

COSHH ASSESSMENT

Determination of Vitamin C in Green Peppers by HPLC

Assessment No.

Assessment Date

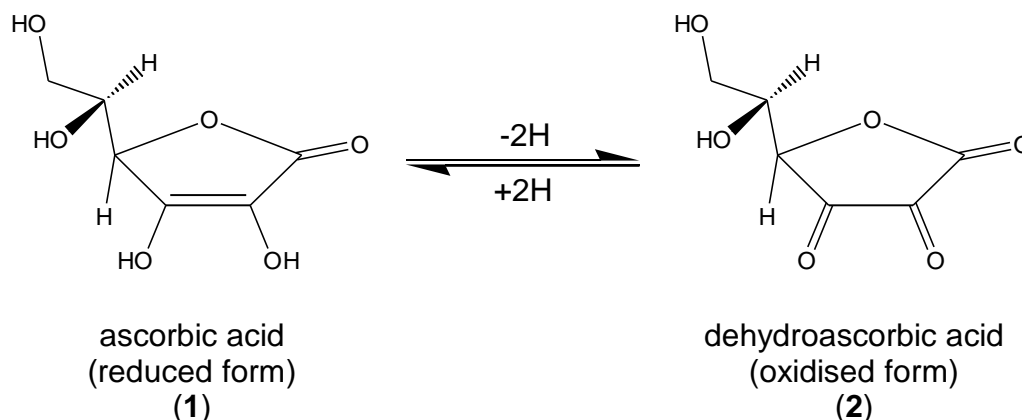
Chemicals – Hazardous in quantities used in experiment	
None	
Chemicals – Hazardous in other circumstances	
5% orthophosphoric acid	Irritating to the eyes and skin. Prolonged exposure may cause burns.
First aid for any of the above chemicals	
Eyes	Irrigate thoroughly with water for at least 10 minutes. OBTAIN MEDICAL ATTENTION.
Lungs	Remove from exposure, rest and keep warm. In severe cases OBTAIN MEDICAL ATTENTION.
Skin	Wash off thoroughly with water. Remove contaminated clothing and wash before re-use. In severe cases OBTAIN MEDICAL ATTENTION.
Mouth	Wash out mouth thoroughly with water and give plenty of water to drink. OBTAIN MEDICAL ATTENTION.

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Introduction

Vitamins are organic compounds required by living organisms in relatively small amounts to maintain normal health. Animals are unable to biosynthesise many vitamins and must have adequate amounts in the diet. Vitamin C (ascorbic acid) cannot be synthesised by humans, other primates and guinea-pigs and these species must ingest ascorbic acid in the diet. The U.K. Food Standards Agency recommends that adults require a daily intake of 40 mg of vitamin C (CMAFNP, 1991), although the amount required depends on gender and age amongst other factors. Since vitamin C is water soluble and cannot be stored in the body it needs to be taken daily.

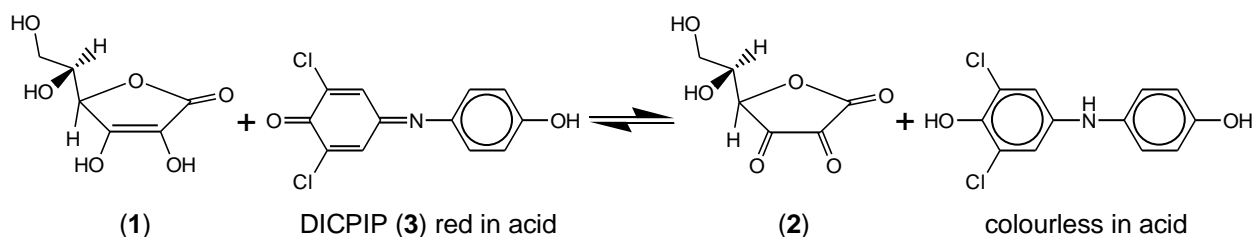
It is believed that vitamin C is involved in several essential redox reactions in both plants and animals (Scheme 1).



Scheme 1: Redox Interconversion of Vitamin C

Vitamin C in body fluids (*e.g.* serum, plasma and urine), fruit juices and fresh fruits can be determined by making use of its redox properties. The vitamin is present in tissues almost entirely in the reduced form (1); however, it is easily oxidised to dehydroascorbic acid (2) when exposed to air or by other chemical oxidants and this behaviour may be used to determine the concentration of ascorbic acid. For example, a common method involves titration with 2,6-dichlorophenolindophenol (3; DICPIP), which acts as an oxidant. DICPIP is a dark blue dye in neutral aqueous solution but in acid solution it is red and when it is reduced (*i.e.* by the ascorbic acid) it is colourless. Thus, when all of the vitamin C has been oxidised by addition of DICPIP, addition of a slight excess of DICPIP produces a red/pink solution.

Nowadays, however, high-performance liquid chromatography (HPLC) is a preferred technique of analysis and has been used for the determination of vitamin C in foodstuffs. HPLC has a number of advantages over titrimetric methods including selectivity, reproducibility and short analysis times. In addition, it is possible to devise methods that simultaneously estimate both L-ascorbic and dehydro-L-ascorbic acids in foods and other biological materials making it a superior to the titrimetric technique in which only L-ascorbic acid can be determined. In this practical, the method you will use only determines L-ascorbic acid.



Scheme 2: Redox Reaction Between Vitamin C and 2,6-Dichlorophenolindophenol

HPLC may be used to separate and determine a wide range of analytes in a variety of matrices. It is a complementary technique to GC and is particularly useful for analytes that are non-volatile and/or polar. A solution of the sample to be analysed is injected into the liquid mobile phase, which is pumped through the column under high pressure to achieve a reasonable flow rate. As a result, HPLC is sometimes referred to as high-pressure liquid chromatography. The stationary phase is held in a stainless steel column and since the analytes partition between the mobile phase and the stationary phase to differing extent, they may be separated from each other. If an analyte is strongly retained by the stationary phase, it moves slowly with the flow of the mobile phase and *vice versa*. In this way, analytes separate into discrete bands that can be detected qualitatively or quantitatively with an appropriate detector (e.g. UV-Vis, fluorescence or refractive index).

Objectives

To determine the concentration of ascorbic acid in raw and variously cooked green peppers.

Experimental

Instrumentation

Isocratic system with a ConstaMetric 3200 solvent delivery system, Rheodyne injector, Supelco Ascentis™ C18 HPLC column (15 cm x 4.6 mm, 5 μm), Merck Hitachi L7420 UV-VIS Detector and a Merck Hitachi D2500 Chromato Integrator.

There may be some variation in the HPLC equipment, so record all the details of the instrumentation that you actually use.

Reagents

These will be prepared for you in advance.

1. Orthophosphoric acid (0.02% v/v)

Pipette 0.2 mL of orthophosphoric acid into a 1000 mL volumetric flask. Make up to the mark with Milli-Q water. Mix. Degas with nitrogen gas for about 5 minutes before use.

2. Orthophosphoric acid (5% v/v)

Pipette 25 mL of orthophosphoric acid into a 500 mL volumetric flask. Make up to the mark with Milli-Q water. Mix.

3. Ascorbic acid standard stock solution (1000 mg/L)

Dissolve accurately approximately 0.1 g of ascorbic acid in about 20 mL of orthophosphoric acid (5% v/v) in a beaker. Transfer the solution to a 100 mL volumetric flask and wash the beaker with about 10 mL of orthophosphoric acid (5% v/v). Add the washings to the flask. Repeat the wash and make up to the mark with orthophosphoric acid (5% v/v). Mix well.

Procedure

1. Preparation of calibration solutions

From the 1000 mg/L ascorbic acid standard stock solution, prepare a calibration series of 0, 20, 40, 60, 80 and 100 mg/L ascorbic acid in orthophosphoric acid (5% v/v) in 50 mL volumetric flasks.

2. Determination of vitamin C in raw and cooked tissue

(a) Quantify the vitamin C in the raw tissue

Take 10 g of tissue, weighed to the nearest 0.1 g, from the edible portion of a pepper. Cut the material into small pieces and transfer it to a mortar. Add about 2 g of sand and 20 mL of orthophosphoric acid (5% w/v) and grind the mixture carefully with a pestle until no obvious lumps of tissue persist (*N.B.* the skin of the pepper usually remains intact and take care not to lose any extract by splashing due to too vigorous grinding). Decant the extract carefully into a 50 mL centrifuge tube, wash the mortar with a further 5 mL portion of orthophosphoric acid (5% w/v) and add the washings to the extract in the centrifuge tube. Remove the solid matter from the combined extract and washings by centrifugation (3000 rpm; 10 min).

N.B. the correct use of the centrifuge will be demonstrated to you but the centrifuge heads **must** be balanced before the centrifuge is operated.

Decant the supernatant quantitatively into a 50 mL volumetric flask and add orthophosphoric acid (5% w/v) to bring the volume to 50 mL. Ensure the diluted sample is well mixed.

Check that the pump tubing is at the bottom of the mobile phase. Select a flow rate of 1.0 mL/min and switch the pump on. Set the detector wavelength to 254 nm and auto zero if necessary after system has equilibrated or as when required.

Injects are made by setting the injection valve to the **LOAD** position, inserting the filled syringe (ensure that there are no air bubbles in the syringe) and filling the 100 μ L sample loop. The loop is full when a drop of liquid emerges from the overflow. When ready, and still holding the syringe in the valve, turn the valve to the **INJECT** position and simultaneously press **ALL START** on the D2500 Chromato Integrator. Remove the syringe and rinse it with Milli-Q water (you should always rinse an injection syringe after use, even if you are going to do a repeat injection with the same solution). When all the components have been eluted, press **1 STOP START** on the D2500 Chromato Integrator.

You should obtain three replicates of each sample. Dilute your samples further if it is required. Identify the chromatographic peak in the samples

by comparing the retention time with that of an authentic sample of L-ascorbic acid.

N.B. The pump must not be allowed to pump air so keep a careful watch on the levels of the mobile phase in the reservoir.

- (b)** Cook the pepper in one of three ways and quantify the vitamin C in the cooked tissue

Baking

Place about 10 g of pepper, weighed to the nearest 0.1 g, in a small beaker and bake in an oven at 180-200°C for 15 minutes (time to nearest 30 seconds). Extract the vitamin C from the cooked tissue, and quantify it, as described above

Boiling

Place a sample of pepper (10±0.1 g) in a beaker of boiling water and boil for 20 minutes. Extract the vitamin C from the cooked tissue, and quantify it, as described above.

If time permits, quantify the amount of vitamin C in the water remaining after the pepper has been cooked. The water will need to be diluted to an appropriate known volume; however, the precise dilution will depend on the amount of water remaining after cooking.

Microwaving

Place a sample of pepper (10±0.1 g) in a small beaker and microwave for one minute. Extract the vitamin C from the cooked tissue, and quantify it, as described above.

Data Analysis

In each case, calculate the vitamin C content of the fruit in g per 100 g of tissue and express the vitamin C content of the cooked tissue as a % of the uncooked control.

Collate all the data for the raw and cooked samples of pepper. What conclusions can be drawn?

References and Resources

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