OXIDATIVE AND TITRIMETRIC DETERMINATION OF ASCORBIC ACID IN SOME HERBAL SAMPLES

BY

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CERTIFICATION

I certify that the project work titled ***Oxidative and Titrimetric Determination of Ascorbic Acid in Some Herbal Samples***, was carried out by NAMI MARCEL GAILA, of the department of chemistry, federal University of Technology, Yola.

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DEDICATION

This research work is dedicated to my dear husband Engr. MARCEL GAILA, my children IBRAHIM, TAMAR, FATIMA and YERIMA and to the memory of my late sister NANA KEVIN PETER. I really appreciate your love, care, support and understanding.

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To my course mates, I really appreciate you all. You are great and wonderful. I pray that we shall all live long to testify the goodness of the lord in Jesus name. It is also my prayer and hope that we all come back for our Ph D program soonest.

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To my friends and well wishers, I want to thank you all for your support and prayers and supports, the blessings of God will forever remain with you in Jesus name.

ABSTRACT

In this work spectrophotometric and titrimetric methods were used to determine the vitamin C contents of some fruits, pharmaceutical and herbal samples. In the spectrophotometric method excess Cu (II) was used to oxidize vitamin C. The excess Cu (II) was then determined by complexation with alizarin red S (ARS) and used to calculate the amount of vitamin C in sample. This method showed an excellent linearity (r^2=1) over a concentration 1.5 -8.2 µg/ml of standard ascorbic acid solution. In this method the amount of vitamin C content ranged from 22.43±0.56-45.78±0.22 mg/100g in fruits 17.88±2.43-60.18±52 mg/100g in fresh leaves, 9.01±0.77-41.67±1.34mg/100g in dry leaves and 116.87±23.67-506.34±56.83 mg/100g in pharmaceutical formulations. Both AOAC titrimetric and direct titration with iodine were also used in the quantitation of vitamin C in the plant samples. The ascorbic acid content determined using AOAC method ranged from 24.88±+0.18-46.22±0.45mg/100g in fruits, 18.06±0.34-67.0±1.09 mg/100g in fresh leaves and 10.05±0.54-42.88±1.50 mg/100g in dry leaves of the plants. The direct titration method showed a range of 26.12±0.66-46.96±0.20 mg/100g in fruits, 18.78±1.32-66.87±2.45mg/100g in fresh leaves and 11.44±1.08-40.36±0.43mg/100g in dry leaves of the plants. All the methods could be used to analyze vitamin C with good precision (RSD 1.06 to 6.46). Hence the direct titrimetric method and the DCIP methods could be reliably used for routine analysis of vitamin C in plant samples in laboratories where expensive equipments are not available. All the methods adopted in this work were validated using recovery (%R) of vitamin C. The %R were found to be high ranging from 91.22±2.89-104.1.11 mg/100g in spectrophotometric method and 97.21±3.32-106.12±3.36 mg/100g in direct titrimetric method.

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CHAPTER ONE
INTRODUCTION

1.1 BACKGROUND OF STUDY

Traditional medicine is vital yet often neglected part of healthcare in the developing world. In Africa and Asia, more than two in three people rely on traditional remedies as their primary healthcare. Pharmaceutical companies and medical researchers in the developed world, faced with rising drug development costs and growing drug resistance, are increasingly turning to traditional medicine to find solutions to the world's most pressing health problems. Integrating modern and traditional medicine faces numerous challenges stemming from the fundamental differences in how each is practiced, evaluated and managed. How can medical researchers and practitioners bridge the gap? As a starting point in closing this gap a study of some relevant constituents of herbs and fruits which could find applications as nutraceuticals should be undertaken.

Nowadays, it is commonly accepted that diet with high contents of fruits and vegetables are protective against severe human diseases, some of which are especially serious, such as cardiovascular diseases and cancer. Owing to the fact that many studies seem to reveal that these protective effects may be a result of the intake of antioxidants, more attention is being focused on potentially antioxidant substances, such as vitamin C and carotene(Mathews 1991, Rock *et al.,*1996;) Halliwell, 1997; Fraser and Brimley, 2004; Melendez-Martinez *et al.,* 2004).

Despite the fact that there are no conclusive in vivo studies in the field yet, consumption of high amount of fruits and vegetables on a daily basis is being recommended (Foods and Nutritional Board 2000). Ascorbic acid is one of the most important organic acids in fruits and vegetables, in relation to the nutritional value of these foodstuffs, Its content has not only been used as a nutritional index, but also for evaluating processing effects, since it is highly unstable (Rock *et al.,* 1996; Rojas Hidalgo, 1998) due to the fact that ascorbic acid is easily oxidized ,many of it functions and activities are known to be based primarily on its properties

as a reversible biological reductant. Aside from its antiscorbutic activity and its likely role in the serious human disease mentioned above, ascorbic acid is involved in many biological processes, such as the inhibition of enzymatic browning and the formation of nitrosamines, reduction of metal ions and improvement of the stability and utilization of folic acid and vitamin E among others. (Rock *et al.,* 1996; Rojas – Hidalgo, 1998)

Ascorbic acid (AA) is also an important vitamin that participates in wide variety of biological events concerning electron transport reactions, hydroxylation the oxidative catabolism of aromatic amino acids and so on. Measuring the concentration of a marker chemical commonly assesses food deterioration and product quality: ascorbic acid is one of such indicator, it is important to direct it selectivity and conveniently in routine analysis. Various methods have been employed for the measurement. Such as spectrometric (Pandey, 1982, Abdelmageed *et a,.l 1995*), thermometric titrimetry (Mayers *et al,. 1987)*, HPLC (Sood *et al* 1976) a kinetic methods (Karayannis *et al,. 1987)*, various modified electrode, (Liu *et al,.* 2000, Han *et al,.* 2001, Ren 2001) sol-gel (Wang, 2000) and a combination of various other techniques.

In order to verify acceptable measurement value, it is therefore important to understand the principles involved in measurement and to select good laboratory techniques for accurate reproducible results for application in areas of food analysis as well as adionostic tool in separation techniques in biochemical and pharmaceutical processes. Ascorbic acid is present in large amount of fruits, tomatoes, leaves, herbs and many commercial beverages.

The present methods used for the determination of ascorbic acid are many. A titrimetric using 2, 6- dichlorophenol indophenol is commonly used in many laboratories (AOAC, 2002). Although the method is rapid, the reagent is unstable and must be standardized for use moreover the method is unsuitable for coloured samples which interfere with the detection of the end point. Ascorbic acid can also be determined by micro-gravimetric method, though this method is very time consuming (AOAC, 2000). Differential-pulse polarography has been used to

determine ascorbic acid at µg/ml level but the method suffers from interference from electro active impurities of the sample.

Other methods are available for the determination of ascorbic acid in food samples. These analytical methods may give over estimates due to the presence of oxidisable species other than ascorbic acid and exclude measurement of Dehyodroasrobic Acid for example the AOAC procedure employing titration with 2, 6-dichorophenol indophenol in acid solution is not applicable to all matrices substances naturally in fruits such as tanning, sulphydry compounds, metals such as copper, iron, and cobalt are oxidized by the dye (Arya *et al,.* 2000).

One of the difficulties of a successful application of spectrophotometric method is that beyond specific limits, the intensity of the absorption is not directly proportional to concentration. Another disadvantage is that well-defined absorption band in the UV region of the spectrum is subject to interference from many other substances, this can be minimized using different extractant like oxalic acid. And these can be taken care of using a different extractant like oxalic acid, the interference can be minimized. The optimal method for ascorbic acid determination are separation techniques, including capillary electrophoresis, gas chromatography and liquid chromatography which however cannot be use for routine analysis of a large number of samples (Versari *et al,.* 2004; Silva, 2005).

The enzymatic procedure that employs ascorbic acid oxidase is highly specific to this compound and utilizes the same as substrate for its reaction. Thus determination of vitamin C in the sample before and after the ascorbic acid oxidase/reaction, using appropriate formation method provides an accurate estimate of the vitamin C content. Hence, if an appropriate method of extraction is adopted and oxidation reaction is also applied the determination of vitamin C could lead to reliable results.

1.2 AIM AND OBJECTIVES OF WORK

The present investigation is carried out to compare the ascorbic acid contents of fruits and herbs used locally as food and traditional medicine. The specific objectives are:

(i) To identify fruits and herbs used as food and traditional medicinal applications.

(ii) To extract vitamin C from the fruits and herbs using meta-phosphoric-acetic method as extractant.

(iii) To determined the ascorbic acid content of the fruits, herbs and pharmaceutical formulations using indirect spectrophotometric and direct titrimetric quantitative methods in the extract.

(iv) To validate the quantitative methods used for the determination of vitamin C in the samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 STRUCTURE AND USES OF ASCORBIC ACID (VITAMIN C)

Ascorbic Acid (vitamin C) is essentially a white crystalline simple sugar derivative which is highly soluble in water and other similar solvents. It has a formula $C_6H_8O_6$. It is found almost entirely in foods of plant origin.

The vitamin is the most sensitive of all the vitamins to processing conditions, especially under neutral and alkaline condition. The ease with which the vitamin oxidizes makes its useful indicator of the nutritional and other quality changes in foods (Wilcox, 2006).

Vitamin C is an antioxidant i.e. it is used as nutrient that blocks some of the damages caused by free radicals, which are by products that react when our body transforms food into energy. The built of this by-product over time is largely responsible aging process and can contribute to the development of various health conditions like arthritis. Antioxidants also help reduce the damage of the body caused by some chemicals and pollutants such as cigarette smoke (Mosure, 2004).

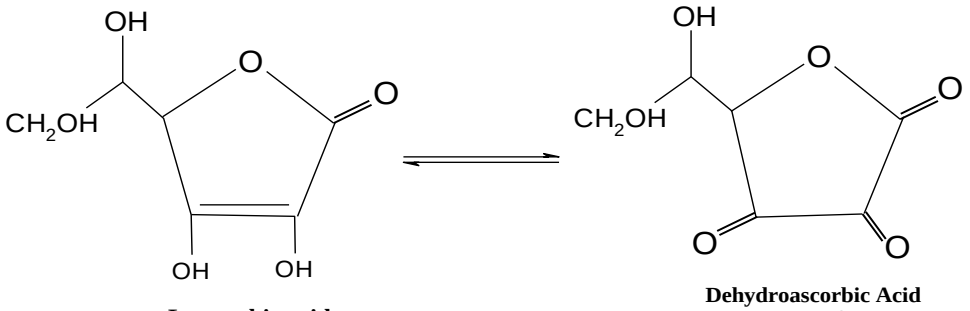
Ascorbic acid (AA) and its oxidized form dehydroascorbic acid (DHA) play an important role in the oxidative stress reactions. (Hagen *et al*, 1999). Generally the total AA is defined as the sum of both AA and its oxidized from. DHA is present in food matrices but it qualification remains, difficult because of the instability of the compound. The qualification of DHA is usually performed after its conversion into AA in the presence of reducing agents (Brauce, *et al., 2003*).

Vitamin C deficiency can lead to dry and splitting hair gingivitis (inflammation of the gum) and bleed gums, dry skin, decreased wound-healing rate, easy bruising, nose bleeding, weakened enamel of the teeth: swollen painful joints, anemia, decrease ability toward off infection and possibly weight gain because of slowed movement rate and energy expenditure. A severe form of vitamin C deficiency is known as scurvy, which mainly effects of malnourished adults (Mosure, 2004).

The body does not manufacture vitamin C on its own, nor does it store, it is therefore important to include vitamin C containing foods in ones daily diet. The most common sources of ascorbic acid are fruits and vegetables. Ascorbic acid was first isolated in pure crystalline form from lemon juice by the American Biochemist C. G. Using W. A. Waugh. In 1932, but it has been known in existence since 1700 A.D. Ascorbic acid helps in keeping enzymes in their get rive state. Oxidation combination ascorbic acid to dehydroascorbic acid which decompose irreversibly by hydrolysis of the lactone ring (Zubay, 1995). Ascorbic acid plays many crucial roles in growth and metabolism. A potent antioxidant, AA had the capacity to eliminate several different reactive oxygen species. In a reduced state, they act as a cofactor maintaining the activity of a number of enzymes (by keeping metal ions in the reduced state) (Arrigoni & De Tullio, 2002; Davel *et al*,. 2002; Klem & Kurrilich, 2000).

2.1.1 Structure of Ascorbic acid

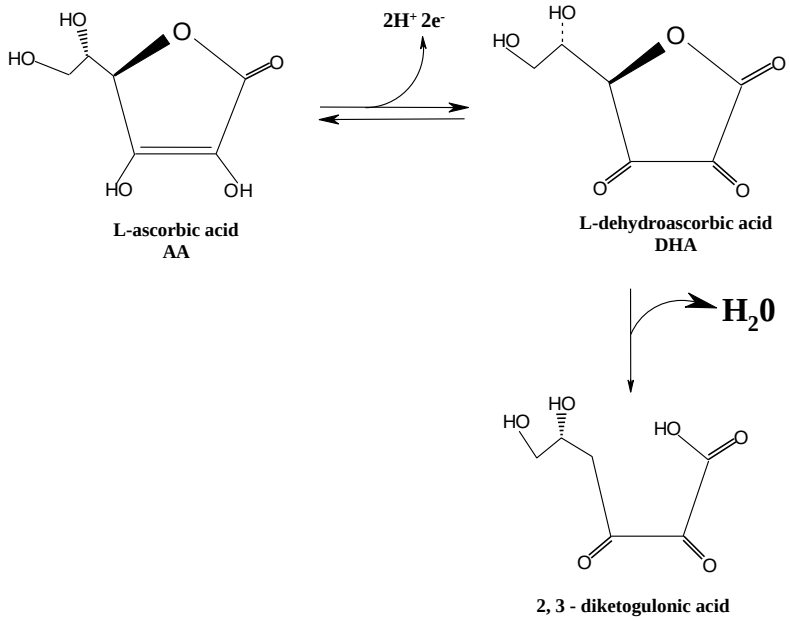
Ascorbic acid is highly soluble compound that has both acidic and strong reducing properties. These qualities are attributable to its enediol structure, which is conjugated with the carbonyl group in a lactone ring. The natural from of the vitamin is L-isomer. The D-isomer has about 10% of the activity of the isomer and is added to foods of non-vitamin purpose (Wilcox; 2006).



L-ascorbic acid **Dehydroascorbic Acid (Oxidized form)**

In solution, the hydroxyl on C_3 readily ionizes (pk_1 = 4.04 at 25^0C) and a solution of the free acid gives a pH of 2.5. The second hydroxyl is much more resistant to

ionization (pk_2 = 11.4). The oxidation pathway of ascorbic acid in solution can be depicted by the reaction outlined below.



L-ascorbic acid
AA

L-dehydroascorbic acid
DHA

2, 3 - diketogulonic acid

2.1.2 Medical Applications

Low level of vitamin C has been associated with a variety of conditions including hypertension, gall bladder disc entry smoke, some cancers, and atherosclerosis (The buildup of plaque in blood vessels that can lead to heart attack, stroke conditions caused by atherosclerotic build up are often collectively referred to as cardiovascular disease). Eating adequate amount of vitamin C in the diet primarily through a lot of fresh fruits and vegetables helps to reduce the risk of developing some of these conditions. There is a little evidence however, that vitamin C supplements can cure any of these disease. As an antioxidant, vitamin C plays an important role in protecting against the following heart disease: Osteoarthritis, Obesity and weight loss, cataracts, age – related muscular degeneration, diabetes, Alzheimer's disease and other type of dementia, high cholesterol, high blood pressure, common cold, cancer etc. (Roberta, 2002).

2.1.3 Stability of Ascorbic Acid and Dehydroascorbic Acid

Stability is a key problem of AA and DHA analysis because the compounds are known to be very unstable in aqueous solution as shown in Figure 1. There are lots of factors that negatively influence there stability (i.e. light, increase in temperatures, increased pH, and the presence of oxygen or metal ions) it is therefore necessary to keep the influences of their variables to a minimum (Song, *et al.,* 2000).

Stability of compounds in solutions, at various conditions (temperature, pH light and presence of divalent cations) and with addition of stabilizing agents has been part of many method of development and validation studies for obtaining good results for vitamin C in samples. The influences of these conditions are reviewed in the subsequent sections.

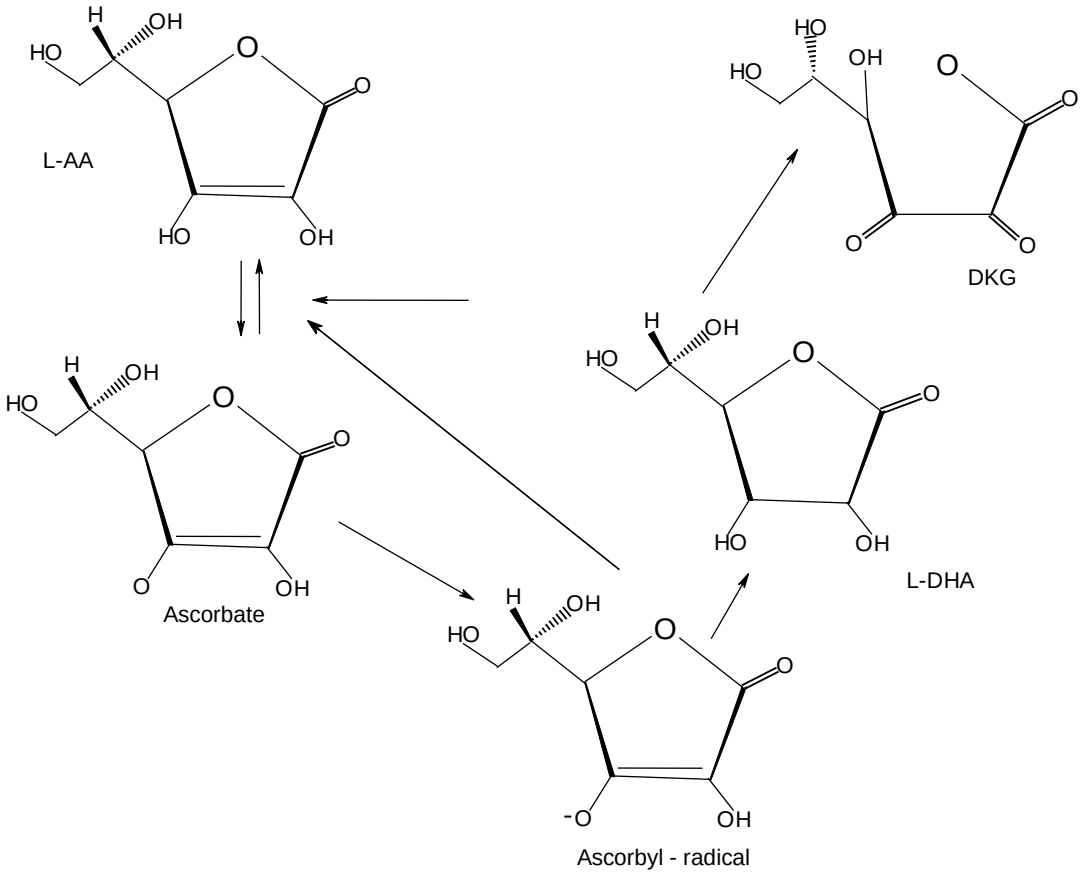


Figure 1: Structure of Ascorbic Acid and its Degradation Products

2.1.4 The Influence of Light

AA and DHA are known to be susceptible to degradation by light. This was studied by Iwase (2000). The effect of the natural light and of UV light (265nm) on the stability of AA in solution was examined in order optimized the choice of glassware. Periodic changes of AA stored in a brown flask and in a plug-free, transparent flask, which was exposed to UV light were compared at room temperature. The results of the experiment demonstrated that degradation of AA was affected by both natural and UV light. After 1 hr of the experiment, the initial concentration of AA decreased to 79% under the influence of natural light. The initial concentration decreased to 84.2% in the transparent flask and to 95.6% in the brown flask (Iwase, 2000). The results from the experiment show that AA is apparently more stable when it is stored in brown volumetric flasks, which appear to protect it from natural light. Some approaches also recommended protection using aluminum foil. Generally, most articles have highlighted the importance of protecting AA solution from natural light.

2.1.5 The Influence of Temperature

Temperature has been described as one of the key factor, which significantly influences the stability of AA and DHA in solution. The effect of the temperature on the stability of AA has been studied in many research groups (Novakova *et al.,* 2008; Iwase, 2000; Gibbons *et al.,* 2001; Chang *et al.,* 2003).

Generally, temperatures 4^0C 10^0C and 25^0C were used in experiments. Decreasing temperature (e.g. in modern auto-samplers) has enable sample cooling during analysis up to 4^0C to improve stability. Stability studies at higher temperatures confirmed a large degree of degradation of AA. The concentration of AA at 60^0C and 80^0C were decreased within to less than 20% of initial concentration at 40^0C the concentration decrease to 75% (Iwase, 2000). The solution of AA at laboratory temperature was stable for 1 hr.

2.1.6 The Influence of pH

AA exhibits higher stability in solution under acidic conditions. At these conditions, the formation of ascorbate, the main degradation product is not

favoured. (See figure 1) most analytical methods included sample preparation extraction at acidic conditions. Some studies involved a comparison of extraction agents of various pH and their impact to AA stability (pH 2.1, pH 2 and 6.9, pH 6.8). Generally, acidic pH around 2.1 was useful for some preparation, ensuring sufficient stability and recovery of AA. M-phosphoric acid (MPA) was the most widely used extractant (Odriozola,*et al*., 2007; Kafkas *et al*., 2006; Karlesen *et al.,* 2005; Romeu, *et al*., 2008; Romeu *et al.,* 2006) sometimes in combination with EDTA (Emadi *et al.,* 2005; Vovk *et al*., 2005) or with some organic addictives (e.g. methanol (Frenich *et al*., 2005) or methanol acetonitrile (Maia *et al*., 2007).

In some cases, extraction was performed at neutral pH using various buffers (NaOH 1M phosphate buffer 5.5, phosphate buffer (Heudi, 2005 and Tai *et al.,* 2006). under hydrophilic interaction liquid chromatography (HILIC) conditions, it was very important to keep organic modifier at high concentration, and extraction was performed using ammonium acetate buffer in order to provide similarity to the HILIC mobile phase (Novakova *et al.,* 2008 and Tai, 2007).

Change in concentration of the organic modifier could significantly influence the partition equilibrium in HILIC and could lead to the shift of retention times and irregular peak shapes. Oxalic acid (Hernandez, *et al.,* 2006). Its mixture with meta-phosphoric acid (MPA) (Kali, *et al.,* 1999) or trichloroacetic acid (Salminen *et al.,* 2008 and Burini, 2007) has been among the extractants used for the sample treatment before analysis of AA and DHA.

2.1.7 The Influence of Concentration

Concentration of AA and DHA in solution could also influence stability. Rumeline *et al* Iwase, *et al.,* and Novakova et al., 2008 studied the stability of AA in solutions of different concentration and shown that the higher the concentration the better the stability, the stability was found to decrease significantly at concentration below 0.1mg/l (Novakova *et al*., 2008).

2.1.8 The Influence of Stabilizing Agents

Stabilizing agents have often been used to improve the stability of AA and DHA. Typically, MPA was able to fulfill the roles of extractant and stabilizer

(Odriozola, *et al.,* 2007; Kafkas *et al.,* 2006; Karlesen *et al*., 2005; Romeu,*et al.,* 2008; Romeu *et al*., 2005). Its combination with EDTA was also found to be efficient (Emadi *et al,* .2005; Lykkesfeldt and Anal., 2000). Among other stabilizers trichloroacetic acid (Burini, 2007), O-phosphoric acid (Novakova *et al*., 2008; Kefkas *et al*., 2006; homocysteine; Howard *et al.,* 1987) Oxalic acid (Novakova *et al.,* 2008), EDTA (Rumelin *et al*., 2000) trichorofioroacetic acid, TCA (Klejdus *et al*., 2004) or their combination TCA+EDTA (Salminen *et al.,* 2008) metabisulfile/glutathione or combination of citric acid/pyrogallol (Muller & Pharm, 2001) also proved to prolong the stability of AA/DHA in solutions. Also less common stabilizing agents (eg L-cysteine, L-methionine, monosodium L-glutanate (MSG amino acid) and guanosine -5- monophosphate (nucleic acid) (Iwase, 2000) were tested.

2.1.9 The Influence of Metal Ions

The presence of metal ions has also been described as one of the factors that could decrease the stability of AA/DHA in solutions, for this reason, EDTA, as a chelating agent, can improve stability (Iwase*,* 2000,). As chelating agents EDTA and MSG were found to be able to assure the stability of AA in the standard solution sufficiently; (Muker *et al*.,) Examines the influence of Cu^{2+}, fe^{2+}, $mg^{2+,}$ $Ca^{2+,}$ Mn^{2+} and metal ions where the amount of each element corresponded to the amount added to multivitamin preparations. Only Cu^{2+} was found to influence the content of AA significantly.

2.2 Sources of Vitamin C

Ascorbic acid occurs mainly in foods of plant origin. Fruits are usually good sources but mainly popular eating apples, pears and plums supply negligible amount. Green vegetables and potatoes are the most important sources of ascorbic acid in the British diet. The amount of ascorbic acid present in vegetables is greatest in the period of active growth during spring and early summer. Storage decreases the ascorbic acid content and this can be clearly seen as given in Table 2.1.

Table 2.1: Average Values for Ascorbic Acid Content of Foods (mg/100g)

Black currants	200	Tomato (raw or juice)	20
Rosehip syrup	175	Liver (Lambs, fried)	19
Sprouts (raw)	87	Potatoes (raw, new)	30
Sprout (boiled)	41	Potatoes (raw Oct. - Nov.)	20
Cauli flower (raw)	64	Potatoes (raw Dec.)	15
Cauli flower (cooked)	20	Potatoes (Jan. – Feb.)	10
Cabbage (raw)	55	Potatoes (March onward)	8
Cabbage (boiled)	20	Potatoes (boiled)	6
Spinach (raw)	60	Apple (raw)	5
" (boiled)	25	Lettuce (raw)	15
Water cress (raw)	60	Bananas (raw)	10
Strawberries	60	Beetroot (boiled)	5
Oranges (raw)	50	Onion (raw)	10
Lemon (juice)	50	Onions (boiled)	6
Grape fruit (raw)	40	Plums (raw)	3
Peas (raw)	25	" (Stewed)	2
" (boiled)	15	Pears (raw)	3
" (dried boiled)	trace	" (stewed)	2
Cow's milk (fresh)	2	" (canned)	1
		Human's milk	5

Potatoes contain less ascorbic acid than green vegetables but eating large quantity of them constitutes an important source of this vitamin. As much as 75 percent of the ascorbic present in green vegetables can be lost during cooking. This loss can be avoided by eating raw green vegetables in salads but the amount which can conveniently be eaten in this way are comparatively small and more ascorbic acid may be obtained by eating larger quantity of cooked vegetables for example 25g of lettuce when property served provides 4mg of ascorbic acid compared with 23mg provided by 100g off cooked cabbage. Raw cabbage is a much better source of ascorbic acid than lettuce and 25g provides about 13mg of the vitamins (Brain & Cameron, 1989).

Cow milk has about one-quarter ascorbic content of human milk and some of this is destroyed during pasteurization. Exposure of milk to sunlight also causes diminution of its ascorbic acid content and this change is brought about by the breakdown products of riboflavin. It is important that babies and particularly those

fed on cow's milk which has been boiled should be provided with other sources of the vitamin.

Concentrated orange juice, rose-hip syrup or black current juices are attractive addition sources of the vitamin. When babies' progress to a mixed diet there is less need for such supplements and at two years of age a normal diet should provide sufficient ascorbic acid. Canned fruits and vegetables vary in their ascorbic acid content but some for example tomatoes are good sources of the vitamin. Some loss of ascorbic acid is inevitable to canning but good quality canned fruits and vegetables often contain more of this vitamin than "fresh" fruits or vegetables cooked at home. This is because they are canned while fresh and cooked under carefully controlled conditions.

Foods such as yeast, egg-yolk, meat and cereals which are rich in vitamin B are usually devoid of ascorbic acid, but liver and kidney are exceptional (Fox & Cameron, 1989).

2.3 TRADITIONAL USES OF THE PLANTS STUDIED

In this study some information was obtained from traditional medicine healers regarding some fruits and herbs that are currently used as food and for traditional medicine formulations. The information obtained was documented in Table 2.2 Some of the plant parts could be taken as normal fruits, used in treating wounds, catarrh and many diseases. This documentary process actually justifies our interest in determining the vitamin C in the herbal remedies which could be potential sources of antioxidants that may be applied in modern medicine.

Table 2.2: Traditional Applications of the Plants Studied

Local Name/Parts of Plant Used	Botanical Name	Area of Study	Traditional Uses
Jinin Kafiri Fruits and leaves	*Haematostaphis bateri*	Hong, M/South, Michika	Fresh leaves prepared as soup. The Bark is taken as blood tonic to cure sickle cell patient.
Kalgo Leaves	*Piliostigma reticulator*	Yola, Mubi	Boiled and used for treatment of teeth ache. Cough
Kalgo Fruit	*Piliostigma reticulator*	Yola, Mubi	The fresh fruit is used for the treatment of toothache.
Tsamiyan Kasa leaves	*Byroscarpus coccineaus*	Hong, Mubi, Yola	The boiled juice is used for the treatment of measles and body rashes
Tsaddan Gida Fruits (Chabule)	*Spondias Purpurea*	Yola	Is taken as normal fruit and the juice is taken at leisure time.
Tsaddan Gida Leaves (Chabule)	*Spondias Purpurea*	Yola	Use in preparing porridge and can be boiled and taken for the treatment of stomach ache
Faron Biri (Yeye) Fruits and leaves	*Lannae acida*	Yola	Fruits is licked or taken as juice while the leaves can be prepared as source of vitamin C
Yakuwan Daji leaves	*Hibiscus asper*	Hong, Michika	Is used to prepare soup, the fruit oil is used to cure rashes and treatment of leprosy

2.4 ANALYTICAL METHODS FOR THE DETERMINATION OF ASCORBIC ACID

2.4.1 Conductometric Method

The conductance of an electrolytic solution at any temperature depends only on the ions present and their concentration. When a solution of an electrolytic is diluted, the conductance will decrease, since fewer ions are present per millitre of solution to carry current (Jeffery *et al,* 1999). Conductivity of solution is usually measured by placing it in a cell carrying a pair of platinum electrodes which are firmly fixed in position. The measurements are made by connecting the cell to a conductivity meter which supplies alternating current at a frequency of about 1000Hz to the cell. The use of alternating current reduces the possibility of

electrolysis occurring and causing polarisation at the electrodes (Shoemaker and Garland, 1967).

It follows that the higher the current obtained the greater the conductivity. The resistance however depends on the distance between the two electrodes and their surfaces which can vary due to deposits of salt or other material (electrolysis). For this reason amperometric system is recommended for solutions with low level of dissolved solids i.e. up to 1gram per litre, (approximately 2000µs/cm).

The 4-ring potentiometric method is based on the principle of induction and eliminates common problems associated with amperometric system such as the effects of polarisation (Shoemaker and Garland, 1967). The two outer rings apply an alternating voltage and induce a current loop in the solution. The two inner loops are dependent on the conductivity of the solution. A PVC shield maintains the current field restrained and constant. It is therefore possible to measure conductivity with ranges up to 200000 µs/cm and 100g/l using the 4-rings method.

In conductivity measurement, the size of the electrode and the distance between them will determine the actual values measured and therefore will differ from instrument to instrument. Theoretically, all instruments will measure the same conductance for any given solution.

2.4.2. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is the most versatile and widely used type of elution chromatography. The technique is used by Chemist to separate and determine species in a variety of organic, inorganic and biological materials. The types of high performance liquid chromatography are often classified by separation mechanism or by the types of stationary phase. These include: partition or liquid – liquid chromatography; adsorption or liquid – solid chromatography; ion – exchange or ion – chromatography; size – exclusion chromatoghraphy; affinity chromatography and chiral chromatography (Skoog and West 2004)

Ascorbic acid AA and dehydroascorbic acid DHA belong to very small polar molecules that are difficult to retain in conventional reversed phased (RP) chromatography systems and separate from the dead volume. The principal approaches to the determination of AA and DHA by LC are: Reversed – phased, ion exchange, Ion exclusion and Ion pair. The mobile phases are often very complex, with more than two components containing various media or reagents. The approach of hydrophilic interaction liquid chromatography (HILIC) which is much simpler and more elegant has become popular recently.

2.4.3 Reversed – Phase Chromatography

Conventional octadecylsilica (ODS) stationary phases are widely used for the determination of AA and DHA. However, RP methods often suffer from poor resolution of AA and the dead retention volume. To get sufficient retention, a very high percentage of water, usually in inorganic/organic acid or organic buffer must be applied in combination with low pH. Neutral forms of acids, which arise in acidic pH are better retained on the ODS stationary phase.

2.4.5 Ion – Pair Chromatography

Ion – pair chromatography is also used vary widely in analyzing AA and DHA. Ion – pairing reagents together with inorganic buffers were often used as additives in analysing AA and DHA. In conventional chromatography, ion-pairing reagents usually decrease column life-time, so optimisation must be due carefully and only low concentrations can be used. They also tend to cause instability in the chromatographic separation or gradually increase pressure with each subsequent injection of sample. There is also a danger of precipitation with the components of the mobile phase. To conclude ion – pairing is not ideal for the modern analytical laboratory.

2.4.6 Ion – Exchange Chromatography

The ion– exchange chromatographic approach was often the first chromatography methods for AA determination. As AA is a weak organic acid, it

can be well retained on anion – exchange stationary – phase type SAX (Strong anion exchange). Recently, the ion – exchange approach was sometimes applied in a modified manner, using an amino- modified stationary phase. Inorganic buffer or acid at low pH (5.0) was typically used as the mobile phase. This approach did not become very popular in analysis of AA and DHA.

2.4.7 Ion – Exclusion Chromatography

This approach was common in the 1009's (55) the stationary phase in ion-exclusion chromatography is often based on sulphonated spherical resins. Ion – exclusion chromatography is a much more elegant approach compared to RP or ion – exchange chromatography. Stationary – phase stability at very low pH is assured by the polymeric base of stationary phase.

2.5 Titrimetric Method

Titrimetric methods include a large and powerful group of quantitative procedures that are based on measuring the amount of a reagent of known concentration that is consumed by an analyte. Volumetric titrimetry involves measuring volume of a solution of known concentration that is needed to react essentially completely with the analyte. Gravimetric titrimetry differs only in that; the mass of the reagent is measured instead of its volume. In coulometric titrimetry, the reagent is a constant direct electrical current of known magnetide that consumes the analyte: here, the time required (and thus the total charge) to complete the electrochemical reaction is measured. (Skoog & West, 2004).

A titration is performed by slowly adding a standard solution from a burette or other liquid-dispensing device to a solution of the analyte until the reaction between the two is judged complete. The volume or mass of reagent needed to complete the titration is determined from the difference between the initial and final readings. It is sometimes necessary to add an excess of the standard titrant and then determine the excess amount by back titration with a second standard titrant.

The equivalent point in a titration is reached when the amount of added titrant is chemically equivalent to the amount of analyte in the sample. We cannot determine the equivalent point of a titration experimentally. Instead, we can only estimate its position by observing some physical change associated with the condition of equivalence. This change is called the end point for the titration. The difference in volume or mass between the equivalence and end-point which is due to inadequacies in the physical changes our ability to observe them is the titration error. Indicators are often added to the analyte solution to produce an observable physical change (the endpoint) at or near the equivalent point.

A primary standard is a highly purified compound that serves as a reference material in volumetric and mass titrimetric method. The accuracy of a method is critically dependent on the properties of this compound. Important requirement for a primary standard are as follows: (i) high purity, atmospheric stability (ii) absence of hydrate water so that the composition of the solid does not change with variation in humidity (iii) modest cost (iv) reasonable solubility in the titration medium (v) the purity of such a secondary standard must be established by careful analysis.

2.5.1 Standard Solutions

Standard solutions play a central role in all titrimetric methods of analysis. The ideal standard solution for a titration method will:

(i) Be sufficiently stable so that it is necessary to determine its concentration only once.

(ii) React more or less completely with the analyte so that satisfactory end point are realized.

(iii) React rapidly with the analyte so that the time required between additions of reagent is minimized.

(iv) Undergo a selective reaction with the analyte that can be described by a balance equation.

The accuracy of a titrimetric method can be no better than the accuracy of the concentration of the standard solution used in the titration. Two basic methods are used to establish the concentration of such solutions. The first is the direct method

in which a carefully weighed quantity of a primary standard is dissolved in a suitable solvent and dilute to an exactly known volume in a volumetric flask. The second is by standardization is used to titrate (i) a weighing quantity of primary standard (ii) a weighed quantity of a secondary standard or (iii) a measured volume of another standard solution. We expressed the concentration of solutions in several ways.

The standard solutions used in titrimetry, either expressed as molarity, or normally. The first term gives the number of moles of reagent contained in one litre of solution and the second gives the number of equivalents of the reagent in the same volume (Skoog & West, 2004).

2.6 Ultraviolet/Visible Absorption Methods

Spectroscopy is an analytical technique concerned with

i). Measurement of the interaction of radiant energy with matter

ii). Instruments of measurement

iii). Interpretation of interaction

For a meaningful analytical work, the use of a single wavelength-monochromatic light is required. The purity of the interacting radiation in terms of monochoromacity has a lot to do with the sensitivity precision and accuracy of photometric analysis. Qualitative and quantitative analysis of substances are achieved from measurement of absorbance or transmittance in UV/Visible region of the spectrum. Absorbing species interact with photon of radiation of particular wavelength depending on the species. Bonding electrons absorb the photons and are excited to higher electronic energy states.

Quantitative aspect of absorption method depends on the fact that a given species absorbs radiation only in specific region and maximally so only at a particular wavelength of the spectrum. The degree of absorption varies with the types of species. Quantitative methods and the application of absorption are of main interest in analytical chemistry. (Ogugbuaja, 2000).

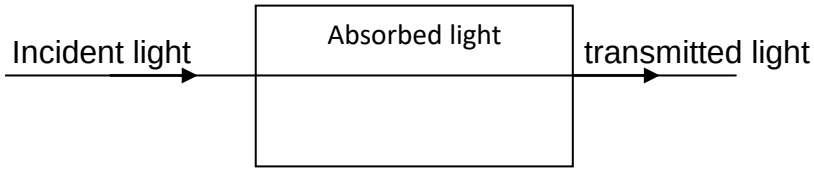
2.6.1. Chromophores

Invariably, not all portions of an absorbing molecule absorbs photon, leading to transition. The type of groups within molecules or ions that are capable of absorbing radiation are known as chromaphores. Chromaphores correspond to the functional groups in organic molecules. Molecules with the same chromaphores usually absorb in the same range, through not exactly at the same wavelength.

Electrons (e) at the ground electronic state absorb photon and are elevated to excited electronic states. Transistors in UV/Visible thus involves pi (π) sigma and non - bonding (n) electrons d and f electrons and charge transfer electrons. Molecules with two or more conjugated double bond absorbs at a longer wavelength than those with one double bond.

2.6.2. Lambert Law

Portion of monochromatic radiation incident on a substance is absorbed by the substances and part is transmitted. 100% absorption of the incident light beam is rarely attained, and is not required.



Substance

L_i and I_t are incident and transmitted light intensities respectively. Lamberts law states that the portion of light absorbed is proportional to the length of radiation transmitted at constant amount of the substance (C) i.e., A α b at constant C and

$A = Kb$ [2.1]

Where K is the proportionality constant and A is absorbance and b is the path length

The mathematical expression of the law is given as

$A = I_t/L_i = -kb$ [2.2]

or

$A = \log I_i/L_t = -\log T = kb$ [2.3]

Where T =Transmittance)

2.6.3 Beer's Law

This law relates the amount of light intensity absorbed (or transmitted) to the concentration of the absorbed species at constant path length i.e., A α C at constant path length (b) and

$A = k^{i}C$ [2.4]

In Beer -Lambert law the two laws were combined i.e., Eqs 2.1 and 2.2 to give

$A= Kk^{i}bc$ [2.5]

$A= \varepsilon bC$ [2.6]

where Kk^{i} is replaced by ε = specific absorbance and C is expressed in mol/dm^3 (molarity).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant Materials

The plant materials used were *Spondias purpurea*: Fresh fruits, fresh leaves and dry leaves; *Haematastaphis bateri*: Fresh fruits, fresh leaves and dry leaves; *Piliostigma reticulator*: Fresh fruits, fresh leaves and dry leaves; *Hibiscus asper*: Fresh leaves, and dry leaves; *Byroscarpus coccineaus*: Fresh and dry leaves; *Lannae acida:* fresh and dry leaves. These samples were collected in the wild area within Adamawa localities as shown in Table 4.1.The samples were washed with distilled water to remove unwanted materials.

3.2 Reagents

All solutions were prepared from analytical reagent grade materials in doubly distilled water. Alizarin red S (ARS), Copper sulphate, acetate buffer (pH = 5.0), potassium thiocyanate, 2,6-dichlorophenolindophenol(DCIP), copper (II) sulphate, acetic acid, sodium acetate meta-phosphoric acid , ascorbic acid, starch, iodine, hydrochloric acid, arsenic trioxide, soluble starch, Potassium iodide, sodium hydroxide, sodium bicarbonate and sulphuric acid.

3.2 PREPARATION OF SOLUTIONS

3.3 Standard Ascorbic Acid Solution

Standard ascorbic acid solution (1.45×10^{-3} M) was prepared daily by dissolving 0.0250 g ascorbic acid in water and diluting to 100 mL. Working solutions of lower concentrations were prepared by appropriate dilution with meta-phosphoric acid – acetic acid solution in 50ml volumetric flask.

3.3.2 Acetate Buffer

Acetate buffer (pH = 5.0) was prepared from concentrated acetic acid and sufficient 2 M sodium hydroxide to give the desired pH.

3.3.3 COPPER (II) SULPHATE SOLUTION

A $3.0x10^{-3}$ M Cu (II) solution was prepared by dissolving 0.070 g $CuSO4.5H_2O$ in water and diluting to 100 mL with water.

3.3.4 ALIZARIN RED SOLUTION

Alizarin red S (ARS) solution (4.0×10^{-3} M) was prepared by dissolving 0.1457 g of the Compound in water and diluting to 100 ml in a volumetric flask. This solution was stable for several months.

3.3.5 Preparation of Thiocyanate Solution

Thiocyanate solution (7.0 M) was prepared by dissolving 0.0335 g potassium thiocyanate (Merck) in water and diluting to 50 ml in a volumetric flask.

3.3.6 PREPARATION OF 2, 6-DICHOLOROPHENOLINDOPHENOL (DCIP)

A concentration of 0.003M 2, 6-dicholorophenolindophenol was prepared by dissolving 0.50g indophenols in 500ml volumetric flask and diluting the solution to mark of the flask with distilled water.

3.3.7 Meta-Phosphoric Acid-Acetic Acid Solution

Meta-phosphoric acid (2%) is prepared by dissolving 15g of meta-phosphoric acid pellet in 40ml glacial acetic acid and 200ml distilled water. The solution is diluted to 500ml volumetric flask then filtered using fluted paper. Meta-phosphoric acid (4%) is also prepared by dissolving 30g of m-phosphoric in 40ml glacial acid in 500ml volumetric flask.

3.3.8 Starch Indicator Solution (1%)

An amount of 0.50 g of soluble starch was added to 50 ml of near-boiling distilled water, mixed thoroughly and allowed to cool before use.

3.3.9 Preparation and Standardization of 0.1 M Iodine Solution

Iodine (14g) was dissolved in potassium iodide solution (100 ml), acidified with hydrochloric acid (1M), diluted with water to 1000 ml and standardized with primary standard arsenic trioxide prior to use. The primary standard arsenic trioxide (150mg) was dissolved in 1 M sodium hydroxide (20 ml) and diluted with water to

40 ml. The solution was acidified with the hydrochloric acid solution using methyl red as an indicator. Sodium bicarbonate (2 g), water (50 ml) and starch (3 ml) was added into the acidified solution prior to titration with iodine solution. The end was reached when the first sign of blue color persisted for 20 seconds of swirling the solution. The titration was repeated three times. Each ml of 0.1 M iodine is equivalent to 4.946 mg arsenic trioxide.

3.4 EXTRACTION OF VITAMIN C FROM PLANT MATERIALS

The extraction of AA from the cells of plants was done by first breaking the tissue in meta-phosphoric-acetic solution medium. The plant parts were readily homogenized by grinding in a 400 ml mortar using a pestle. For accurate measurement of the amount of AA, the extraction was done to avoid lost of the acid. Many plant materials are known to contain an enzyme called ascorbic acid oxidase, which catalyses the oxidation of ascorbic acid to dehydroascorbic acid (Hernandez, *e*t *al*., 2006).

The grinding process normally disrupts the tissue and cells of plants. However some cell components usually separated by membranes get to mix together. In this case, ascorbic acid oxidase will convert the ascorbic acid present in the tissue to dehydroascorbic acid. Grinding the tissue in 2% meta-phosphoric acid will inactivate the oxidase, and thus prevent the loss of ascorbic acid to oxidation. Hence extraction of AA from the samples was done by grinding 5g of each sample in 30 ml of 2% meta-phosphoric–acetic acid solution. The solution was then filtered through Watman filter paper into a conical flask and kept in a cupboard at room temperature.

3.5 PROCEDURES FOR ANALYTICAL METHODS FOR THE DETERMINATION OF ASCORBIC ACID

In this work, the analyses of ascorbic acid were carried out by the modification of the indirect spectrophotometric method as outlined previously by Raghu *et al***,** (2007) and Nejati-Yazdinejad, (2007). A JENWAY 6405 UV/VIS spectrophotometer was used for this analysis. The titrimetric method based on

AOAC (2002) and direct titrimetric method by Suntornsuk *et al.,* 2002 was also adopted.

3.6 Indirect Spectrophotometric Method

3.6.1 Determination of Ascorbic Acid in Standard Ascorbic acid Solutions

A 3 ml aliquot of solution containing 15–25 µg of standard ascorbic acid was added into a 50 ml volumetric flask. Then 2.0 ml of $3.0x10^{-3}$ M Cu (II) solution, 5.0 ml of $4x10^{-3}$ M ARS solution, 5.0 ml of 7.0 M thiocyanate solution and 1.0 ml of acetate buffer solution were added. The contents of the flask were mixed and left undisturbed for a few minutes. Then the solution was diluted to the mark with water and the absorbance of 5 ml of the solution was recorded at 510 nm against a reagent blank (ARS solution). This procedure was repeated three times. The absorbance and the concentration will be used to prepare the calibration curves.

3.6.2 Determination of Ascorbic Acid in Plant Materials

The procedure in section 3.6.1 was carried out with extracts of the plants and their absorbance measured. The concentration of AA of the samples was determined by extrapolating the absorbance of the samples in the calibration curve prepared from the standard ascorbic acid.

3.6.3 Determination of Ascorbic Acid in Pharmaceutical products

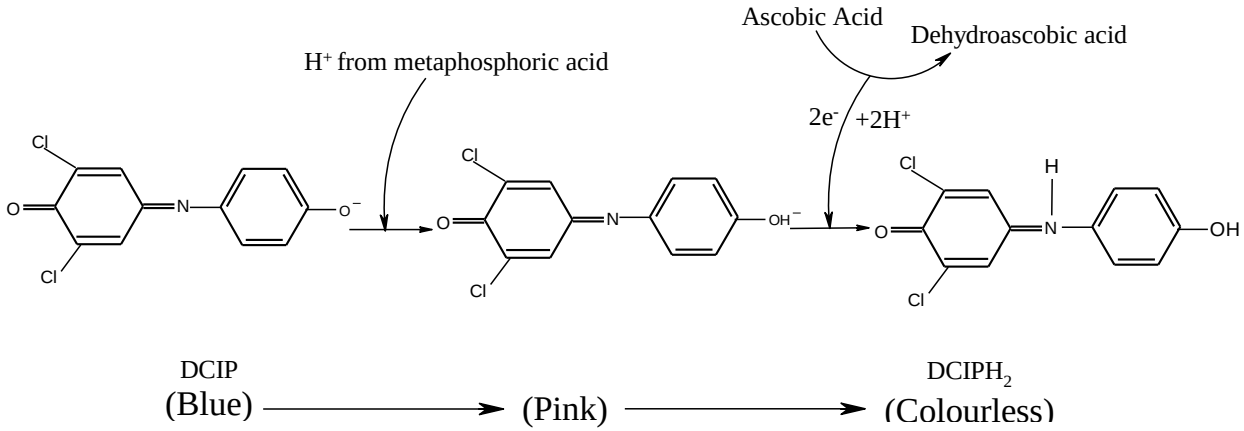
A known portion (0.5g) of the powdered tablets is dissolved in distilled water, filtered into a 50 ml standard flask and diluted to volume with distilled water. The procedure in section 3.6.1 was carried out with this solution and their absorbance measured. The concentration of AA of the samples was determined by extrapolating the absorbance of the samples in the calibration curve prepared from the standard ascorbic acid.

3.6.4 AOAC DCIP TITRIMETRIC METHOD

Principle

The hydrogen atoms of the two enol groups of AA may be readily oxidized making the acid a strong reducing agent. This property can be used to measure the

amount of AA present in a plant sample. The DCIP dye is blue in alkali, pink in acid and can be reduced by ascorbic to a colorless form. If a drop of the blue dye is added to acidified extract, the drop will turn pink and then colorless as shown in the reaction scheme shown in Figure 3.1



When the entire AA in the extract has been converted to dehydroascorbic acid, no more electrons will be available to reduce a drop of DCIP to the colorless form and the solution will remain pink. Therefore, the amount of AA in the extract can be measured by titration against a dilute DCIP dye.

Standardization of DCIP Solution

The DCIP solution was standardized against a known amount of standard ascorbic acid solution. This was accomplished by titrating the dye into a standard solution containing 1.0 mL of AA solution (a stock solution containing 4.0 mg/mL) and 9 mL of 4% meta-phosphoric-acetic acid solution. The end point of the titration was defined as a pink colour that persisted for close to 20 seconds when swirled. The amount of AA equivalent to 1.0mL of dye was calculated as

$$\frac{\text{AA (mg)}}{\text{1.0 Ml dye}} = \frac{\text{4.0mg AA}}{\text{dye titrated (ml)}} \qquad [3.1]$$

3.6.5 Determination of Ascorbic Acid in Standard Ascorbic acid Solutions

The standard ascorbic acid was analysed by taking 1.0 ml of vitamin C in 4% meta-phosphoric –acetic acid solution into a 50ml conical flask. The solution was then titrated by drop wise addition of DCIP solution until a distinct rose pink colour persists for 20 seconds. In a similar fashion, three blanks containing 5.0 ml

of 4% meta-phosphoric-acetic acid solution and 1.0 ml of distilled water was titrated. The average result of each three measurements was calculated.

The average amount of ascorbic acid was obtained as

$$\text{mg of AA in the aliquot} = \text{average amount of dye titrated (ml)} \times \frac{\text{AA (mg)}}{\text{1.0 ml dye}} \quad [3.2]$$

The amount of AA in 100 g of the sample was calculated as

$$\frac{\text{AA (mg)}}{\text{1.0 ml dye}} = \text{mg AA in the aliquot} \times \frac{\text{Total volume of sample (ml)}}{\text{Volume of liquout (ml)}} = \frac{100}{\text{weight of vol. (g)}} \quad [3.3]$$

3.6.6 Determination of Ascorbic Acid in Plant Materials and Pharmaceutical Products

For the plant extract the following procedures was followed: 15ml of the aliquots of the plant extract was diluted in beaker by adding 50ml of meta-phosphoric acid–acetic solution and then filtered through a Whatman filter paper. A portion of 10.0ml of the filtrate was taken into a 50ml conical flask and titrated three times with DCIP previously described. The amount of the ascorbic acid in the unknown samples was calculated using Eqs 3.1 – 3.3

For the pharmaceutical products, a known portion (0.5g) of the powdered tablets was dissolved in distilled water, filtered into a 50 ml standard flask and diluted to volume with distilled water. The previous procedures in sections 3.7.2.2-3.7.2.3 were followed to obtain the amount of AA in the products.

3.6.7 BY DIRET TITRIMETRIC METHOD

3.6.8 Determination of Ascorbic Acid in Plant Samples

The vitamin C content in the plant samples was determined by direct titration with iodine according to the method of Suntornsuk, *et al* 2002. In this method each 25ml of the extracts was transferred into a 250ml Erlenmeyer flask. Twenty-five milliliter (25ml) of 1 M sulfuric acid was added, mixed, diluted with 50 ml of water and 3 mL of starch was added as an indicator. The solution was directly titrated with 0.1 M iodine previously standardized with primary standard arsenic trioxide. A blank titration was performed prior to the titration of each Sample (n=5). Each ml of 0.1 M iodine is equivalent to 8.806 mg ascorbic acid.

3.6.9 Determination of Ascorbic Acid in Pharmaceutical products

A known portion (0.5g) of the powdered tablets was dissolved in distilled water, filtered into a 50 mL standard flask and diluted to volume with distilled water. The procedure in section 3.7.3.1 was carried out to obtain the amount of AA in the products.

3.7 VALIDATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF VITAMIN C

3.7.1 Linearity of Methods

The linearity of the methods was determined by adding standard ascorbic acid at 100% of the amount AA found in the samples into the samples. Triplicate determinations using spectrophotometric, AOAC and direct titration methods were made for each standard added solution as described previously. The linear regression line was plotted between the amount of standard ascorbic acid found and the amount of standard ascorbic acid added using Microsoft EXCEL®. The regression equation, intercept and regression coefficient (r^2) values were obtained.

3.7.2 Precision and Recovery Test

The precision was studied by determining vitamin C content in samples by performing five determinations on each sample using the various methods adopted in this work. Precision was expressed as % relative standard deviation (%R.S.D.). The accuracy of each method was calculated from percentages of recovery using standard addition method. Standard ascorbic acid was added at two different concentration levels (100 and 250 µg of AA to the samples. Vitamin C in the added samples was determined by the various methods as described in the previously.

$$\% \text{ Recovery} = \frac{\text{Amount of Standard Recovered}}{\text{Amount of Standard Added}} \times 100 \qquad [3.4]$$

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 ANALYSIS OF VITAMIN C IN SAMPLES

4.1 Calibration Curves for the Determination of AA by Spectrophotometric Method

The spectrophotometric method adopted in this work was found to be a simple technique for the determination of ascorbic acid. The procedure was based on the reducing effect of ascorbic acid on Cu (II) ion and the chelating ability of alizarin red S (ARS) with excess of Cu (II) ion to provide a coloured solution suitable for UV spectrometry. For a sample containing ascorbic acid and large amount of Cu (II), the Cu (II) will be reduced to Cu (I). The excess oxidant, Cu (II), is determined by complexation with ARS. The reaction step could be represented as follows:

$$2Cu^{2+} + H_2R \rightarrow 2\ Cu^{+} + R + 2H^{+} \qquad [4.1]$$

$$Cu^{2+}_{(excess)} + 2H_2L \rightarrow [CuL_2] + 4H^{+} \qquad [4.2]$$

In the above reaction, H_2R, R and H_2L are ascorbic acid, dehydroascorbic acid and ARS, respectively. In the oxidation reaction in equation 4.1, Cu (I) ion is stabilized by thiocyanate ion (Nejati-Yazdinejad, 2007). This effect will affect the completion of oxidation of ascorbic acid by Cu (II) ion.

A series of standard solutions of ascorbic acid were analysed under the optimized conditions to test the linearity of the calibration graph (Muller & Miller, 2001). Figure 4.1 shows the linear range. In this graph abscorbance is drawn vs concentration of ascorbic acid, linearity was found to be perfect in the range of 1.5 – 8.2µg/ml with a correlation coefficient of (r^2) of 1 and the following calibration equation was obtained.

$A = 0.0192C + 0.0001$ with $r^2 = 1$

where A is absorbance of the sample and C is the concentration.

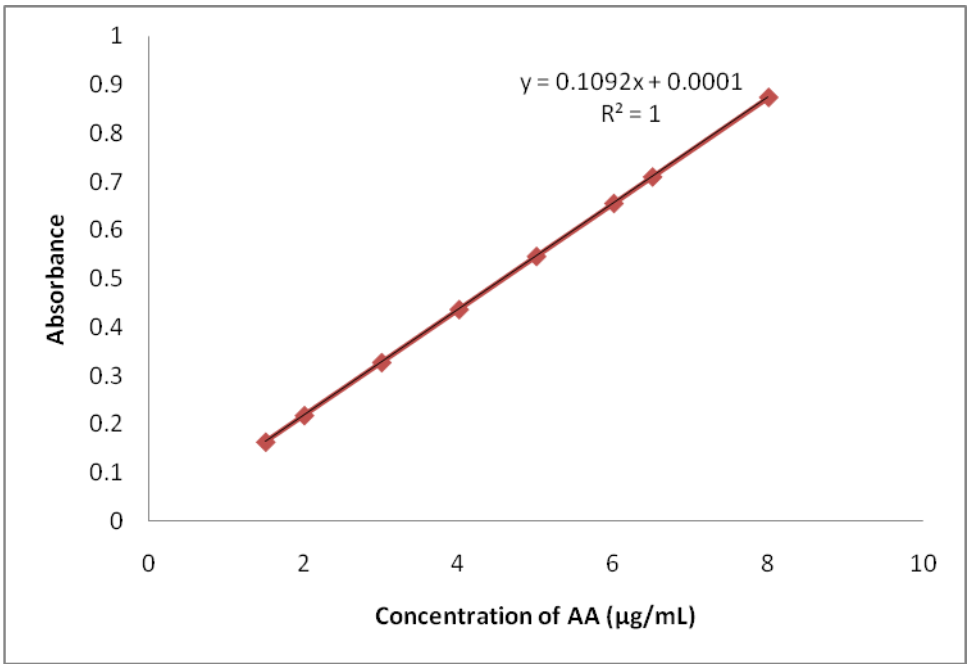


Figure 4.1: Calibration Curve for the Determination of ascorbic acid

Table 4.1: Vitamin C Content of Samples as Determined by Various Methods

Sample	Amount of Vitamin C (mg/100g) [a]		
	Direct Method	Spectrophotometric Method	DCIP Titrimetric Method
Spondles purpurea			
Fresh fruits	46.96±0.20	45.78±0.22	46.22±0.45
Fresh leaves	18.78±1.23	17.89±0.13	18.06±0.34
Dry leaves	16.26±0.26	15.98±0.28	16.01±0.12
Haematastaphis bateri			
Fresh fruit	30.46±0.24	28.33±0.11	29.87±0.18
Fresh leaves	23.14±1.04	20.22±1.00	21.89±0.24
Dry leaves	18.1±1.44	16.76±1.33	15.97±0.98
Piliostigma reticulator			
Fresh fruits	26.12±0.66	22.43±0.56	24.88±0.71
Fresh leaves	20.66±0.16	17.88±2.43	18.98±0.21
Dry leaves	11.44±1.08	9.01±0.77	10.05±0.54
Hibiscus asper			
Fresh leaves	40.22±9.66	34.66±5.78	36.18±6.64
Dry leaves	26.23±0.45	20.67±2.83	22.06±1.89
Byroscarpus coccineaus			
Fresh leaves	51.67±0.67	46.88±0.78	49.89±0.54
Dry leaves	35.67±1.33	33.87±0.54	35.05±0.33
Lannae acida			
Fresh leaves	66.87±2.45	60.18±52	67.0±1.09
Dry leaves	40.36±0.43	41.67±1.34	42.88±1.50

[a]Values are mean±S.D

4.2 VITAMIN C CONTENT OF THE SAMPLES

4.2.1 (VITAMIN C CONTENT OF FRESH FRUITS)

Table 4.1 presents the vitamin C contents of fruits of *Spondia purpurea,Haematostaphis bateri, Piliostigma reticulator, Hibiscus asper, Byroscapus coccineus* and *Lannae acida* as determined by spectrophotometric, Dichloro-Indophenol (DCIP), titrimetric and Direct titrimetric methods. The value obtained in *Lannae acida* fresh leaves was 67.0± 1.09mg/100g as obtained by DCIP titrimetric method was found to be highest when determined unlike the other two methods. The values of vitamin C obtained for direct Titrimetric method and spectrophotometric methods were 66.87± 2.45mg/100g and 60.18± 52.0 mg/100g respectively. Similarly, the vitamin C content of *Haematostaphis bateri* fresh fruits were 28.33±0.11 mg/100g, 29.87±0.18 mg/100g and 30.46±0.24 mg/100g using DCIP titrimetric, Direct titrimetric and spectrophotometric methods respectively.

Also the vitamin C content of *Piliostigma reticulator* fresh fruits were 26.12±0.66mg/100g (spectrophotometric method), 22.43±0.56 mg/100g (DCIP method) and 24.88±0.71 mg/100g (Direct titrimetric method). Thus vitamin C content of *Piliostigma reticulator* was found to be the lowest using spectrophotometric method as compared to the values obtained from the other methods; this could be due to the presence of some metal ions or other interferences.

Table 4.2: Vitamin C Content of Pharmaceutical Formulations as Determined by Various Methods

	Amount of Vitamin C (mg/100) [a]		
Sample	Spectrophotometric Method	DCIP Titrimetric Method	Direct Titrimetric Method
Cecon Vitamin C	486.87±98.00	466.98±67.89	480.88±30.78 (500)
Ascorbon Vitamin C	506.34±56.83	456.87±56.67	460.78±87.14 (500)
Polybion Forte C	232±89.67	287.55±33.69	289.45±17.91 (500)
Stress Capsule (Multivitamin)	216.98±78.79	223.67±45.89	236.65±89.76 (500)
Vitera Multivitamin With minerals	116.87±23.67	113.66±45.86	122.66±63.33 (150)

[a]Values are mean±S.D

[b]Reported values of vitamin C by manufacturers

4.3 VITAMIN C CONTENT OF SOME PHARMACEUTICAL FORMULATION

The results obtained from Table 4.2 indicate that the vitamin C contents of some pharmaceutical formulations were statistically higher than those obtained from the plant samples using the same methods. For instance Ascobon vitamin C has the range of 456.87± 56.67mg/100g to 506.34±56.83mg/100g of vitamin C content as the highest content ascorbic acid, whereas vitera multivitamin with minerals has a range of 113.66±45.86mg/100g to 122.66±63.33mg/100g of vitamin C content as the lowest value obtained for the pharmaceutical formulations.

The discrepancy so far obtained in the ascorbic acid content variation may be attributed to the formulation ingredients available in the pharmaceutical formulations as compared to the crude nature of the plant samples.

Table 4.3: Recovery of Vitamin C from Samples (R %) Using Spectrophotometric Method

	100µg AA added		250µg AA added	
Sample	(R %)	RSD (%)	(R%)	RSD (%)
Spondles purpurea				
Fresh fruits	96.16±5.20	5.08	94.82±2.15	2.26
Fresh leaves	98.27±4.20	3.65	95.87±4.42	4.41
Dry leaves	97.66±4.11	4.06	96.21±2.14	1.56
Haematastaphis bateri				
Fresh fruit	96.92±4.50	4.43	97.06±4.66	4.57
Fresh leaves	95.73±5.04	5.18	99.86±4.35	4.76
Dry leaves	97.23±6.47	6.46	94.97±4.32	3.65
Piliostigma reticulator				
Fresh fruits	97.12±5.45	5.21	94.76±7.02	6.99
Fresh leaves	93.45±3.67	3.56	93.98±2.26	2.18
Dry leaves	104.00±1.11	1.06	100.00±5.00	4.87
Hibiscus asper				
Fresh leaves	95.32±1.89	2.04	96.13±1.77	1.87
Dry leaves	93.34±1.39	1.28	92.82±2.33	2.36
Byroscarpus coccineaus				
Fresh leaves	91.67±2.33	2.00	93.44±3.87	3.79
Dry leaves	93.34±1.39	1.28	92.82±2.33	2.36
Lannae acida				
Fresh leaves	90.45±2.48	3.01	91.22±2.89	2.76
Dry leaves	91.31±3.22	3.15	91.42±2.50	2.45

4.4 RECOVERY OF VITAMIN C OF SOME PLANT SAMPLES

Recovery test was conducted on six different plants using the three different methods used in this work. The result of the recovery test is presented in Table 4.3, 4.4, and 4.5. Generally there was good indication of recovery of ascorbic acid in all the plant samples. In this analysis highest amount of vitamin C was recovered using the direct titrimetric method in most of the plant samples. Among the tested samples, fresh fruit and fresh leaves of *Haematostaphis bateri*, fresh fruits of *Spondias purpurea* and dry leaves of *Philostigma reticulator* showed the highest vitamin C content using direct titrimetric method.

A notable discrepancy was observed between the fresh and dry leaves in most of the samples. This could be as a result of degradation of vitamin C during the drying process. In addition, the direct titrimetric method is reliable and cost effective in terms of the instrumentation and reagent.

Table 4.4: Recovery of Vitamin C from Samples (R %) Using DCIP Titrimetric Method

	100µg AA added		250µg AA added	
Sample	(R %)	RSD (%)	(R %)	RSD (%)
Spondies purpurea				
Fresh fruits	98.36±3.65	3.68	97.43±2.98	2.85
Fresh leaves	97.43±4.06	4.01	96.86±3.42	3.37
Dry leaves	97.48±3.31	2.98	97.28±3.76	3.62
Haematastaphis bateri				
Fresh fruit	97.65±1.22	1.11	98.09±1.55	1.32
Fresh leaves	98.63±2.50	2.48	99.22±3.35	2.25
Dry leaves	97.00±1.47	1.43	98.97±1.32	1.40
Piliostigma reticulator				
Fresh fruits	95.56±2.33	2.23	96.76±3.02	2.86
Fresh leaves	95.75±2.67	2.58	96.27±2.16	2.10
Dry leaves	96.50±2.44	2.46	98.23±2.76	2.72
Hibiscus asper				
Fresh leaves	95.45±4.65	4.63	95.18±4.08	4.00
Dry leaves	95.87±3.98	3.78	96.56±3.45	3.40
Byroscarpus coccineaus				
Fresh leaves	94.56±2.30	2.11	94.45±3.03	2.99
Dry leaves	94.35±2.43	2.40	95.86±2.96	2.95
Lannae acida				
Fresh leaves	96.45±3.48	3.43	97.66±3.54	3.46
Dry leaves	97.31±3.06	3.00	97.82±2.56	2.60

4.4.1 RECOVERY OF VITAMIN C FROM SAMPLES(R %) USING SPECTROPHOTOMETRIC METHOD

Table 4.3 presents the recovery of vitamin C from samples using spectrophotometric method. All the samples showed a value of recovery of vitamin C ranging from 90.45±2.48 mg/100g to 104.00±1.11 mg/100g. All the results from the analyses on recovery of vitamin C content were in close agreement.

However, using the spectrophotometric method the result of the recovery test showed that *Philiostigma reticulator* dry leaves has the highest value of vitamin C (104.00±1.11 mg/100g) whereas the *Lannae acida* fresh leaves has 90.45±2.48mg/100g as the lowest recovered vitamin C content value.

4.4.2 RECOVERY OF VITAMIN C FROM SAMPLES (R %) USING DCIP TITRIMETRIC METHOD

Also Table 4.4 shows the results of recovery values of vitamin C of the plant samples using DCIP titrimetric method. The values obtained in all the plant samples when 100µg ascorbic acid was added ranged from 94.35±2.43mg/100g to 98.63±2.50mg/100g. The range of recovery of vitamin C from sample when the 250µg ascorbic acid was added was 94.45±3.03mg/100g to 99.22±2.35mg/100g.

In this method of analysis fresh leaves of *Haematastaphis bateri* had the highest recovery rate of 98.63±2.50 whereas *Byroscarpus coccineas* dry leaves has the lowest value of 94.35±2.43 for 100µg Ascorbic Acid added.

4.4.3 RECOVERY OF VITAMIN C FROM SAMPLES (R %) USING DIRECT TITRIMETRIC METHOD

The results obtained using this method is indicated in Table 4.5 As usual there was little difference in the results obtained when 100µg and 250µg Ascorbic Acid were added. The vitamin C recovered from sample when 100µg Ascorbic Acid was added ranged from 98.32±2.23mg/100g to 106.12±3.66mg/100g while the value recovered when 250µg ascorbic acid was added range from 98.32±3.93mg/100g to 102.11±3.33mg/100g.

The highest value obtained when 100µg ascorbic acid was added was 106.12±3.66mg/100g for *Haematastaphis bateri* fresh fruit. On the other hand Lannae *acida f*resh leaves yield the lowest value of 95.47±2.23mg/100g. Similarly, when 250µg ascorbic acid was added for the recovery of vitamin C, Haematastaphis bateri fresh fruit gave the highest value of 102.11±3.33mg/100g while *Byroscarpus coccineus* dry leaves gave the lowest value of 98.32±3.93mg/100g.

Table 4.5: Recovery of Vitamin C from Samples (R %) Using Direct Titrimetric Method

	100µg AA added		250µg AA added	
Sample	(R %)	RSD (%)	(R %)	RSD (%)
Spondles purpurea				
Fresh fruits	101.16±5.11	5.08	100.2±3.53	3.43
Fresh leaves	99.33±3.30	3.25	99.34±4.02	3.96
Dry leaves	99.61±4.21	4.18	99.76±2.78	2.77
Haematastaphis bateri				
Fresh fruit	106.12±3.66	3.43	102.11±3.33	3.35
Fresh leaves	103.73±3.66	4.00	99.99±4.22	4.14
Dry leaves	99.43±4.57	4.43	99.92±3.42	3.33
Piliostigma reticulator				
Fresh fruits	99.45±3.35	3.33	99.78±4.11	4.09
Fresh leaves	98.56±4.62	4.59	98.45±3.46	3.48
Dry leaves	100.03±1.23	1.15	100.34±3.11	3.07
Hibiscus asper				
Fresh leaves	99.52±2.66	2.45	99.29±2.55	2.53
Dry leaves	99.26±2.05	2.00	99.43±2.23	2.21
Byroscarpus coccineaus				
Fresh leaves	98.57±3.43	3.40	98.34±3.00	3.01
Dry leaves	98.44±4.29	2.26	98.32±3.93	3.87
Lannae acida				
Fresh leaves	95.47±2.23	2.20	99.23±2.04	1.99
Dry leaves	97.21±3.32	3.25	99.24±2.76	2.80

CHAPTER FIVE

SUMMARY, RECOMMENDATION AND CONCLUSION

5.1 SUMMARY

A Spectrophotometric, AOAC and direct titrimetric methods are described. The linearity of the methods was determined by adding standard Ascorbic acid at 100% of the amount of AA found in the samples .Triplicate determinations using spectrophotometric, AOAC, and Direct Titration methods were made for each standard added solution as described previously. The linear regression line was plotted between the amounts of standard Ascorbic Acid added using Microsoft excel. The regression equation, intercept and regression coefficient (r^2) values were calculated/obtained/determined.The spectrophotometric procedure involves the use of Cu (II) as vitamin C oxidant. After the completion of oxidation reaction excess of Cu (II) was determined by complexation with alizarin Red S (ARS). Thiocyanate ion is used as stabilizing agent for Cu (II) which is the product of oxidation reaction .This method is used for the determination of vitamin C in fruits, leaves and pharmaceutical product. Linearity was found to be perfect. The proposed method is simple, safe, inexpensive and rapid.

Direct titration with iodine was utilized for the determination of vitamin C in the plant samples. The method was validated and showed good precision, accuracy and recovery. With these methods analysis of vitamin C in herbal juice can be performed in laboratories where expensive equipments such as HPLC, gas chromatography, Infrrared spectrophometric and NMR are not available.

Titration with 2, 6-dichlorophenolindophenol (DCIP) is rapid, easy and cheap method that can be employed in the determination of ascorbic acid not only in food stuff but also in pharmaceutical product.

5.3 CONCLUSION

This work shows that the proposed methods can be applied to the determination of ascorbic acid in different food type products. The significant features of the proposed procedure for the determination of ascorbic acid with other methods such as fluorimeteric, enzymetic chromatographic and spectrophotometeric methods are simplicity and excellent precision and sensitivity. The high sensitivity of the methods could provide analysis of real samples and possible pharmaceutical products with small content of ascorbic acid. The methods were validated and showed good precision, accuracy and recovery. Direct titration and titration with 2,6 – Dichlorophenol indophenol (DCIP) are rapid, easy and cheap methods that can be employed in the determination of ascorbic acid, not only in foodstuff but also in pharmaceutical products.

5.2 RECOMMENDATION

i. This work shows that the proposal methods can be applied to the determination of ascorbic acid in different food type product.

ii. To reduce the instability of vitamin C, it can be recommended that the decrease in temperature, protection of samples from light and adding various stabilizing agent among which are: Phosphoric acid EDTA and Oxalic acid may be employed for accurate determination of ascorbic acid.

iii. The recommended daily intake of vitamin C from the samples studied should be worked out.

iv. Studies of the chemical constituent of the plants should be studied.

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