JIMMA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF CHEMISTRY

A RESEARCH PAPER ON

TRACING THE LEVEL OF ASCORBIC ACID ELECTROCHEMICALLY IN ORANGE MARKET CHAIN AROUND JIMMA TOWN

October, 2014
Jimma, Ethiopia

TRACING THE LEVEL OF ASCORBIC ACID ELECTROCHEMICALLY IN ORANGE MARKET CHAIN AROUND JIMMA TOWN

A Thesis Research Submitted to School of Graduate Studies Jimma University in Partial Fulfillment of the Requirements for The Degree of Master of Science in Chemistry.

By

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October, 2014
Jimma, Ethiopia

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List of abbreviations

AA	ascorbic acid
AuNPs	gold nanoparticles
CNTs	carbon nanotubes
CV	cyclic voltammetry
GCE	glassy carbon electrode
ME	mercaptoethanol
MFP	meat, fish and poultry
PD	phenylenediamine
SWV	square-wave voltammetry

Acknowledgements

First of all I would like to express my sincere gratitude to my advisor Dr. Tesfaye Refera and Shimeles Addisu for their endless support and guidance during the course of this work. Next I am happy to give great thanks to Mr. Fikadu Melak for his polite support and advice all my work. Finally I would like to express my appreciation to the department of chemistry.

ABSTRACT

Orange is one of the important fruit crops around Jimma town and the juice from orange fruit is one of the natural good antioxidant sources due to the presence of ascorbic acid (vitamin C). The objective of the present work was to assess the degradation kinetics of ascorbic acid in orange fruit starting from farm to consumer under different storage conditions around Jimma town (Ascolla, Dedo; Alemayehumecha, Yebu) using random nano/hole p-phenylenediamine film grafted glassy carbon electrode. The anodic peak current for ascorbic acid occurs at about 0.3 V on the modified electrode while it occurs at 0.62 V on a bare glassy carbon electrode versus Ag/AgCl. The calibration graph from amperometric determination at applied potential of 0.35 V, shows a linear dependence between current and ascorbic acid concentration in the range of 5 x 10^{-6} M to 10^{-2} M R^2 = 0.9999). The results of amperometric determination of ascorbic acid were compared with those obtained by the standard iodometric titration method and were found to be in good agreement. The loss in concentration of ascorbic acid under different storage conditions was studied. The concentration was determined amperometrically by standard addition method using the modified electrode. The degradation kinetics of ascorbic acid in orange juices during thermal treatment from 30-50^{0}C follows the first order kinetics. The value of the activation energy and Arrhenius constant were 3.39 KJ/mol and 0.96 min^{-1}, respectively. From the analysis of the results, appropriate storage and transportation means of orange fruit was recommended.

1. Introduction

1.1. Ascorbic acid (Vitamin C)

Ascorbic acid (AA) is an organic compound of carbon, hydrogen and oxygen. Pure ascorbic acid is a white solid and is made synthetically from the sugar dextrose. It is used both in vitamin supplements and as a food preservative. Vitamin C is used as a main characteristic description of both ascorbic acid (reduced form) and dehydroascorbic acid (oxidized form). It is widely distributed and occurs in large quantities from plant origins that are most important for animal nutrition, particularly in green feeds and silages. Moreover, Vitamin C is highly important to play a critical role in the body immune system because it involved in the synthesis of collagen and incorporating plasma iron in to ferritin. It also required for neutrophil function and glucocorticoids circulating reduction, not only this but also reduces oxidized form of tocopherol to its active form in liver. Vitamin C is the lactone 2, 3-dienol-L-gluconic acid and is an odorless, and white crystalline having the chemical formula $C_6H_8O_6$. It is the L-enantiomic form of AA which also encompasses the oxidation product of dehydroascorbic acid. It participates in numerous biochemical reactions and important for everybody process from repair [1]. Vitamin C helps in the metabolism of tyrosine, folic acid and tryptophan. It also helps in the metabolism of cholesterol, increasing its elimination and thereby assisting lower blood cholesterol[2].

In addition to the synthesizing amino acids (carnitine and the catecholamines) that regulate the nervous system, Vitamin C, helps to absorb iron that break down histamine and the inflammatory component of many allergic reactions [3]. Absorption of iron, especially the non-heme variety found in plants and drinking water is enhanced by Vitamin C. It has been shown to facilitate iron absorption by its ability to reduce ferric iron to the ferrous form [4]. Ordinarily our absorption of iron is quite poor, putting us at risk of iron deficiency anemia. One milligram of ascorbic acid is approximately equivalent in enhancing power to 1 g of cooked MFP (iron present in meat, fish and poultry) or 1.3 g of raw MFP [5].

A recommended daily intake of AA is about 70–90 mg. Inadequate intake will result in the symptoms of scurvy, gingival bleeding, and so on; excess AA intake will also lead to urinary stone, diarrhea and stomach convulsion [6]. AA is a well-known antioxidant, which helps the

human body to reduce oxidative damage and protects food quality by preventing oxidative deterioration [7-9]. The overall oxidation of AA is [8]

$$C_6H_8O_6 \longrightarrow C_6H_6O_6 + 2H^+ + 2e^-$$

Ascorbic acid (vitamin C) is one of the important water soluble vitamins. It is essential for collagen, carnitine and neurotransmitters biosynthesis [10]. Most plants and animals synthesize ascorbic acid for their own requirement. However, apes and humans cannot synthesize ascorbic acid due to lack of an enzyme gulonolactone oxidase. Hence, ascorbic acid has to be supplemented mainly through fruits, vegetables and tablets [11].

Vitamin C helps maintain capillaries, bones, and teeth and aids in the absorption of iron. Ascorbic acid is necessary to maintain the enzyme prolyl hydroxylase in an active form, most likely by keeping its iron atom in a reduced state. The precursor molecule to the protein collagen, procollagen, contains an unusual amino acid sequence in that every third amino acid is a glycine and contains a high frequency of two amino acids not found in any other proteins – hydroxylproline and hydroxylysine. The latter two amino acids are converted from proline and lysine, respectively, after the procollagen molecule has been synthesized. The hydroxylation of proline and lysine in procollagen is carried out by the enzyme prolyl hydroxylase using ascorbic acid as a cofactor. The natural form of the vitamin is the L-isomer [12, 13].

Ascorbic acid is a powerful antioxidant because it can donate a hydrogen atom and form a relatively stable ascorbyl free radical. As a scavenger of reactive oxygen and nitrogen oxide species, ascorbic acid has been shown to be effective against the superoxide radical ion, hydrogen peroxide, the hydroxyl radical and singlet oxygen [14].

1.2. Sources of ascorbic acid

Foods that are plant output are the main dietary sources of vitamin C, vitamin E and carotenoids, which could act as efficient scavengers of radicals and oxidants. Vitamin C (chemical names: ascorbic acid and ascorbate) is a six-carbon lactones which is synthesized from glucose by many animals. Vitamin C is synthesized in the liver in some mammals and in the kidney in birds and reptiles. However, several species including humans, non-human primates, guinea pigs, Indian fruit bats, and Nepalese red-vented bulbuls are unable to synthesize vitamin C. When there is

insufficient vitamin C in the diet, humans suffer from the potentially lethal deficiency disease scurvy[15]. Humans and primates lack the terminal enzyme in the biosynthetic pathway of ascorbic acid, l-gulonolactone oxidase, because the gene encoding for the enzyme has undergone substantial mutation so that no protein is produced[16].

Ascorbate is found in many fruits and vegetables[17]. Citrus fruits and juices are particularly rich sources of vitamin C but other fruits including cantaloupe and honeydew melons, cherries, kiwi fruits, mangoes, papaya, strawberries, tangelo, tomatoes, and water melon also contain variable amounts of vitamin C. Vegetables such as cabbage, broccoli, Brussels sprouts, bean sprouts, cauliflower, kale, mustard greens, red and green peppers, peas, and potatoes may be more important sources of vitamin C than fruits, given that the vegetable supply often extends for longer periods during the year than does the fruit supply.

An orange (Citrus sinensis) is a type of citrus fruit which people often eat. Oranges are a very good source of vitamins, especially vitamin C. Orange juice is an important part of many people's breakfast. The "sweet orange", which is the kind that is most often eaten today, grew first in Asia but now grows in many parts of the world. Peoples around Jimma town are known cultivators of orange. Due to the advantage of orange fruit as antioxidant (reducing agent), scurvy protection and others in the presence of vitamin C, the study of the degradation of this vitamin in orange fruit is interesting. In this investigation cyclic voltammetry was used in the determination of ascorbic acid levels of orange fruit samples collected around Jimma town.

1.3. Statement of the problem

Orange fruit grows around Jimma in small scale farm lands as a source of income by subsistent farmers from the sale of the fruits. The climate of Jimma and its surrounding is hot and there could be a problem of degradation of ascorbic acid in orange fruit during transportation, storage and processing. To determine the level of ascorbic acid degradation, there is need to determine the degradation of ascorbic acid to the weather condition of Jimma.

During this study, answers for the following questions will be provided.

- Is AA in orange fruit degrading through market chain? And by how much?
- Which step(s) is/are more important in the market chain in degrading the level of ascorbic acid?
- What is the rate of degradation?

1.4. Objective of the study

1.4.1. General objective

- To amperometrically determine the level of ascorbic acid in orange fruits collected from Jimma town and its surrounding.

1.4.2. Specific objective

- Determine the degradation of ascorbic acid by comparing the level of ascorbic acid from fruit harvest, transportation to market, and storage in consumers' house.
- Determine the rate of degradation of ascorbic acid with temperature and storage time.
- Recommend the best way of transportation, storage and processing of orange fruits.

1.5. Significance of the study

This study was used to demonstrate the level of degradation of ascorbic acid in orange fruit due to transportation, storage and air dehydration around Jimma town. The result can be used to consumers and producers of orange to pay attention for degradation problem and look for preventive measures.

2. Literature Review

2.1. History of ascorbic acid

Scurvy is a disease which is the result of vitamin C (L-AA) deficiency was described by the ancient Greeks, Egyptians and Romans and it has been associated with invading armies, navies and explorers. Even at the end of the last century, scurvy was rife amongst the gold miners of California and Alaska. The first systematic study of this problem was carried out by the naval surgeon James Lind in the 1750's, who found that the disease could be prevented and cured by the daily administration of fresh citrus fruits. By Holst and Frölich[18] demonstration, scurvy could be produced in the guinea pig which indicates that scurvy was the result of a dietary deficiency. Svent-Györgi isolated a compound from vegetables that he named 'crystalline hexuronic acid',[19] and which was subsequently shown to be the same as the vitamin C. Methods had been devised to synthesize the compound by the mid-1930's, and it soon became widely available at low cost. It was soon established that the substance was virtually nontoxic at any dosage. Scurvy has been known since ancient time. People in parts of the world assumed that it was caused by a lack of fresh plat foods. In 1932, before the discovery of vitamin C (ascorbic acid), nutrition experts recognized that something in citrus fruits could prevent scurvy, a disease that killed as many as 2 million sailors between 1500 and 1800[20]. The structure of vitamin C is shown in Figure 1.

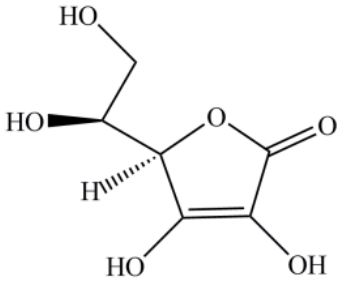


Figure 1: Structural formula of ascorbic acid

2.2. Determination of ascorbic acid

An accurate and specific determination of the nutrients content of fruits is extremely important to understand the relationship of dietary intake and human health. A wide variety of foods such as fruits, vegetables, and organ meats are the best sources of vitamin C, however, muscle, meats and most seeds do not contain significant amounts of Vitamin C. For better utilization of fruits

and vegetables as a human food, clear understanding of their nutrition value as well as the content of vitamin C estimation is essential.

The development of reliable, rapid and preferably portable analytical methods to quantify AA during the production and quality-control stages and in clinical applications is important due to the increasing use of AA in the food, pharmaceutical and cosmetic industries and its significance in biomedical science [9, 21, 22]. Traditional procedures for AA determination are generally based on enzymatic methods [23], on titration with oxidizing agents, like iodine or 2, 6-dichlorophenolindophenol and HPLC analysis with fluorimetric [24] or UV-Vis detection [25] and spectrophotometry [26].

Previous studies have shown during fruits and vegetable preparation, losses of Vitamin C may be high, and this may sometimes exceed those caused by drying operation. Early report showed that losses of the vitamin C during preparation of apple flakes were 8% during slicing, 62% from blanching; 10% from pureeing and 5% from drum drying. Reports have also shown that Vitamin C degradation could be as high as 80-95% during air-dehydration of fruits. This limits the air-dehydration of fruits [27]. The removal of moisture during drying is attributed to the changes on the dried products. To alleviate this problem of air-dehydration of fruit, pretreatment of fruit slices with sucrose-osmosis and sulphiting (SO_2) have been proved effective [28, 29].

2.2.1. Determination of ascorbic acid by spectrophotometric method

Direct UV use for the determination of ascorbic acid has not been easy because of its instability in aqueous solutions. Due to the oxidation of L-ascorbic acid to dehydroascorbic acid which is a reversible reaction and subsequently to 2, 3-diketo-L-gulonic acid which is irreversible, the L-ascorbic acid becomes instable. These reactions can be inhibited by the stabilizers sodium oxalate (0.0056 M). The proposed method was applied to the determination of the ascorbic acid contents in commercial pharmaceutical preparations (tablets). The results obtained are 80 mg/tablet[30].

For the determination of the total vitamin C (ascorbic acid + dehydroascorbic acid) in various fruits and vegetables a simple UV- spectrophotometric method is used. It involves the oxidation of ascorbic acid to dehydroascorbic acid by bromine water in presence of acetic acid. By using

simple UV- spectrophotometric method, the content of vitamin C was 1.868 to 51.74 mg/10g in fruits and 0.841 to 17.416 mg/10g in vegetables [31].

2.2.2. Determination of ascorbic acid electrochemically

Because of the oxidation of ascorbic acid to dehydroascorbic acid, electrochemical methods can be useful in the determination of vitamin C levels in foods. Electrochemical methods traditionally have found important applications in sample analysis and in organic and inorganic synthesis. The electrode surface employed in such determinations can be a powerful tool in such applications [32]. This method is fast, sensitive, selective and gives linear response at low concentration range.

To develop chemical sensors for electrochemical detection of AA, there is a considerable interest. AA can be easily oxidized electrochemically at conventional electrodes that have been used to detect AA [33, 34]. However, direct oxidation of ascorbic acid at bare electrode requires high over-potential which results in electrode fouling by its oxidation products with poor reproducibility, low selectivity and sensitivity. In addition, some of biological molecules, e.g. dopamine and uric acid, undergo oxidation within same potential window as AA. To resolve these problems, chemically modified electrodes have been developed and reported with various functional materials such as conducting polymers [35-39], ionic liquid [40, 41], metal nanoparticles [36], carbon nanotubes [37, 41] and macrocyclic compounds [42, 43].

Electrochemical techniques, particularly cyclic voltammetry (CV) and square-wave voltammetry (SWV), have been employed as alternative tools for the evaluation of antioxidant activity [44]. These methods are attractive because of the speed of analysis, simplicity and low cost of the instrumental requirements. Ascorbic acid oxidation at a bare glassy carbon electrode (GCE) generally occurs at a relatively high oxidation potential, indicating a slow electron transfer rate at the GCE [45]. Such sluggish electrode kinetics may also be due to electrode fouling caused by the deposition of oxidation product(s) of AA on the electrode surface, which results in poor selectivity and reproducibility, thus limiting the use of bare GCEs in quantitative measurements. Presently there are increasing reports on the use of carbon nanotubes (CNTs) in electroanalysis [46].

Kumar, S. with his colleagues developed an analytical method for the analysis of ascorbic acid (AA) by square-wave voltammetry (SWV) using multiwalled carbon nanotubes to modify the surface of a glassy carbon electrode to enhance its electroactivity. Nafion served to immobilize the carbon nanotubes on the electrode surface [47].

The amount of ascorbic acid can be also determined by a redox titration with a standardized solution of iodine. The iodine is reduced by the ascorbic acid to form iodide. As shown in the other half of this redox equation.

$$C_6H_8O_6 + I_2 \longrightarrow C_6H_6O_6 + 2I^- + 2H^+$$

Although titrimetric methods are simple to use in the determination of vitamin C, difficulties are encountered with commonly used titrants and interferences often occur with coloured samples. In addition direct spectrophotometric determination of ascorbic acid in the UV region is prone to matrix effect since many organic compounds in complex samples may also exhibit ultraviolet absorbance[48]. The spectrophotometric method suffers from poor selectivity due to interference from other compounds present in commercial fruit juices (e.g., sugars or glucuronic acid) while citrate may affect enzymatic methods [26].

In this study, the use of a random nano arrayed modified electrode has been reported for the direct electroanalysis of ascorbic acid in orange fruit collected from surroundings of Jimma town. The modified electrode showed superior selectivity and sensitivity towards electroanalysis of AA.

3. Materials and Methods

3.1. Chemicals and reagents

Potassiumteterachloroaurate [$KAuCl_4$, 99.99%, Aldrich], p-Phenylenediamine [$C_6H_8N_2$, 100 %, Aldrich], Sodium nitrite [$NaNO_2$, 96%, Wardle], Sodium perchlorate [$NaClO_4$, 99+%, Aldrich], Potassium nitrate [KNO_3, 99%, Nice], Potassium hexacyanoferrate [$K_3Fe(CN)_6$, 99%, BDH Laboratory], Ruthenium(III) chloride [$RuCl_3.xH_2O$, 99.98%, Aldrich], 2-Mercaptoethanol [$HSCH_2CH_2OH$, 100 %, Aldrich], Hydrochloric acid [HCl, 37%, Riedel-De Haen], Sulphuric acid, [H_2SO_4, 98%, Merck], Hydroquinone [$C_6H_6O_2$, 99%, Kiran], Potassium iodide [KI, 99%, Nice], Potassium iodate [KIO_3, 99.5%, Nice], Potassium chloride [KCl, 99%, Finkem], Sodium acetate [$C_2H_3NaO_2.3H_2O$, 99.8%, Chem. Rein], Sodium hydroxide [NaOH, >97%. Aldrich], Glacial acetic acid [CH_3COOH, 100%, BDHL Laboratory] and Citric acid [$C_6H_8O_7$, 99%, Wardle] were used. Deionized water (Milli Pore, conductivity 18.2 MΩ cm^{-1}) was used to prepare aqueous solutions.

3.2. Electrochemical measurements

All electrochemical measurements were carried out with Epsilon electrochemical analyzer (Bioanalytical System Inc. USA model BAS Epsilon-EC-Ver 1.40.67) and a conventional three-electrode system. The working electrode was a glassy carbon electrode (3 mm diameter, BAS Model MF-2012). A platinum wire (1mm, model MW-4130) and a commercial Ag/AgCl saturated NaCl electrode (BAS, model MF-2053) were used as auxiliary and reference electrodes, respectively. All potentials are reported with respect to Ag/AgCl reference electrode at room temperature.

3.3. Experimental procedures

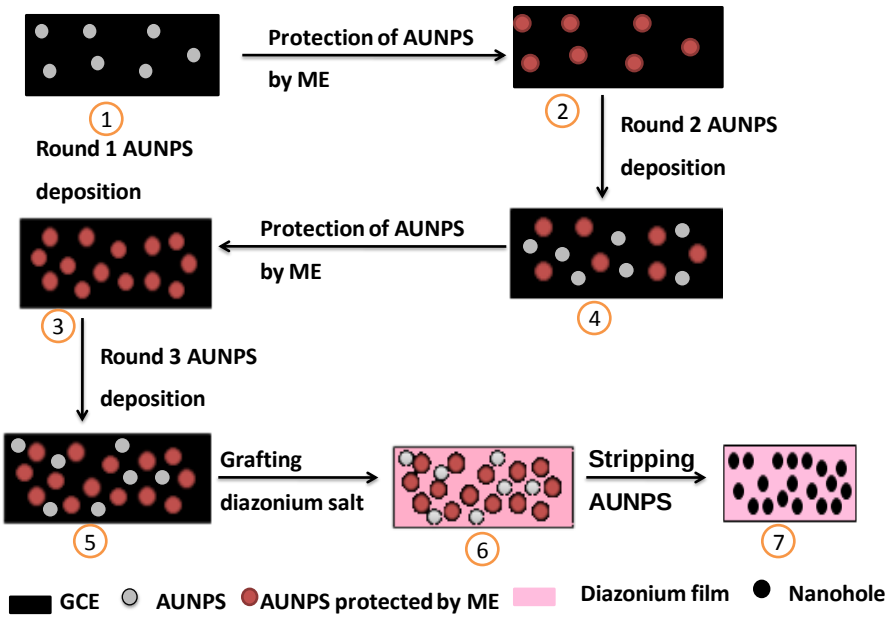
3.3.1. Electrode pretreatment

Glassy carbon electrodes (GCE) were polished, cleaned and electrochemically conditioned before any electrochemical measurement was made. The GCE was hand polished with slurry of alumina on a piece of micro-fabric cloth until there were no visible markings or scratches. The GCE was ultrasonicated (Elmasonic S 10 sonicator) in Millipore (MQ) water for 15 minutes to

remove the alumina from the GCE surface. After the cleaning step, the GCE was electrochemically conditioned in 1M sodium perchlorate.

3.3.2. Fabrication of randomly nano arrayed electrodes

The procedure developed by Negasa, B. [49] was used to modify the GC electrodes for electroanalysis of ascorbic acid. The electrode modification was undertaken in seven steps (scheme 1). These steps were: (i) Sequential Electro deposition of gold nanoparticles (AuNPs) [50,] (step 1 to 5), (ii) Grafting of diazonium films (step 6) and (iii) Stripping of the deposited AuNPs (step 7).



Scheme 1: Schematic representation the glassy carbon electrode surface modification used in this work. The sequential electrodeposition/protection procedure for nanoparticles deposition on GCE (steps 1-5), grafting of the gold deposited GC electrode (step 6) and striping of the aminophenylene nanohole GC electrode (step 7).

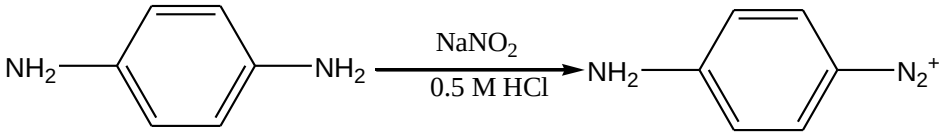
3.3.2.1. Sequential electrodeposition of gold nanoparticles

Gold nanoparticles were electrodeposited on GCE following the literature [50] procedure. This was done first by adding 10 µL of 0.5 M $KAuCl_4$ in 5 mL of 0.5 M H_2SO_4 in an electrochemical

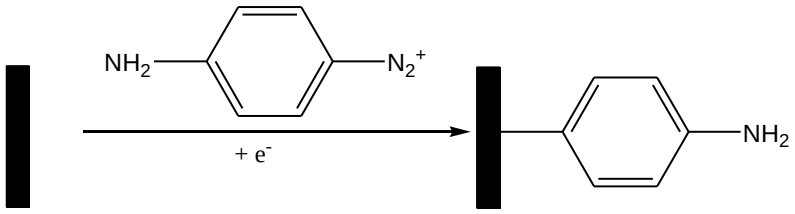
cell. Then chronoamperometry (CA) was run with general parameters of initial potential 1.1 V for 5 s then stepped to 0 V for 5 s. After each deposition step the electrode was protected by 2 mM 2-mercaptoethanol (ME) in order to prevent the crystal growth of gold nanoparticles on the GCE. To make it the gold nanoparticles deposited GCE was kept upside down in a micropipette tip that filled with 2 mM 2-ME for at least 2 hour at room temperature.

3.3.2.2. Modification of surfaces with diazonium film

Diazonium salts derived from p-phenylenediamine was used to insulate the GCE. This was done by reduction of in situ generated amino benzene monodiazonium cations onto the gold nanoparticles deposited GCE using the method developed by Belanger and co-workers [51]. Briefly, 10 mL solution of 3 mM p-phenylenediamine and 10 mL of 3 mM sodium nitrite in 0.5 M HCl were kept separately in ice jacketed beaker for 1 hour. Then, p-phenylenediamine was reacted with an equal amount of sodium nitrite in 0.5 M HCl for a few seconds in an electrochemical cell at room temperature to generate aminophenyl diazonium cation in situ (Scheme 2a). Immediately, the gold nanoparticles deposited GC electrode was inserted into the cell, and grafting was performed electrochemically using cyclic voltammetry method from potential window of 0.6 V to -0.2 V at a scan rate of 0.1 V/s for 3 cycles. The electrochemically reduced aminophenyl diazonium cations were then grafted onto the gold nanoparticles deposited GC electrode (Scheme 2b).



(a)



(b)

Scheme 2: a) In situ generation of diazonium cations b) grafting of aminophenyl films onto gold nanoparticles deposited GC electrode.

3.3.2.3. Stripping of deposited gold nanoparticles

Following the sequential electronucleation of gold nanoparticles, the electrode surface was passivated by the diazonium film. Nanohole were produced on this modified electrode by stripping the deposited AuNPs in 0.1 M KCl using CV in a potential range of 0 V to 1.4 V for three cycles.

3.3.3. Electrochemical characterization of randomly nanoarrayed electrodes

The modified electrodes prepared as such were electrochemically characterized by CV using hydroquinone (neutral redox probe) and $K_3Fe(CN)_6$ (negative redox probe). The signals of these probes at nanohole electrodes were compared to signals at bare GCE.

3.4. Preparation of solutions

3.4.1. Preparation of acetate buffer solutions

Acetate buffer solution of 0.1 M of pH 5 were prepared by dissolving 0.1 M of glacial acetic acid and 0.1 M of sodium hydroxide by using calibrated pH meter.

3.4.2. Preparation of ascorbic acid solutions

Stock solutions of 0.5 M ascorbic acid was prepared in 0.1 M acetate buffer solutions (pH 5) and other intermediate solutions of ascorbic acid were prepared by appropriate dilution of the stock solution in acetate buffer solution. Amperometric method was used for detection of the samples. Construction of calibration curve of the samples, average values of triplicate measurements were taken for each concentration.

3.4.3. Preparation of solutions of interferents

For interference study of ascorbic acid, solutions of 0.5 M glucose, 0.5 M citric acid and 0.5 M tartaric acid were prepared in 0.1 M acetate buffer solution (pH 5). The study of interferences was conducted amperometrically at nanohole PD grafted and bare GCE at potentials of 0.35V and 0.65 V respectively.

3.4.4. Procedure for preparation and analysis of real samples

Orange fruits were obtained from farms around Jimma town (Ascolla, Dedo). Dedo is one of the woredas in the Oromia Region of Ethiopia. Part of the Jimma Zone, Dedo is bordered on the south by the Gojeb River which separates it from the Southern Nations, Nationalities and Peoples Region, on the west by Gera, on the north by Kersa, and on the east by Omo Nada. The major town in Dedo is Sheki. Located in the Jimma Zone of the Oromia Region, it sits at a latitude and longitude of 7°25′N 37°00′E and an elevation of 1560 meters above sea level.

Fresh juices of oranges were prepared by squeezing oranges fruit into a glass beaker then the juices were filtered through filter paper to remove the fiber and pulp. Then, 1 mL juice was diluted with 4 mL of 0.1 M acetate buffer (pH 5) and chronoamperometric measurements were done three times at 0.35 V at nanohole PD grafted GCE and average results of triplicate measurements were taken.

3.4.5. Determination of ascorbic acid amperometrically

Concentration ascorbic acid was determined amperometrically by standard addition method. This was done by sequential addition of the orange juice and 1 mM standard ascorbic acid solution in a well stirred solution of 0.1 M acetate buffer by keeping the aminophenyl grafted nanohole GCE at potentials of 0.35 V.

3.5. Determination of ascorbic acid by titration

In this study the procedure was taken from the literature of Izuagie, A. A. et al [52].

3.5.1. Solution preparation

Iodine solution: 5.005 g of KI and 0.303 g of KIO_3 were dissolved in 200 mL distilled water in 1000 mL flask. Then 30 mL of 3 M H_2SO_4 was added. Finally, 500 mL of distilled water was added to the flask. A change in colour from transparent to brown orange was noticed.
Vitamin C standard solution: 0.252 g of vitamin C powder was dissolved in distilled water up to a final solution volume of 250 mL. 1% starch indicator: 0.50 g of soluble starch was mixed with 50 mL of hot water and then cooled down to room temperature.

3.5.2. Titration process

Before titrating the samples, the iodine solution was titrated against the standard vitamin C solution by carrying out the following procedure.

20.00 mL of the standard vitamin C solution was poured into the erlenmeyer flask using the pipette. The burette was filled with the iodine solution. Before starting the titration, 10 drops of starch solution was added. Once everything is ready, the stop cock was opened and mixes the solution with the substance that contains vitamin C slowly until it was changed colour (normally deep blue or grey). This would mean the ascorbic acid has been fully oxidized and now iodine is in excess. The titration was repeated with a second and a third sample in order to reduce random error and to establish a mean value for the calculations. In the same manner the samples were titrated.

3.6. Kinetic study

Thermal degradation kinetics of ascorbic acid was studied by isothermal heating at 30, 40 and 50^0C respectively. Orange juice samples were taken in sealed glass tubes and heated by placing them in a hot water bath. In regular time interval of 30 min, the tubes were taken out and rapidly cooled by plunging in to ice water and analyzed for ascorbic acid content.

4. Result and Discussion

4.1. **Electrochemical deposition of gold nanoparticles**

Electrochemical deposition is a rapid and easy procedure for the production of nanoparticles on conducting surfaces, and many reports have been devoted to the electrodeposition of Au onto glassy carbon electrode (GCE) [53]. In this study gold nanoparticles were deposited on GCE from a solution of 0.1 mM $KAuCl_4$ by using amperometry by stepping the potential from 1.1 V for 5 s to 0 V for 5 s. The deposition of gold on GCE was confirmed by running CV in 0.5 M H_2SO_4. As shown in the figure below (Figure 2) there is a difference between two scans, this approves the deposition of Au onto glassy carbon electrode. Figure 2, scan A shows the cyclic voltammogram of the supporting electrolyte at GCE before Au deposition. However, Figure 2 scan B shows the cyclic voltammogram of the supporting electrolyte after the deposition of Au onto GCE. The appearance of sharp cathodic peak around 0.95 V is an indication of gold electrodeposition onto the GCE surface that corresponds to gold oxide reduction. Earlier studies also reported that the reduction of gold oxide takes place around the same [50].

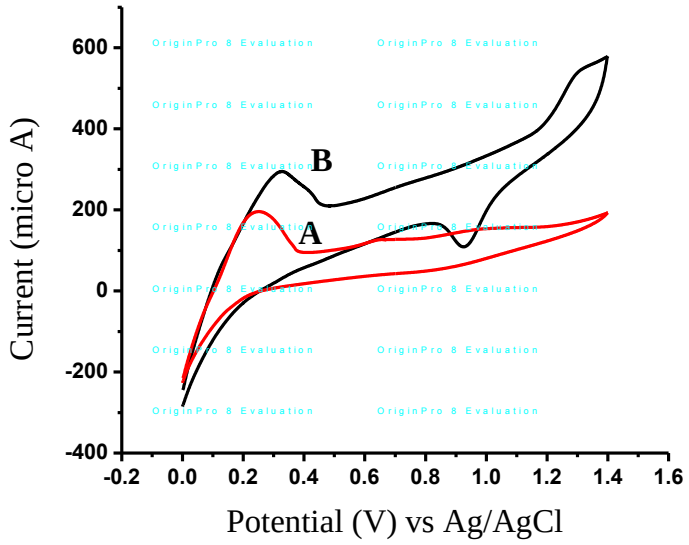


Figure 2: Cyclic Voltammogram of 0.5 M H_2SO_4 A) at bare GCE; B) at gold electrodeposited GCE (v = 50 mV/s)

4.2. Grafting of p-phenylenediamine film on GCE

Generation of an organic radical in solution near a surface can lead to attack of the radical on the surface, resulting in the formation of a covalently bonded film at the surface [54]. The most common method used to generate radicals is the reduction of aryldiazonium salts. Grafting using diazonium salt solution can be carried out either electrochemically [55] or non-electrochemically [56]. In the electrochemical method, the aryldiazonium cation is reduced by application of an external potential, leading, via homolytic dediazoniation, to an aryl radical that grafts onto the electrode surface through a covalent bond. Electrografting of a wide range of aryldiazonium salts have been shown to proceed at carbon [54], metal [57] and semiconductor surfaces [58]. In this study electrochemically induced modification of GCE with p-PD film was conducted in situ by recording cyclic voltammetric scans in the presence of 3 mM *p*-phenylenediamine and 3 mM $NaNO_2$ in 0.5 M HCl on AuNPs deposited GCE (Figure 3A). Finally the AuNPs was stripped by 0.1 M KCl (Figure 3B).

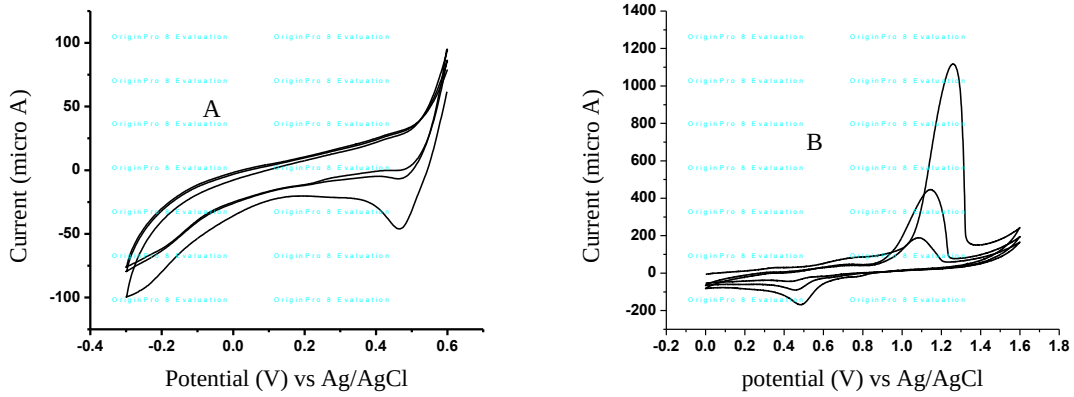


Figure 3: (A) Electrochemical reduction of in situ synthesized diazonium from 3 mM *p*-PD and, 3 mM $NaNO_2$ in 0.5 M HCl solution. (B) Cyclic voltammogram of AuNPs nucleated p-PD modified GCE in 0.1 M KCl (v = 100 mV/s in all case)

4.3. Electrochemical characterization of p-phenylenediamine grafted glassy carbon electrode

The film produced by electro grafting of diazonium generated from p-PD was studied by CV using different redox probes.

4.3.1. Cyclic Voltammetry of $K_3Fe(CN)_6$

The CV of PD film modified GCE is shown in Figure 4 that illustrated by looking at the one electron reduction of ferricyanide to ferrocyanide. The ferricyanide/ferrocyanide redox couple exhibits nearly a reversible electrode reaction. Thus, the couple has been a popular choice through years to characterize electrode modifications. As depicted in Figure 4, the redox peaks current of ferricyanide at nanohole PD grafted GCE was observed to be higher than that of bare GCE; this is due to electrostatic interaction between the positively charged film PD with negatively charged ferricyanide. This statement is supported by earlier studies of Bikila [49].

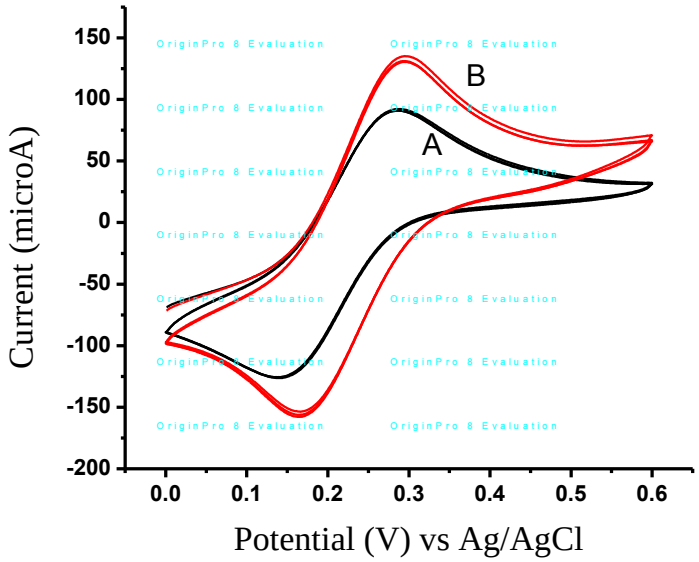


Figure 4: Cyclic voltammogram of 10 mmol/L $K_3Fe(CN)_6$ in 0.1 mol/L KNO_3 at (A) bare GCE, (B) nanohole PD grafted GCE; Scan rate 100 mV/s.

4.3.2. Cyclic voltammetry of hydroquinone

Figure 5 shows cyclic voltammogram of HQ at bare GCE and nanohole PD grafted GCE. Theoretically electrochemical response of HQ should not be affected by positive or negative charged films formed on electrodes; because HQ is neutral and is not attracted nor repulsed by charged films; but at nanohole PD grafted GCE redox peak of HQ increased relative to at bare GCE due to holes formed at modified electrode that could change diffusion from planar to 3D.

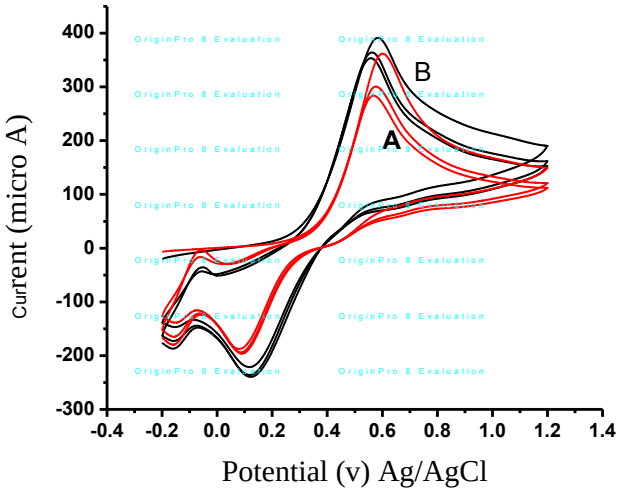


Figure 5: Cyclic voltammogram of 10 mM HQ in 0.1 M KNO_3 at (A) bare GCE, (B) PD grafted GCE; scan rate 100 mV/s.

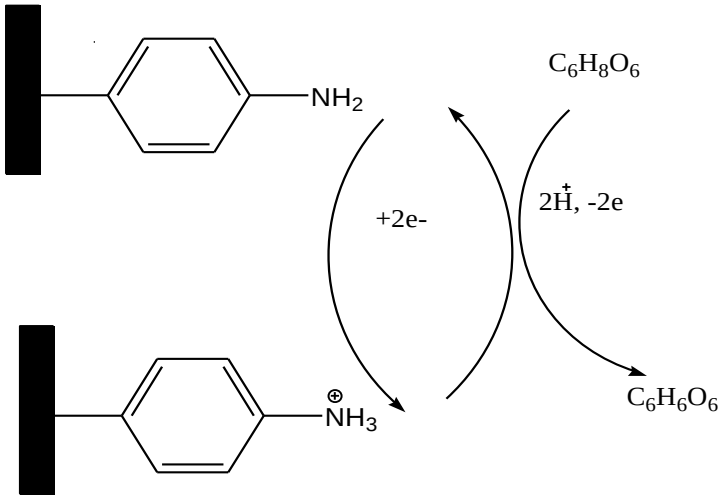
4.4. Electrochemical oxidation of AA at the p-PD grafted nanohole GCE

Because of the enhancement of ferrocyanide at the p-PD grafted nanohole GCE due to the anionic form has attracted to determine the ascorbic acid at this electrode.

To investigate the electrocatalytic activity of the p-PD grafted nanohole GCE toward the oxidation of AA, the electrochemical behaviors of AA were studied in different electrodes (bare and modified) by cyclic voltammetry in 0.1 M acetate buffer (pH 5.0) at a scan rate of 100 mV s^{-1}, as shown in Figure 6. Figure 6A shows the voltammetric response of AA at bare GCE and a broad anodic peak of AA is irreversible observed at about 0.62 V. Compared with the bare GCE, the oxidation peak current of AA increase significantly at p-PD grafted nanostructured GCE and the oxidation peak potential shifted to 0.30 V (Fig. 6B). The obviously increased peak current and the decrease in the anodic over potential for AA demonstrate an efficient oxidation of AA at the p-PD grafted nanohole GCE. The oxidation of ascorbic acid at the modified glassy carbon electrode is in agreement with previously studies (315 mV) [47] (Scheme 3).

The oxidation of AA was reported to proceed via two consecutive one-electron processes, involving a predissociation of proton to give the monoanionic species followed by a one-electron, one-proton oxidation of the monoanionic species to form a radical anion, which then

undergoes a second one-electron oxidation to dehydroAA [59, 60]. The latter is rapidly protonated and then dehydrated to form the final electro inactive product of 2, 3-diketogulonic acid. Therefore only the anodic anion peak of AA can be observed and the cathodic peak cannot be observed even at high scan rate, which is in agreement with the results obtained by Wehmeyer and Wightman [61].



Shcheme 3: proposed mechanism of electro-oxidation of ascorbic acid at nanohole p-PD grafted GCE

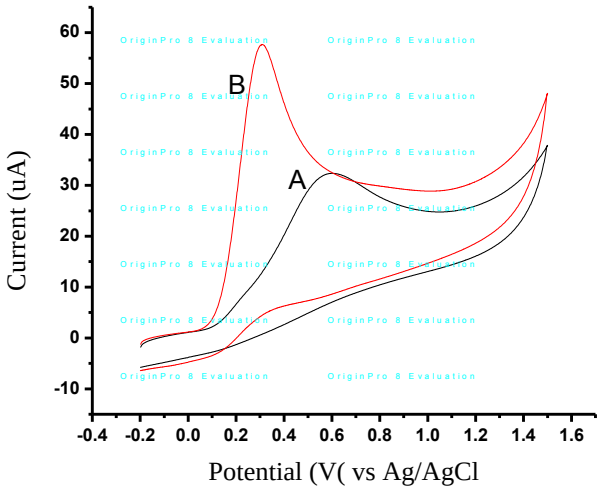


Figure 6: Cyclic voltammogram of 2mM AA in 0.1 M acetate buffer at (A) a bare GCE; (B) a p-PD grafted nanostructured GCE; Scan rate: 25 mV s^{-1}.

4.5. Amperometric response of AA at p-PD grafted nanohole GCE

An Amperometric measurement of AA was carried out in 450 rpm stirred solution and the results demonstrated that the p-PD grafted nanohole GCE has a good response for ten sequential additions of 10 μM AA (Fig. 7). Keeping the p-PD grafted nanohole GCE at 0.35V, after spiking the AA solution, the oxidation current increased and reached the steady-state current fastly, within a few seconds, indicating a fast reaction at the electrode, the raw data are given in Figure 8 and 9. Figure 7 further shows that steady-state current increased linearly with the AA concentration. The linear range was from $5x10^{-6}$ M to 10^{-2} M with sensitivity of 0.04869 μA $(\mu M)^{-1}$ and an intercept of -0.6973 μA with a correlation coefficient, r, of 0.9999. The limit of detection (LOD), defined as the ratio of three times standard deviation of the blank and the slop, was found to be 3.62×10^{-7} M.

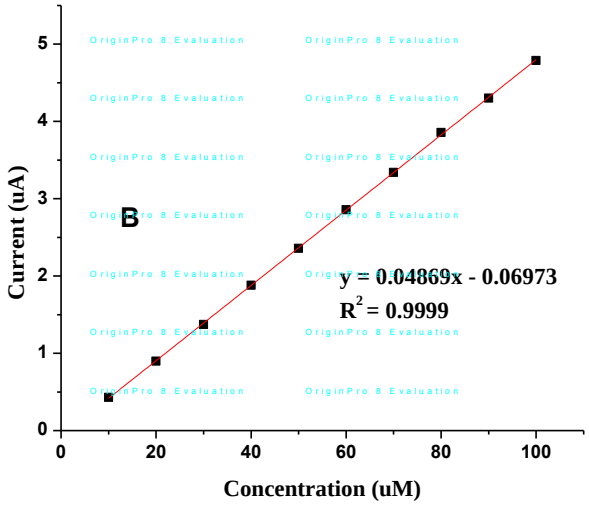


Figure 7: Calibration curve for determination of AA at A) bare GCE B) nanohole PD grafted GCE; in 0.1 M sodium acetate buffer (pH 5) (the average of triplicate measurement)

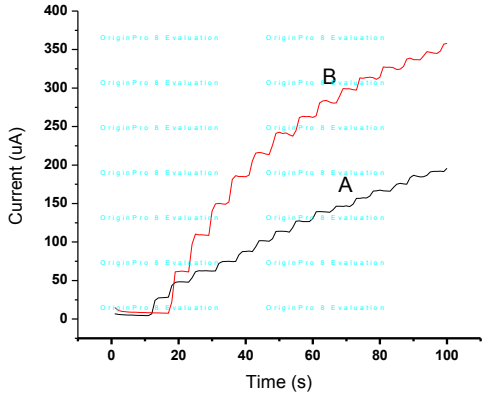


Figure 8: Amperometric current vs. time curve upon additions of 1mM AAs into a stirred system of 0.1 mol/L sodium acetate buffer (pH 5) at A) bare GCE B) nanohole PD grafted GCE

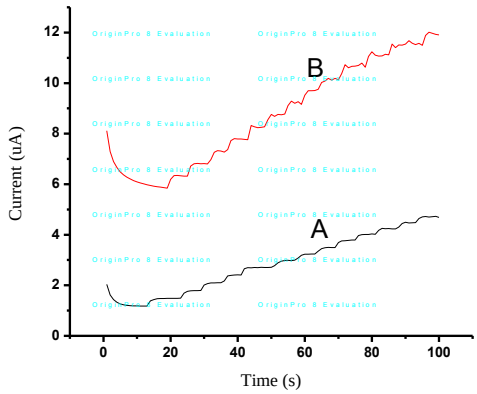


Figure 9: Amperometric current vs. time curve upon additions of 10µM AAs into a stirred system of 0.1 mol/L sodium acetate buffer (pH 5) at A) bare GCE B) nanohole PD grafted GCE

Table 1: Triplicate measurement of ascorbic acid amperometrically at PD modified GCE

Concentration (µM)	Current response (µA)	RSD/%
10	0.430	15.74
20	0.899	6.54
30	1.373	6.18
40	1.879	3.92
50	2.359	3.76
60	2.857	3.66
70	3.339	4.11
80	3.856	2.79
90	4.301	0.32
100	4.789	0.35

4.5.1. Study of interference

The influence of possible interferents, such as glucose (GLU), citric acid (CA) and tartaric acid (TA) which may co-exist in fruit may interfere with detection of AA. Electro-oxidation of AA in the presence of above possible interfering substances, under similar conditions was studied at a fixed concentration of 1 mM AA and 400-fold excesses of interfering species at nanohole PD grafted GCE. But, as shown in the Figure 10a these substances could not interfere with the determination of ascorbic acid in orange fruit. However, at bare GCE amperometric determination of ascorbic acid was interfered by interferents (Figure 10b). These results indicate that the selective and sensitive determination of AA can be achieved on the nanohole p-PD grafted GCE.

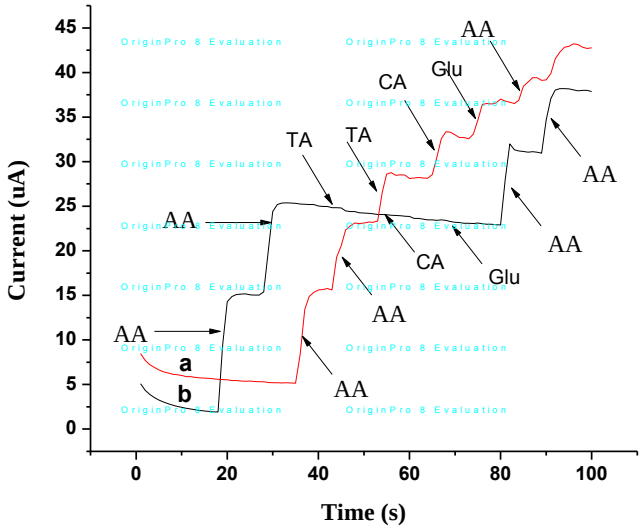


Figure 10: Amperometric response of a) 1 mM AA and interfering species at bare GCE b) 1 mmol/L AA and 400-fold excesses of interfering species at nanohole p-PD grafted GCE at 0.35 V

4.6. Amperometric determination of ascorbic acid in orange under different storage conditions

Ascorbic acid was determined amperometrically at PD nanohole grafted GEC starting from the day collected from the farm to various days stored in different conditions.

To determine the concentration of vitamin C in orange fruit juice, standard addition method was used. The concentration of ascorbic acid in the orange juice samples was obtained from calibration curves using the following formula.

$$|a| = \frac{b}{m}$$

Where, *a* is x-intercept, b is y-intercept and m is the slope of the calibration curve.

The concentration of ascorbic acid was measured starting from fresh level at farm through transportation and different storage conditions. The orange samples which were collected from the farm were preserved in portable fridge and transported to the laboratory to amperometrically analyze the level of ascorbic acid. The results of the concentrations of AA in fresh orange juices determined at farm site and in laboratory from the time of arrival to six days are shown in Table 2. As has been seen in Table 2, the concentration of AA was the highest in fresh juices and started to gradually decrease on storage. This indicates the concentration of AA in orange fruit is decreased upon transportation may be due to air dehydration. The result obtained in fresh juices is comparable with reported values [62].

Table 2: Determination of ascorbic acid in orange fruit juice at nanohole PD grafted GCE

Time (days)	**0**	**1/6**	**1/2**	**1**	**2**	**4**	**6**
Conc. (mM)	2.30	2.17	1.82	1.35	1.10	0.95	0.83
SD	0.04	0.03	0.11	0.04	0.06	0.04	0.04

($t_{critical}$ = 2.78, p = 0.05)

As shown in the above table (Table 2) the concentration of ascorbic acid in orange fruit juice for each step the difference was statistically significant. The calculated t-value at fresh level with laboratory after transportation from farm, laboratory after transportation from farm with overnight in the laboratory, overnight with one day, one day with two days, two days with four days and four days with six days was found 4.5, 5.38, 7.23, 6.25, 3.75 and 3.64 respectively. This shows the difference in concentration of ascorbic acid at each step is significant.

Percent loss of ascorbic acid during transportation was 6 %, after overnight 21 %, after one day 41.3 %, after two days 52.2 %, after four days 59 % and after six days 64 %.

The degradation of AA concentration in orange fruit juice during storage under refrigerated conditions for 24 hrs and 6 days of storage is presented in figure 12. The loss of concentration increased with storage period under refrigerated conditions. The trend in loss of the concentrations of AA in orange fruit juice on storage in refrigerator is in agreement with the earlier report by Singh and Reddy [63].

Table 3: Concentration of ascorbic acid in different time storage in refrigerator

Times (Day)	0	1	6
Concentration (mM)	2.30	1.98	1.18

4.7. Stability and reproducibility of nanohole p-PD grafted GCE

The prepared nanohole PD grafted GCE was examined its stability and reproducibility. To test its stability, the prepared electrode was kept in acetate buffer (pH 5) for two weeks and then, when 2 mM AA was measured the response of the electrode was 56 μA, which is in close proximity with the value of the fresh electrode (59 μA). Therefore, the prepared electrode has good stability. The reproducibility of the electrode was tested by preparing three different electrodes under the same conditions to measure the peak current standard ascorbic acid (2 mM), the results obtained were 54, 59 and 61 μA (SD = 3.6), indicating the reproducibility of the prepared electrode.

Table 4: Comparison of the Limit of Detection of the Fabricated Electrodes with Some Literatures

Electrodes	LOD	Linear range
Nanohole PD grafted GCE	3.62×10^{-7} M	$5x10^{-6}$ M to 10^{-2} M
MWCNT-GCE[64]	1.4 μM	0.0047–5.0 mM
Cobalt (II) phthalocyanine–multi-walled carbon	1.0×10^{-6} M	1.0×10^{-5} M to

nanotubes modified glassy carbon (CoPc–MWNTs/GC) electrode[65]		2.6×10^{-3} M
poly (Evans Blue) modified glassy carbon electrode[39]	2.5×10^{-7} M	1.0×10^{-6} to 3.0×10^{-5} M

4.8. Determination of ascorbic acid by iodometric titration

Ascorbic acid can be determined by titration from different citrus fruits [-]. A titration is a means of quantitative analysis in which the substance to be measured (in a liquid solution) is reacted stoichiometrically with another reagent (called a titrant) until it has completely reacted. The end of the reaction is usually signaled with the appearance of a color from another non-interfering substance called an indicator. In this case the titrant is iodine solution and soluble starch *used* as an indicator. The concentration of ascorbic acid was found 1.85 mM.

The statistical study of precision and accuracy of the proposed method was made from *F*-test and the *t*-test, respectively. The *t*-test was applied to the results obtained by the nanohole PD grafted GCE and the iodine method, and it showed that calculated *t* values (0.56) were lower than the tabulated *t* value ($t = 2.78$, $P = 0.05$). This suggested that at 95 % confidence level differences between the results obtained by the two methods were statistically not significant. The *F*-test revealed that there is no difference between the precision of the two methods. In every case, the calculated value of *F* (13.44) was lower than the critical value ($F = 19.00$, $P = 0.05$). Thus, the proposed method can be successfully applied to real samples (Table 5).

Table 5: Determination of AA in orange fruit using nanohole PD grafted GCE and titration

Concentration of ascorbic acid (mM)	
Titration	Amperometric
1.85±0.03	1.82±0.11

4.9. Kinetics of ascorbic acid degradation in orange fruit juice

Prolonged exposure of fruit juices to high temperature results in degradation reaction and ultimately in poor quality of final products. One of the commonly occurring degradation in fruit

juices is ascorbic acid loss. A general reaction rate expression for degradation kinetics can be written as follows[66-68]

$$d[C]/dt = k[C]^m$$

where 'C' is the quantitative value of the degraded product under consideration, 'k' is the reaction rate constants and m is the reaction order. The rate of degradation of ascorbic is first order and the equation of first order reaction is obtained upon integration of the above equation and can be written as

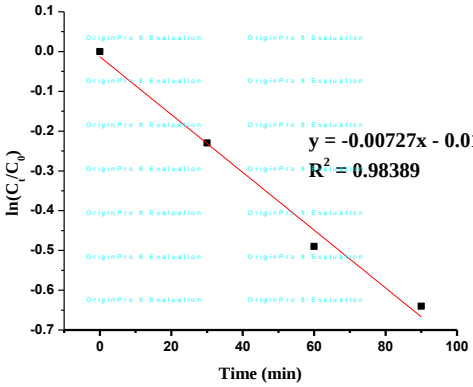
$$\ln([C]_t/[C]_0) = -kt.$$

$[C]_0$ is the value of the product at time 0 and $[C]_t$ is the value after reaction time t.

The plots of $\ln([C]_t/[C]_0)$ versus t are shown in Figure 14A-C and the first order rate constant k were calculated from the slope of the straight line. In all cases, a correlation coefficient > 0.9 confirms that the degradation of ascorbic acid in orange juice follows a first order reaction at all the temperatures. Earlier studies also reported that the ascorbic acid degradation follows first order kinetics in aqueous solution [69, 70]. $t_{1/2}$, the time required for ascorbic acid to degrade 50% of its original value was calculated from the rate constants by using 0.693/k.

Table 6: Effect of thermal treatment on ascorbic acid concentration (mM) in orange juice

	Temperature (°C)		
Time (min)	30	40	50
0	1.64	1.12	0.85
30	1.30	0.89	0.67
60	1.00	0.58	0.47
90	0.86	0.45	0.24



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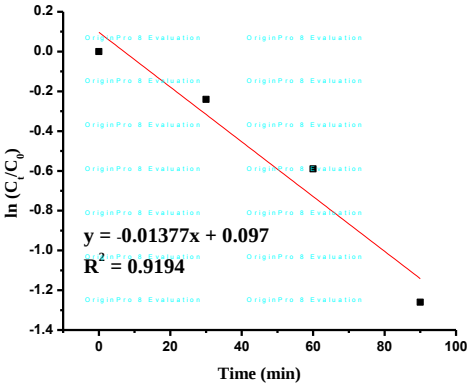

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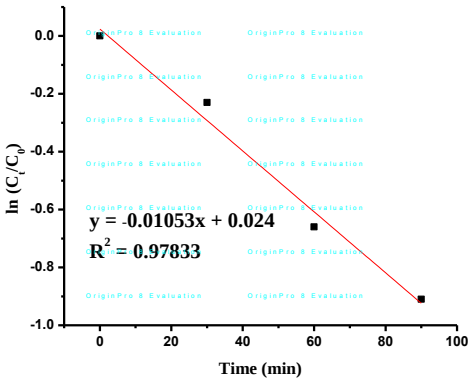

B

Figure 11: Kinetics of ascorbic acid content of orange juice at A) 30°C B) 40°C and C) 50°C

The relationship of reaction rate to temperature was quantified by the Arrhenius relationship

$$k = A_0 \exp^{(Ea/RT)}$$

where Ea is the activation energy of the reaction, R is the universal gas constant, T is the absolute temperature and A_0 is pre exponential constant. As shown in Figure 15, the value of Ea, calculated from the slope of the graph, is 3.39KJ/mol and A_0 is 0.96 min^{-1}.

Table 7: kinetic parameters for ascorbic acid degradation in orange fruit juice

Temperature (°C)	30	40	50
Temperature (K)	103.15	113.15	123.15
1/T(K)	$9.69x10^{-3}$	$8.84x10^{-3}$	$8.12x10^{-3}$
k	$7.27x10^{-3}$	$1.05x10^{-2}$	$1.38x10^{-2}$
lnk	-4.92	-4.56	-4.28
R^2	0.98389	0.97833	0.9194
$t_{1/2}$ (min)	95.8	66.0	50.2

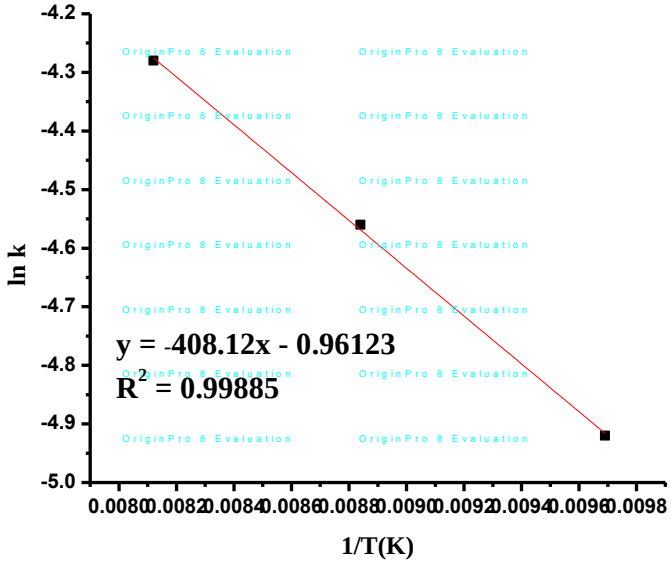

Figure 12: Arrhenius plot of the ascorbic acid degradation rate of orange fruit juice

5. Conclusion and recommendation

Form the study, the amount of ascorbic acid in orange fruit was found to be high when it is fresh and as soon as collected from the tree. Then the level gradually decreases with temperature, air dehydration, transportation and storage. During storage of orange fruit in refrigerator, the decrease in the amount of ascorbic acid is least amount. However, the oranges have to be used immediately after taking out from the fridge. This indicates the influence of storage on the fruit affecting the ascorbic acid content.

The society should use the orange fruit immediately after it is collected from the tree to get the required ascorbic acid (vitamin C) from the fruit. Based on the WHO guideline the recommended daily intake of AA is about 70–90 mg, therefore to get these amounts of AA people of Jimma and its surrounding should consume 170–220 mL of orange juices per day. As has been observed to protect the degradation of AA from the orange juices it's better to preserve the fruits in cool environment instead of storing in a deep freeze until consumption.

6. References

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