

## Citrus Fruit Ascorbic Acid Extraction and Quantification by HPLC

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**[Abstract]** Citrus are among the most relevant sources of vitamin C (ascorbic acid + dehydroascorbic acid). Recent studies have revealed that it increases in the peel as fruit ripens and remains constant or even decreases in the pulp tissue. Moreover, important differences on ascorbic acid content exist among citrus varieties in both tissues. Here we describe a simple method for vitamin C analysis/quantification in the peel and pulp tissues of citrus fruit.

### Materials and Reagents

1. Citrus fruit tissue (ground frozen flavedo or pulp tissue)
2. Metaphosphoric acid (MPA) (65%) (Sigma-Adrich, catalog number: 79615)
3. Orthophosphoric acid (Sigma-Adrich, catalog number: W290017)
4. Tris base (Sigma-Adrich, catalog number: T1503)
5. Methanol HPLC-grade (Sigma-Adrich, catalog number: 34860)
6. Ascorbic acid (Sigma-Adrich, catalog number: A902902)
7. C18 Sep Pak (Waters, catalog number: WAT091139)
8. DTT (Sigma-Aldrich, catalog number: D0632)
9. Sterile Mili-Q water
10. 0.1% MPA (see Recipes)
11. 2% MPA (see Recipes)
12. 8.5% orthophosphoric acid (see Recipes)
13. 400 mM Tris base (see Recipes)
14. 200 mM DTT in 400 mM Tris base (see Recipes)
15. Methanol:Milli-Q water (15:85, v/v) (see Recipes)
16. Ascorbic acid stock solution I for standard curve (see Recipes)

### Equipment

1. Disposable 15 ml plastic tubes
2. 0.45 µm nylon filter (25 mm diameter) (Análisis Vínicos, catalog number: E0036)
3. Refrigerated centrifuge (15 ml tubes)
4. Polytron (Kinematica AG, model: PT-1035 GT; <http://www.kinematica.ch/en.html>)
5. HPLC system with a photodiode array detector (PDA, Dionex)

6. Ultrabase C<sub>18</sub> column (100 x 4.6 mm, 2.5 µm)

## **Software**

1. Chromeleon version 6.80 (Dionex)

## **Procedure**

### **A. Ascorbic acid extraction**

1. Resuspend frozen ground citrus flavedo or pulp tissue (0.5 g) in 4 ml 0.1% MPA in a 15 ml disposable plastic tube and keep it on ice. The frozen tissue was previously ground using mortar and pestle chilled with liquid nitrogen.
2. Homogenize the tissue for 1 min using a Polytron homogenizer at maximum speed (30,000 rpm).
3. Centrifuge the homogenate for 10 min at 4,000 x g at 4 °C.
4. Collect the supernatant and keep it in a fresh 15 ml disposable plastic tube on ice.
5. Activate the Sep Pak C<sub>18</sub> cartridges using 4 ml methanol and subsequently wash them with 4 ml Milli-Q water and 4 ml 2% MPA. Discard the solution from the washing steps.
6. For the ascorbic acid determination in flavedo take 1 ml of supernatant and mix it with 3 ml 0.1% MPA (1:4 dilution). For ascorbic acid determination in pulp do not dilute it, just continue with the protocol using the whole supernatant.
7. Filter the solutions obtained in step A4 (pulp) or A6 (flavedo) through a previously activated C<sub>18</sub> Sep Pak cartridge using a syringe. Collect the filtrate in a fresh 15 ml disposable plastic tube.
8. Wash the C<sub>18</sub> Sep Pak cartridge with 4 ml 2% MPA. Collect the eluate together with the solution from step A7.
9. Filter the solution from step A8 through a 0.45 µm nylon filter using a syringe. This step is added to make sure the solution is clean of any particles that could damage the HPLC column.
10. Wash the filter with 4 ml 2% MPA and collect it together with the filtrate solution from step A9.
11. Measure the final volume and take it into account together with the initial dilution (in the case of flavedo samples) for the calculations of ascorbic acid concentration.
12. Prepare an amber HPLC vial with 1 ml of the final extract (from step A10) for the ascorbic acid measurement.

### **B. Dehydroascorbic acid extraction**

Dehydroascorbic acid is converted to ascorbic acid by the addition of DTT, then the amount of total ascorbic acid (dehydroascorbic acid + ascorbic acid) is measured and the

dehydroascorbic acid concentration results from the subtraction of ascorbic acid to total ascorbic acid (see step D3).

1. Transfer a 200 µl aliquot of the final extract (step A10) to an amber HPLC vial.
2. Incubate it for 15 min at room temperature with 100 µl of 200 mM DTT in 400 mM Tris base (pH 8.5).
3. Stop the reaction by acidification with 100 µl of 8.5 % orthophosphoric acid.

#### C. Ascorbic acid and dehydroascorbic acid HPLC measurements

1. To quantify the ascorbic acid concentration inject 10 µl of the solution obtained from step A10 in a HPLC equipped with a photodiode array detector and an Ultrabase C<sub>18</sub> column (100 x 4.6 mm, 2.5 µm). Set column temperature at 35 °C. Use an isocratic mobile phase of methanol: Milli-Q water (pH 2.5) (15:85, v/v, pH adjusted with MPA) at 0.2 ml/min flow.
2. Quantify the area under the peak that displays a maximum at 248 nm (ascorbic acid absorbs between 210 and 300 nm with a maximum at 248 nm). This area is applied to the formula described in D2 to calculate the ascorbic acid concentration of the sample. Measure the spectrum between 200 and 450 nm with a PDA detector to confirm the quantification of pure ascorbic acid.
3. To obtain the dehydroascorbic acid concentration inject 10 µl of the solution obtained from step B3 using the same equipment and conditions described in step C1.
4. Quantify the area under the peak that displays a maximum at 248 nm. Apply this area to the formula described in D3 to calculate the dehydroascorbic acid concentration of the sample. Measure the spectrum between 200 and 450 nm with a PDA detector to confirm the quantification of pure ascorbic acid.

#### D. Ascorbic acid and dehydroascorbic acid quantification

1. Prepare a calibration curve using a series of ascorbic acid dilutions as described in Table 1 and measure the peak area following the same procedure as described in section C.
2. Adjust the linear function of ascorbic acid concentration vs. peak area and use the slope to calculate the concentration of the unknown samples as described in the formula:

$$\text{Ascorbic acid concentration (}\mu\text{g/gFW)} = \frac{\text{curve slope} \times \text{peak area from C2 (}\mu\text{g/ml)} \times \text{final volume of the eluate (ml)}}{\text{Sample weight (g)}}$$

3. Dehydroascorbic acid concentration:

$$\text{Dehydroascorbic acid concentration (}\mu\text{g/gFW)} = \left[ \frac{\text{curve slope} \times \text{peak area from C4 (}\mu\text{g/ml)} \times 2 \times \text{final volume of the eluate (ml)}}{\text{Sample weight (g)}} \right] - \text{ascorbic acid concentration (D2)}$$

The x2 is included in the formula because it is the dilution factor due to the addition of 200  $\mu$ ls of extra volume in steps B2-3.

## **Recipes**

1. 0.1% MPA  
1.5 g of MPA 65%  
900 ml of Milli-Q water and after completely dissolved complete to 1,000 ml with Milli-Q water  
Solution can be stored at room temperature indefinitely
2. 2% MPA  
31 g of MPA 65%  
900 ml of Milli-Q water and after completely dissolved complete to 1,000 ml with Milli-Q water  
Solution can be stored at room temperature indefinitely
3. 8.5% orthophosphoric acid  
10 ml of 85% orthophosphoric acid  
90 ml of Milli-Q water and store at room temperature indefinitely
4. 400 mM Tris base (pH 9.0)  
Dissolve 12.1 g of Tris base in 200 ml of Milli-Q water  
Add Milli-Q water to a final volume of 250 ml, stored at 4 °C for three months
5. 200 mM DTT in 400 mM Tris base (pH 8.5)  
Dissolve 0.031 g of DTT in 1 ml of 400 mM Tris base solution and vortex  
Do not store, this solution must be prepared fresh daily
6. Methanol:Milli-Q water (pH 2.5) (15:85, v/v)  
Mix 150 ml of methanol HPLC grade with 850 ml of Milli-Q water at pH 2.5 (pH is adjusted with 2% MPA)
7. Ascorbic acid stock solution I for standard curve (100 mg/ml in 2% MPA)

**Table 1. Calibration curve preparation**

Ascorbic acid solution	Final concentration	Composition
Stock solution I	100 mg/ml	100 mg ascorbic acid + 1 ml 2% MPA
Stock solution II	1 mg/ml	990 µl MPA 2 % + 10 µl stock solution I
A	100 µg/ml	900 µl MPA 2 % + 100 µl stock solution II
B	50 µg/ml	500 µl MPA 2 % + 500 µl solution A
C	25 µg/ml	500 µl MPA 2 % + 500 µl solution B
D	12.5 µg/ml	500 µl MPA 2 % + 500 µl solution C
E	6.25 µg/ml	500 µl MPA 2 % + 500 µl solution D
F	3.125 µg/ml	500 µl MPA 2 % + 500 µl solution E
G	1.5625 µg/ml	500 µl MPA 2 % + 500 µl solution F
H	0 µg/ml	500 µl MPA 2 %

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### **References**

1. Alós, E., Rodrigo, M. J. and Zacarías, L. (2014). [Differential transcriptional regulation of L-ascorbic acid content in peel and pulp of citrus fruits during development and maturation](#). *Planta* 239(5): 1113-1128.