

Quantitative Determination of Ascorbate from the Green Alga *Chlamydomonas reinhardtii* by HPLC

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[Abstract] Ascorbate (Asc, also called vitamin C) is of vital importance to the cellular functions of both animals and plants. During evolution, Asc has become one of the most abundant metabolites in seed plants; however, Asc contents in cyanobacteria, green algae and bryophytes are very low. Here we describe a sensitive and reliable HPLC method for the quantitative determination of cellular Asc content in the green alga *Chlamydomonas reinhardtii*.

Keywords: Ascorbate, Cell counter, *Chlamydomonas reinhardtii*, HPLC, Dehydroascorbate

[Background] Previous protocols for the determination of cellular Asc content were developed for higher plants, of which the amounts of cellular Asc are high enough for the analysis. Those protocols are not suitable for green algae, since both the amounts of the plant material and the levels of the cellular Asc are usually limited. Therefore, there was a need to develop a novel method with sensitivity improved to the μM range to measure the cellular Asc in green algae.

Materials and Reagents

1. 15-ml conical centrifuge tubes (Corning, Falcon®)
2. Polypropylene microcentrifuge tubes of 1.5 ml (Eppendorf)
3. 0.3 ml polypropylene chromatographic vials (Focus Technology, model: VP91) with polypropylene cap and PTFE/silicon septa (Focus Technology, model: SC9291)
4. 4 mm hydrophilic PTFE syringe filter with a 0.22 μm pore size (Nantong Filterbio Membrane, catalog number: FBS4PTFE022L)
5. 47 mm hydrophilic PTFE membrane with a 0.45 μm pore size (Nantong Filterbio Membrane, catalog number: FBM047PTFE045L)
6. Glass beads 212-300 μm (Sigma-Aldrich, catalog number: G9143-250G)
7. 1 ml disposable polypropylene syringe (B. Braun Medical, catalog number: 9166017V)
8. Millex-GS 33 mm sterile syringe filter with a 0.22 μm pore size (EMD Millipore, catalog number: SLGS033SS)
9. Milli-Q water
10. Liquid nitrogen
11. Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) (Carl Roth, catalog number: HN95.2)

12. Potassium dihydrogen phosphate (KH_2PO_4) (Carl Roth, catalog number: 3904.1)
13. Orthophosphoric acid (H_3PO_4) (Sigma-Aldrich, catalog number: W290017)
14. EDTA (Sigma-Aldrich, catalog number: EDS-100G)
15. Sodium chloride (NaCl) (Duchefa Biochemie, catalog number: S0520.1000)
16. Potassium chloride (KCl) (Duchefa Biochemie, catalog number: P0515.1000)
17. Di-sodium hydrogen phosphate (Na_2HPO_4) (Carl Roth, catalog number: P030.2)
18. Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: M2393-100g)
19. Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (Duchefa Biochemie, catalog number: C0504.1000)
20. Hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: 258148-2.5L-D)
21. Sodium L-Asc (Sigma-Aldrich, catalog number: 4034-100G)
22. Acetonitrile HPLC gradient grade (VWR, HiPerSolv Chromanorm[®], catalog number: 83639.320)
23. Phosphate-buffered saline (PBS) (see Recipes)
24. Extraction buffer (see Recipes)
25. HPLC mobile phase A (see Recipes)
26. HPLC mobile phase B (see Recipes)
27. Sodium L-Asc standard solutions (see Recipes)

Equipment

1. Scepter[™] handheld automated cell counter (EMD Millipore, catalog number: PHCC00000)
2. Scepter[™] Sensors – 40 μm (EMD Millipore, catalog number: PHCC40050)
3. Refrigerated table centrifuge and microcentrifuge
4. HPLC system (Shimadzu Scientific Instruments) equipped with:
 - a. LC pump (Shimadzu Scientific Instruments, model: LC-20AD)
 - b. Thermostated autosampler (Shimadzu Scientific Instruments, model: SIL-20AC)
 - c. Photo Diode Array detector (Shimadzu Scientific Instruments, model: SPD-M30A)
 - d. Column oven (Shimadzu Scientific Instruments, model: CTO-20AC)
 - e. Synergy hydro 4u Hydro – RP 80 Å 250 x 4.6 mm (Phenomenex, catalog number: 00G-4375-E0) directly mounted with a guard column AQ C18 4.0 x 3.0 mm (Phenomenex, catalog number: KJ0-4282)

Software

1. LabSolution (Shimadzu Scientific Instruments)

Procedure

- A. Sample preparation

1. Prepare liquid *Chlamydomonas* cultures and collect 1 ml sample when the cultures are in the exponential growth phase. Mix the sample with 19 ml of phosphate-buffered saline (PBS). Use a Scepter cell counter to determine cell number and cell volume by following the instructions displayed on the screen. Briefly, depress the plunger, submerge the tip, and release the plunger to soak up 50 μ l liquid culture into the tip. The Scepter cell counter detects each cell passing through the orifice of the tip, calculates cell density, and displays a histogram of cell diameter or volume on the screen (Johnston, 2010).
 2. Based on the calculated cell density, harvest 2×10^6 cells, place it in a 15-ml conical centrifuge tube and centrifuge it at 12,000 $\times g$ for 1 min at room temperature.
 3. Gently re-suspend the pelleted cells in 1 ml Milli-Q water and repeat the centrifugation step.
 4. Discard the supernatant and freeze the pellet in liquid nitrogen. The samples can be stored at -80 $^{\circ}$ C for several months.
 5. Add 200 μ l of extraction buffer to the frozen pellet and mix it by vigorous shaking. The extraction buffer contains TCEP to keep Asc in the reduced form.
 6. Transfer the resuspended cells into a 1.5 ml microcentrifuge tube containing 100 μ l glass beads and vortex at maximum speed for 30 sec. Incubate the sample at room temperature for two minutes (during which the sample becomes brownish), then place it on ice.
 7. Centrifuge the sample at 19,000 $\times g$ at 4 $^{\circ}$ C for 30 min.
 8. Collect 120 μ l of the supernatant (without stirring up the pellet) and filter it into chromatographic vials using 4 mm hydrophilic PTFE syringe filters with a pore size of 0.22 μ m. The samples are stable for 24 h at 4 $^{\circ}$ C and can be stored at -80 $^{\circ}$ C for several months; however, freeze-thaw cycles should be avoided.
- B. Determination of Asc concentration by HPLC (see Nováková *et al.*, 2008a for theoretical basics)
1. Ascorbate can be efficiently separated chromatographically using a Synergy hydro 4u Hydro – RP 80 Å 250 \times 4.6 mm column. Attach the column fitted with a suitable guard column (AQ C18 4.0 \times 3.0 mm) to the HPLC. Equilibrate the column with mobile phase A for at least 30 min at a flow rate of 1 ml/min.
 2. Set the tray temperature of the autosampler at 4 $^{\circ}$ C and the column oven temperature at 20 $^{\circ}$ C.
 3. While waiting for the temperature and baseline to stabilize, prepare the sample sequence table. Insert the chromatographic vials containing the samples into the tray of the autosampler.
 4. Inject 20 μ l sample. For the elution of Asc, use an isocratic mobile phase of 50 mM KH_2PO_4 (pH 2.5, adjusted with orthophosphoric acid) for 5 min at a flow rate of 1 ml/min then apply a short acetonitrile (mobile phase B) gradient from 0% to 30% between 3.5 and 6 min to elute the less polar components from the column. Between 8 and 9 min decrease the percentage of mobile phase B to 0% and equilibrate the column to the mobile phase A for an additional 9 min. The total running time is 18 min.

- Record the signal between 190 and 500 nm at a sampling frequency of 4 Hz and 1,024 spectral resolution with a slit set to 8 nm. The Asc peak appears at approximately 5.1 min with an absorption maximum at around 244 nm.

Data analysis

- Prepare a two-fold serial dilution between 0.2 and 50 μM Asc dissolved in the extraction buffer. Determine the peak areas of Asc in the HPLC chromatograms by the LabSolution software (Shimadzu Scientific Instruments) and use linear fitting to obtain a calibration curve (Figure 1).

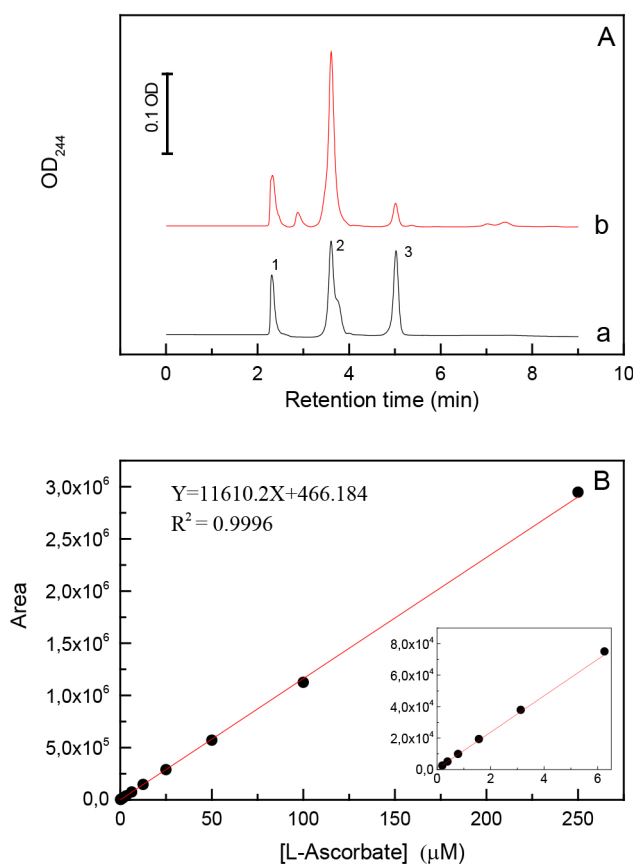


Figure 1. Typical HPLC chromatograms recorded at 244 nm and a calibration curve for the determination of L-Asc concentration. A. Chromatographic profiles of (a) 62.5 μM L-Asc dissolved in extraction buffer (95% orthophosphoric acid, 2 mM EDTA, 10 mM TCEP, pH 2.5) and (b) *C. reinhardtii* cell extract. The identified peaks are (1) orthophosphoric acid; (2) EDTA; (3) L-Asc. B. Calibration curve for the determination of L-Asc concentration calculated based on linear fitting of the peak areas and using 1/area weighting. The LOD and LOQ values are 0.2 and 1.5 μM , respectively.

- Calculate the cellular Asc concentration using the following formula:

$$\text{Cellular Asc concentration } (\mu\text{M}) = \frac{\text{Asc concentration of the extract } (\mu\text{M}) \times \text{Extract volume } (\mu\text{L})}{\text{Cell volume (fL)} \times \text{Amount of cells} \times 10^{-9}}$$

Notes

1. The average cell volume for *C. reinhardtii* is about 200 fl. The typical Asc concentration of CC124 *C. reinhardtii* cells grown in liquid Tris-acetate-phosphate medium for three days at approximately 80 $\mu\text{mole photons m}^{-2} \text{ s}^{-1}$ is about 40 μM . The cellular Asc content, however, strongly depends on the algal strain, the growth medium, the age of the cultures, growth light and stress conditions (Nagy *et al.*, 2016; Vidal-Meireles *et al.*, unpublished).
2. The extraction buffer contains TCEP, which keeps Asc in the more stable reduced form; therefore, by the method described above, the total cellular Asc concentration (dehydroascorbate + Asc) is determined. However, if there is a need to determine the amount of dehydroascorbate within the cell, omit TCEP from the extraction buffer. In this case, however, the samples should be processed and measured within less than 2 h, and cannot be frozen. Dehydroascorbate does not absorb at 244 nm, thus the ratio of Asc to dehydroascorbate is calculated based on the difference in absorption at 244 nm in the presence or absence of TCEP.

Recipes

1. Phosphate-buffered saline (PBS, 1 L; based on Chazotte, 2012)
 - 8 g NaCl
 - 0.2 g KCl
 - 1.44 g Na_2HPO_4
 - 0.24 g KH_2PO_4
 - 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
 - Milli-Q water

Dissolve the salts in Milli-Q water and adjust the pH to 7.2 with HCl
 Complete the volume to 990 ml by adding Milli-Q water and dispense the solution into aliquots
 Sterilize the solution by autoclaving for 20 min at 121 °C, 15 psi on liquid cycle
 Dissolve 0.133 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 10 ml of Milli-Q water, filter-sterilize it and add it to the solution after autoclaving
 Store the sterile PBS at room temperature
2. Extraction buffer (100 ml; based on Nováková *et al.*, 2008b)
 - 74.448 mg EDTA
 - 286.65 mg TCEP
 - Milli-Q water

Dissolve the salts completely in Milli-Q water
 Add 5 ml of 98% orthophosphoric acid
 Dispense the solution into aliquots, and store at -80 °C until use

3. HPLC mobile phase A (1 L)
 - 6.8045 g KH₂PO₄
 - Milli-Q water
 - Adjust the pH to 2.5 with 98% orthophosphoric acid
 - Filter the solution using 0.45 µm hydrophilic PTFE membrane
 - Note: This solution must be freshly prepared.*
4. HPLC mobile phase B (1 L)
 - 100% acetonitrile
 - Degas by sonication for 20 min at room temperature
5. Sodium L-Asc standard solutions
 - Dissolve 49.5 mg sodium L-Asc in 1 ml of extraction buffer to obtain a 50 mM sodium L-Asc solution
 - Dilute 10 µl aliquot in 10 ml extraction buffer
 - Prepare a two-fold dilution series from this 50 µM stock solution down to 0.2 µM
 - Note: This solution must be freshly prepared.*

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