arabidopsis* leaf samples before
extraction. The increment was linear (Figure 4) and the R^2 value 0.9999. Extracts were
stored at 8 °C for nine days and re-injected after 3, 6 and 9 days to check the storage life
(Figure 5). The total AA content did not change during the nine days, but the reduced
AA content dropped to 70 % on the 6th day. Plant extracts were stored at -80 °C for a
month and leaf tissues for a week. The total AA content of the plant extract decreased
by 10 % and the reduced AA content by approx. 40 % during storage (Figure 6). The
total AA content of frozen leaf tissues decreased by 12 % and the reduced content by 15
% after a week is storage (Figure 7.).

3.3. Application of the method to plants during stress

3.3.1. Determination of ascorbic acid in wild type and AA-deficient Arabidopsis mutants

To test the method, the AA contents of AA-deficient (*vtc2-1*) and wild-type (Col-0) *Arabidopsis* plants were measured. The total AA content was less than 1 μmol g^{-1} FW in the seedlings of Col-0 (Figure 8a), and approx. 12 % of this value in *vtc2-1*. The amount of DHA was one order of magnitude less than that of the reduced form in Col-0. Although the AA level was lower in the *vtc2-1* mutants, the DHA/AA ratio was much higher in these plants than in Col-0 (Figure 8b).

3.3.2. Determination of ascorbic acid in wheat plants during Cd stress

The amount of total AA in wheat leaves was approx. 2.4 μmol g^{-1} FW, while it was only approx. 0.8 μmol g^{-1} FW in the roots under control conditions. The levels of both DHA and AA increased significantly during Cd stress in the leaves, but not in the roots (Figure 9a). In contrast the DHA/AA ratio was low in the leaves and did not change during Cd stress, while this ratio was higher in the roots and increased during stress (Figure 9b).

4. Discussion

For the measurement of antioxidants in biological materials it is desirable to have a single, reliable and relatively inexpensive method. Numerous methods have been reported for the determination of AA. Every reported method is shown to find application in the analysis of one or the other type of samples mainly from pharmaceuticals, fruits, vegetables and biological fluids (for example blood) (2). These samples contain much higher amounts of AA and only its total amount has been measured. For plant physiology experiments it is also important to detect the reduced and oxidised forms separately to obtain a picture of the plant redox status. The

successful determination of AA and DHA depends on proper sample handling, the quantitative reduction of the compound and the accurate quantification of both the reduced form and total AA. In addition, although electrochemical detectors may be more suitable for detecting the low amounts expected in physiological samples, they are not always available in the laboratory. The most commonly used detector is the UV-visible detector. Several organic solvents (e.g. methanol and acetonitrile) were used in recent studies, but in routine analysis these mobile phases are expensive, and are also considered as significant pollutants. In the present method the sample preparation is simple and prevents AA oxidation and degradation, while the analytical parameters are sufficient. Storage was also tested and it was found that the total AA extracts could be stored for more than a week at 8 °C and the reduced form was also stable for three days. By contrast, degradation was observed in leaf tissue samples stored at -80 °C for a week. Plant extracts can be stored for a few weeks at -80 °C.

In leaves of *Arabidopsis thaliana* the ascorbate concentration is about 5 μmol g^{-1} FW (15), but this value depends on the type of tissue, plant age, the time of day when samples were taken, and light intensity (13). In the present case the total AA level of the *Arabidopsis* seedlings was less than 1 μmol g^{-1} FW, but this was extracted from young seedlings grown at relatively low light intensity. In the case of wheat it was found that the oxidative stress caused by Cd elevated the level of AA in the leaves, while the DHA/AA ratio increased in the roots. DHA is reduced to AA by dehydroascorbate reductase (DHAR) in a reaction requiring glutathione as an electron donor. The capacity to efficiently recycle DHA into AA can be critical under stress conditions, when AA is being rapidly consumed. The ratio of AA/DHA may reflect the survival potential of a plant under stress conditions (38). Changes in the AA/DHA ratio were reported during senescence and aluminium, salt and drought stress (4; 25; 17; 20).

It can be concluded that a simple, economical, selective, precise, and stable HPLC method has been developed for the detection of ascorbate in plant tissue. Statistical analysis proved that the method is precise, reproducible, selective and specific for the analysis of AA. The sensitivity, the short retention time and the simple isocratic elution mean that the method is suitable for the routine quantification of AA in the case of a high daily sample number. The method has been found to be better than previous methods, because of the use of an economical, readily available mobile phase, UV detection and the lack of complicated extraction procedures.

References

1. Agarwal, S. (2007) Increased antioxidant activity in *Cassia* seedlings under UV-B radiation. *Biol. Plant.* 51, 157-160.

2. Arya, S.P., Mahajan, M., Jain, P. (2000) Non-spectrophotometric methods for determination of Vitamin C. *Anal. Chim. Acta* 417, 1-14.

3. Attolico, A.D., De Tullio, M.C. (2006) Increased ascorbate content delays flowering in long-day grown *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol. Biochem.* 44, 462–466.

4. Bartoli, C. G., Pastori, G. M., Foyer, C. H. (2000) Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol.* 123, 335-343.

5. Chen, T.H.H., Murata, N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.* 5, 250-257.

6. Conklin, P.L., Williams, E.H., Last, R.L. (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant (ozone/ultraviolet B/sulfur dioxide/reactive oxygen detoxification/vitamin C). *Proc. Natl. Acad. Sci USA.* 93, 9970-9974.

7. Davey, M.W., Van Montagu, M., Inze, D., Sanmartin, M., Kanellis, A., Smirnoff, N., Benzie, I.J.J., Strain, J.J., Favell, D., Fletcher, J. (2000) Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. J. Sci. Food Agric, 80, 825-860.

8. Demirevska-Kepova, K., Simova-Stoilova, L., Stoyanova Z.P., Feller, U. (2006) Cadmium stress in barley: growth, leaf pigment, and protein composition and detoxification of reactive oxygen species. *J. Plant Nutr.* 29, 451-468.

9. Fercha, A., Hocine, G., Mebarek, B. (2011) Improvement of salt tolerance in durum wheat by ascorbic acid application. *J. Stress Physiol. Biochem.* 7, 27-37.

10. Foyer, C.H., Noctor, G. (2005) Redox homeostis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17, 1866-1875.

11. Furusawa, N. (2001) Rapid high-performance liquid chromatographic identification/quantification of total vitamin C in fruit drinks. *Food Control* 12, 27-29.

12. Gallie D.R. (2013) L-ascorbic acid: A multifunctional molecule supporting plant growth and development. *Scientifica*, 2013, 1-24.

13. Gazdik, Z., Zitka, O., Petrlova, J., Adam, V., Zehnalek, J., Horna, A., Reznicek, V., Beklova, M., Kizek, R. (2008) Determination of vitamin C (ascorbic acid) using high performance liquid chromatography coupled with electrochemical detection. *Sensors* 8, 7097-7112.

14. Gest, N., Gautier, H., Stevens, R. (2013) Ascorbate as seen through plant evolution: the rise of a successful molecule? *J. Exp. Bot.* 64, 33-53.

15. Karau, G.M., Njagi, E.N.M., Machocho, A.K., Wangai, L. N. (2012) Phytonutrient, mineral composition and *in vitro* antioxidant activity of leaf and stem bark powders of *Pappea capensis* (L.). *Pak. J. Nutrition* 11, 123-132.

16. Khan, T.A., Mazid, M., Mohammad, F. (2012) Potential of ascorbic acid against oxidative burst in plants under biotic stress: A Review. *J. Ind. Res. & Technology* 2, 72-80.

17. Kotchoni, S.O., Larrimore, K.E., Mukherjee, M., Kempinski, C.F., Barth, C. (2009) Alterations in the endogenous ascorbic acid content affect flowering time in *Arabidopsis. Plant Physiol.* 149, 803-815.

18. Li, Q., Krauss, M.R. Hempfling, W.P. (2006) Wounding of root or basal stalk prior to harvest affects pre-harvest antioxidant accumulation and tobacco-specific nitrosamine formation during air curing of burley tobacco (*Nicotiana tabacum* L.). *J. Agr. Crop Sci.* 192, 267-277.

19. Li, Q., Li, Y., Li, C., Yu, X. (2012) Enhanced ascorbic acid accumulation through overexpression of dehydroascorbate reductase confers tolerance to methyl viologen and salt stresses in tomato. *Czech J. Genet. Plant Breed.* 48, 74–86.

20. López-Carbonell, M., Munné-Bosch, S., Alegre, L. (2006) The ascorbate-deficient vtc-1 *Arabidopsis* mutant shows altered ABA accumulation in leaves and chloroplasts. *J. Plant Growth Regul.* 25, 137-144.

21. Nayyar, H., Chander, S. 2004 Protective effects of polyamines against oxidative stress induced by water and cold stress in chickpea. *J. Agr. Crop Sci.* 190, 355-365.

22. Niu, Y., Wang, Y., Li, P., Zhang, F., Liu, H., Zheng, G. (2012) Drought stress induces oxidative stress and the antioxidant defense system in ascorbate-deficient *vtc1* mutants of *Arabidopsis thaliana. Acta Physiol. Plant.* 35, 1189-1200.

23. Novákova, L., Solich, P., Solichova, D. (2008) HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids. *Trends Anal. Chem.* 27, 942-958.

24. Panda, S.K., Khan, M.H. (2009) Growth, oxidative damage and antioxidant responses in Greengram (*Vigna radiata* L.) under short-term salinity stress and its recovery. *J. Agr. Crop Sci.* 195, 442-454.

25. Pisoschi, A.M., Danet, A.F., Kalinowski, S. (2008) Ascorbic acid determination in commercial fruit juice samples by cyclic voltammetry. *J. Autom. Methods*

Manag. Chem. 2008, Article ID 937651, 8 pages, doi:10.1155/2008/937651, http://www.hindawi.com/journals/jammc

26. Prasad, B.B., Tiwari, K., Singh, M., Sharma, P.S., Patel, A.K., Srivastava, S. (2008) Molecularly imprinted polymer-based solid-phase microextraction fiber coupled with molecularly imprinted polymer-based sensor for ultratrace analysis of ascorbic acid. *J. Chromatogr. A* 1198, 59-66.

27. Ribeiro, C., Cambraia, J., Peixoto, P.H.P., da Fonseca, J.É.M. (2012) Antioxidant system response induced by aluminum in two rice cultivars. *Braz. J. Plant Physiol.* 24, 107-116.

28. Sawant, L., Prabhakar, B., Mahajan, A., Pai, N., Pandita, N. (2011) Development and validation of HPLC method for quantification of phytoconstituents in *Phyllanthus emblica*. *J. Chem. Pharm. Res.* 3, 937-944.

29. Shekhovtsova, T.N., Muginova, S.V., Luchinina, J.A., Galimova, A.Z. (2006) Enzymatic methods in food analysis: determination of ascorbic acid. *Anal. Chim. Acta* 573-574, 125-132.

30. Smirnoff, N. (1996) The function and metabolism of ascorbic acid in plants. *Annals Bot.* 78, 661-669.

31. Smirnoff, N. (2000) Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Curr. Opin. Plant Biol.* 3, 229-235.

32. Sommano, S., Caffin, N., McDonald, J., Kerven, G. (2011) Measurement of ascorbic acid in Australian native plants. *IFRJ* 18, 1017-1020.

33. Soni, H., Singhai, A.K., Sharma, S. (2012) Quantification of ascorbic acid in leaves of *Annona squamosa*. *Int. J. Pharm. Pharm. Sci.* 4, 144-147.

34. Soni, H., Singhai, A.K., Sharma, S., Nayak, G., Swarnkar, P. (2012) Quantification of ascorbic acid in salad components. *Int. J. Curr. Pharm. Res.* 4, 43-47.

35. Streb, P., Aubert, S., Gout, E., Bligny R. (2003) Reversibility of cold- and light-stress tolerance and accompanying changes of metabolite and antioxidant levels in the two high mountain plant species *Soldanella alpina* and *Ranunculus glacialis. J. Exp. Bot.* 54, 405-418.

36. Streb, P, Feierabend, J., Bligny, R. (1997) Resistance of photoinhibition of photosystem II and catalase and antioxidative protection in high mountain plants. *Plant Cell Environ.* 20, 1030-1040.

37. Szarka, A., Tomasskovics, B., Bánhegyi, G. (2012) The ascorbate-glutathione-α-tocopherol triad in abiotic stress response. *Int. J. Mol. Sci.* 13, 4458-4483.

38. Vermeir, S., Hertog, M.L., Schenk, A., Beullens, K., Nicolai, B.M., Lammertyn, J. (2008) Evaluation and optimization of high-throughput enzymatic assays for fast L-ascorbic acid quantification in fruit and vegetables. *Anal. Chim. Acta* 618, 94-101.

39. Wingsle, G., Moritz, T. (1997) Analysis of ascorbate and dehydroascorbate in plant extracts by high-resolution selected ion monitoring gas chromatography-mass spectrometry. *J. Chromatogr. A* 782, 95-103.

40. Yang, Y., Han, C., Liu, Q., Lin, B., Wang, J. (2008) Effect of drought and low light on growth and enzymatic antioxidant system of *Picea asperata* seedlings. *Acta Physiol. Plant.* 30, 433-440.

41. Zhang, J., Kirkham, M. B. (1996) Antioxidant responses to drought in sunflower and sorghum seedlings. *New Phytol.* 132, 361-373.

42. Zhou, Y., Tao, Q.C., Wang, Z.N., Fan, R., Li, Y., Sun, X.F., Tang, K.X. (2012) Engineering ascorbic acid biosynthetic pathway in Arabidopsis leaves by single and double gene transformation. *Biol. Plant.* 56, 451-457.

Legends to figures

Figure 1. Spectrum of ascorbic acid with a maximum at 244 nm.

Figure 2. Calibration curve of ascorbic acid in the concentration range 5-800 μM. Values on the X-axis are the amount of ascorbic acid (in pmols) after the injection of 5 μl of standards to the column.

Figure 3. Representative chromatograms of a) standard ascorbic acid (injection volume 2 μl); b) Arabidopsis Columbia-0 (wild type) leaf sample (injection volume 20 μl); c) Arabidopsis *vtc2-1* (ascorbic acid-deficient) mutant leaf sample (injection volume 20 μl); d) wheat leaf sample (injection volume 10 μl) and e) wheat root sample (injection volume 10 μl).

Figure 4. Results of the recovery study using the standard addition method. The recovery of ascorbate was calculated by comparing the area before and after the addition of 0, 1.5, 3 μmol standard solution to the leaf sample. Values on the X-axis are the amount of ascorbic acid (in pmols) after the injection of 5 μl of samples to the column. (n=5)

Figure 5. Changes in the amount of ascorbic acid in plant leaf extracts after 0, 3, 6 and 9 days of storage at 8 °C. Values are expressed as a percentage of the control (100%). (n=3)

Figure 6. Changes in the amount of ascorbic acid after 3-week storage of plant leaf extract at -80 °C. Values are expressed as a percentage of the control (100%). *

significant difference between the control and frozen plant extracts at the p ≤ 0.05 level. (n=3)

Figure 7. Changes in the amount of ascorbic acid after 7-day storage of plant leaf tissue at -80 °C. Values are expressed as a percentage of the control (100%). (n=3)

Figure 8. Reduced (AA) and oxidised (DHA) ascorbic acid content (a) and DHA/AA ratio (b) of wild type (Columbia-0) and ascorbic acid-deficient mutant (*vtc2-1*) *Arabidopsis* plants. *** significant difference between the control and 1 mM Cd-treated plants at the p ≤ 0.001 level (n=5)

Figure 9. Reduced (AA) and oxidised (DHA) ascorbic acid content (a) and DHA/AA ratio (b) in the leaves and roots of wheat plants after 1 mM Cd treatment for seven days. *, ** significant difference between the control and 1 mM Cd-treated plants at the p ≤ 0.05 and 0.01 levels, respectively. (n=5)

$y = 488.44x - 11034$
$R^2 = 0.9996$

$y = 5*10^6x + 846697$

$R^2 = 0.9999$

(a)

AA content (nmol g^{-1} FW)

□ Col-0   ■ vtc2-1

***

AA   DHA

(b)

DHA/AA ratio

Col-0   vtc2-1