Experiment 1: Determination of Vitamin C (Ascorbic Acid) Concentration in Fruit Juice

Introduction

Vitamin C (L-Ascorbic acid)

Vitamin C is a highly water-soluble compound that has both acidic and strong reducing properties. It naturally occurs in many plants and animals except in humans. The natural vitamin exists in L-ascorbic acid form. The D-isomer (i.e., D-ascorbic acid), which is the mirror image of the same molecular structure, has only about 10% of the activity of the L-isomer. L-ascorbic acid is a weak sugar acid which is structurally related to glucose attached to a hydrogen ion. It is a strong reducing agent, which carries out its reducing function and easily converts to its oxidized form, the L-dehydroascorbic acid, when oxidative stress is present. Due to this characteristic, L-ascorbic acid is commonly applied in food industry as a food additive functioning as a versatile antioxidant to protect foods from deterioration by oxidation.

Vitamin C is an essential nutrient in humans as it functions as a cofactor in several vital enzymatic reactions. It is widely known that deficiency of Vitamin C would lead to scurvy in humans. Vitamin C also has other beneficial effects to our body, such as preventing common cold/heart diseases and strengthening human immune system. However, human beings cannot synthesis Vitamin C by themselves and should obtain it from other sources. The richest natural sources of Vitamin C are fruits and vegetables, for example, blackcurrant, blueberry, orange, lime, lemon, strawberry, cabbage and malt. It is noted that Vitamin C can be chemically decomposed under certain conditions, such as heating and oxidation, many of which may occur during the cooking of food.

In general, the recommendation for vitamin C intake in humans is around 60–95 milligrams per day and the maximum upper intake level is 2000 milligrams per day. Vitamin C exhibits remarkably low acute toxicity. However, it is reported that a long-term overdose of this vitamin may cause diarrhea, iron overload disorders and kidney stone formation.
**Titration**

In this experiment, we use titration method to determine the concentration of Vitamin C in freshly prepared and packaged fruit juice samples. Titration (or called as volumetric analysis) is a common laboratory method of quantitative analysis that can be used to determine the concentration of a known analyte (or called reactant). A titrant (or called reagent) of known concentration is used to react with a solution of the analyte of unknown concentration. Using a calibrated burette, it is possible to determine the exact amount of titrant that has been consumed when the endpoint is reached. The endpoint is the point at which the titration is complete, as determined by the color change of an indicator.

**Objectives**

- To familiarize with the titration method and its application on food analysis
- To understand the method and principle of determining Vitamin C concentration in food
- To investigate the heat stability of Vitamin C in a commercial fruit beverage under different heating conditions

**Materials**

Fruit Juice/Beverage Samples

Ascorbic Acid Standard Solution (100mg L-ascorbic acid in 100ml of 0.5% (w/v) Oxalic acid)

0.5%(w/v) Oxalic Acid (Extracting Media)

2,6-Dichloro-Indophenol (DCIP) Solution

30% Hydrogen Peroxide

Titration Set (Burette, Stand, Clamp, Tile and funnel) (x1)

250ml Conical Flask (x3)

Buchner Funnel and Filter Paper (x1)

Glass Stopper Bottles (100ml)

Pipetman 1000 and Pipette Tips (x1)

25ml Measuring Cylinder (x1)

250ml Beaker (x1)

Test Tube (Large: 16x150mm) (x3)

Test Tube Rack

Hot Plate

Glass Thermometer

Timer

Holding Clamp

Knife

Latex Glove and Heat Protective Glove
Methods

Part A. Standardization of DCIP (Titrant) Solution

1. Using a 25ml measuring cylinder, measure 25ml of 0.5% oxalic acid and transfer it into a 250ml conical flask.
2. Using a pipetman, add 1ml of ascorbic acid standard solution into a 250ml conical flask and mix it well with the oxalic acid.
3. With a titration set, carry out a Trial Run of titration. Titrate the ascorbic acid solution rapidly with the DCIP solution. Add the DCIP solution through the burette and vortex the solution well. You may observe the DCIP solution changes to pink color when contacts with the ascorbic acid solution and then becomes colorless after shaking well. The end point is reached once a drop of DCIP solution is added and a distinct light rose-pink color persists in the solution even after mixing thoroughly. You may add the DCIP solution in exceed as in this trial run, you are only needed to be familiar with the color of the end point and to estimate the volume of DCIP solution required to bring the Vitamin C solution to the end point.
4. After the trial run, conduct another three actual titrations to the ascorbic acid standard solution and average the results. At this time, add the DCIP solution drop by drop carefully when the volume of DCIP solution added is close to the end point volume. (You may prepare three ascorbic acid solutions at the same time with three conical flasks).
5. Record the volume of DCIP solution added. Estimate the reading of the burette up to TWO decimal places (e.g., 6.15ml or 6.20ml, but not 6.2ml). This is equal to the volume of DCIP solution required to completely react with 1mg of ascorbic acid.

Part B. Determination of the Vitamin C Concentration in a Fresh Lemon Juice

1. Cut a lemon in half with knife and squeeze the juice out.
2. With the aid of a Buchner funnel and filter paper, separate the flesh and seed from the juice. (~10ml lemon juice should be collected)
3. Pipette 1ml of the lemon juice into a 250ml conical flask, which contains 25ml of 0.5% oxalic acid, and mix the solution well.
4. Titrate the lemon juice solution with the DCIP solution.
5. Triplicate the test and average the results.
6. Calculate the Vitamin C concentration in the fresh lemon juice by the following equation,

\[
\text{Conc. of Vitamin C in juice (mg/ml)} = \frac{\text{DCIP (ml) used to titrate Lemon Juice}}{\text{DCIP (ml) used to titrate Standard}} \times 1\text{mg/ml}
\]

*assuming that the amount of titrant for the blank (1ml H₂O + 25ml of 0.5% Oxalic acid) is very little and insignificant.
**Part C. Determination of the Vitamin C Concentration of an Apple Juice Beverage and Investigation of its Heat Stability under Different Conditions**

Caution: Beware of the hot plate and hot-water bath as they may cause severe burn. Pay attention when handling the hot glassware. Wear protective glove when handling the test tube in hot water.

**Part C1.**

1. Open the package of the beverage (apple juice) and transfer ~16ml of them into a test tube (16x150mm).
2. Pipette 1ml of the beverage into 25ml of 0.5% oxalic acid in a conical flask.
3. Titrate the sample with the DCIP solution using the procedures previously mentioned. Obtain 3 titration readings in total and average the results.
4. Put ~200ml water into a 250ml beaker. Put it on the hot plate and turn on the heater. Let the water heat up to boil.
5. Put the test tube containing the apple juice sample into the boiling water bath. Put a thermometer into the test tube and let the juice sample heat up to ~100°C.
6. When the sample temperature has reached to 100°C, start to count the time. Collect the apple juice sample at 15min, 30min and 45min intervals. Conduct the DCIP titration thrice (3 times) each similarly as above.
7. Compare the Vitamin C concentration of the apple juice beverage at different time intervals after boiling with the unboiled one.

**Part C2.**

1. Transfer ~10ml of apple juice sample into a new test tube.
2. Pipette 0.5ml of 30% hydrogen peroxide into the sample and mix them well with vortex.
3. Titrate the sample immediately with the DCIP solution similarly as above. Obtain 3 titration readings in total and average the results.
4. Then, put the test tube, which contains the rest of sample, into the boiling hot-water bath set in part C1. Let the test tube heat for 10min.
5. Collect the heated sample and conduct the titrations similarly as above. Record the readings.
6. Compare the Vitamin C concentration of the apple juice sample before and after heating.
Results

Part A. Standardization of DCIP (Titrant) Solution

<table>
<thead>
<tr>
<th></th>
<th>Initial Reading</th>
<th>Final Reading</th>
<th>Volume of DCIP (ml) used to titrate with 1ml of the Ascorbic Acid Standard Solution (1mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
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<tr>
<td>Trial 2</td>
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<td></td>
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<tr>
<td>Trial 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
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</tbody>
</table>

Part B. Vitamin C Concentration in the Fresh Lemon Juice

<table>
<thead>
<tr>
<th></th>
<th>Initial Reading</th>
<th>Final Reading</th>
<th>Volume of DCIP (ml) used to titrate with 1ml of Lemon Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td></td>
<td></td>
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<td>Trial 2</td>
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<td>Trial 3</td>
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<tr>
<td><strong>Average</strong></td>
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</tbody>
</table>

The Vitamin C concentration of the fresh lemon juice is _________ (mg/ml).

Part C1. Vitamin C Concentrations of the Apple Juice with Different Heating Time

<table>
<thead>
<tr>
<th></th>
<th>Volume of DCIP (ml) used to titrate with 1ml of Apple Juice when</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Without Heating</td>
</tr>
<tr>
<td></td>
<td>Heating (100°C) for 15min</td>
</tr>
<tr>
<td></td>
<td>Heating (100°C) for 30min</td>
</tr>
<tr>
<td></td>
<td>Heating (100°C) for 45min</td>
</tr>
<tr>
<td>Trial 1</td>
<td></td>
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<tr>
<td>Trial 2</td>
<td></td>
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<tr>
<td>Trial 3</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
</tr>
</tbody>
</table>

The Vitamin C Concentration of the apple juice without heating is ________ (mg/ml).

Discuss the heating effect on Vitamin C concentration in the apple juice:

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________
## Part C2. Vitamin C Concentrations of the Apple Juice added with Hydrogen Peroxide

<table>
<thead>
<tr>
<th>Volume of DCIP (ml) used to titrate with 1ml of Apple Juice added with 30 % Hydrogen peroxide</th>
<th>without Heat</th>
<th>Heating (100°C) for 10min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td></td>
<td></td>
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<tr>
<td>Trial 2</td>
<td></td>
<td></td>
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<tr>
<td>Trial 3</td>
<td></td>
<td></td>
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<tr>
<td><strong>Average</strong></td>
<td></td>
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</tbody>
</table>

Discuss the effect of hydrogen peroxide on Vitamin C concentration in the Apple Juice (before and after heating)

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**Discussions**

1. What are the Vitamin C concentrations of the fresh lemon juice and the apple juice beverage? Which one is higher?
2. Is there any change in the Vitamin C concentration of the apple juice after heating in 100°C for a period? Why?
3. What is the effect of heating (100°C) on the Vitamin C content in the apple juice beverage?
4. Is there any change after adding the hydrogen peroxide? If yes, what are the reasons?
5. It is common to know that the content of Vitamin C in foods (especially the vegetables) will be greatly reduced after cooking for a period of time. If it is true, what are the explanations for this phenomenon?
6. Suggest some factors that may affect the Vitamin C content in Food.
7. Suggest some possible methods that can preserve the Vitamin C content in food after cooking.
8. Suggest some limitations of the DCIP titration methods in determination of Vitamin C content of foods.
Experiment 2: Studies of Natural Food Pigments in Plants: Chlorophylls and Anthocyanins

Introduction

Chlorophylls
The quality of food is generally based on color, flavor, aroma, texture and nutritive values. However, no matter how nutritious and tasty of a food, it is unlikely to be eaten unless it has the “right” color. The colors of food are contributed by natural pigments, or by artificial colorants/color additives. Pigment is a term generally referring to normal constituents of cells or tissues that impart color. Chlorophylls, anthocyanins, flavonoids, proanthocyanidins, tannins, betalains, quinones, xanthones, carotenoids, myoglobin and hemoglobin are the common natural food pigments occurring in plants and animals.

Chlorophyll is a green pigment found in most plants and algae. Chlorophyll absorbs light most strongly in the blue and red but poorly in the green portions of the electromagnetic spectrum, hence we can perceive the green color of chlorophyll-containing tissues like plant leaves.

In plant cells, these chlorophylls are located within plastid bodies called chloroplast. Chlorophylls are vital for photosynthesis, which allows plants to obtain energy from light. Chlorophyll is a chlorin pigment, which is structurally similar to other porphyrin pigments such as heme. At the center of the chlorin ring is a magnesium ion. The chlorin ring can have several different side chains, usually including a long phytol chain.

There are several forms of chlorophyll existing in nature, such as chlorophylls a, b, c and d, bacteriochlorophylls a and b. In foods we are mainly concerned with chlorophyll a and b which occur in the approximate ratio of 3:1 in terrestrial higher plants. Chlorophylls a and b are soluble in alcohols, ether, benzene and acetone, but are insoluble in water. Chemically, the chlorophylls may be altered in many ways, but in food processing the most common alternation is **pheophytinization**, which is the replacement of the central magnesium by hydrogen and the consequent formation of the dull olive-brown phenophytins.
**Anthocyanins**

Anthocyanins are another group of pigments commonly found in edible plants. They are a group of water-soluble vacuolar flavonoid pigments that are widespread in fruits, vegetables and flowers. They are synthesized by organisms of the plant kingdom and have been observed to occur in all tissues of higher plants, providing attractive color of leaves, stems, roots, flowers, and fruits.

All anthocyanins are derivatives of the basic flavylium cation structure. More than 550 anthocyanins are known but only few (e.g., Pelargonidin, Cyanidin, Delphinidin, Peonidin, Petunidin and Malvidin) are important in food as others are comparatively rare and are found in non-edible flowers and leaves. The flavylium nucleus of the anthocyanin pigment is electron-deficient and therefore highly reactive. The reactions usually involve decolorization of the pigments and are usually undesirable in fruit and vegetable processing.

One of the interesting properties of anthocyanins is that they show a marked color change with different pH, being red in acids and blue in bases. It involves different chemical structural changes in response to the pH variation. Some sciences teachers may use the anthocyanins (in red cabbage) as a pH indicator for teaching in school.

In nature, attractive coloration of flowers and fruits by anthocyanin pigments helps attract pollinators and hence facilitates plant pollinations. Besides this function, anthocyanins in photosynthetic tissues can protect cells from strong light intensity damage by absorbing blue-green light, thereby protecting the tissues from photoinhibition. Apart from these, anthocyanins are also powerful antioxidants as they have a strong reducing property. This antioxidant property remains even after the anthocyanins in plant are consumed by another organism. There are some supporting evidences shown in recent researches that anthocyanins in berries have potential health effects against cancer, aging, neurological diseases, inflammation, diabetes and bacterial infections.

Anthocyanins are commonly found in vegetables and fruits in reddish color, such as blackcurrant, raspberry, cherry, eggplant, blue grape, red/black beans and red cabbage, and the amounts are relatively large. One kilogram of blackberry contains approximately 1.15g and red/black legumes can contain up to 20g/kg.
**Thin layer chromatography (TLC)**

Thin layer chromatography is a fast, simple and generic chromatography technique applied to separate chemical compounds from a mixture. It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel or cellulose, immobilized onto a flat and inert carrier plate. A sample mixture, which consists of the different chemical compounds to be separated, is applied on the stationary phase and then dissolved in an appropriate solvent (mobile phase). The mixture will be carried upward alone the plate due to the capillary action and the components will be separated based on the different polarities of the individual compounds.

This is a fundamental technique for analytical chemistry and has a wide range of uses in food related analyses, for instance, determination of the plant pigments, detection of pesticides or insecticides in food, analyzing the colorant or dye composition and authentication of Chinese medicinal materials.

![TLC Plate Development Sequence](image)

**Objectives**

To understand and familiarize with the TLC analysis on natural food pigment

To investigate the cooking effects on the color of plant pigments (chlorophylls)

To study the effect of pH on food anthocyanins

**Materials**

- Green Vegetable Leave Samples
- 85% Aqueous Acetone
- Calcium Carbonate
- 150ml Beaker (x3)
- Glass-Rod (x3)
- Funnel and Filter Paper (x3)
- 100ml Conical Flask (x3)
- Pre-coated Silica Gel TLC Plates (x1)
- Glass Capillary (x3)
- Pencil and Ruler (x1)
- TLC Developing Solvent: (Petroleum Ether-Isopropanol-Water, 100:10:0.25 v/v/v)
- TLC Developing Tank (x1)
- Hair Dryer (x1)
- UV-Light (x1)
- Forceps (x1)
- 250ml Beaker (x3)
- 50ml Beaker (x3)
- Hot Plate (x1)
- White Vinegar (food grade)
- Sodium Bicarbonate (food grade)
- Grape/Blueberry Juice
- pH Buffer of 2.0, 3.0, 5.0, 7.0, 9.0 and 10.0
- Test-Tube (x10)
- Pipetman 1000 and Tips (x1)
- Pipetman 5000 and Tips (x1)
- Vortex-Mixer
- Timer
Methods

Part A. Extraction of Chlorophylls from Fresh Spinach Leave and Thin Layer Chromatography Separation of the Green Pigment Extracts

Caution: The solvent is organic and volatile. You should handle it very carefully and should not sniff deeply on it. Collect the used solvent in waste bottle after using.

1. Cut spinach leave into fine pieces. Weigh an approximately 15g portion
2. Place them in a 100ml beaker and add a small quantity (c.a. 0.1g) of calcium carbonate. Grind tissue with a glass rod for a short time.
3. Add 30ml 85% aqueous acetone to the tissue and continue to grind until a fine pulp mixture is formed.
4. Transfer the pulp mixture to a funnel with filter paper and filter. Collect the pigment extract.
5. With the aid of a glass capillary tube, spot a small aliquot of the pigment extract on a silica gel TLC Plate. The spot should be approximate 1.5 cm from the end of the TLC plate. Keep the sample spot as small as possible (no more than 5mm in diameter).
6. If necessary, dry the spot with a hair dryer during spotting. Re-apply aliquots of the pigment extract if the color of the spot is too pale, until the spot is dark enough.
7. Repeat Step1-6 with another vegetable leave sample. Spot the pigment extract on the same TLC plate. It should be noted that the two spots should locate on the same horizontal level and at least 1-1.5cm apart from each others.
8. When the sample spots are thoroughly dry, place the TLC plate in a developing chamber containing the running solvent (petroleum ether-isopropanol-water, 100:10:0.25 v/v/v) at a level just below the sample spots. The height of the solvent is crucial as the sample spots must not drip into the solvent.
9. Cover the chamber with a lid. Let the solvent run upwards and do not disturb the chamber.
10. When the solvent front has approached near the top end of the TLC plate, use a forceps to remove the TLC plate from the developing chamber.
11. Allow the TLC plate to air dry (hair dryer may be used but the warm air should be applied gently)
12. View the TLC plate both visually by eye and under UV light. Record the relative position and color of all the spots on the TLC plate.
13. Compare the pigment spectrum of the samples in the different lanes.
Part B. Effects of Cooking in Blank Water, Acid and Alkali on the Color of Spinach Leave

Caution: Beware of the hot plate and hot-water bath as it may cause severe burn. Pay attention when handling the glassware. Wear protective glove. Adjust the heater a bit weaker when the water is boiling violently.

1. Cut spinach leaves into fine pieces and weigh an approximately 15g portion. Prepare a total of three portions.
2. Pour water into three 250ml beakers (each should contain ~200ml water). Heat them on the hot plate up to boiling.
3. Add 1 tablespoon of white vinegar into one of the beaker and label it as acidic water.
4. Add 1/4 teaspoon of sodium bicarbonate into another beaker and label it as alkali water.
5. Put the three portions of spinach leave into the three beakers containing blank water, acidic water and alkali water. Ensure that all of leaves are totally submerged.
6. Boil them for 5min and stir them with individual glass rods occasionally.
7. Take the spinach leave out and transfer them to individual beakers. Let them cool down and slightly squeeze the leave to remove the exceed water.
8. Compare and describe the color of each cooked spinach leave.

Part C. Effect of pH on the Color of the Anthocyanins in Different Grape Juices

1. Place 10ml portions of buffer solutions of the following pH values: 2.0, 3.0, 5.0, 7.0, 9.0 and 10.0 in separate test tubes.
2. To each buffer solution, add 1ml of the grape juice and mix well. Stand for 3min and then observe any color change.
3. Repeat test by using a white grape juice sample. Record the results.
Results

Part A. Pigments Separation by TLC

On the right hand side, draw a picture to record the pigment separation result on the TLC plate in scale. Based on references, try to identify all the spots as much as possible.

Part B. Effects of Cooking in Different pH Conditions on the Color of Spinach Leaves

Describe the colors of spinach leaves after boiling in

A. Hot Water

B. Acidic Water

C. Alkali Water

Part C. Effect of pH on the Color of the Anthocyanins in Grape Juices

Describe the colors of grape juices at different pH

<table>
<thead>
<tr>
<th></th>
<th>Color of Juice Samples at Buffer</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pH 2.0</td>
</tr>
<tr>
<td>Grape Juice (at 0min)</td>
<td></td>
</tr>
<tr>
<td>Grape Juice (at 10min)</td>
<td></td>
</tr>
<tr>
<td>White Grape Juice (at 0min)</td>
<td></td>
</tr>
<tr>
<td>White Grape Juice (at 10min)</td>
<td></td>
</tr>
</tbody>
</table>

Discussions

1. Suggest some limitations of TLC method in food pigment analysis.
2. What are the effects of adding acid and alkaline on the color of spinach leave during cooking in water? Provide explanations to these effects?
3. Suggest some possible methods to preserve the green color of vegetable after cooking.
4. Suggest other factors that may cause discoloration of vegetables in food process.
5. Why people can determine the maturity (or degree of sourness) of some fruits (e.g. Strawberries) by just observing their color?
References, Relative Websites and Suggest Readings