Sudan University of Science and Technology

College of Graduate Studies

Development of Methods for the Determination of Vitamin C Content in some Dry Fruits and Leaves, Laloub and Mesquite

(*Balanites aegyptiaca* and *Prosopis juliflor*a)

تطوير طرق لتعيين فيتامين (ج) في بعض الثماروالأوراق الجافة (اللالوب والمسكيت)

A thesis submitted in fulfillment for the degree of

Doctor of Philosophy in Chemistry

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Declaration

I, the signing here –under, declare that I'm the sole author of the Ph.D.thesis entitled

Development of methods for the determination of vitamin C content in some dry fruits and leaves, laloub and mesquite (*Balanites aegyptiaca* and *Prosopis juliflor*a)

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Dedication

For the spirit of my mother and father

To my brothers and sisters

To my husband

To my son

Published Papers

- **Determination of ascorbic acid content in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves) by using high –performance liquid chromatography**

- **Development of indirect UV spectrophotometric method for the determination of vitamin C content in *Prosopis juliflora and Balanites aegyptiaca* using standard ceric (IV) sulphate**

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Abstract

In this study, vitamin C has been determined by iodine titration in which starch was used as indicator in which four solvents were used to extract vitamin C from fruits and leaves samples of *Balanites aegyptiaca* and *Prosopis juliflora*. When it was extracted in acetic acid it gave maximum percentage of vitamin C in *Balanites aegyptiaca* leaves, BL (54.7) and minimum percentage for BF (1.76). However, when it was extracted with a mixture of 0.3M metaphosphoric acid and 1.4M acetic acid, the highest percentage was in BF (71.39) and the lowest percentage (15.8) for *Prosopis juliflora* leaves, PL .In contrast, when the vitamin was extracted with a mixture of 4.5% metaphosphoric acid and 1% sulphuric acid, the results were 52.75% for BL and 7.07% for the *Prosopis juliflora* fruits (PF).

HPLC was used with two percentages of extracting solution: first when the percentages of metaphosphoric acid were 2%, it gave highest value in PF fruits (16.128 ppm) followed by BL (7.727 ppm) and PL 5.597 ppm ,but the lowest value of the vitamin was found in BF (3.794 ppm). But when the percentage of metaphosphoric acid was 4.5% it gave highest value of vitamin C in BL (461.5 ppm), followed by BF (391ppm), while PL gave 315.1 ppm and PF gave the lowest value 216.5 ppm.The HPLC method was validated for linearity, ruggedness, inter –and intra precision and recovery

A UV spectrophotometric method for the determination of cerium has been developed. In order to establish the optimum experimental conditions, the effect of various parameters comprising absorption, wavelength, temperature, reaction time after preparation and pH was investigated. It was found that an increase in temperature (20, 30, 40 ˚C) or increase in reaction time (5, 10, 20, 30 and 40 min) increased the absorbance of ceric (IV) sulphate solution. Absorbance was

measured at five wavelengths (300,323,325,332 and 340 nm) and the maximum absorbance was obtained at 323nm.The absorbance of ceric (IV) sulphate was also measured in 4M, 5M and 6M H_2SO_4 .The maximum absorbance was obtained in 5M H_2SO_4.

A simple, rapid and accurate method for the determination of vitamin C content using ceric (IV) sulphate was developed by indirect UV spectrophotometry.The method has been applied for the determination of vitamin C content in the dry fruits and leaves samples. Three extracting solutions were used: 4.5% metaphosphoric acid, acetic acid and distilled water. The first extracting solution gave comparatively high concentration of vitamin C in BL(465 ppm),followed in BF(380 ppm).In PL also it comparatively high content (315 ppm) was obtained, followed in PF(216 ppm).The second extracting solution gave high content(441 ppm) in BL ,followed in BF(361ppm),and in PF(306 ppm) followed in PL(198 ppm).The third extracting solution gave high content in PL(423 ppm) followed in PF(326 ppm),and in BL(283 ppm),and in BF(179 ppm).

مستخلص البحث

تم في هذه الدراسة تقدير فيتامين (ج) حامض الأسكوربيك عن طريق المعايرات باليود في وجود النشأ وذلك بإستخدام أربعة مذيبات لإستخلاص فيتامين (ج) من العينات وهي ثمار وأوراق نباتي اللالوب والمسكيت حيث أعطي الإستخلاص بحامض الإيثانويك أعلي نسبة مئوية للفيتامين في أوراق اللالوب (54.7) وأدني قيمة لثماره (1.76) أما بالنسبة للإستخلاص بحامض ميتافسفوريك (0.3م) وحامض الإيثانويك (1.4م) حيث أعطي أعلي نسبة لثمار اللالوب(71.39) وأدني نسبة لأوراق المسكيت (15.8) وعند الأستخلاص بخليط من (4.5%) حامض ميتافسفوريك و(1%) حامض الكبريتيك أعطي نتائج (52.75) لأوراق اللالوب و (7.07) لثماره.

وتم إستخلاص الفيتامين بحامض ميتافسفوريك بنسبة 2% حيث أستخدم جهاز HPLC والذي أعطي أعلي قيمة لثمار المسكيت (16.128 جزء في المليون) وتليها أوراق اللالوب (7.727 جزء في المليون) كما أعطت أوراق المسكيت نسبة (5.597 جزء في المليون) وأدني نسبة للفيتامين كانت في ثمار اللالوب (3.794 جزء في المليون).أما عند إستخدام 4.5% حامض ميتافسفوريك أعطي أعلي قيمة لأوراق اللالوب (461.5 جزء في المليون) أما ثمار اللالوب (391 جزء في المليون) تليها أوراق المسكيت(315.1 جزء في المليون) كما أعطت ثمار المسكيت أدني قيمة للفيتامين (216.5جزء في المليون).

تم تطوير طريقة مضوائية طيفية لتعيين السيريوم وتمت دراسة ظروف التجربة المثلي بعدة متغيرات وهي التركيز والطول الموجي ودرجة الحرارة والزمن ودرجة الحموضة.تم قياس الإمتصاص عند درجات الحرارة 20، 30 و40 درجة مئوية ووجد أن الإمتصاص يزداد بإزدياد درجة الحرارة وتم قياس الإمتصاص بعد 5،10، 20,30 و40 دقيقة ووجد أيضاً أن الإمتصاص يزداد مع الزمن .أما بالنسبة للطول الموجي أختيرت خمس أطوال موجية (300 ،323 ، 325 ، 332 ، 340 نانومتر) ووجد أن إزدياد الإمتصاص يزداد بإزدياد الطول الموجي من 300 إلي 332 ثم يقل عند 340 نانومتر عند درجة حموضة 4م أما بالنسبة لحموضة 5م يزداد من 300 الي 323 ثم يقل من 325و 340 وعند 6م كسابقه. وأخيراً أثر الحمضية علي إمتصاص السيريوم تمت بتغيير من 4م إلي 6م حيث يزداد الإمتصاص بسرعة من 4م الي 5م ثم ينخفض في 6م لذلك تم إختيار تركيز 5م لوجود أعلي إمتصاص.

في هذه الدراسة تم تطوير طريقة سهلة ودقيقة وسريعة لتعيين فيتامين (ج) في ثمار وأوراق اللالوب والمسكيت الجافة بإستخدام وفرة من محلول كبريتات السيريوم(+4) القياسي وإضافة محاليل قياسية متدرجة من محلول فيتامين (ج) وقياس السيريوم (+4) المتبقي وذلك بإستخدام طريقة المضوائية الطيفية للأشعة فوق البنفسجية غير المباشرة. وتم تقدير نسبة الفيتامين في العينات بإستخدام ثلاثة مستخلصات وهي حامض ميتافسفوريك وحامض الإيثانويك والماء المقطر فعند الإستخلاص بنسبة (4.5%) من حامض ميتافسفوريك أعطي أعلي تركيز لفيتامين (ج) في أوراق اللالوب حيث أعطي قيمة 465 جزء في المليون وتليها 380 لثماره ، أما بالنسبة لنبات المسكيت فأعطي 216 جزء في المليون للثمار وقيمة 315 للأوراق. وبالمقارنة مع الإستخلاص بحامض الإيثانويك وجد أيضا أن للأوراق أعلي قيمة للفيتامين وهي 441 جزء في المليون أما الثمار 361 .أما نبات المسكيت فقيمة الفيتامين 306 جزء في المليون للثمار و198 جزء في المليون للأوراق. وأما الإستخلاص بالماء المقطر فوجد أنه أعطي 423 جزء في المليون لأوراق اللالوب و326 جزء في المليون لثماره ونبات المسكيت وجد قيمة الفيتامين 283 جزء في المليون للأوراق و179 جزء في المليون للثمار.

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List of abbreviations

Avg	Average
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
DCIP	Dichloroindophenol
DNPH	Dinitrophenylhydrazine
EDTA	Ethylene diamine tetra acetic acid
Em	Emission wavelength
Ex	Excitation wavelength
FIA	Flow injection analysis
HPLC	High – performance liquid chromatography
ICH	International conference on harmonization
ISO	International organization for standardization
LC	Liquid chromatography
LC-MS	Liguid chromatography-mass spectrophotometry
LOD	Limit of detection
LOQ	Limit of quantification
MEECC	Micro emulsion electrokinetic capillary chromatography
MECC	Micellar electrokinetic capillary chromatography
OPD	O-Phenylenediamine
RSD	Relative standard deviation
S	Slop of the calibration curve
SD	Standard deviation
SIA	Sequential injection analysis
SPE	Solid phase extraction
UNIDO	United nation for industrial development organization

Chapter one

Introduction and literature review

1. Introduction and literature review

1.1 Introduction to vitamins

Nowadays the different malignant tumors and cardiovascular diseases are widespread throughout the world, in several times the improper manner of living and malnutrition are in the background of these diseases. Inappropriate food choices and economic constrains leading to unbalanced diets are unlikely to provide adequate levels of all micronutrients, which are essential for the normal growth and maintenance of life.

The group of vitamins belongs to these essential compounds. The term “vitamin” (vita=life) also indicates their important role in the preservation of health. Vitamins are a diverse group of organic compounds that are, in very small amounts, essential for the normal functioning of several physiological processes.

Lack of them can generate several serious diseases. Vitamin malnutrition is a widespread problem throughout the world in both developing and industrialized countries, with serious health and economic implications.

The best way of preventing micronutrient malnutrition is to ensure consumption of a balanced diet that is adequate in every nutrient. Thus consumer education programmes to encourage changes in dietary patterns would be the best long-term solution. However, factors affecting food choice are complex (e.g.: bad habits, economic constrains), and therefore programmes to effect changes in dietary patterns may take a relatively long time. For this reason food fortification can be an important tool in helping populations to meet their dietary requirements. In growing numbers of countries fortification of staple foods (salt, flour) with micronutrients becomes an important part of their strategy to improve the nutritional status of the population. Due to the

spread of nutrition science knowledge among the population, there is a growing segment of the society that strives for health-conscious nutrition. To take such a demand of the people into consideration a growing number of products with health-promoting properties are developed and put into trade circulation by the pharmaceutical and food industry. Because of this reason an expanding scale of various dietary supplements and fortified foods with micronutritions are commercially available.

Coming outand fast spreading of the fortified food products require the development of modern, accurate, timesaving, low cost analytical methods for quality control purposes. (Moreno and Salvado 2000)

1.2 Classification of vitamins

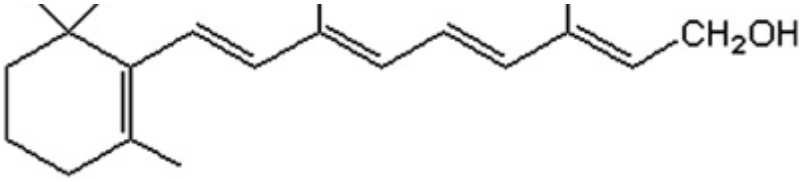
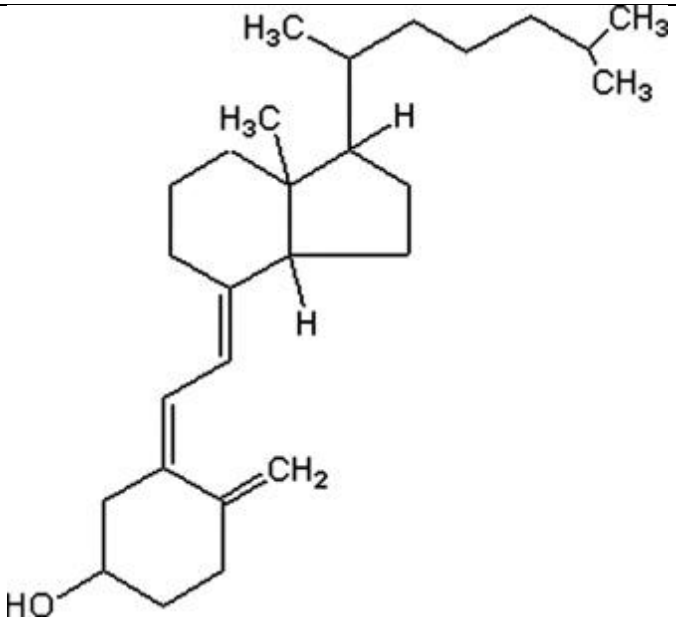
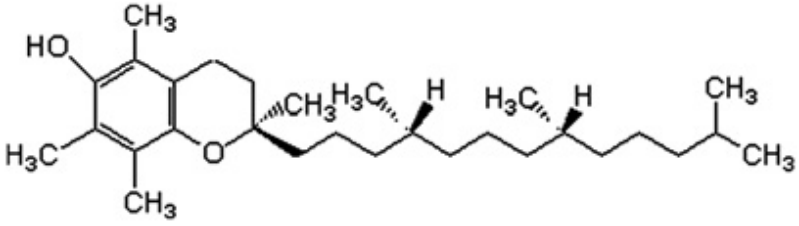
Vitamins are usually classified into two different groups according to their solubility: fat-soluble vitamins (vitamins A, D, E, and K) and water soluble vitamins (thiamin (vitamin B1), riboflavin (vitamin B2), pantothenic acid (B5), and folic acid, cyanocobalamin (Vitamin (B12), biotin, pyridoxine vitamin (B6), niacin (B3) and ascorbic acid (vitamin C).

1.2.1 Fat-soluble vitamins

Table 1.1 shows the chemical structures of the fat-soluble vitamins as well as the main food sources for these compounds.

All - Trans retinol, vitamin A, is closely related to carotenoids, given the particular provitamin A activity of some of them, more importantly b-carotene or b-cryptoxanthin. In fact, as observed in Table 1.1, basically a molecule of vitamin A is half of a b-carotene molecule. Nevertheless, a wider group of retinoids are commonly considered as vitamin A, including some all-trans retinol esterified with fatty acids, which are more stable and more soluble in oils, in which they are often commercially incorporated. (Herrero, Cifuentes, and Iba´n˜ ez 2012)

Table 1.1fat soluble vitamins structures and main sources:

Vitamin	Chemical structure	Good food sources	Biological functions
A	 All-trans-retinol	Meat, milk, dairy products, butter, eggs, fish oils	Effects on visual cycle; effects on immune response and gene expression during embryonic development
D	 Vitamin D3	Fatty fish, liver, eggs, milk, dairy products	Maintaining calcium homeostasis; mobilizationof calcium and phosphorous in bone;absorption of calcium
E		Nuts, beans, vegetable oils	Antioxidant protecting from lipid peroxidation

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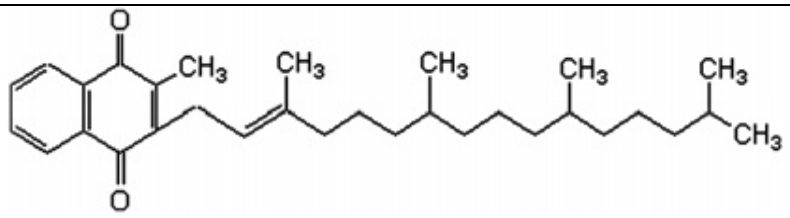
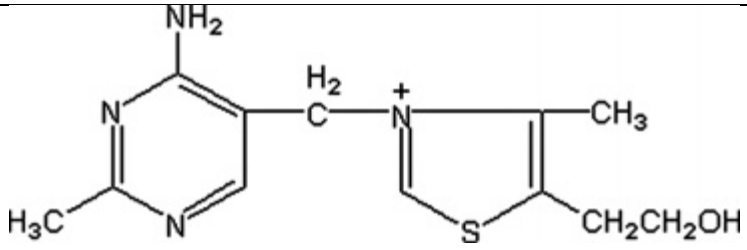
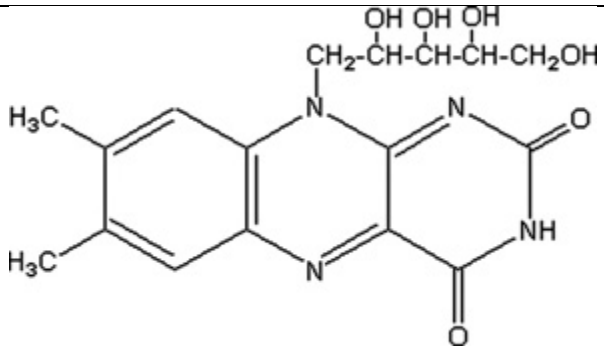
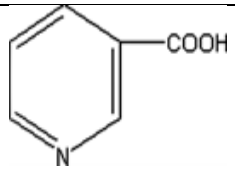
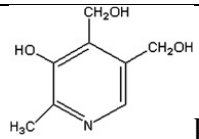
	a-Tocopherola-		
K	 Phylloquinone	Legumes, vegetable oils, leafy green vegetables, cheese	Antihemorrhagic

1.2.2 Water-soluble vitamins

Table 1.2 water-soluble vitamin chemical structure, main biological functions, and good food sources

Vitamin	Chemical structure	Good food sources	Biological functions
Thiamin (B1)		Yeast, whole grains, nuts, wheat bran, liver,legumes	Coenzyme in carbohydrate and amino acid metabolism

(continued)

Riboflavin (B2)		Yeast, liver, kidney,wheat bran, eggs, meat, milk, cheese	Structure of FMN and FAD, participation in oxidation–reduction reactions; rolein energy productionin cells
Niacin (B3)	 Nicotinic acid	Red meat, poultry, liver, legumes	NAD and NADP cofactors in several oxidation–reduction reactions
Vitamin B6	 pyridoxine	Yeast, wheat bran, liver, whole grains, nuts	Cofactor for more than 100 enzymes; essential for normal growth, development

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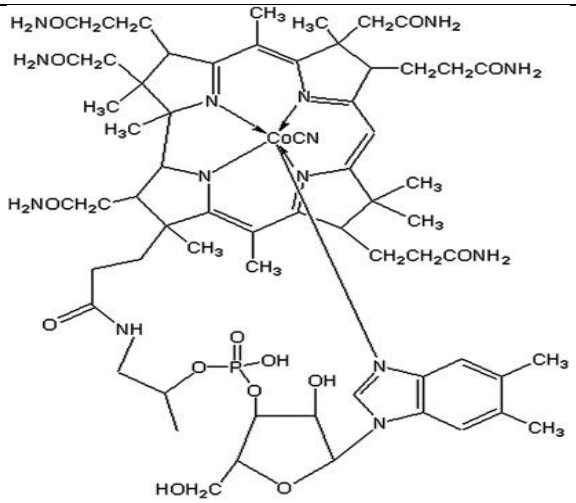
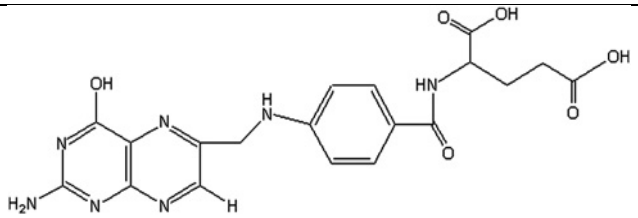
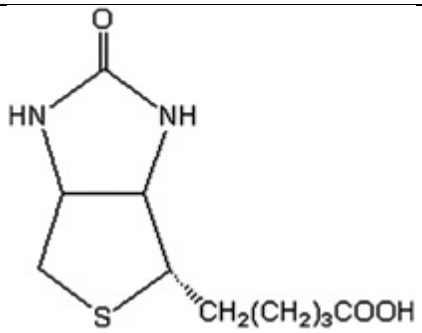
Vitamin B12	 Cyanocobalamin	Meats, seafoods, eggs, milk	Coenzyme for several enzymes
Folate	 Folic acid	Legumes, leafy green vegetables, citrus fruits, liver	Cofactors, serve as acceptors and donors of one-carbon units in reactions involved in amino acid and nucleotide metabolism
Biotin		Liver, eggs, soy beans,	Cofactor for 4 carboxylases

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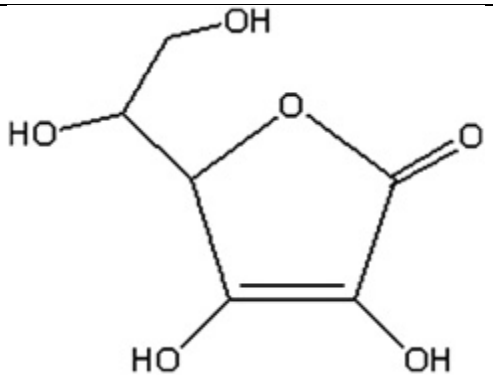
Vitamin C	 L-Ascorbic acid	Fresh fruits, green vegetables	Very important antioxidant; electron donor

1.3 Methods of vitamins analysis

Many vitamins have sensitivity to different conditions, regardless of the type of applied assay. Therefore, certain precautions need to be taken to prevent any deterioration throughout the analytical process. For example, in bioassays, some steps need to be followed with the test material throughout the feeding period, while in microbiological and physicochemical methods; they are required during extraction and analytical procedure (Blake 2007, Ball 2006).

Various vitamins belong to different classes of organic substances, so analysis of vitamins is performed by different chemical, physical, and biological methods (Kozlov, Solunina, Lyubareva and Nadtochii 2003).

Vitamin analysis methods can be classified as follows: (Pegg, Landen and Eitenmiller 2010).

•Bioassays involving humans and animals

•Microbiological assays using organisms, bacteria, and yeast

•Physicochemical assays, including spectrophotometric, fluorometric, chromatographic, enzymatic, immunological, and radiometric methods

1.3.1 Bioassay methods

Bioassays are procedures that can determine the concentration of purity or biologicalactivity of a substance such as vitamin, hormone, and plant growth factor (EPA 2000).

They measure the enzyme under the influence of a vitamin and the phenotypiceffects of their deficiency (Pegg, Landen and Eitenmiller 2010).

Bioassay methods are rarely used clinically for vitamin analysis; they are most commonly used for the analysis of vitamins B12 and D (Ball 1998).

There are two types of bioassays:

•In vivo bioassays. Although this method is close to reality, it has many disadvantages, including that it is not applicable for all vitamins and is time consuming and is an indirect method and expensive, and has no clinical application (Carlucci and Bowes 1972).

•In vitro bioassays. Compared with in vivo bioassays, this method is easier, less time consuming, inexpensive, can be used to analyze several vitamins, and not close to reality, and the cell cultures are at risk of contamination (Gregory and Litherland 1986).

1.3.2 Microbiological assay methods

This is a method of measuring compounds, such as vitamins and amino acids, using microorganisms. It determines or estimates the concentration or potency of an antibiotic by means of measuring and comparing the area of zone of inhibition or turbidity produced by the test substance with that of standard over a suitable microbe under standard conditions. The applicability of microbiological assays is limited to water-soluble vitamins; they are most commonly applied to niacin, cyanocobalamin, and pantothenic acid (Blake 2007).These methods are highly sensitive and specific for each vitamin. Although they are somewhat time consuming, they can generally be used for

the analysis of a relatively wide array of biological matrices without major modifications (Blake 2007). An example of microbiological assays mentioned in the literature is for the determination of vitamins in tarhana (a traditional Turkish cereal food) (Ibanoglu, Ainsworth, Wilson and Hayes 1995).

1.3.3 Physicochemical assay methods

Because of their relative simplicity, accuracy, and precision, the physicochemical methods, in particular the chromatographic methods using high-performance liquid chromatography (HPLC) are the preferred method for vitamin analysis.

HPLC has been used increasingly in the analysis of food samples to separate and detect additives and contaminants. HPLC can separate a large number of compounds both rapidly and at high sensitivity, reduce separation times, and reduce volume of the sample needed. HPLC is ideally suited for compounds of limited thermal stability, but requires sample pretreatment such as extraction and filtration. In addition, HPLC requires careful selection of mobile phase and sample pumping rate (Lee 2000).

Today, HPLC is used as a reference technique to analyze any type of vitamin (Leenheer, Lambert and Ruyter 1985), this technique possesses simultaneous detection and determination of vitamins in one sample (Kozlov, Solunina, Lyubareva and Nadtochii 2003).

HPLC is often used for the simultaneous qualitative and quantitative analysis of water-soluble vitamin and fat-soluble vitamin in biological matrices such as plasma and urine (Gentili, Caretti, D'Ascenzo, Marchese, Perret, Corcia, and Rocca 2008, Luttseva and Maslov 1999, Luttseva, Maslov and Seredenko 2001).

1.4 Ascorbic acid (vitamin C)

1.4.1Chemistry of ascorbic acid

L-Ascorbic acid, also known as; L-xyloascorbic acid, 3-oxo-L-gulofuranolactone (enol form), L-3-ketothreohexuronic acid lactone, antisorbutic vitamin

Vitamin C: has the Chemical formula: $C_6H_8O_6$

Molecular weight: 176.12

Melting point: 190 - 192˚C

This water-soluble vitamin is important in forming collagen, a protein that gives structure to bones, cartilage, muscle, and blood vessels. It also helps maintain capillaries, bones, and teeth and aids in the absorption of iron. Ascorbic acid, a reducing agent, 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 - hydroxyproline and hydroxylysine. These 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. Ascorbic acid plays an important role as a component of enzymes involved in the synthesis of collagen and carnitine; however, its most vital role is as a water-soluble vitamin in the human body (Sies, Stahl 1995; Levine et al 1995).

Ascorbic acid is a powerful antioxidant because it can donate a hydrogen atom and form a relatively stable ascorbyl free radical

$RO\bullet + C_6H_7O_6^- \rightarrow ROH + C_6H_6O_6\bullet^-$ (Hickey and Roberts 2004).

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 (Weber, Bendich and Schalch 1996).

Ascorbate Oxidase

$L\text{-Ascorbic acid} + 1/2\ O_2 \rightarrow l\text{-Dehydroascorbic acid} + H_2O$

Ascorbate Peroxidase

$L\text{-Ascorbic acid} + H_2O_2 \rightarrow l\text{-Dehydroascorbic acid} + 2H_2O$

Ascorbic acid protects folic acid reductase, which converts folic acid to folinic acid, and may help release free folic acid from its conjugates in food. Ascorbic acid facilitates the absorption of iron.

1.4.2 Deficiencies

Severe deficiency of ascorbic acid causes scurvy. Symptoms appear when the serum level falls below 0.2 mg/dl. A total body pool of less than 300 mg is associated with symptoms of scurvy, while maximum body pools are limited to about 2 g (IOM 2000).

Several symptoms of ascorbic acid deficiency have been recognized including follicular hyperkeratosis, swollen and inflamed gums, loosening of teeth, dryness of the mouth and eyes, loss of hair and dry itchy skin. These symptoms reflect the role of ascorbic acid in the maintenance of collagen and blood vessel integrity. It is an acute or chronic disease characterized by hemorrhagic manifestations and abnormal osteoid and dentin formation. The psychological manifestations of scurvy include depression and hysteria. This potentially fatal disease can be prevented with as little as 10 mg ascorbic acid per day, an amount easily obtained through consumption of fresh fruits and vegetables.

1.4.3 Methods of vitamin C analysis

Vitamin C is the only water-soluble vitamin not assayed microbiologically. Rapid advances were made after the guinea pig bioassay was developed in 1922 to measure antiscorbutic activity (Sherman and Campbell 1922). Methodology has advanced from the bioassay to instrumentally advance spectrophotometric, fluorometric, electrochemical, and chemiluminescence methods.

Chromatographic procedures, primarily liquid chromatography, and capillary electrophoresis provide excellent means to resolve l-ascorbic acid, l-dehydroascorbic acid, and d-isoascorbic acid. These separation techniques used with ultraviolet/visible (UV/visible), fluorescence, or electrochemical detectors provide selective and sensitive means to quantify l-ascorbic acid and its isomers from complex biological matrices.

Liquid chromatography coupled to mass spectrometry (LC-MS) has been used less frequently for vitamin C analysis compared to its use in other water-soluble vitamin studies.

Pachla et al (1985) classified vitamin C methods into spectroscopic, electrochemical, enzymatic, and chromatographic techniques. Capillary electrophoretic methods must now be added to this classification. In addition, chemiluminescence methods have been successfully applied to vitamin C analysis since 1985.

Several of these procedures represent the classical approaches to vitamin C analysis used in laboratories worldwide. The basic chemistry of these procedures forms the basis of newer, more instrumentally advanced methods.

1.4.4 Extraction procedures

Owing to the labile nature of vitamin C, extraction procedures are designed to stabilize the vitamin. Cooke and Moxon (1985) reviewed the literature up to 1981 and found that 20 or more extraction solutions were used by various researchers working with a large number of biological matrices. Extraction solutions should maintain an acidic environment, chelate metals, inactivate ascorbic acid oxidase, limit soluble oxygen, and precipitate starch and proteins.

Choice of the extraction solution depends upon the sample matrix and the determinative procedure. Extractants that usually limit l-ascorbic acid destruction to less than 5% include 3%–6% metaphosphoric acid containing acetic or sulfuric acid or 0.005 M ethylenediaminetetraacetic acid (EDTA).Cooke and Moxon (1985). Metaphosphoric acid, while not compatible to some LC procedures, has been the most commonly used extractant. Metaphosphoric acid inhibits l-ascorbic acid oxidase, inhibits metal catalysis, and precipitates proteins that aid in extract clarification. (Pelletier1984).Starch is problematic in that it interferes with colorimetric titrations and fluorometric assays. Addition of ethanol or acetone to the metaphosphoric extract precipitates solubilized starch. (Remmers 1968)(Pelletier, Leduc, Tremblay and Brassard 1977) .This step is necessary for analysis of many vegetables including potatoes, legumes, and corn by spectroscopic methods. Acetone is also useful to remove metabisulfite and sulfur dioxide from dehydrated fruit products and fruit juices. These reducing agents interfere with the 2, 6-dichloroindophenol (DCIP) titration based on reduction of the oxidized dye. EDTA is active as a chelator in vitamin C extractants. It is effective for copper chelation in metaphosphoric acid and trichloroacetic acid, but ineffective for oxalic acid chelation.

All extraction procedures should be completed rapidly in subdued light to limit light catalyzed oxidative reactions. Particle size reduction methods should avoid heat buildup. Whenever possible, the sample should be over layered with nitrogen during initial sample compositing procedures. (Eitenmiller and Landen 1995) Freeze-drying is not recommended for sample concentration or preservation since vitamin C stability decreases in the porous matrix. (Cooke and Moxon 1985) When high moisture samples are blended, the stabilizing extractant should be added before blending. Stability of total ascorbic acid (L-ascorbic acid + L-dehydroascorbic acid) in serum can be extended for long periods under proper conditions. Addition of dithiothreitol or metaphosphoric acid (50 g L^{-1}) effectively stabilizes the vitamin when the serum is held frozen at −70°C. (Margolis, Paule and Ziegler1990), (Margolis and Duewer 1996).

Ascorbic Acid in Serum is stabilized with metaphosphoric acid at 50 g L−1. (Margolis, Vangel and Duewer 2003). Stability in the blood from the time it is drawn to the time the plasma is prepared, stabilized, and analyzed is problematic. Terzuoli et al (2004) recommended a lithium heparin treatment with samples kept at room temperature for transport of no more than 2 h from the time the blood is drawn for maximal l-ascorbic acid stability. Ching et al. (2002) recommended dipotassium EDTA over lithium hepararin treatment with separation within 2 h to minimize degradation.

Margolis and Park (2001) showed that degradation can occur upon placement of l-ascorbic acid solutions in auto sampler vials before analysis. They showed that the inside surface of the glassware can contain materials such as trace amounts of metals that can rapidly degrade l-ascorbic acid. The authors stated that the sample tubes for collection, processing, and storage are also subject to this problem. They evaluated nine lots of vials from five suppliers and found high variation in the stability of l-ascorbic acid stored in the different

vials. Effective limitation of the degradation involved soaking the vials in 0.5 mol L−1 NaOH for 30 min; rinsing with distilled, deionized water; soaking in 1 mol L−1 HCl; and again rinsing with distilled, deionized water. Sodium thiosulfate (0.04% w/v) was an effective stabilizer for l-ascorbic acid dissolved in distilled water before UV analysis. (Kwakye 2000) It was more effective than either sodium metabisulphite and sodium sulphite. Rates of decomposition within 60 min after dissolution were sodium thiosulphate (0.04%) < sodium metabisulphite (0.4%) < sodium sulphite (0.16%).

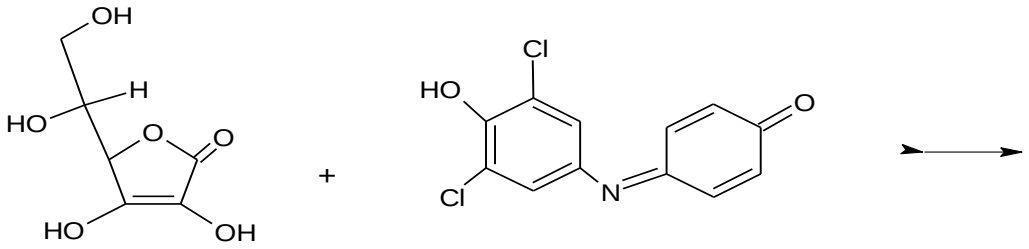
Rizzolo et al (2002) showed that handling and storage of fresh fruit was critical to stability of l-ascorbic acid before analysis. These authors sampled pears by avoidance of extensive cutting to minimize induction of ascorbate oxidase and other oxidases that catalyze L-ascorbic acid oxidation, freezing each sample in liquid nitrogen and storing samples before extraction at −80°C. Hernandez et al (2006) compared 3% metaphosphoric acid–8% acetic acid to 0.1 oxalic acid for extraction of ascorbic acid from tropical fruits. The metaphosphoric–acetic extractant was suitable for all fruits studied; whereas, oxalic acid gave variable results.

1.5 Classical approaches to vitamin C analysis

1.5.1 Oxidation–reduction methods

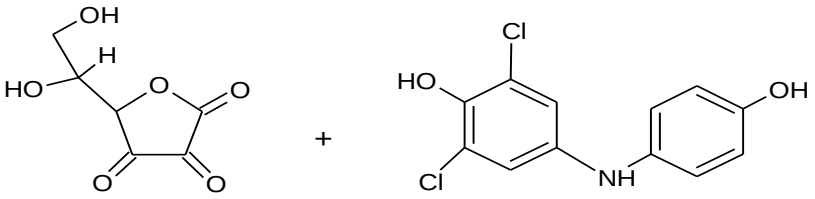
1.5.1.1 2, 6-Dichloroindophenol titration (DCIP)

The DCIP titration was introduced byTillmans in (1930) DCIP is reduced by L-ascorbic acid to a colorless solution from the deep blue color of the oxidized dye (l-a scorbic acid is oxidized to dehydroascorbic acid and excess dye remains pink in acid solution, forming the visual end point of the titration.



Ascorbic Acid

2,6 - DCIP



Dihydroascorbic acid

Leuco dye

Absorbance at 518 nm can be used alternatively to visual end point determination.

Several important deficiencies exist with the method. Most importantly, the titration is limited to quantitation of L-ascorbic acid. Dehydroascorbic acid will not be measured unless it is reduced to ascorbic acid. The titration will not distinguish between l-ascorbic acid and isoascorbic acid. The method cannot be used for vitamin C analysis of processed and cured meats containing isoascorbic acid. DCIP titration can be used for fresh juices and multivitamins that do not contain excessive amounts of copper or iron. (Pelletier, Augustin, Klein, Becker and Venugopal, John Wiley and Sons 1984). For processed or cooked food known to contain copper, iron, or tin, other methods capable of measuring dehydroascorbic acid in addition to L-ascorbic acid should be used to quantitate total vitamin C. (Eitenmiller, Landen, Jeon, Ikins and Marcel Dekker 1995)

Highly colored extracts from fruit and vegetables can mask the color change at the titration's end point.

Reduction of DCIP is not limited to L-ascorbic acid, and any reducing substance present in the sample can reduce the dye. Such interferences can lead to erroneously high measurements, if not recognized. Substances that can interfere include cuprous, ferrous, and stannous ions, sulfite, thiosulfate, tannins, betanin, cysteine, glutathione, and reductones generated by nonenzymatic browning.

Several method modifications have been introduced to eliminate or minimize the effects of interferences on the DCIP titration. (Pelletier, Klein, Becker, Venugopal, BJohn Wiley and Sons1984), (Pachla, Reynoldsand Kissinger1985), (Cooke, Moxon, Counselland Horning 1985)

A solid-phase extraction (SPE) procedure was developed that expands the DCIP titrationto highly colored multivitamins, soft drinks, and fruits and vegetables (Verma, Jain, Sahasrabuddhey, Gupta and Mishra1996).

Further, the cleanup step removes copper, iron, sulfite, and other interfering reducing substances, suchas cysteine and glutathione.

C18 silica impregnated with 2, 29-bipyridyl-2,9-dimethyl-1,10-phenathroline (neocuproine) and N-ethylmaleimide removes Fe(II) and Cu (I) and sulfhydryl compounds, respectively. The method provides for determination of L-ascorbic acid and L-dehydroascorbic acid by reducing the L-dehydroascorbic acid back to L-ascorbicacid with cysteine before the SPE step. This procedure is simple and increases the sensitivity of the DCIP procedure. Incorporation of the SPE step into existing regulatory methods could decrease problems associated with existing titration procedures (AOAC 1990) (AOAC 2005).

Ascorbic Acid in vitamin preparations and Juices, 2, 6-Dichloroindophenol titrimetric Method, AOAC Official Methods of Analysis,

AOAC (2005). ANP (1990) was recommended for the analysis of l-ascorbic acid in beverages and juices for nutritional labeling purposes (BP 2007).

However, the method is routinely applied world wide to other food matrices because of its simplicity. If the deficiencies of the methodare recognized by the analyst, the procedure provides reliable measures for l-ascorbic acid provided that the food does not contain appreciable quantities of reducing substances and l-dehydroascorbic acid (Eitenmiller and Landen, Jeon and Ikins (1995).

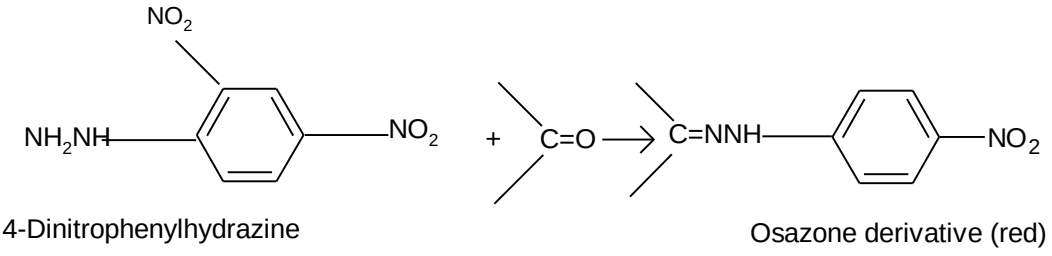
Application of the SPE cleanup discussed previously could greatly expand the use of the DCIP titration since many laboratories still must rely on simple, non instrumental approaches to food analysis

The method includes the following steps:

1. Extraction
2. Titration

1.5.1. 2 Dinitrophenylhydrazine(DNPH)

2, 4-Dinitrophenylhydrazine(DNPH) reacts with ketone groups of dehydroascorbic acid under acidic conditions to form a red osazone derivative .



2,4-Dinitrophenylhydrazine

Osazone derivative (red)

DNPH is useful for the analysis of total vitamin C if appreciable quantities of sugars are not present in the product. L-Ascorbic acid is oxidized to l-dehydroascorbic acid by Norit or DCIP. Derivatization is completed with the addition of DNPH and the color is produced upon acidification with sulfuric

acid. Maximum absorbance occurs between 500 and 550 nm. Most methods measure the DNPH derivative at 520 nm.

Specificity of the reaction for L-dehydroascorbic acid in complex matrices was attributed to the ability of DNPH to react faster with l-dehydroascorbic acid compared with other carbohydrates; color is produced more easily with DNPH derivatives of 5- and 6- carbon sugar-like compounds and through the ability to minimize formation of non ascorbic acid chromogens by carrying out the reaction at low temperatures (Pachla, Reynolds and Kissinger 1985). Despite the specificity afforded by such factors, DNPH has not been used recently as extensively for food analysis as O-Phenylenediamine (OPD) derivatization. The methods do not compare in simplicity and specificity to the microfluorometric method for total vitamin C assay.

1.5.2 Metal ion reduction.

L-Ascorbic acid in biological samples can be measured by redox reactions in which l-ascorbic acid is used to reduce metal ions to produce colored products. Pachla etal (1985) reviewed metal ion redox methods in detail. These methods rely upon the formation of a stable colored complex between the reduced ion and a chelating agent. Reduction of Fe (III) to Fe (II) by l-ascorbic acid is the most common reaction; although many metal ion redox reactions have been utilized for l-ascorbic acid analysis. A chelator is added, which complexes with the reduced metal. The reduced metal–chelator complex is then measured by spectroscopic methods. With Fe (II), the most common chelating agents are 2, 29-dipyridine, 2, 4, 6-tripyridyl-5-triazine, and ferrozine (Pachla, Reynolds and Kissinger1985).

More recent approaches to metal redox applications for l-ascorbic acid have entailed flow injection and sequential injection techniques with

spectrophotometric methods designed to quantitate l-ascorbic acid and l-dehydroascorbic acid.

A simple flow injection technique developed for pharmaceutical products used Fe (III) and hexacyanoferrate (III) as the chromogenic complexing reagents to produce Prussian blue (Nobrega and Lopes 1996).

The reaction sequence is the following:

1. Oxidation of Fe (II)

$Fe^{2+} + [Fe\ (CN)_6]^{3-} \rightarrow Fe^{3+} + [Fe(CN)_6]^{4-}$

2. Formation of hexacyanoferrate (II) ferric complex

$4Fe^{3+} + 3[Fe\ (CN)_6]^{4-} \rightarrow Fe^{4}[Fe\ (CN)_6]^{3}$Prussian blue

Ascorbic acid reduces Fe (III) to Fe (II) resulting in the formation of a deep blue soluble complex. When excess hexacyanoferrate (III) is present, the formation of Prussian blue is measured at 700 nm.

1.5.3 Derivatization methods

1.5.3.1 O-Phenylenediamine(OPD)

Theo-phenylenediamine (OPD) condensation reaction with l-dehydroascorbic acid represents one of the most useful derivatization reactions to quantitate total vitamin C. The condensation reaction produces a highly fluorescent quinoxaline product (Ex λ = 350, Em λ = 430). AOAC International Method was developed by Deutsch and Weeks (1965) for analysis of total vitamin C (l-ascorbic acid +dehydroascorbic acid).

This assay improved the scope and specificity of vitamin C methods existing at the time (Eitenmiller and Landen 1995).

AOAC Official Method Vitamin C (Total) in Vitamin Preparations,

Microfluorometric Method—the AOAC Task Force on Methods for nutrition Labeling recommended that method is used for most food matrices (AOAC 1993).

The method includes the following steps:

1. Extraction
2. Quinoxaline Fluorescent Derivative Formation
3. Blank Correction

1.5.4 Enzymatic methods

Enzyme conversions of L -ascorbic acid to L -dehydroascorbic acid coupled to a determinative step such as direct spectrophotometric assay following decrease of L-ascorbic acid, OPD **(**o-Phenylenediamine**)**, other derivatization reactions, and electrochemical determination of oxygen uptake during the reaction have been used to assay l-ascorbic acid in biological samples.

Ascorbate oxidase and ascorbate peroxidase activity represented by the following equations convert the l-ascorbic acid to the dehydro form (Ball 1994).

Ascorbate Oxidase

L-Ascorbic acid + (½)O_2→ L-Dehydroascorbic acid + H_2O

Ascorbate Peroxidase

L-Ascorbic acid + H_2O_2→ L-Dehydroascorbic acid + $2H_2O$

A variety of enzyme sources have been used for the enzymatic conversion. An ascorbate oxidase spatula is available from Boehringer–Mannheim to convert l-ascorbic acid and isoascorbic acid to the dehydro forms before OPD derivatization and LC quantitation ofthe quinoxaline derivatives (Speek, Schrijver and Schreurs 1984).

Total vitamin C and isoascorbic acid can be quantitated at levels as low as 0.2 μg g^{-1}. L-Dehydroascorbic acid can be quantified by omitting the enzymatic oxidation.

Ascorbate peroxidase oxidation of l-ascorbic acid to dehydroascorbic acid has been applied to the spectrophotometric assay of total vitamin C in foods. In more recently published methods, quiacol peroxidase from horseradish has been used (Casella, Gullotti, Marchesinib and Petrarulo 1989) (Tsumura, Ohsako, Haraguchi, Kumagai, Sakurai, and Ishii 1993).

This peroxidase is commercially available (Sigma Chemical Co.) and catalyzes the oxidation of l-ascorbic acid aswell as quiacol. The direct spectrophotometric assay developed by Tsumura et al (1993) was tested on a wide variety of foods and no interferences were apparent. The method was more precise when compared to assays using DCIP and DNPH.

1.6 Spectrophotometric methods

Spectrophotometric methods for the determination of ascorbic acid can be subdivided into the following two groups: methods based on measuring the intrinsic absorption of ascorbic acid and methods based on measuring the light absorption of products that result from the reduction of various regents by ascorbic acid.

Direct UV spectrophotometry belongs to the former group. Abdel-Hamid et al (1985) proposed a procedure for determining ascorbic acid in the presence of its degradation products. They used the first-derivative spectral peaks at λ= 215 nm for ascorbic acid. Calibration curves were linear over concentration ranges of 1.5–3.0 mg/L for ascorbic acid.

Brodnjak-Voncina and Dobcnik (1986) used absorption at 245nm (ascorbic acid) as an analytical signal. Matrix effects were taken into account with the use of reference solutions prepared by boiling with a 1 M alkali solution (ascorbic acid). Calibration curves were linear over the following ranges, mg/L: 2–50 for ascorbic acid.

The determination of ascorbic acid in non alcoholic beverages, fruits, fruit juices, and drugs requires the preliminary removal of carbon dioxide and solids by degasification and centrifugation, respectively (Fung, Luk (1985),(Lau, Luk, and Wong (1986),(Lau, Luk, and Wong (1987).

The determination sensitivity was improved by the successive addition of Cu (II) (for the catalytic oxidation of ascorbic acid) and a Trilon B solution (Lau, Luk, and Wong (1986), Lau, Luk, and Wong (1987)).The calibration curve was linear up to an ascorbic acid concentration of 120 mg/L when the absorbance was measured at 267 nm.

A procedure for determining ascorbic acid in pharmaceuticals by flow-injection analysis (FIA) was proposed (Verma, Jain, Verma, and Chaurasia, 1991).This procedure is based on the measurement of the intrinsic absorption of ascorbic acid at λ= 245 nmin an acidic antioxidant medium (a 2-mercaptoethanol solution in H_2SO_4). To prepare a reference solution, asample was treated with NaOH and held at 298 K for10 min for the complete degradation of the ascorbic acid by the dissolved oxygen.

The ascorbic acid concentration in a sample was found by a calibration curve plotted on the ΔA_{245} _ c_{Asc} coordinates, where ΔA_{245} is the difference between the absorbance of test solutions before and after treatment with NaOH and $c_A$is the concentration of ascorbic acid. The calibration curve was linear up to an ascorbic acid concentration of 20 mg/L.The productivity of analysis was up to 180 samples per hour.

As a rule, classical reduction procedures for the spectrophotometric determination of ascorbic acid with the use of aniline, 2, 6-dichlorophenolindophenol, Folin's reagent, and hydroxylamine (Korenman 1975) include a time consuming and labor-intensive stage of converting

ascorbic acid into a chromophoric product. Moreover, these methods are characterized by a low selectivity.

For example, the determination of ascorbic acid with Folin's reagent is based on the reduction of a mixture of molybdophosphoric and urano phosphoric acids to molybdenum blue. Other reducing agents (amidopyrine, phenol, tannin, reducing sugars, ydroquinone, pyrocatechol, and resorcinol) interfere with the determination.

Several improved procedures with the use of the above reagents, in particular, 2, 6-dichlorophenolindophenol, were proposed (Chen, Xiao, and Wang, 1994, Davies, and Masten, 1991, Srivastava, Kochar, and Verma1985, Shvartsman, Iorga, and Byrsanu, 1990, Karayannis, and Farasoglou, 1987, Chen, Xiao and Wang 1994).

The determination is based on the decolorization of the reagent on the reduction by ascorbic acid. The interference of Fe (II) and Fe (III) (<400μmol) was removed by the addition of Trilon B (Davies and Masten1991).The sulfhydryl groups of cysteine or glutathione were masked by the pretreatment of a sample with acrylonitrile in a phosphate buffer solution at pH 7.0 (Srivastava, Kochar and Verma 1985).

Unreacted 2, 6-dichlorophenolindophenol was extracted with an organic solvent (butyl acetate or amyl acetate) and the light absorption of the extract was measured at 540 nm. The method determined the concentration of ascorbic acid in the range of 4–8 mg/L.

A kinetic procedure was proposed for the analysis of orange juice, parsley, and potato samples (Karayannis and Farasoglou 1987).The parsley and potato samples were homogenized with a solution of $H_2C_2O_4$. Their resulting suspensions as well asorange juice were centrifuged, and a4×10^{-5}M 2, 6-dichlorophenolindophenol solution in0.2 M $H_2C_2O_4$ was added to the

supernatant liquids of all samples. The light absorption was measured at 522 nm. The concentration range of ascorbic acid was 3.5–88 mg/L.

A number of methods for the determination of ascorbic acid were proposed based on the reaction of Fe (III) reduction followed by the detection of the resulting Fe (II). Thus, Arya and Mahajan (1997) described an extraction–spectrophotometry procedure based on the extraction of Fe (II) with chloroform as acomplex with quinaldic acid and pyridine followed bythe photometric measurement of the extract. The calibration curve was linear in the ascorbic acid concentration range of 2.5–25 mg/L.

The procedure of Arya, Mahajan and Jain (1998) is different in that the Fe(II) formed by the reaction with ascorbic acid was extracted with a trioctylamine solution in chloroform as a complex with picolinic acid and the absorption of the extract was measured at λ= 470 nm.

Another version of this procedure (Arya and Mahajan 1996) is based on the extraction of Fe (II) complex with picolinic acid and pyridine. The light absorption of the yellow complex was measured at 400 nm. The calibration graph was linear in an ascorbic acid concentration range of 0.4– 5.6 mg/L. The procedures of Arya and Mahajan (1997), Arya, Mahajan and Jain (1998) and Arya and Mahajan (1996) were applied to the analysis of pharmaceuticals, biological samples, and foods and satisfactory results were obtained.

Nobrega and Lopes (1996) used hexacyanoferrate(III)λ= 700 nm;$\varepsilon=3.0\times10^{4}$L-mol^{-1}cm $^{-1)}$ for the detection of Fe(II) formed by the reaction with ascorbic acid.

Under conditions of FIA, an alkaline oxalate solution was used for washing off a colored product adsorbed onthe walls of a cell. Calibration graphs were linear for 5–100µM ascorbic acid. The productivity was 140

determinations per hour, and the consumption of reagents was no higher than 0.5 mL per determination.

Ferreiraet al (1997) proposed the photometry of iron (II) as a complex with 2-(5-bromo-2-pyridylazo)-5-thylaminophenol (560nm ($\varepsilon = 1.31\times10^5$ L $mol^{-1}cm^{-1}$; ($\varepsilon_{748} = 5.69\times10^4$ L $mol^{-1}cm^{-1}$). The developed method is highly sensitive: the detection limit is 0.015 (560 nm) or0.044 mg/L (748 nm). Koch and Peisker (1990) and Lauand Luk (1987) determined the concentration of Fe (II) by the reaction with o-phenanthroline in a sodium acetate buffer solution in the presence of EDTA (for maskingan excess of Fe (III)). Background correction was achieved by the pretreatment of a sample with a Cu (II) solution (Lauand Luk (1987).

In the extraction–spectrophotometric determination of ascorbic acid (Koch and Peisker (1990) $NaClO_4$ was added to the reaction mixture; the resulting mixture was extracted with nitrobenzene for 30 s; the extract was separated from an aqueous phase and dried. The absorbance was measured at 515 nm with reference to nitrobenzene. The detection limit of ascorbic acid was 0.012 mg/L at a sample volume of 25 ml. The analytical range was 2–10 μg of ascorbic acid in a sample the ascorbic acid in fresh fruit juices and fruit drinks.

Yamane and Ogawa (1987) described a procedure to determine by flow-injection analysis. The solutions of Fe (III) and o-phenanthroline were passed at flow rates of 0.55 and 0.25 ml/min, respectively, and 100 μL of the test solution was injected into the flow. The absorbance at 510 nm was measured in a continuous mode.

The detection limit was equal to 0.04 mg/L; the calibration curve was rectilinear up to an ascorbic acid concentration of 1.8 mg/L. The productivity of the procedure was about 70 determinations per hour.

Besada (1987) analyzed tablets and ampoule solutions containing vitamin C as described below. A solution containing o-phenanthroline, HCl, and $FeNH_4(SO_4)_2$ were added to a sample; the mixture was allowed to stand for 1 min and then diluted with distilled water toa total volume of 25 ml. The light absorption was measured at 510 nm. The calibration graph for ascorbic acid was linear in the range of 0.8–2.4 mg/L. Nicotinic acid; nicotinamide; thiourea; methionine; starch; glucose; fructose; mannose; sucrose; citric acid; and $Ca^{2+,}$ $Mg^{2+,}$ and Cu^{2+} caused no interference with the determination.Skaltsa et al (1987) proposed to detect iron (II) formed in a reaction with ascorbic acid as a complex with2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine.

Ascorbic acid was determined in plants as described below. A sample of acomminuted plant material was mixed with an acetate buffer solution for 3 min in a nitrogen atmosphere; and the mixture was filtered through a paper filter. The filtrate and a standard solution of ascorbic acid were treated with a solution of ascorbate oxidase, stirred, and thermostatted at 310 K for 15 min. Next, equal volumes of a mixture containing acetate buffer solution,2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine, HCl, and $FeCl_3$ were added to each solution. The absorbance of both solutions was measured at 593 nm. The calibration curve was linear over an ascorbic acid concentration range of 10–100 mg/L.

Procedures based on Cu (II) reduction followed by the detection of univalent copper were proposed for the determination of ascorbic acid. Thus, Baker and Lowe (1985) bound Cu (I) ions by neocuproine in a colored complex and measured the light absorption of this complex at 450 nm. The method determined 2–20µg of ascorbic acid. Cysteine, N-ethylmaleimide, and iodo acetic acid interfered with the determination.

Tutem et al (1992) proposed a simple method for determining ascorbic acid by a reaction with Cu (II) inthe presence of 5-(4-hydroxy-3-thoxybenzylidene)

rhodanine in a water–dioxane mixture atpH 3.8. The photometry of the product was performed at 473 nm ($\varepsilon = 2.6\times10^4$ L mol^{-1} cm^{-1}). Beer's law was obeyed in the concentration range 1.1–6.6 mg/L. The method was applied to the analysis for ascorbic acid in pharmaceutical preparations.

A procedure for the photometric determination of ascorbic acid in trace amounts by the reduction of Ag (I) (gelatin complex) in an aqueous solution at pH 7.5–10.0 with the formation of a yellow silver sol was described (Pal and Maity 1985).For this purpose, a solution of $AgNO_3$was mixed with a gelatin solution and pH 8.0 was adjusted by adding a solution of NaOH. A sample of ascorbic acid was added, and the absorbance at 415 nm was measured after 20 min. The linearity range was1–10 mg/L. Glycine, alanine, sucrose, fructose, citric acid, tartaric acid, oxalic acid, maleic acid, succinic acid, and various reducing agents caused no interference with the determination.

Pal (1999) photochemically reduced Ag (I) with ascorbic acid in a Triton X-100 solution to form a yellow silver sol. The absorbance at 415 nm was measured ($\varepsilon = 1.43\times104$ L mol^{-1} cm^{-1}).

Ascorbic acid can be determined within a range of 0.4–13 mg/L. The method was proposed for the analysisof pharmaceutical preparations containing vitamin C.Lazaro et al. (1986) described the FIA determination of ascorbic acid with spectrophotometric measurement.

The determination was based on the oxidation of HI with chloramine T in an acidic medium followed by the photometric determination of a complex of $I_2$with starch. In the presence of ascorbic acid, $I_2$was also consumed in the oxidation of ascorbic acid to decrease the concentration of the colored complex.

The determination was performed as described below. A solution of ascorbic acid was mixed with H_2SO_4 and a starch–KI solution; the mixture was injected into the flow of achloramine T solution, which was passed through a

coiland directed to a flow cell. The light absorption was measured at 650 nm. The decrease in the absorbance was proportional to the concentration of ascorbic acidin the range of 15–150 mg/L. The same indicator system was proposed for the simultaneous determination of ascorbic acid and sulfite ions in soft drinks. For this purpose, two micro samples, one of which was premixed with a NaOH solution, were synchronously introduced at different points of a flow. As the result ofthe successive mixing of each sample zone with the flow of a reagent (a chloramine T solution containing a KI–starch indicator mixture at pH 10.82) and then with the flow of an H_2SO_4 solution, both ascorbic acid and SO_3^{2-}only reacted with the reagent. The amount of resulting colored products was measured by photometry at 581 nm as peaks whose heights were proportional to the total concentration of ascorbic acid and SO_3^{2-} the concentration of in samples.The analytical range for ascorbic acid and was 44 – 140 mg/L.

A spectrophotometric procedure was proposed forthe FIA determination of ascorbic acid; this procedureis based on the generation of triiodide ions or a triiodide complex with starch directly in a flow-injection system (Hernandez-Mendez, Alonso-Mateos, Almendral-Parra, and Garcia-de-Maria (1986).

A decrease in the absorbance of solutions at 380 and 580 nm was proportional to the concentration of ascorbic acid. The solutions of KI–starch and KIO_3 in H_2SO_4 were passed through a coil (3 m×0.7 mm) at aflow rate of 1.5 ml /min and mixed with an aqueous sample flow. The mixed flow was passed through a coil (130 cm×0.5 mm) and directed to a flow cell for photometry.

The linearity range of calibration graphs for ascorbic acid varied from 0.1–7 to 2–40 mg/L, depending on the concentration of iodate. The productivity of this procedure was 300 samples per hour. The procedure was tested in the

analysis of pharmaceutical preparations containing vitamin C and pineapple juice and jam.

Ciesielski (1986) described a procedure for the simultaneous determination of cysteine, glutathione, and ascorbic acid traces in a mixture with the use of the iodine–azide reaction induced by analytes. A sample was introduced into a solution containing KI and a phosphate buffer solution with pH 5.8. The ascorbic acid content was found from a decrease in the light absorption (350 nm). Another sample was added to a solution containing $NaN_3$and KI (pH 8.0–8.5), and the resulting solution was mixed with formaldehyde, which blocked the effect of cysteine. After 1 min, HCl and I_2 solutions were introduced, and photometric measurements were performed after 5 more minutes. The glutathione content was found by a decrease in the absorbance.

A third sample was added to a solution containing NaN_3, KI, and HCl (pH 5.8). A solution of I_2 was added to the resulting solution, and the light absorbance of the solution was measured after 1 min. The cysteine content of the sample was calculated from the data obtained. The method made it possible to determine10–30mg of ascorbic acid, 0.05–0.25mg of cysteine, and 0.4–2mg of glutathione in 10 ml of the solution.

A simple and selective procedure was developed for the determination of ascorbic acid with diazotized 1-aminoanthraquinonezinc chloride (Fast Red AL salt) in an alkaline medium (λ= 630 nm; ε= 4.07×103 L molÐ1 cmÐ1) Backheef, Emara, Askal and Saleh (1991).The calibration curce was linear within an ascorbic acid concentration range of 5–25 mg/L.

Other vitamins and dehydroascorbic acid caused no interference with the determination. This procedure was applied to the determination of ascorbic acid in pharmaceutical preparations and juices.A procedure based on the reduction

of ascorbic acid with a ternary Mo (VI)–Sb (III)–P (V) heteropoly acid was described (Chen, Xiao and Wang (1994).

Optimum conditions of the determination are the following: reagent concentrations (10^{-4} M) of 3.0 for MoO_4^{2-}, and 0.45 for Sb (III); H+ concentration of 0.5 M; and a temperature of 298 K. The color (e710 = 3.5 ´ 103 L molĐ1 cmĐ1) was developed in 35 min. The linearity of the calibration graphs was retained within the limits of 1–50 mg/L; the detection limit was 0.2 mg/L. The procedure was tested in the analysis of vitamin C tablets.

A procedure was proposed for the spectrophotometric determination of ascorbic acid with a tungstophosphate–vanadate reagent, which consisted of a mixture of tungstophosphoric acid and sodium vanadate with an acetate buffer solution (pH 1.5) (Muralikrishna and Murty 1989).

The concentration of ascorbic acid was directly proportional to the value of DA360 = A1 Đ A2, where A1 and A2 is the absorbance of the reagent in the absence and in the presence of ascorbic acid. The procedure was tested in the determination of ascorbic acid (50Đ60 mg) in tablets, syrups, and fruit juices.

A procedure was proposed for the determination of ascorbic acid in pharmaceuticals, fruit juices, vegetables,fruits, and infant milk powder is based on the reduction reaction of 4-chloro-7-nitrobenzofurazane in an alkaline medium with the formation of a colored product (λ_{max} = 582 nm) (Abdelmageed, Khashaba, Askal, Saleh and Rafaaf 1995).

The calibration graph for ascorbic acid was linear over a range of 5–20 mg/L.Dehydroascorbic acid, all other vitamins, and mineral components of multivitamin preparations, rutin, salicylamide, acetylsalicylic acid, paracetamol, caffeine, etc, did not interfere with the determination.Dabrowski and Hinterleitner (1989) described the spectrophotometric determination of ascorbic

acid andits derivatives in the extracts of animal tissues and feed.For this purpose, five series of solutions were prepared.

1.7 selecting an analytical method

A method is the application of a technique to a specific analyte in a specific matrix. To select an analytical method intelligently, it is essential to define clearly the nature of the analytical problem. Such a definition requires answers to the following questions:

- What accuracy is required?
- How much samples are available?
- What is the concentration range of the analyte?
- What components of the sample might cause interference?

1.7.1 Method development

1.7.1. 1 Specificity

The specificity describes the ability of the method to measure unequivocally the analyte of interest in the presence of all other components as interfering analytes (Riley and Rosanske 1996).

1.7.1. 2 Linearity

The mathematical relationship between the measured response and the concentration of the analyte in the matrix has to be established. Whenever possible, a suitable standard should be used for the quality control of the calibration curves.

1.7.1. 3 Accuracy

Accuracy is a measure of how closely the result of an experiment agrees with the expected result. The difference between the obtained result and the expected result is usually divided by the expected result and reported asa percent relative error.

$$\% \text{ Error} = \frac{\text{obtained result} - \text{expected result} \times 100}{\text{expected result}}$$

Analytical methods may be divided into three groups based on the magnitude of their relative errors. When an experimental result is within 1% of the correct result, the analytical method is highly accurate.Methods resulting in relative errors between 1% and 5% are moderately accurate, but methods of low accuracy produce relative errors greater than5%.

1.7.1.4 Precision

When a sample is analyzed several times, the individual results are rarely the same. Instead, the results are randomly scattered. Precision is a measure of this variability.The closer the agreement between individual analyses, the more precise the results.

Precision is a measure of the spread of data about a central value and may be expressed as the range, the standard deviation, or the variance. Precision is commonly divided into two categories: repeatability and reproducibility.

Repeatability is the precision obtained when all measurements are made by the same analyst during a single period of laboratory work, using the same solutions and equipment.

Reproducibility, on the other hand, is the precision obtained under any other set of conditions, including that between analysts, or between laboratory sessions for a single analyst. Since reproducibility includes additional sources of variability, the reproducibility of an analysis can be no better than its repeatability ((Riley and Rosanske (1996).

1.7.1.5 Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD (lowest amount of an analyte in a sample which can be detected but not necessarily quantitated) and LOQ (Lowest amount of an analyte in a sample

which can be quantitatively determined with a suitable precision and accuracy) were determined according to the International Conference of Harmonization (ICH) guidelines for validation of analytical procedures.

The following formula was used: LOD or LOQ = k SDa/b, where k = 3.3for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope (ICH (2005).

1.7.1.6 Standard deviation

The absolute standard deviation**,** SD, describes the spread of individual measurements about the mean, and is defined as a statistical measure of the "average" deviation of data from the data's mean value.

SD is often expressed in a relative manner. Calculations are made therefore of the relative standard deviation (or RSD).

$$RSD = \frac{SD}{X-}$$

Where X–is the mean.

The percent relative standard deviation is obtained by multiplying RSD by 100%.

1.8 Ultraviolet and visible absorption spectroscopy

Analysts have developed large number of instrumental techniques and these techniques are extremely sensitive and can yield results with a high degree of accuracy. Among these instrumental analytical techniques, ultraviolet and visible (UV-Vis) absorption spectroscopy technique occupies a unique position, because of its simplicity, sensitivity, accuracy and rapidity (Dehahay 1967).

The ultraviolet and visible (UV-Vis) absorption spectroscopy is used to measure the absorption of light, when it is passed through a sample, in the

visible and "near" ultraviolet region, that is, in the 200-750 nm range (Morrison and Boyd's 1992).

The ultraviolet and visible (UV-Vis) provides information about compounds with conjugated double bonds. Ultraviolet light and visible light have enough energy to cause an electronic transition (the promotion of an electron from one orbital to another of higher energy). Depending on the energy needed for the electronic transition, a molecule will absorb either ultraviolet or visible light. If it absorbs ultraviolet light, a UV spectrum is obtained; if it absorbs visible light, a visible spectrum is obtained.

Ultraviolet light is electromagnetic radiation with wavelengths ranging from 180 to 400 nm (nanometers); visible light has wavelengths ranging from 400 to 780 nm. (One nanometer is or10 Å.) Wavelength (λ) is inversely related to the energy: The shorter the wavelength, the greater is the energy. Ultraviolet light, therefore, has greater energy than visible light.

$E = hc/\lambda$

Where:

h Plank's constant

c velocity of light

λ Wavelength

Many types of transitions between quantized energy levels account for molecular UV/Vis spectra. As electron is held tightly, and a good deal of energy is required to excite it: energy corresponding to ultraviolet light of short wavelength, in a region - "far" ultraviolet - outside the range of the usual spectrometer. It is chiefly excitations of the comparatively loosely held n and л electrons that appear in the (near) ultraviolet spectrum, and, of these, only jumps to the lower more stable excited states (Figure 1).

The electronic transitions of most important are:

(a) n л*, in which the electron of an un shared pair goes to an unstable (antibonding) лorbital.

(b) л л*, in which an electron goes from a stable (bonding) л orbital to an unstable (antibonding) л orbital, that because they involve functional groups that are characteristic of the analyte, and wavelengths that are easily accessible (Morrison and Boyd's (1992).The approximate wavelength ranges for these absorptions, as well as apartial list of bonds, functional groups, or molecules that give rise to these transitions is shown in (Table 1.3). The specific bonds or functional groups of organic compounds (ketones, amines, nitrogen derivatives, etc.), responsible for the absorption of a particular wavelength of light in UV/Vis are called chromophores (Harvey 2000).

Table 1.3 Electronic transitions involving n, s, and p Molecular Orbitals

Transition	Wavelength range (nm)	Examples
s s *	< 200	C–C, C- H
n s*	160-260	H_2O, CH_3OH, CH_3Cl
л л*	200-500	C=C, C=O, C=N, C=C
n л*	250-600	C=O, C=N, N=N, N=O

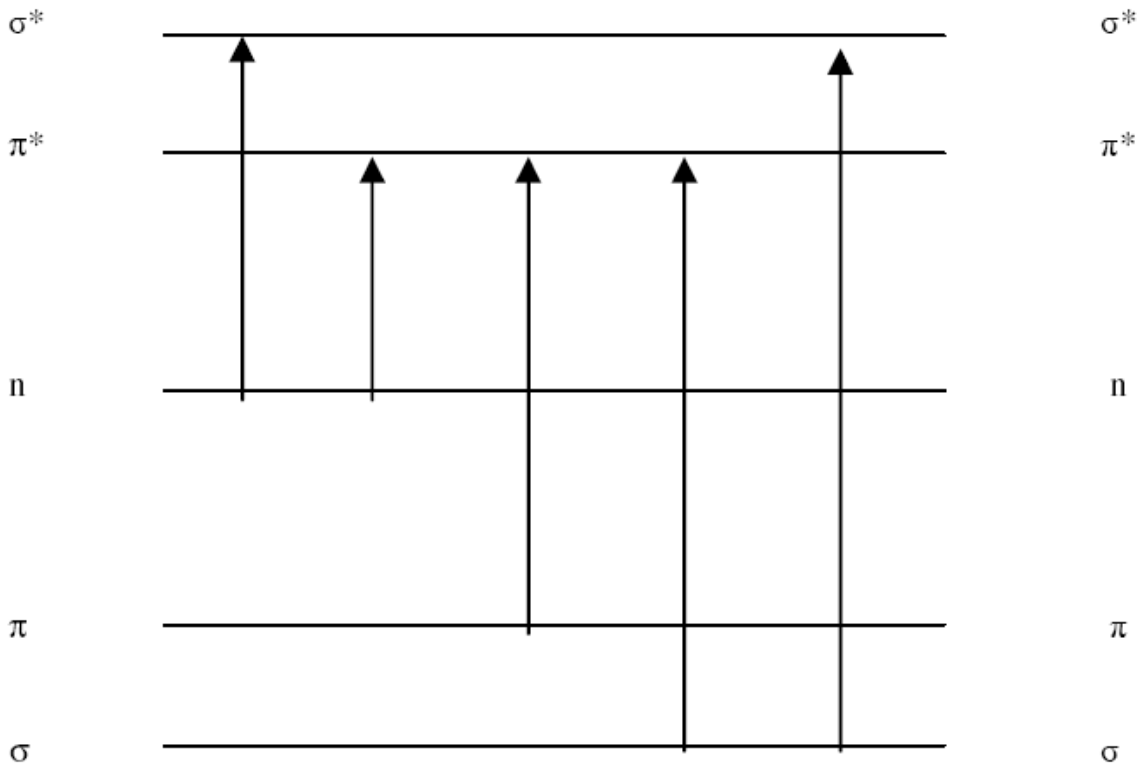


Figure1. 1 Electronic energy level and transition state

The basis of spectrophotometric methods is the simple relationship between the color of a substance and its electronic structure. A molecule or an ion exhibits absorption in the visible or ultra-violet region when the radiation causes an electronic transition in molecules containing one or more chromophoric groups.

The color of a molecule may be intensified by substituents called auxochromic groups, which displace the absorption maxima towards longer wavelength (bathochromic shift). The color determining factors in many molecules is the introduction of conjugated double bonds by means of electron donor or electron acceptor groups (Blaedel and Meloche 1964).

The quantitative applicability of the absorption method is based on the fact that the number of photons absorbed is directly proportional to the number or concentration of atoms, ions or molecules.

Absorption spectroscopy is one of the most useful tools available to the chemist for quantitative analysis.

Important characteristics of spectrophotometric methods include:

- Wide applicability: Numerous inorganic and organic species absorb in the ultraviolet and visible ranges and are thus susceptible to quantitative determination.
- High sensitivity: Having calibration graphs that are linear over a wider range. Avery extensive range of concentration of substances (10^{-2}–10^{-8}M) may be covered.
- Moderate to high selectivity: It may be possible to locate a wavelength region in which the only absorbing component in a sample is the substance being determined.
- Ease and convenience: Spectrophotometric measurements are easily and rapidly performed with modern instruments (Skoog, Holler, Grouch and David 2007).

1.-9 Beer's law in chemical analysis

The Beer –Lambert Law is used to determine the concentration of analyte by measuring the absorbance at various wavelengths. Beer –Lambert's Law is the relationship between a sample's absorbance and the concentration of the absorbing species. It can be written as:

A = e bc

Where:

'A' is the absorbance,

'c' is the concentration of the sample (compound) and expressed as mole/L

'b' is pathlength of cell and expressed in units cm.

‘e’ is the molar absorbtivity (or extinction coefficient in the older literature)and has the units l $mol^{-1}cm^{-1}$.

Beer-Lambert’s law offers a valuable and simple method for quantitative analysis. In practice, a calibration curve is constructed by plotting absorbance vs. concentration and the concentration of unknown with ‘X’ absorbance is determined by finding the concentration corresponding to the measured absorbance on the calibration curve (Harris 2003).

1.10 Advances in the analysis of vitamin C

1.10.1 Spectroscopic and electrochemical detection combined with flow injection and sequential injection analysis

Development of spectroscopic methods using spectrophotometric, fluorescence, and chemiluminescence detection and methods based on electrochemical detection are rapidly advancing. Some recent reviews include those by Arya et al (1998, 2000), Zaporozhets and Krushshinskaya (2002) and Yebra-Biurrun (2000).

Many of the spectrophotometric methods utilizethe redox properties of l-ascorbic acid; thus, the reduction of Fe (III) to Fe (II) and Cu (II) toCu (I) are frequently incorporated into newer methods. Metal ion reduction is usually coupled with the complex of the reduced metal with various dyes exhibiting color change upon complexation. Color changes, while easily measured spectrophotometrically, can be used for simple, visual tests or development of test strips for approximation of vitamin C content. Dyes that have been used include 2, 2-dipyridyl, pyridine-2, 6-dicarboxylic acid, p-carboxyphenylfluorone, 4-(2-pyridylazo) resorcinol, 1, 10-phenanthroline, 2, 4, 6-tri (2-pyridyl)-1, 3,5-trianzine, ferrozine, and many others.

It is very evident from recent literature that the most dramatic change in l-ascorbic acid analysis has been the combination of flow injection analysis (FIA) and more sophisticated sequential injection analysis (SIA) with proven approaches of vitamin C analysis to provide rapid analytical methods. The review paper by Yebra-Biurrun (2000) showed the trend very clearly. The author summarized the advantages of FIA as follows:

1. High sample throughput
2. Low sample volume
3. Low consumption of reagents
4. High reproducibility
5. Simple automation
6. Low contamination risks
7. Selectivity through kinetic discrimination
8. Small bench space needs
9. Limited need for lab ware

1.10.2 Capillary electrophoresis (CE)

Capillary electrophoresis (CE) has been extensively used for analysis of fat- and water soluble vitamins. The methodology is widely used for assay of vitamin C in pharmaceuticals and fruit and vegetable products. Heiger (1992) reviewed CE principles, instrumentation, and modes of operation. Since its introduction, two basic techniques have been most used for vitamin C analysis, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). Each technique is highly versatile, faster, more efficient and cost-effective compared to more traditional methods (Trenerry 2001).

A disadvantage is lower sensitivity compared to LC. However, for higher concentration matrices, supplements and fruit and vegetables, CE procedures are highly useful. Further, CE is very amenable to multi analyte assays; CZE is

normally applied to water-soluble analytes and is subject to column fouling by macromolecular components. This is proven to be a challenge for food analysis application. MECC was developed to overcome such problems and can be used toresolve neutral analytes on the basis of partitioning between the aqueous electrolyte and a pseudo stationary phase of charged molecules (Pedersen, Naess, Moestue and Rasmussen 2000; Sanchez and Salvado 2002).

Therefore, MECC is more adaptable to complex food matrices. However, hydrophobic analytes, such as fat-soluble vitamins, can precipitate out during electrophoresis owing to low solubility in aqueous MECC buffers.

Microemulsion electrokinetic capillary chromatography (MEECC) was developed to overcome deficiencies of MECC with hydrophobic analytes (Sanchez and Salvado 2002).For vitamin C, CZE and MECC techniques are widely used for pharmaceutical analysis.

1.10.3 Liquid chromatography (LC)

Liquid chromatography (LC) methods have been applied to pharmaceuticals and many types' of biological samples for analysis of L-ascorbic acid and related compounds. The need to simultaneously assay L -ascorbic acid, L -dehydroascorbic acid and isoascorbic acid from food has led to the development of excellent procedures for accurate assay of total vitamin C in the presence of isoascorbic acid. (Ball 1994; Nyyssonen, Salonen and Parviainen, 2000; Lee, and Coates 1999).

1.10.3.1 Extraction procedures for the analysis of vitamin C by LC

Most extraction procedures used for biological samples in conjunction with spectrophotometric oxidation–reduction based methods or derivatization procedures are compatible with resolution and detection modes used for LC analysis of vitamin C.The analyst must determine extractant compatibility to all components of the LC system. Recent methodology showed that

metaphosphoric acid, mixtures of metaphosphoric acid with glacial acetic acid, trichloroacetic acid, citric acid, mixtures of citric acid and glacial acetic acid, sulfuric acid, and phosphoric acid are usually compatible to LC supports and mobile phases. If the assay requires quantitation of total ascorbic acid, the resolution system must be capable of resolving l-ascorbic acid from l-dehydroascorbic acid with use of a detection mode capable of detecting both forms of vitamin C. Alternatively, a reducing agent such as dithiothreitol, cysteine, or homocysteine can be added to the extractant toreduce the dehydro form to l-ascorbic acid. Conversely, the l-ascorbic acid can be oxidized to the dehydro form by Norit or enzyme treatment. Total vitamin C assayed as L-dehydroascorbic acid permits use of (OPD) o-Phenylenediamine derivatization with fluorescence detection to quantitate total vitamin C.Metal chelators, usually (EDTA) ethylenediaminetetraacetic acid can be added to the extractant to inhibit metal catalyzed oxidation. Use of metaphosphoric acid as the primary component of the extractant has significant metal chelation properties. Any additions to the extractant must be compatible with the resolution and detection modes of the system. Metaphosphoric acid, with or without glacial acetic acid, has been the most common extractant used to extract vitamin C in LC-based methods.

1.11 *Prosopis juliflora*

1.11 .1 Scientific classification

Kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked): Rosids

Order: Fabales

Family: Fabaceae

Subfamily: Mimosoideae

Tribe: Mimoseae

Genus: *Prosopis*

Species: **P. *juliflora***

1.11. 2 Mesquite (Prosopis spp.) in Sudan: history, distribution

Prosopis spp. (mesquite) is ever green leguminous trees or shrubs. The genus comprises 44 species of which 40 are natives to the Americas. Of the remaining species P. Africana is indigenous to Africa, whereas P. kodziana, P. farcta and P. cineraria are natives to the Middle East and Pakistan (Bukart 1976). Prosopis species grow in arrays of environments and are not restricted by soil type, pH, salinity or fertility. In Sudan the tree flowers year-round. The fruiting period, which peaks in December to June, coincides with the dry season (El Tayeb, Mahir and El Hassan, 2001). Mesquite leaves are unpalatable, while pods, renowned for high sugar (16%) and protein (12%) contents are attractive to animals (Mohamed 2002). Self incompatibility promotes hybridization and results in genetic variability which confers plasticity and allows colonization of a wide range of habitats (Hunziker, et al 1986). Taxonomy of mesquite is complex and often confusing (Abdel Bari1986). The identity of the prevalent species in Sudan is controversial. The species, when, introduced, was claimed to be P. juliflora (Broun and Massey 1929). However, it was, later, identified as P. chilensis (Wunder1966). This identity, confirmed by Abdel Bari (1986), was refuted by Elfadl (1997) and Mohamed (2001) who ascertained the species as P. juliflora. The tree is a copious seed producer. In South Africa over six hundred thousand seeds were reported to be produced per tree per annum (Zimmermann

1991). The seeds, characterized by coat imposed dormancy, germinate in flushes and establish a huge persistent seed bank in soil. Goats, sheep, cows and feral animals, attracted by the green foliage, eat ripened pods and liberate the seeds. The seeds encapsulated in animal droppings, are spread into new sites over long distances (Fisherm, et al 1959). The pods are also transported by floodwaters and run-off. Following germination mesquite seedlings grow vigorously (Mohamed 2001). The rapidly growing root system and un-palatability of the foliage increase seedling survival rate and competitiveness particularly in heavy grazed areas and/or on uncultivated fallows (Mohamed 2001). The high coppicing ability of mesquite ensures recovery of the plant when cut and often results in a multi stem tree. Mesquite species have attracted attention, because of plasticity, ability to survive in hospitable environments and capacity to provide fuel, timber, fodder and edible pods.

The most common species P. juliflora, native to central and South America, was introduced into several countries in the tropics and sub-tropics (Bukart 1976). The tree, often multi-stemmed with a spreading crown of pendulous branches hanging down to the ground, has a high capacity to fix sand dunes.

Mesquite (P. Juliflora) was introduced into Sudan in 1917 from South Africa and Egypt and planted in Khartoum .The success attained in establishment of the tree and its abilities to tolerate drought and fix sand dunes provided the impetus for introduction of the tree into various agro ecologies with emphasis on dry areas (Broun and Massey 1929). In 1938 the plant was introduced into Sinar, Fwar, EL foung (central Sudan), Elghaba, Lietti basin (northern Sudan), Sinkat, ELgalabat, Portsudan (eastern Sudan), Kordofan and Darfur (western Sudan). Late in 1947 and subsequently in 1965 mesquite was re-introduced into eastern Sudan, where it was planted in a green

belt around Kassala (Abdel Barie 1986). In New Halfa mesquite was introduced to protect the research farm at inception in 1966 (El Tayeb, et al 2001). The prevailing drought in the 1970 s rejuvenated the interest in mesquite and further introductions, into eastern Sudan, were made to protect residential and cultivated areas. In 1974 mesquite seeds were broadcast by airplanes in around Kassala and further planted in protected forests (Elsidig, Abdelsalam and Abdelmagid1998). In the period 1978-1981 the tree was planted as shelterbelts at Portsudan and Tokar.

Moreover, introductions were made into the White Nile province, western and central Sudan. The tree was planted in shelter belts around farms, irrigated schemes and along the Nile (Luukkanen ,et al 1983) Several species of mesquite (P. chilensis, P. valutina, P. glandulosa var. terreyona, P.alba, P. pallida and P. articulata) were introduced, in the period 1978-1986, with the objective of selecting suitable species for the different ecological zones. Some of the species selected, had their seeds multiplied and distributed in western Sudan around El Obeid, and various other locations (Elfadl 1997). Mesquite seedlings failed to establish on sand dunes, but were well established within oases leading to lowering of water tables and suppression of native vegetation.

At present mesquite has become a noxious weed in Sudan (El houri 1986). It has invaded both natural and managed habitats, including water courses, flood plains, highways, degraded abandoned land and irrigated areas. The weed is more of a problem within central, northern and eastern Sudan. In the sandy soils of western Sudan, a part from localized foci, no problems of weedy invasion were reported (Elfadl and Luukkanen 2003). Mesquite tends to establish, successfully, on clay or alluvial soils which have good water retention (Luukkanen, et al 1983). Currently mesquite infestations cover over 230 thousand hectare. The bulk of mesquite infestation (>90%) is in eastern Sudan,

where livestock keeping and subsistence cultivation constitute the main source of income. The plant is found in the Gash delta from Kassala northwards passing Wager and southwards up to the borders with Eriteria, in Atbara River, a long Khor Baraka extending from the delta up to 130 kilometers upstream and in water collection pits a long Kassala-Gadarif and Portsudan highway. Rate of spread of the weed in eastern Sudan, as revealed by aerial photographs, successively taken, in 1962, 1978 and 1992 and a survey undertaken in 1996 was initially low. However, a substantial increase in rate of spread, 371 hectares per annum, was observed during 1978-1992. In 1992-1996 the average rate of spread increased to 460 hectares per annum (Elsidig, et al 1998). In most of the infested sites mesquite forms impenetrable thickets that smothered and excluded native vegetation and substantially changed community structure. Indigenous tree species were replaced by mesquite (Elsidig, et al 1998).

1.11. 3 Fruits (pod)

The fruits of the P. juliflora are indehiscent pods, generally pale yellow in colour. A pod consists of three separable components: exo- and mesocarp (pulp), endocarp (fibrous hulls) and seeds. The seeds are enclosed in the endocarp, which can be opened by hand only with difficulty. There is an average of 25 seeds per pod (Solano, 1989; Bravo, Grados, Saura-Calixto1994).

The seeds are small and very hard, approximately 5 mm in diameter; ovoid in shape and weigh about 40 mg. Seeds are made up of three parts, an episperm being the thin, brown seed coat, the endosperm which is adhered to the seed coat, and the cotyledon. The pulp represents 56% of the total weight of the fruit. The main soluble component of the pulp is sucrose (46%), representing over 90% of total soluble sugars, while the reducing sugars, glucose, fructose and xylose, are present in very small amounts (Cruz, Del,

Amadò, 1987; Sáenz, Serra, Escriche, Fito, Mulet, asiecznik, Felker, Harris, Harsh, Cruz, Tewari, Cadoret and Maldonado 2001)

Talpada (1985) found that sugar content of P. juliflora pods varied from 13% to 20% in different seasons and years showing a strong environmental effect on pod compositions. Soluble sugars from the pericarp of P. juliflora comprises of 75% sucrose, 12% being fructose, 5% glucose, 5% inositol and 1% raffinose (Marangoni and Alli 1988)

1.11 .4 Leaves

The composition of the leaves of P. juliflora can be divided into basic extractives (Protien (26.3%), Fibre (24.8%), Extract (8.5%), and Ash (1.4%), and Nitrogen free extract (31.8%) **(**Vimal, Tyagi and Patel 1986) and mineral elements (macronutrients and micronutrients) (N, P, K, Ca, Mg, Na) (Sharma 1968; Singh, Abrol, Cheema1988; Singh, Abrol, Cheema 1990; Patel 1986**).**

Leaves are composed principally of lignin and cellulose in the cell walls to give rigidity to the leaf structure.Comparison of the aproximate analysis of various authors show high levels of crude protein (14- 26%) and crude fibre (21-25%), with ether extract (fat) of 3-9%, nitrogen free extract (carbohydrate) at 30-46% and highly variable levels of ash (1- 16%). Elemental mineral content assessed in juliflora but it is the bark, stem wood and root wood With P. juliflora, Kazmi and Singh (1992) found 3-8% tannin in bark and root wood, Patel **(**1986) found 3-8% tannin in bark and up to 9% in wood, while Vimal and Tyagi **(**1986) found 6-7% tannin in root wood. Tan extract from all plant parts of P. juliflora is yellowish. Tannin in P. juliflora roots was found to be unsuitable for tanning purposes by Vimal and Tyagi **(**1986). Tannins are also used in the petroleum industry, mixed with sodium hydroxide, to reduce the viscosity of drill mud, and also for making ink.

1.12 *Balanites aegyptiaca*

1.12 .1 Scientific classification

Common name: Hingota

Kingdom: Plantae

Subkingdom: Tracheobionta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Sapindales

Family: ZygophyMaceae

Genus: *Balanites*

Species: *aegyptiaca*

1.12.2The species and botanical history

Balanites aegyptiaca (L.) Delile (the desert date or heglig tree) was first described as agihalid in 1592 by prosper Alpinio (Sands 2001). In 1753 Linnaeus described it as Ximenia aegyptica. In 1813 Delile replaced Agihalid (derived from the Arabic name for the tree 'Heglig') by Balanites (from the Greek for acorn, referring to the fruit).

According to Sands (2001) and Hall and Walker (1991), the placement of the genus Balanites was debatable along its history. It was originally placed in Zygophyllaceae then shifted to Olacaceae, Simaroubaceae, and finally Balanitaceae. Based on floral anatomy, embryology, taxonomy and pollen orphology, Singh et al., (2002) supported the retention of the genus under

Zygophyllaceae. While Boeswinkel, (1994) supported the recognition of a separate family Balanitaceae based on its unique ovule and seed characters. The whole genus was recently revised by Sands (2001). Nine species were identified as follows:

1. Balanites wilsoniana.
2. Balanites maughamii.
3. Balanites triflora.
4. Balanites roxburghii.
5. Balanites aegyptiaca.
6. Balanites pedicellaris.
7. Balanites angolensis.
8. Balanites rotundifolia.
9. Balanites glabra.

Although the main species that occurs in Sudan is *Balanites aegyptiaca*, however, Balanites rotundifolia is recorded in southern parts of Sudan 21 (Sands 2001).

1.12.3 Description

Balanites aegyptiaca (L.) Del.(Arabic, Heglig) was described by Elamin,(1990); Souane,(1984); Sahni (1968) as a multi-branched, medium sized ever green tree up to 15 meter high with spreading spherical crown. Bark, dark brown to grey deeply fissured. Leaves, compound and spirally arranged on the shoots with two separate leaflets, are ovate to orbiculate rhomboid, grey green in colour. Thorns are stout, green and straight. Flowers, yellowish with different sizes and shapes. Flowering occurs during October to March and fruiting occurs during November to April.

1.12.4 Distribution

Balanites aegyptiaca is one of the most widely distributed of Africa's trees (Sands, 2001; Hall and Walker 1991). It is indigenous to all dry lands south of Sahara and extending southwards. It is found in many African countries from Mauritania in the west to Somalia in the east and from Egypt in the north and southwards to Zimbabwe (Shanks, 1991; Sidiyene1996). In these areas it is found in areas with rainfall between 400-800 mm per annum, or where rainfall was augmented with water from other sources. Associated woody species are Combretaceae, especially Combretum and Mimosaceae, especially Acacias. It is also found in the Arabian Peninsula and adjacent parts of the Middle East (Hall 1992). In Sudan it is more likely that the species has the widest natural range (Suliman and Jackson 1959). It is distributed from Egyptian frontier up to high rainfall Savannah and from flat areas on clay and sand to high altitudinal areas (Badim, et al 1989). It is a characteristic of dark cracking clay under rainfall of 500 mm and above.

It is normally associated with Acacia seyal and Combretum spp. On sandy soil it is more sporadic and tends to occur as scattered tree. It occurs on hard surfaced soil and on the slopes at the foot of rocky hills. Also it is found in the fringe of ironstone region and in wadis in the Red Sea coast up to the northern frontier of the Sudan (Suliman and Jackson 1959).

Balanites aegyptiaca is widely distributed in Sudan. Its range stretches from the Egyptian frontiers and Red Sea coast to the Southern part of the Sudan, being absent only from the wettest parts of the country - i.e., when rainfall is above 1100 mm/annum (Suliman and Jackson, 1959; Von Maydell 1986). It is characteristic of dark cracking clay under a rainfall of 500 mm and above, where it is commonly associated with Acacia seyal. On sandy soils of Kordofan and Darfur it occurs as a scattered tree where rain fall exceeds 250 mm. It is

also found on hard surfaced soil at the foot slopes of rocky hills. Many studies have indicated that *Balanites aegyptiaca* has very wide range of variation in phenotypic traits such as fruit and seed size and shape ,crown shape, tree height, time of flowering and fruiting, spine lengths and position (Sayda, 2002; Sands, 2001; Hall,1992; Hall and Walker, 1991, Von Maydell 1986).

1.12.5 Reproductive biology

There is no definite time for flowering. Flowering behavior varies highly (Von Maydell1986) but mainly between October and February and sometimes in April (Badim, et al 1989). Fruiting occurs from December to January and occasionally later from March to July. In the Red Sea area of the Sudan fruiting is in August (Badi, et al 1989).

Pollination is by insect activity. The tree starts fruiting for the first time at age 5-7 years. However, under green house conditions 1-year old cloned material and 2-3 years old seed derived stock plants were observed to flower (Simon, et al 1992).

1.12.6 Uses

B. aegyptiaca has a wide range of uses and it has been utilized over thousands of years (Von Maydell 1986). The fleshy pulp of the fruit is eaten fresh or dried. The seed kernel produces good quality oil, which is used for cooking and oil making and also used as snacks (nuts). The fresh and dried leaves and sprout are eaten by livestock. The wood is used for furniture and hand tools. The tree is used as a shade for humans and animals.

1.12.6.1 Uses of fruits and seed kernel

The fruit pulp contains 64-72% carbohydrates (mainly sugars), plus crude protein, steroidal saponins, CO_2 , vitamin C, ethanol and other minerals, and the seed kernel consists of 30% protein and 51% saturated oil, a proportion

very similar to those of the sesame seeds and groundnuts. It was estimated by UNIDO that Sudan could earn up to 80 million dollars per year with total revenue of 25 million dollars (FAO1985). Mohamed et al (2002) found that the seed kernel contains 49% crude oil and 32% crude protein and the sapogenic contents of the full fat, defatted and testa flours were 1.5, 2.7, and 3.0%, respectively. The seed contains 440g/kg lipids of highly saturated fatty acid profile (Kapseu and Kayen 1997).78.2% protein, content and 53.7% recovery were obtained by isoelectric precipitation followed by aqueous ethanol treatment (Mohamed et al2000). The edible mesocarp contents 1.2 - 1.5% protein and 35 - 37% total sugars and the kernel contain 45 - 46% oil. In Nigeria fruits, leaves and nuts of Aduwa (balanites) are widely used by the rural people especially during the dry season and drought periods (Lockett, et al 2000).

1.13 Objectives of the study

The official analytical methods of water-soluble vitamins in most cases are based on outdated procedures, which are complicated, time-consuming, and inaccurate.

For these reasons there is increasing need for the development of accurate, time-saving, low cost, modern, multicomponent methods.

General objectives:

- Development of a sample preparation procedure for the vitamin C extraction
- Development of modern analytical methods, which allow, fast, routine determination of vitamin C which could be frequently used infood enrichment.
- Application of the developed methods for the most, commercially available vitamin in fruits and leaves.

Specific objectives:

- Development of HPLC method for the determination of vitamin C in the fruits and leaves of *Balanites aegyptiaca* and *Prosopis juliflora*.
- Development a new spectrophotometric method for determination of ascorbic acid.
- Development of indirect spectrophotometric method for determination of ascorbic acid.
- Application of the developal methods for the analysis of vitamin C in the fruits and leaves of *Balanites aegyptiaca* and *Prosopis juliflora*.
- Comparison of the values obtained from the different methods

Chapter two

Materials and methods

2. Materials and methods

2. 1 Materials

Both leaves of *Balanites aegyptiaca* (BL) and *Prosopis juliflora* (PL) and fruits of the latter (PF) were collected from the trees grown in the different parts of Khartoum State (Sudan). However, fruits of the former (BF) were purchased from Khartoum State market.

2.1.1 Chemicals

Glacial acetic acid (PC)(INDIA), nitric acid ,trichloro acetic acid, petroleum ether,methyl red (Sigma-Aldrich) ,sulphuric acid (LOBACHEMIE) (INDIA) ,Sodium hydroxide,boric acid (CDH), bromocresoal (TCI) chemicals (India),hydrochloric acid (LOBACHEMIE), starch (BDH) (Poole England) , potassium iodide (S d fine - CHEM Limited) ,iodine (FINKEM) , acetic acid (Fluka) (chemika), metaphosphoric acid (Sigma – Aldrich (Johannesburg , South Africa), L-Ascorbic acid (AA) powdered dop (ANKARA .TORKIYE), methylene blue (BDH) (Poole England), methanol (SHAM LAB),ceric (IV) sulphate (BDH) (Poole England), chloroform(ACS)**.**

2.1.2 Apparatus

Electronic analytical balance, crucible,Oven, furnace, glass wares(Pyrex) , beakers (25ml to 500ml) , pipettes (0.1ml-10ml) ,volumetric flasks (5ml-1litre), reflex apparatus,round bottle,soxhlet,, conical flasks(50ml-500ml),burette.

2.1.3 Equipment

HPLC (Shimadzu CLASS-VP V 6.14SPI)

UV/VIS spectro star Nano (BMG LABTECH) with 1cm matched quartz cell

2.2 Methods

2.2.1 Approximate analysis of *Balanites aegyptiaca* (leaves, fruits (cortex and fruits) and *Prosopis juliflora* (leaves and fruits)

2.2.1.1 Moisture content

The crucible was weighed in which 2 g from sample were added, placed in the oven at 105C˚ for 18 hours (overnight) and then cooled and weighed.

$$\text{Moisture} = \frac{\text{weight of crucible + weight of sample – weight after oven}}{\text{weight of sample}} \times 100$$

see table 3.1

2.2.1.2 Crude fiber

Preparation reagent of fiber.In a volumetric flask 450 ml distilled water, 500 ml glacial acetic acid, 50ml nitric acid and 20g of trichloro acetic acid were added.2g of sample was added in a beaker which was placed in reflex apparatus for one hour, filtered and transferred to a crucible that was kept in oven at 105C˚ over night , cooled , weighed , placed in multi furnace at 550C˚ for three hours and then cooled and weighed.

$$\text{Crude fiber} = \frac{\text{weight of sample after oven – weight of sample after furnace}}{\text{weight of sample}} \times 100$$

see table 3.1

2.2.1.3 Ether extract

A round bottle was weighed before extraction, 2g of sample was weighed and placed in to column of soxhlet, 150 ml of petroleum ether was added then open the soxhlet apparatus for 6 hours, collected petroleum ether, cooled the round bottle and weighed

$$\text{Ether extract} = \frac{(\text{weight of round bottle after} - \text{weight of round bottle before})}{\text{weight of sample}} \times 100$$

see table 3.1

2.2.1.4 Ash

2g of sample was weighed in a crucible, placed in muffle furnace oven at 550C° for 3 hours, cooled and weighed.

$$\text{Ash} = \frac{(\text{weight after furnace} - \text{weight of crucible})}{\text{weight sample}} \times 100$$

see table 3.1

2.2.1.5 Crude protein

By Kejldahl method measured in three stages.

Stage one: digestion, 1g of sample was weighed and placed in Kejldahl flask, 25ml H_2SO_4 was added, placed in digestion tubes (250 ml) up to be clear, placed in volumetric flask and completed to 100 ml.

Stage two: distillation, 5 ml distilled water was added to 10 ml sodium hydroxide solution, resaved in conical flask with 25 ml boric acid and10 ml indicator (methyl red and bromocresoal) then completed to 75 ml.

Stage three: titration, 0.02 HCl N was the titrant used.The end point was read when the colour began to change.

Cp =N×6.25

$$N = \frac{T\times14\times0.02\times20\times100}{\text{weight of sample}} \times1000$$

NFE = DM – (Cp+ CF+EE+Ash)

NFE ($C_6H_{12}O_6$)

NFE: Nitrogen Free Extract

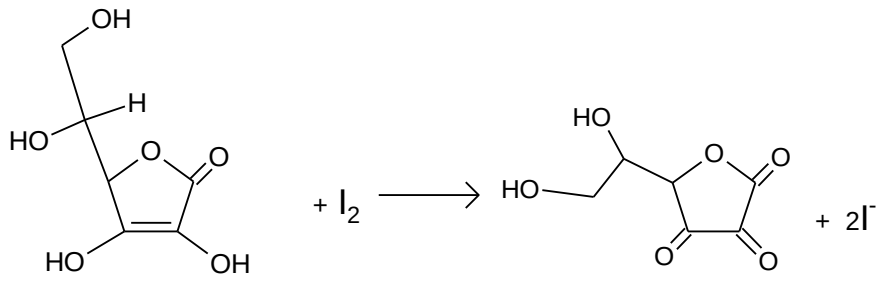
see table 3.1

2.2.2 Determination of vitamin C in (*Balanites aegyptiaca* and *Prosopis juliflora*(fruits and leaves),extracted with acetic acid,using iodine titration method

Determination of the concentration of vitamin C by iodine titration

The redox titration using iodine, which oxidized ascorbic acid to dehydroascorbic acid, while the iodine was reduced to iodide ions.

Ascorbic acid + $I_2 \rightarrow 2I^-$ + dehydroascorbic acid



Due to this reaction ,the iodine formed is immediately reduced to iodide as long as there is any ascorbic acid present .Once all the ascorbic acid has been oxidized the excess iodine is free to react with the starch indicator ,forming the blue-black starch –iodine complex.

Preparation of solutions

Iodine solution ($0.005 molL^{-1}$)

2g of potassium iodide, 1.3 g of iodine were weighed into a beaker100ml; few ml of distilled water was added and swirled for few minutes until iodine was dissolved. Iodine solution was transferred to 1L volumetric flask, making sure to rinse all traces of solution into the volumetric flask using distilled water .The the solution was up to the 1 L mark with distilled water.

Starch indicator solution (0.5%).

0.25g of soluble starch was weighed and 50 ml of near boiling water was added to it stirred to dissolved and cooled before used.

Preparation of samples

BF: *Balanites aegyptiaca* fruits BL: *Balanites aegyptiaca* leaves

PF:*Prosopis juliflora* fruits PL *: Prosopis juliflora l*eaves

Weights of samples taken:

BF =5.0061 g BL =5.0010g

PF =5.0016g PL =5.0010 g

15 ml acetic acid was added to the sample then stirred , 50 ml distilled water was added, filtered through filter paper 15cm then filtered with Buchner funnel.

Blank

60 ml distilled water and 2 ml starch were mixed and titrated.

Method

10 ml of the filtrate, 2 ml starch were mixed with 50 ml distilled water and then titrated.

2.2.3 Determination of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves), extract with 0.3 M metaphosphoric acid and 1.4 M acetic acid, using iodine titration method

Preparation of solutions

Extract solution

50ml distilled water was added to 1.92g metaphosphoric acid, mixed and 1.4 ml acetic acid was added .

Iodine solution (0.005molL^{-1})

As previous one

Starch indicator solution (0.5%).

As previous one

Weights of samples taken:

BF =5.0426 g BL =5.000g

PF =5.0021g PL =5.0021 g

50ml of extract solution was added to the sample then stirred for 15 min and filtered.

Blank

10ml of distilled water was added to 2ml starch then titrated.

Method

10ml filtrate was added to 2 ml starch then titrated.

2.2.4 Determination of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves), extracted with 4.5% metaphosphoric acid and 1% sulphuric acid, using iodine titration method

Preparation of solutions

Metaphosphoric acid 4.5%

4.5 g Metaphosphoric acid was weighed, 100 ml distilled water was added.

1% Sulphuric acid

1ml sulphuric acid was completed to 100 ml with distilled water.

Iodine solution ($0.005 molL^{-1}$)

As previous one

Starch indicator solution (0.5%).

As previous one

Preparation of samples

Weights of samples taken:

BF =5.0046 g BL =5.0041g

PF =5.0070g PL =5.0007 g

30 ml of extract solution (metaphosphoric and sulphuric acid) was added to 5g sample, filtered through filter paper 11cm followed by 0.45µm membrane.

Blank

5ml distilled water and 2 ml starch was mixed then titrated.

Method

2 ml starch was added to5 ml filtrate then titrated.

2.2.5 Determination of vitamin C in (*Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves), extracted with acetic acid, using iodine titration method

Preparation of solution

Iodine solution (0.005 mol L^{-1})

Potassium iodide (2.0 g) and iodine (1.3 g) were dissolved in100 ml distilled water. This solution was diluted ten times. The concentration of prepared iodine solution was more accurately determined by titration with a standard solution of AA.

Starch indicator solution (0.5%)

As previous one

Preparation of samples

Weights of samples taken:

BF =2.5 g BL =2.5 g

PF =2.5 g PL =2.5 g

Four samples of selected plants of each *Prosopis juliflor*a and *Balanites aegyptiaca* (fruits and leaves) were used for the determination of ascorbic acid. The fruits and leaves of samples were coarsely powdered and 2 ml of glacial acetic acid was added. The mixture was stirred for about 20 minutes and rapidly filtered using Buchner funnel. The volume of the samples was then made up to 100 ml with distilled water. The samples were analyzed titrimetrically in very short time after sample preparation.

Method

Titrimetric determination of ascorbic acid was done according to the following procedure: 10 ml of the filtrate, 50 ml of distilled water and 1 ml of 0.5% starch solution were mixed and immediately titrated to the end-point with the standardized iodine solution(0.005 molL-1) using 0.5% starch. The titrations were repeated in triplicates and blank determinations were carried out following the above procedure using 10 ml of distilled water instead of the filtrate. Results are expressed in mg of ascorbic acid per 100 g of dry sample.

2.2.6 Determination of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves), extracted with distilled water, using iodine titration method

Preparation of solution

Starch indicator solution

As previous one

Iodine solution

As previous one

Preparation of samples

Weights of samples taken:

BF =5.058 g BL =5.008g

PF =5.007g PL =5.007g

Four samples of selected plants *Prosopis juliflora* and *Balanites aegyptiaca* (fruits and leaves) were used for the determination of ascorbic acid. 50 ml distilled water was added to 5 g of each fruits and leaves samples which were coarsely powdered and the volume of the samples was made up to 250 ml with distilled water the mixture was stirred and filtrated through cheese close and filter paper No.4

Proceduer

Titrimetric determination of ascorbic acid was done according to the following procedure: 25 ml of the filtrate, 1 ml of starch 1% solution were mixed and immediately titrated to the end-point with the standardized using 1% starch. The titrations were repeated in triplicates and blank determinations were carried out followed the above procedure using 25 ml of distilled water instead of the filtrate. Results are expressed in mg of ascorbic acid per 100 g of dry sample.

2.3 Spectrophotometricdetermination of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves)

Preparation of solution

Stock solution of ascorbic acid

Containing 0.1 $molL^{-1}$ of ascorbic acid was prepared by dissolving appropriate amount (0.4g) of ascorbic acid in distilled water and stored in a glass stopped bottle at 4C˚ in the dark. Solutions of variable concentrations were prepared by diluting the stock standard solution in water before use.

Methylene blue solution (MB)

(0.0004 mol dm^{-3}) was prepared by dissolving 0.0126 g of methylene blue in 100 ml distilled water.

Preparation of samples

Four samples of selected plants of each *Prosopis juliflora* and *Balanites aegyptiaca* (fruits and leaves) were used for the determination of ascorbic acid.

2 ml of glacial acetic acid was added to 2.5 g of fruits and leaves samples which were coarsely powdered. The mixture was stirred for about 20 minutes and rapidly filtered using Buchner funnel, transferred into100 ml volumetric flask and diluted to the mark with distilled water. The samples were then analyzed spectrophotometrically.

Procedure

The spectrophotometric study was carried out by UV/VIS portable spectrophotometer (Shimadzu, Japan) to determine the amounts of ascorbic acid in the samples. Fifty microliters of a sample solution was mixed with 125 µl of MB (0.0004 mol dm^{-3}) solution and diluted up to 10 ml with distilled water. The absorption was measured at

λ_{max} = 665 nm. All analyses were carried out in triplicates. Results were expressed in mg of ascorbic acid per 100 g of dry sample.

2.4 High-performance liquid chromatographic determination of vitamin C in *Balanites aegyptiaca* and *prosopis juliflora* (fruits, leaves), by extraction with 2% metaphosphoric acid

Method of extraction of vitamin C

Vitamin C was extracted accordingly to the combination of the methods of Amin and Cheah (2003) and Abdulnabi, et al (1996) with slight modification.

Extract solution

2% metaphosphoric acid

Sample pretreatment

50ml extract solution was added to 5g sample in a conical flask (wrapped with aluminum foil) and the solution mixture was agitated at 100 rpm with the aid of orbital shaker for 15 min at room temperature.The mixture was filtered through a whatman No.4 filter paper to obtain a clear extract the ratio of the sample to extraction solution was 1to 1.Sample was extracted in triplicates.

Mobile phase preparation

One liter of mobile phase solution was prepared from 0.1% acetic acid and methanol in the ratio of 95:5

[Acetic acid glacial, HPLC grade CH_3COOH: M = 60.05 D=1.05g/cm Acedimetric=99.8%]

[Methanol SHAMLAB: Methanol HPLC grade/isocratic CH_4O M=32.04 D=0.79 g/cm Purity=99.7%]

Stock solution (100ppm)

0.01g standard in 100 ml volumetric flask

Table 2.1 HPLC conditions for analysis of vitamin C extracted with 2%metaphosphoric acid

Parameters	Conditions
Mobile phases	Acetic acid /methanol (95:5)
Flow rate	1.5 ml/min
Detection wave length	254 nm
Column	C_{18}

2.5 High-performance liquid chromatographic determination of vitamin C in *Balanites aegyptiaca* and *prosopis juliflora* (fruits, leaves), by extraction with 4.5% metaphosphoric acid

Method

Vitamin C was extracted by 4.5% metaphosphoric acid (w/v) as described by Contreras-Oliva et al. (2010) but without the addition of sulphuric acid. 5 g sample was added with 50 ml of 4.5% metaphosphoric acid. The mixture was placed in a conical flask (wrapped with aluminium foil) and shaked in an orbital shaker for 30 min at room temperature. The mixture was then filtered through a nylon 0.45µm syringe filter. 1ml from the extract was completed to 10 ml with 4.5% metaphosphoric acid solution and then injected into the HPLC column.

Mobile phase preparation

One liter of mobile phase solution was prepared from acetic acid 0.1% acetic acid and methanol in the ratio of 95:5

[Acetic acid glacial, HPLC grade CH_3COOH: M = 60.05 D=1.05g/cm Acedimetric=99.8%]

[Methanol SHAMLAB: Methanol HPLC grade/isocratic CH_4O M=32.04 D=0.79 g/cm Purity=99.7%].

Stock solution (1000 ppm)

0.05g standard in 50 ml volumetric flask

Table 2.2 HPLC conditions for analysis of vitamin C extracted with 4.5% metaphosphoric acid

Parameters	**Conditions**
Mobile phases	Acetic acid /methanol (95:5)
Flow rate	0.7ml/min
Detection wave length	254 nm
Column	C_{18}

2.6 Spectrophotometric determination of ceric sulphate using ascorbic acid

2.6.1 Optimization of spectrophotometric conditions for analysis of ceric sulphate

To determine the absorption maximum, standard solutions of ceric sulphate of concentration of 20 to 160 ppm were prepared. Scanning of the absorption of the solution complex in a wavelength range from 280 nm to 1000 nm showed a maximum absorbance (λ_{max}) at 323 nm

2.6.2 Preparation of standard solution of ceric sulphte Sulphuric acid (4M, 5M and 6M)

Ceric sulphate (1000ppm)

0.1g ceric sulphate was weighed, dissolved with 1ml of (4M, 5M or 6M) sulphuric acid and completed to the mark with distilled water in 100-ml volumetric flask.

Series of Ce concentration from 20 to 160 ppm were prepared.

Blank : 0.5ml (4M, 5M or 6M) H_2SO_4 was diluted to 50 ml with distilled water.

2.7 Spectrophotometric determination of vitamin C (ascorbic acid) in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits, leaves) using ceric (IV) sulphate

Ascorbic acid (100 ppm)

0.01g ascorbic acidwas weighed and dissolved with distilled water and completed to the mark in 100-ml volumetric flask.

Blank

0.5ml $H_2SO_4$5M was diluted to the volume 50 ml with distilled water.

Method

5ml Ceric (IV) sulphate (150 ppm) was added to different volumes of ascorbic acid (100ppm) and completed the volume to25 ml with distilled water.

Abbreviations of sample

BF: *Balanites aegyptiaca* fruits BL: *Balanites aegyptiaca* leaves

PF: *Prosopis juliflora* fruits PL: Prosopis *juliflora* leave

2.7.1 Preparation of extract solutions

Metaphosphoric acid (4.5%)

Acetic acid

2.7.2 Method of extraction of vitamin C from *Balanites aegyptiaca* and *Prosopis juliflora* (Fruits and leaves)

Preparation of samples extracted with distilled water

1g of each sample was weighed to which 25 ml distilled water was added and after 30 min was filtered through filter paper 11cm.

Preparation of samples extracted with metaphosphoric acid

1g of each sample was weighed to which 25 ml metaphosphoric acids (4.5%) was added and after 30 min was filtered throw filter paper 11cm.

Preparation of samples extracted with acetic acid

1g of each samplewas weighed to which 25 ml of acetic acid was added and after 30 min was filtered throw filter paper 11cm.

Method (1)

5ml Ce (150ppm) was mixed with 1 ml filtrate of the samples (which extracted with distilled water) completed the volume to50 ml with distilled water; the absorbance was measured at 323 nm.

Method (2)

5ml Ce (150ppm) was mixed with 1 ml filtrate of the samples (which extracted with metaphosphoric acid (4.5%) completed the volume to50 ml with distilled water; the absorbance was measured at 323 nm.

Method (3)

5ml Ce (150ppm) was mixed with 1 ml filtrate of the samples (which extracted with acetic acid completed the volume to50 ml with distilled water; the absorbance was measured at 323 nm.

Chapter three

Results and discussion

3.1 Main constituents of *Balanites aegyptiaca* (fruits, cortex and leaves) and *Prosopis juliflora* (fruits, leaves)

The main constituents of *Balanites aegyptiaca* (fruits, cortex and leaves) and *Prosopis juliflora* (fruits, leaves) were determined and are shown in Figure 3.1 and Table 3.1

Table 3.1 Main constituents of of *Balanites aegyptiaca* (leaves, cortex, fruits) and *Prosopis juliflora* (leaves, fruits)

Plant sample / Constituents percentage	DM	CP	CF	E.E	Ash	NFE
B.a Leave	94.71^{b}	9.63^{a}	19.27^{c}	4.22^{a}	13.72^{a}	47.88^{e}
B.a Cortex	90.20^{d}	1.92^{e}	24.53^{a}	2.01^{c}	3.32^{e}	58.43^{c}
B.a Fruit	80.92^{e}	3.35^{c}	5.96^{e}	0.97^{e}	6.84^{c}	63.81^{b}
P.j Leave	95.28^{a}	9.27^{b}	21.35^{b}	3.96^{b}	11.53^{b}	49.18^{d}
P.j Fruit	90.83^{c}	2.95^{b}	14.63^{d}	1.32^{d}	4.76^{d}	67.18^{a}
±SEM	0.02	0.01	0.02	0.02	0.02	0.04

DM= Dry Matter CP = Crude Protein CF = Crude Fiber

E.E = Ether Extract NFE = Nitrogen Free Extract SEM = Standard error of mean

B.a: *Balanites aegyptiaca* P.j*: Prosopis juliflora*

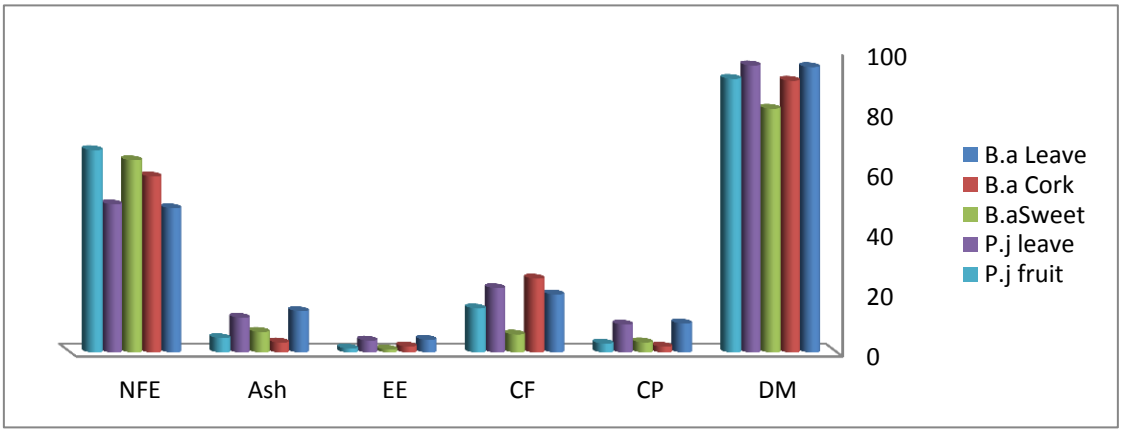


Figure 3.1 Main constituents of *Balanites aegyptiaca* and *Prosopis juliflora*

The highest value of dry matter was observed in *Prosopis juliflora* leaves (95.28%) followed by that in *Balanites aegyptiaca* leaves (94.71%), *Prosopis juliflora* fruits (90.83%) and *Balanites aegyptiaca* cortex (90.20%), and the lowest value was observed in *Balanites aegyptiaca* fruits (80.92%).

The highest value of crude protein was observed in *Balanites aegyptiaca* leaves (9.63%) followed by that in *Prosopis juliflora* leaves (9.27%), *Balanites aegyptiaca* fruits (3.35%) and *Prosopis juliflora* fruits (2.95%) and the lowest value was observed in *Balanites aegyptiaca* cortex (1.92%).The highest value of crude fiber was obtained in *Balanites aegyptiaca* cortex (24.53%) followed by that in *Prosopis juliflora* leaves (21.35%), *Balanites aegyptiaca* leaves (19.27%),*Prosopis juliflora* fruits (14.63%) and the lowest value was determined in *Balanites aegyptiaca* fruits (5.96%).

Ether extract percentage of the tested samples were measured and the results were in the range of 0.97% to 4.22%.The lowest value of ether extract was detected in *Balanites aegyptiaca* fruits, while the highest level of ether extract was detected in *Balanites aegyptiaca* leaves .

Balanites aegyptiaca leaves showed high amount of ash content (13.72%) as compared with that of *Balanites aegyptiaca* cortex which had the lowest value of ash (3.32%).

Nitrogen free extract of *Prosopis juliflora* fruits was found to be 7.18% followed with that in *Balanites aegyptiaca* fruits (63.81%),*Balanites aegyptiaca* cortex (58.43%), while that of *Balanites aegyptiaca* leaves showed low result(47.88%) followed by that of *Prosopis juliflora* leaves (49.18%).

Using acetic acid solution, the iodimetric titration method for the determination of milligrams per 100g sample *Balanites aegyptiaca* gave the highest results for its leaves (54.7%) and the lowest for its fruits (1.76%).In contrast, using 0.3M metaphosphoric acid and 1.4M acetic acid, its fruits showed

the highest value (71.39%), but the lowest value was showed by the leaves of *Prosopis juliflora* (15.8%).

However, extracting the same plants with a solution mixture of 4.5%metaphosphoric acid and 1% sulphuric acid, the fruits of *Balanites aegypticae* gave also the highest results (52.75%), and the leaves of *Prosopis juliflora* gave also the lowest results (7.03%).

3.2 High- performance liquid chromatographic determination of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits and leaves) using 2% metaphosphoric acid

Chromatograms of standard 1, standard 2 and standard 3 of ascorbic acid are shown in Figure 3.2, 3.3and 3.4 respectively

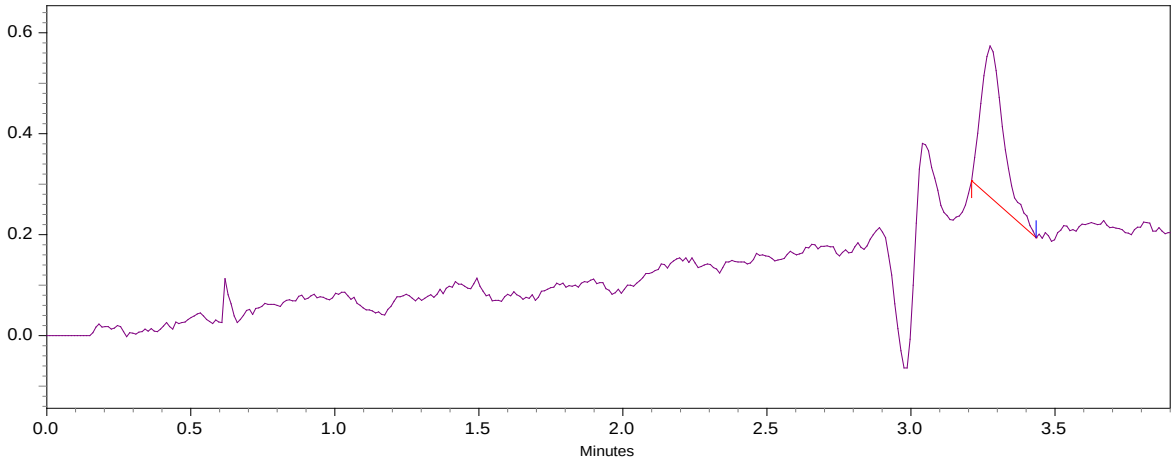


Figure 3.2 Chromatogram of standard 1of ascorbic acid

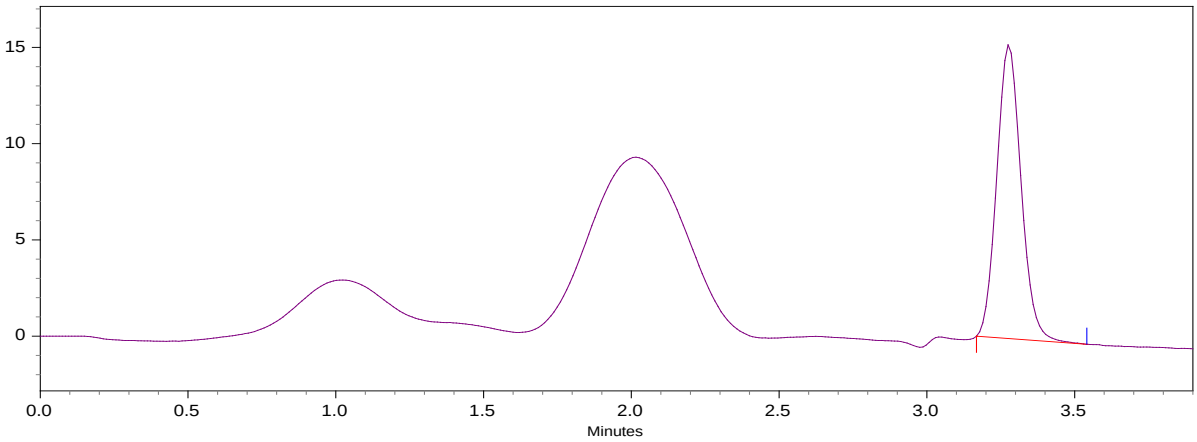


Figure 3.3 Chromatogram of standard 2of ascorbic acid

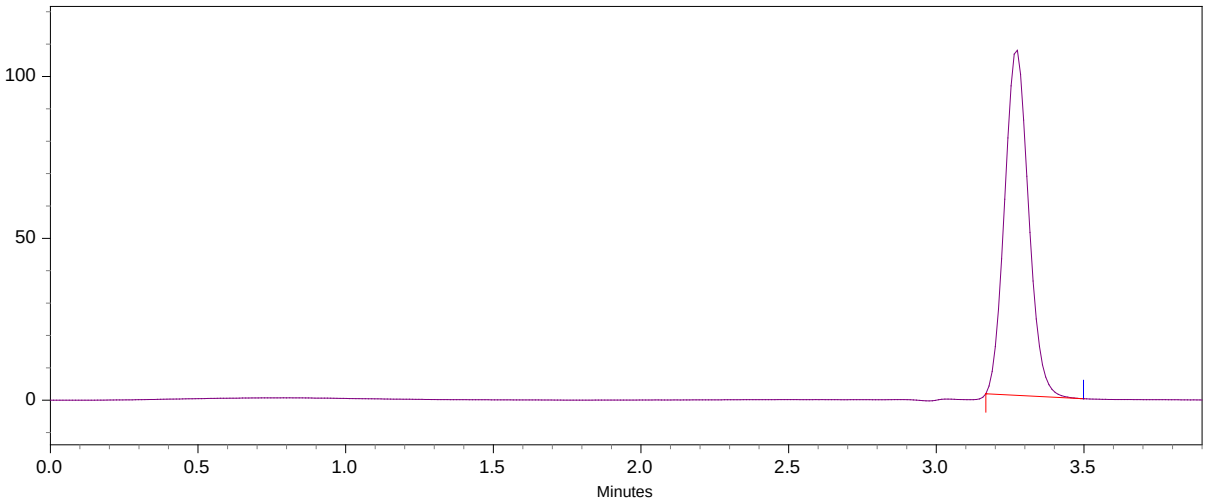


Figure 3.4 Chromatogram of standard 3of ascorbic acid

Table 3.2 Retention times and peak areas of standard solutions of ascorbic acid

Ascorbic acid standards	Retention. time	Area	Concentration ppm
Ascorbic std.1	3.275	1611	2.400
Ascorbic std.2	3.275	87304	8.000
Ascorbic std. 3	3.275	603732	40.000

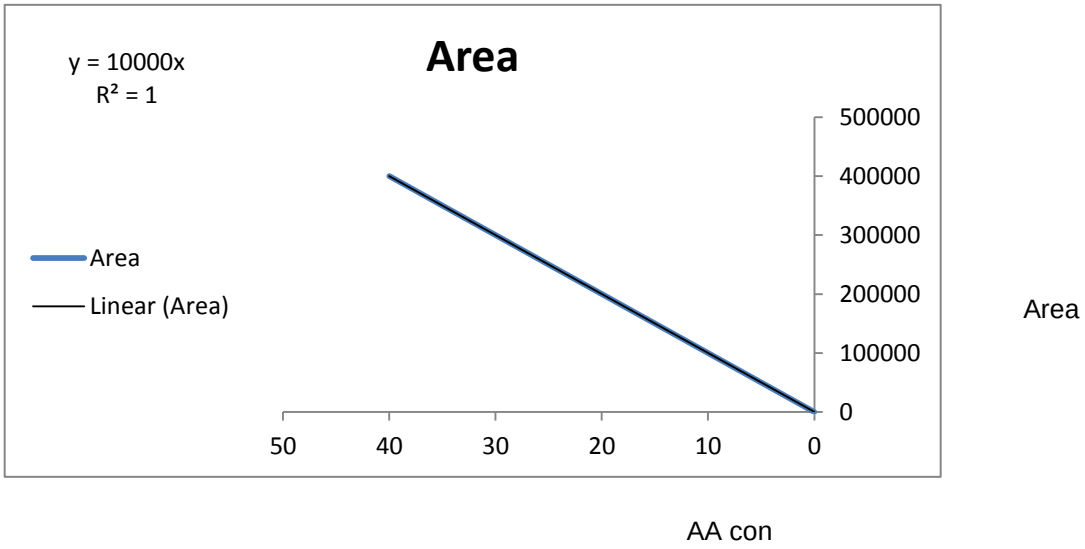


Figure 3.5 Analytical calibration curve of chromatic peak areas against ascorbic acid concentrations

The HPLC curves of *Balanites aegyptiaca* and *Prosopis juliflora* (fruits, leaves) are shown in Figures 3.6, 3.7, 3.8 and 3.9 and their data in Table 3.4

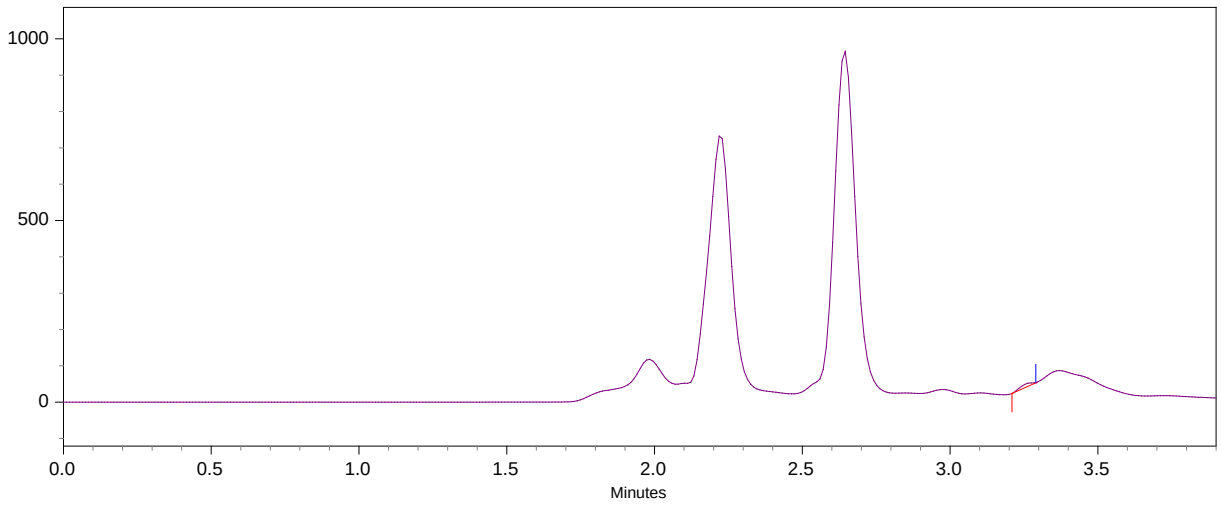


Figure 3.6 Chromatogram of *Balanites aegyptiaca* fruits

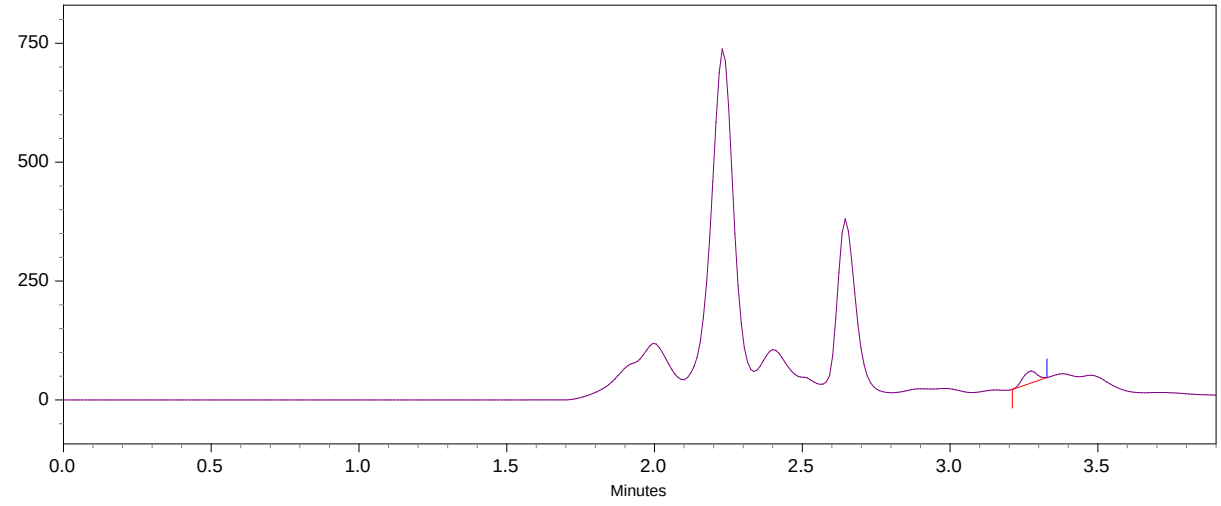


Figure 3.7 Chromatogram of *Balanites aegyptiaca* leaves

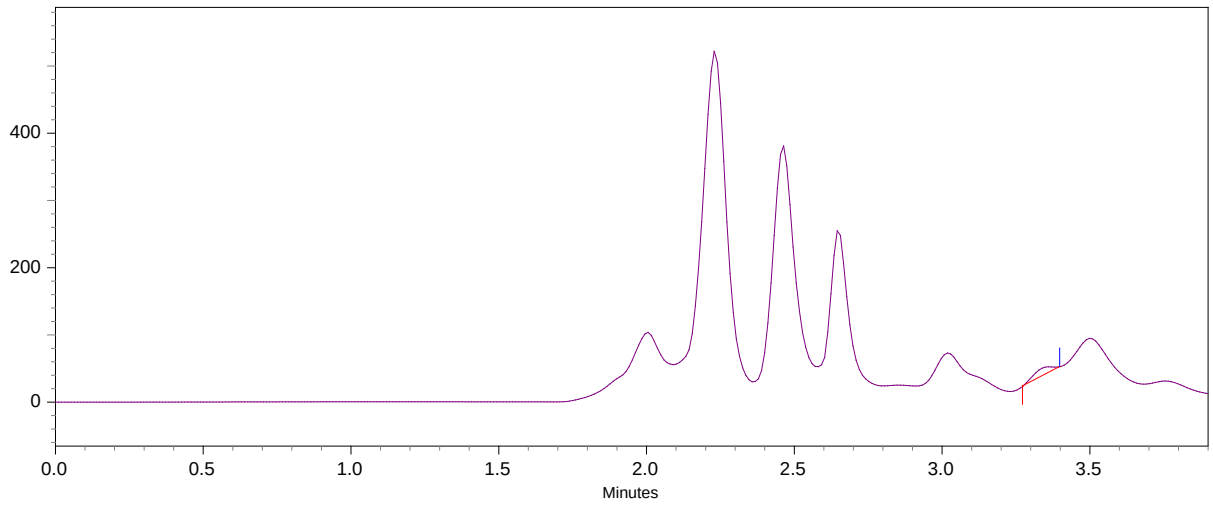


Figure 3.8 Chromatogram of *Prosopis juliflora* fruits

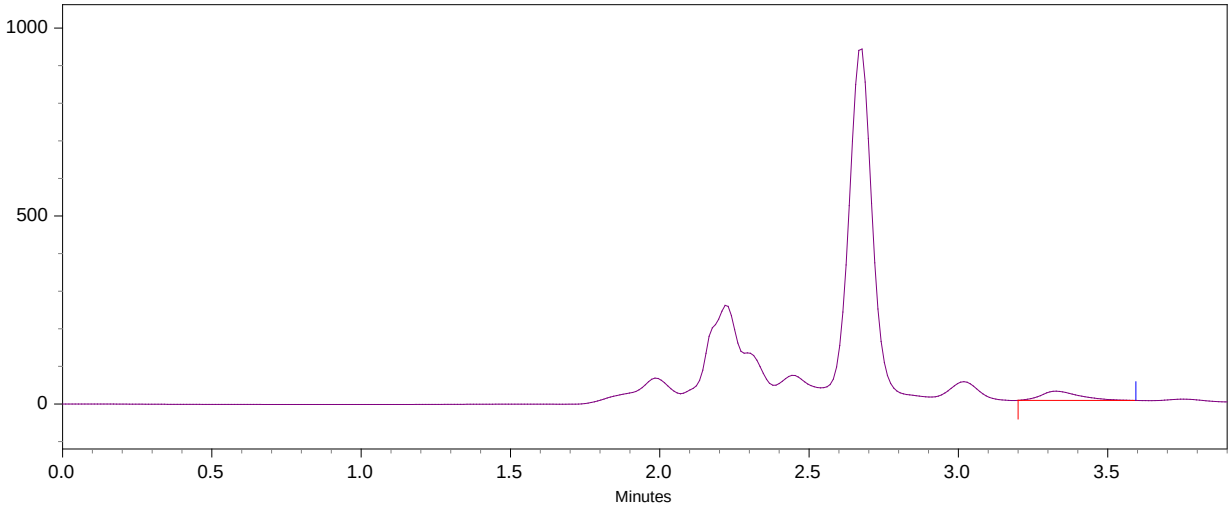


Figure 3.9 Chromatogram of *Prosopis juliflora* leaves

Table3.3 Chromatographic retention times and peak areas of *Balanites aegyptiaca and Prosopis juliflora* (fruits and leaves)

Samples	Retention .times	Area	Concentration ppm
BF	3.275	22043	37.94
BL	3.275	85202	77.27
PF	3.328	220093	161.28
PL	3.360	50997	55.97

BF:*Balanites aegyptiaca* fruits BL: *Balanites aegyptiaca* leaves

PF:*Prosopis juliflorae* fruits PF:*Prosopis juliflora* leaves

3.3 High- performance liquid chromatic determination of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits, leaves) using 4.5% metaphosphoric acid

Chromatographic peak areas of standard solutions of vitamin C are recorded in Table 3.4 and plotted in Figure 3.10

Table 3.4 Chromatographic peak areas of standard solutions of vitamin C

Concentration ppm	Area
2.000	15808
5.000	262123
10.000	623171
15.000	1053818
20.000	1415528

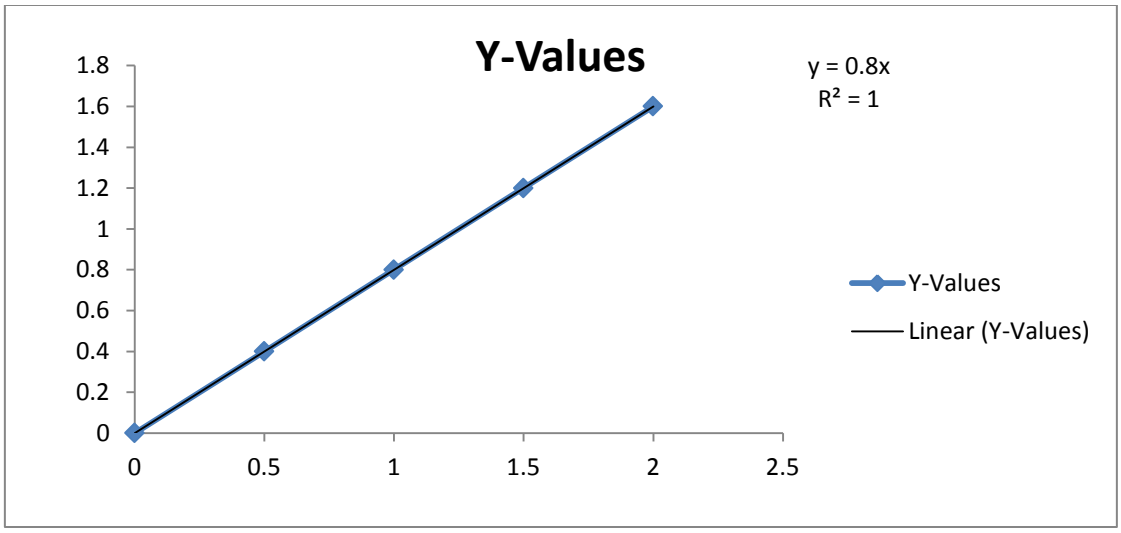


Figure 3.10 Analytical calibration curve of chromatic peak areas against ascorbic acid concentrations.

Linearity was determined by constructing the calibration curves of five standard solutions of vitamin C (Figure 3.11) using the chromatic peak area of each against its concentration.

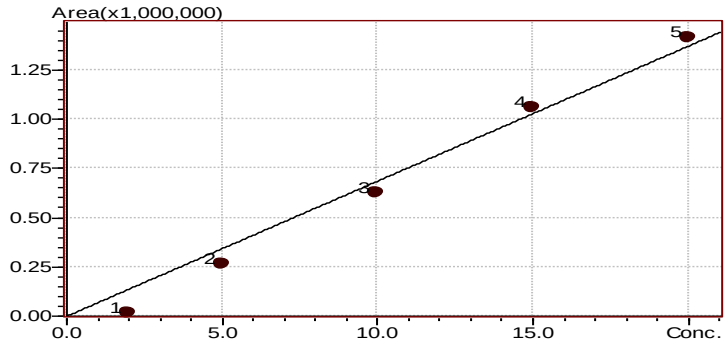


Figure 3.11 Linearity of standard vitamin C using HPLC method

Table 3.5 Linearity of standard vitamin C using HPLC method

Vitamin C mg/L	Theoretical plate	Tailing Factor	Area	Height	Conc.
Vitamin C 2	8704.408	1.289	15808	3490	1.965
Vitamin C 5	8576.184	1.273	262123	55637	5.121
Vitamin C 10	7661.369	1.271	623171	127571	9.748
Vitamin C 15	7504.457	1.270	1053818	211121	15.266
Vitamin C 20	7220.297	1.280	1415528	279018	19.900
Average	7933.343	1.277	674090	135367	10.400
%RSD	8.395	0.649	84.558	82.744	70.225
Maximum	8704.408	1.289	1415528	279018	19.900
Minimum	7220.297	1.270	15808	3490	1.965
Standard Deviation	665.983	0.008	569993	112008	7.303

3.4 High-performance liquid chromatic identification of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora* (fruits, leaves)

Vitamin C constituent in samples 1, 2, 3 and 4 were identified by comparison of the retention time of the standard vitamin C (Figure 3.12) with those of the samples (Figures 3.13, 3.14, 3.15 and 3.16 respectively).

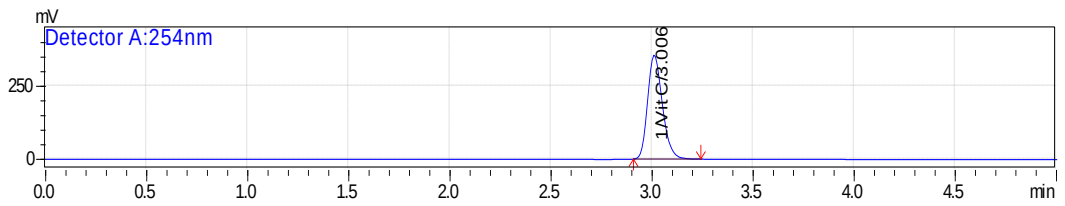


Figure 3.12 Retention time of vitamin C in Standard

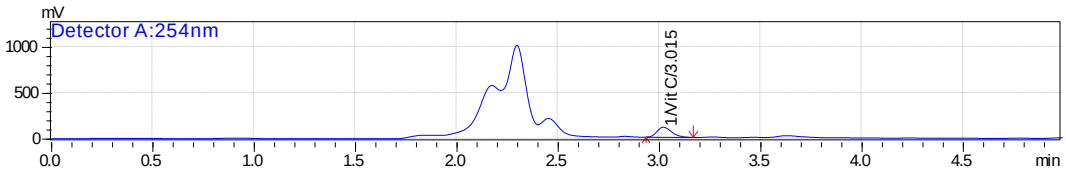


Figure 3.13 Retention time of vitamin C Sample1

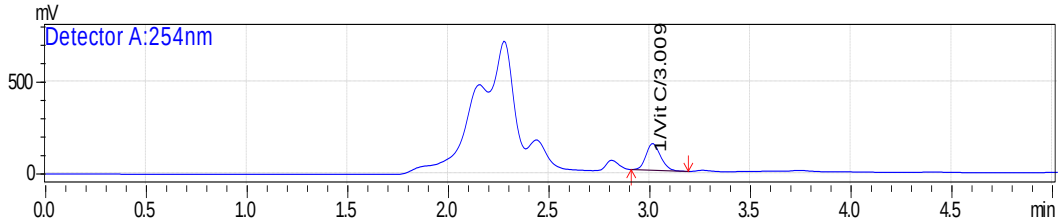


Figure 3.14 Retention time of vitamin C Sample2

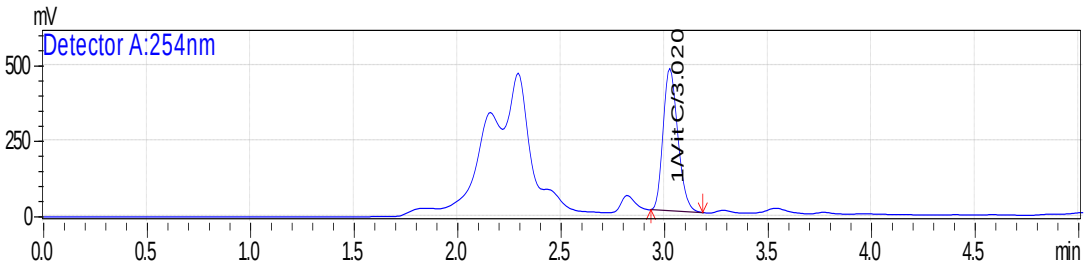


Figure 3.15 Retention time of vitamin C Sample3

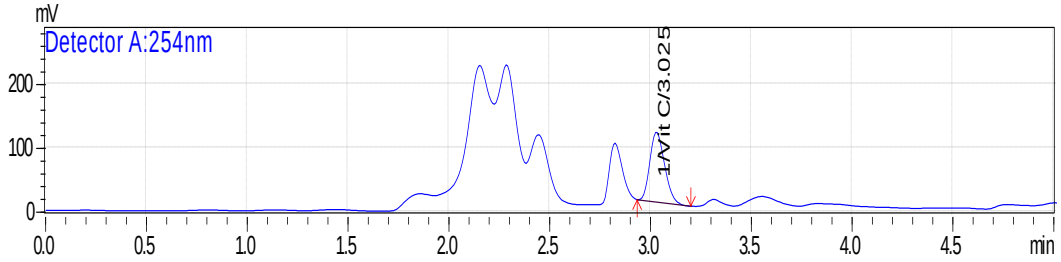


Figure 3.16 Retention time of vitamin C Sample4

Table 3.6 Chromatographic retention times and peaks area of *Balanites aegyptiaca* and *Prosopis juliflora* (fruits, leaves)

Vitamin C	Retention.Time	Area	Height	Concentration ppm
Vitamin C standard	3.006	1819307	355884	25.074
Vitamin C sample1	3.015	552386	110254	8.841
Vitamin C sample2	3.009	728038	144959	11.091
Vitamin C sample3	3.020	2345784	472169	31.820
Vitamin C sample4	3.025	560630	109183	8.946
Average	3.015	120229	238490	17.154
%RSD	0.253	68.992	69.629	61.902
Maximum	3.025	2345784	472169	31.820
Minimum	3.006	552386	109183	8.841
Standard Deviation	0.008	828749	166057	10.619

Sample 1:*Balanites aegyptiaca* fruits Sample 2: *Balanites aegyptiaca* leaves
Sample 3: *Prosopis juliflora* fruits Sample 4: *Prosopis juliflora* leaves

High- performance liquid chromatography analysis of vitamin C *Balanites aegyptiaca* and *Prosopis juliflora* gave concentration of 8.841 ppm and 31.820 ppm for their fruits and 11.091 ppm and 8.946 ppm for their leaves respectively.

Linearity of high-performance liquid chromatography method the determination of *in* vitamin C *in Balanites aegyptiaca* and *Prosopis juliflora* (fruits, leaves) using 4.5% metaphosphoric acid extract was obtained by injecting five standard solutions of the vitamin and recording the peak areas in Table 3.7

Table 3.7 Standard solution of vitamin C against chromatographic peak areas

Concentration ppm	Area
5.000	457361
15.000	1340471
20.000	1803273
30.000	2634934
50.000	4429232

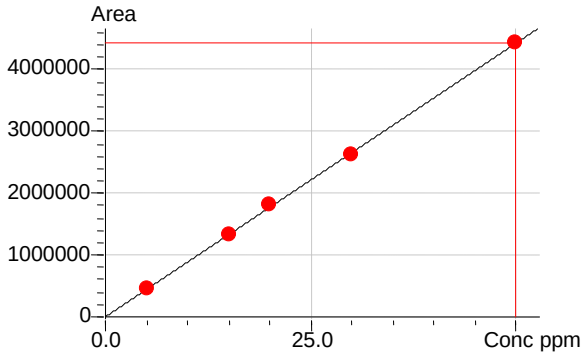


Figure 3.17 Linearity of calibration curve of determination of vitamin C using HPLC method (4.5% metaphosphoric acid)

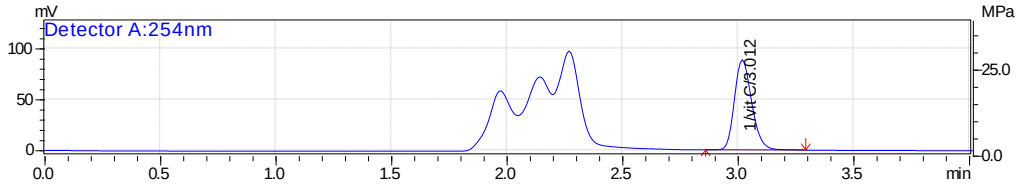


Figure 3.18 Linearity of vitamin C concentration 5

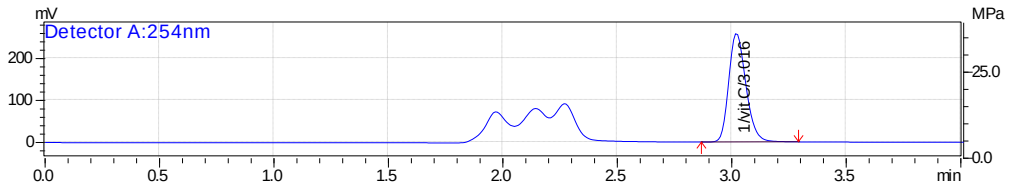


Figure 3.19 Linearity of vitamin C concentration 15

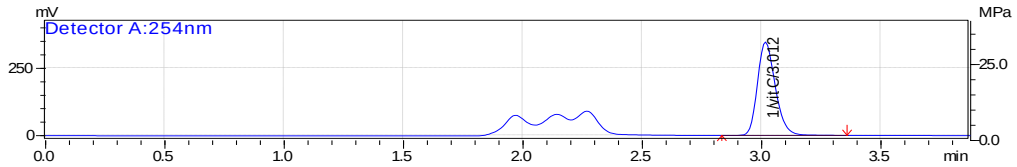


Figure3. 20 Linearity of vitamin C concentration 20

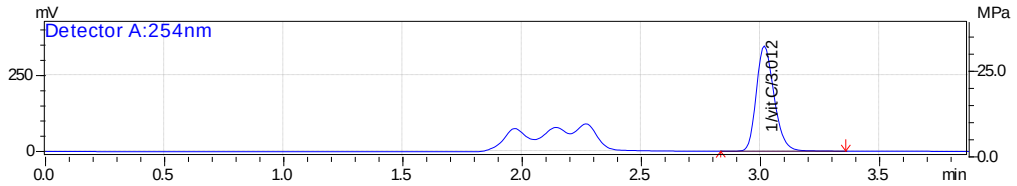


Figure 3.21 Linearity of vitamin C concentration 30

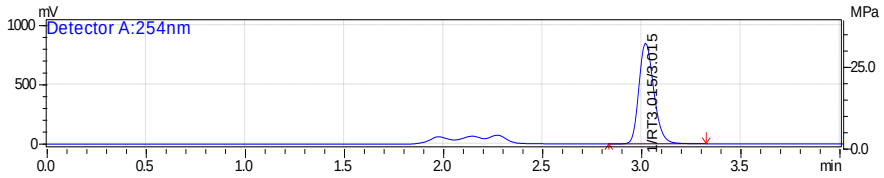


Figure 3.22 Linearity of vitamin C concentration 50

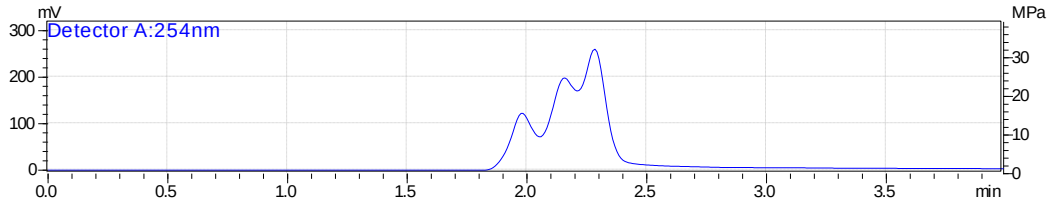


Figure3. 23 Linearity of vitamin C Blank

Table 3.8 Linearity of standard vitamin C using HPLC method

Vitamin C ppm	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Vitamin C 5	3.012	457361	88656	6547.463	1.303	5.000
Vitamin C 15	3.016	1340471	257976	6442.799	1.315	14.965
Vitamin C 20	3.012	1803273	346418	6474.955	1.310	20.050
Vitamin C 30	3.006	2634934	505863	6418.280	1.306	29.701
Vitamin C 50	2.998	4429232	850467	6322.306	1.311	49.972
Average	3.009	2133054	409876	6441.161	1.309	23.938
%RSD	0.229	70.592	70.460	1.278	0.345	71.302
Maximum	3.016	4429232	850467	6547.463	1.315	49.972
Minimum	2.998	457361	88656	6322.306	1.303	5.000
Standard Deviation	0.007	1505756	288798	82.303	0.005	17.068

3.4.1 Vitamin C standard linearity concentration 5 ppm

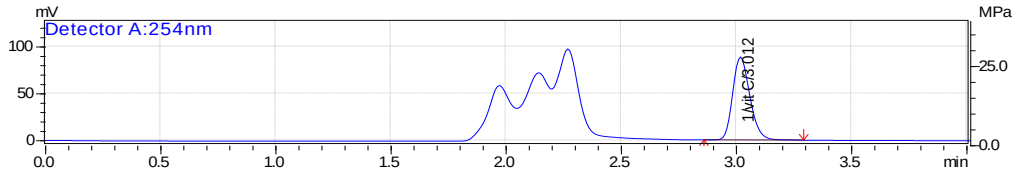


Figure3. 24 Linearity of vitamin C concentration 5 injection1

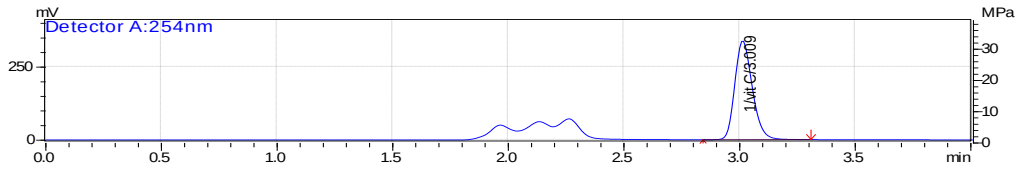


Figure 3.25 Linearity of vitamin C concentration 5 injection 2

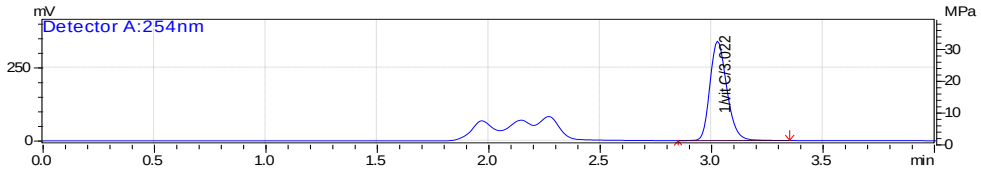


Figure 3.26 Linearity of vitamin C concentration 5 injection 3

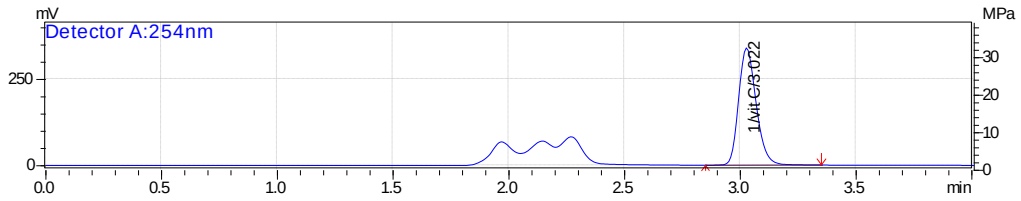


Figure 3.27 Linearity of vitamin C concentration 5 injection 4

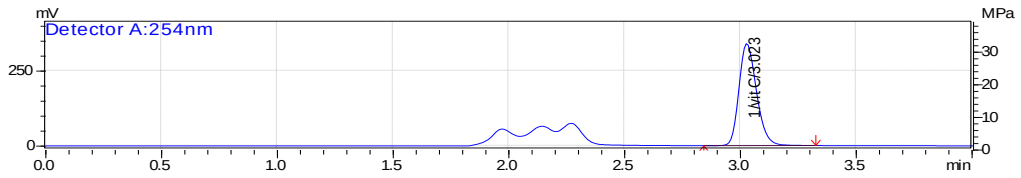


Figure 3.28 Linearity of vitamin C concentration 5 injection 5

Table 3.9 Linearity of vitamin C standard 5 ret. time (3.012) using HPLC method

Vitamin C standard 5	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Vitamin C linearity 5injection 1	3.012	456454	88470	6545.998	1.305	0.000
Vitamin C linearity 5injection 2	3.012	457361	88656	6547.463	1.303	5.000
Vitamin C linearity 5injection 3	3.010	456965	88070	6483.750	1.309	0.000
Average	3.011	456927	88399	6525.737	1.306	5.000
%RSD	0.057	0.100	0.339	0.557	0.225	0.000
Maximum	3.012	457361	88656	6547.463	1.309	5.000
Minimum	3.010	456454	88070	6483.750	1.303	5.000
Standard Deviation	0.002	455	299	36.369	0.003	0.000

Slope=88311 SD=455

Limit of detection (LOD) = 3.3× (SD/S)

3.3×455/88311=0.017µg/ml

Limit of quantification (LOQ) = 10× (SD/S)

10×455/88311=0.052 µg/ml

Limit ofdetection (LOD) and limit of quantification (LOQ) were determined from the regression equation of calibration curve

SD: Standard deviation of the response signal from the regression line

S: Slope from the linear regression analysis

3.4.2 Vitamin C Standard Recovery

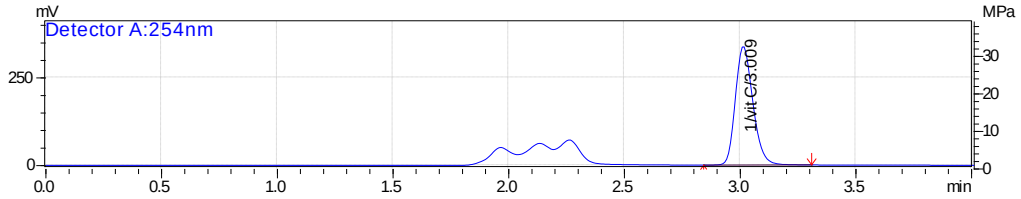


Figure 3.29 Vitamin C RE 20 injec 1

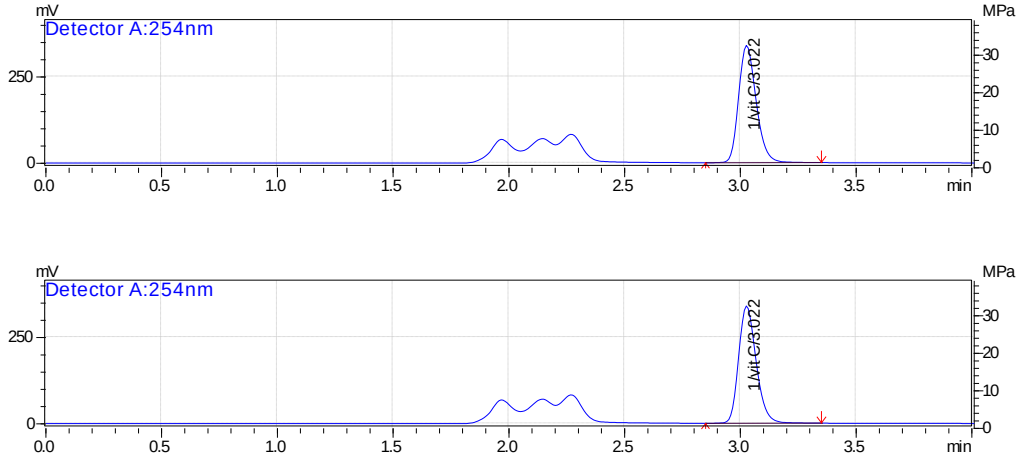


Figure 3.30 Vitamin C RE 20 injec 5

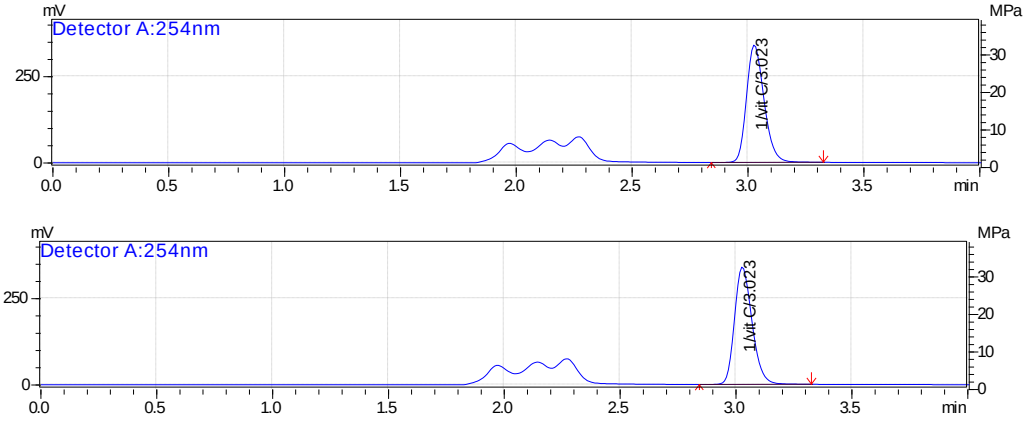


Figure 3.31Vitamin C RE 20 injec 6

Table 3.10 Vitamin C Recovery for Standard 20

Vitamin C Recovery 20	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Vitamin C rec 20 inj 1	3.009	1790422	342922	6369.338	1.313	20.200
Vitamin C rec 20 inj2	3.015	1781254	342364	6391.518	1.312	20.097
Vitamin C rec 20 inj3	3.009	1777675	337458	6318.815	1.315	20.056
Vitamin C rec 20 inj4	3.016	1797249	340405	6286.306	1.310	20.277
Vitamin C rec 20 inj5	3.022	1786878	340408	6414.993	1.308	20.160
Vitamin C rec 20 inj6	3.023	1788930	340304	6293.512	1.308	20.183
Average	3.016	1787068	340644	6345.747	1.311	20.162
%RSD	0.202	0.388	0.565	0.847	0.221	0.388
Maximum	3.023	1797249	342922	6414.993	1.315	20.277
Minimum	3.009	1777675	337458	6286.306	1.308	20.056
Standard Deviation	0.006	6935	1925	53.728	0.003	0.078

3.4.3Vitamin C standard ruggedness

3.4.3 .1 Vitamin C standard ruggedness at decreased wavelength (253nm)

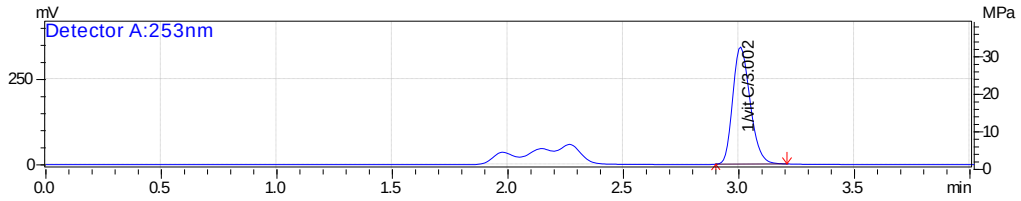


Figure 3.32 Chromatogram of vitamin C standard at decreased wavelength detection (253nm)

Table 3.11 Ruggedness results of Vitamin C standard at decreased wavelength detection (253nm)

Vitamin C rug(253 nm)	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Rug injection 1	3.011	1788425	343059	6387.691	1.313	20.178
Rug injection 2	3.002	1789552	343392	6339.397	1.314	20.190
Rug injection 3	3.011	1789127	343247	6383.805	1.316	20.185
Average	3.008	1789035	343233	6370.298	1.314	20.184
%RSD	0.169	0.032	0.049	0.421	0.132	0.032
Maximum	3.011	1789552	343392	6387.691	1.316	20.190
Minimum	3.002	1788425	343059	6339.397	1.313	20.178
Standard Deviation	0.005	569	167	26.831	0.002	0.006

3.4.3.2 Vitamin C standard ruggedness at increased wavelength (255nm)

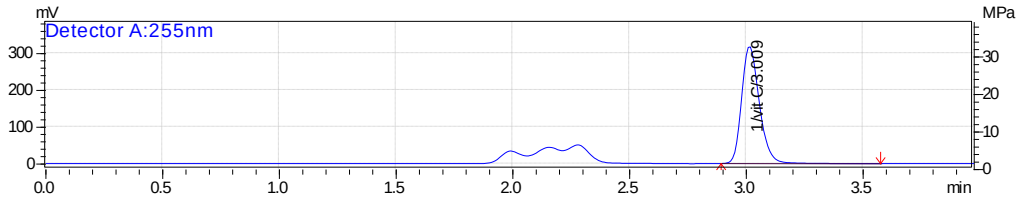


Figure 3.33 Chromatogram of vitamin C standard at increased wavelength detection (255nm)

Table 3.12 Ruggedness results of Vitamin C standard at increased wavelength detection (255nm)

Vitamin C rug (255nm)	Ret.time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Rug injection 1	3.000	1709141	317309	6220.147	1.324	19.283
Rug injection 2	3.009	1685081	316773	6314.373	1.326	19.012
Rug injection 3	2.993	1701398	318865	6251.510	1.328	19.196
Average	3.001	1698540	317649	6262.010	1.326	19.163
%RSD	0.272	0.723	0.342	0.766	0.181	0.723
Maximum	3.009	1709141	318865	6314.373	1.328	19.283
Minimum	2.993	1685081	316773	6220.147	1.324	19.012
Standard Deviation	0.008	12282	1087	47.982	0.002	0.139

3.4.3.3Vitamin C standard ruggedness (flow rate 0.6, 0.8) cm^3/min

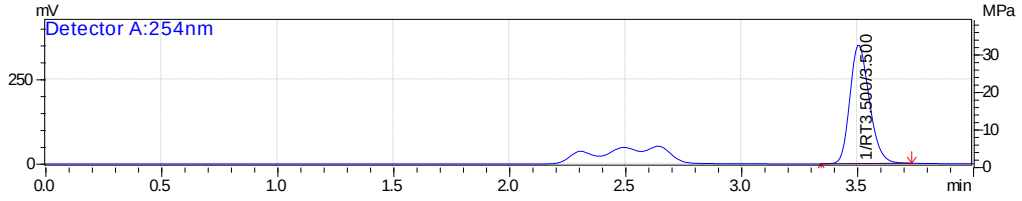


Figure 3.34 Chromatogram of vitamin C standard at decreased flow rate (0.6) m^3/min

Table 3.13 Ruggedness results of Vitamin C standard at decreased flow rate (0.6) m^3/min

Vitamin C(rug flow rate 0.6)	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Rug injection 1	3.497	2048257	353094	7142.412	1.283	0.000
Rug injection 2	3.500	2048712	349867	7140.828	1.291	0.000
Rug injection 3	3.508	2048362	348334	7144.450	1.292	0.000
Average	3.502	2048444	350432	7142.563	1.288	0.000
%RSD	0.168	0.012	0.693	0.025	0.389	0.000
Maximum	3.508	2048712	353094	7144.450	1.292	0.000
Minimum	3.497	2048257	34334	7140.828	1.283	0.000
Standard Deviation	0.006	239	2430	1.816	0.005	0.000

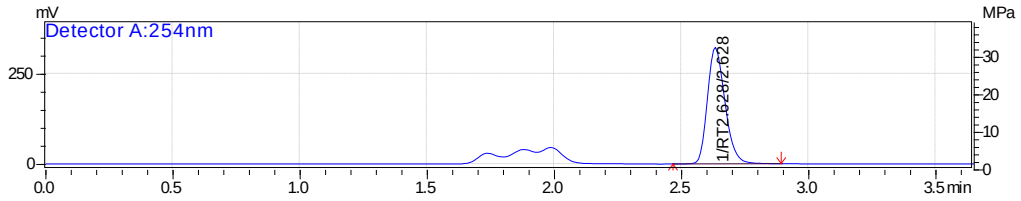


Figure 3.35 Chromatogram of vitamin C standard at increased flow rate (0.8) m^3/min

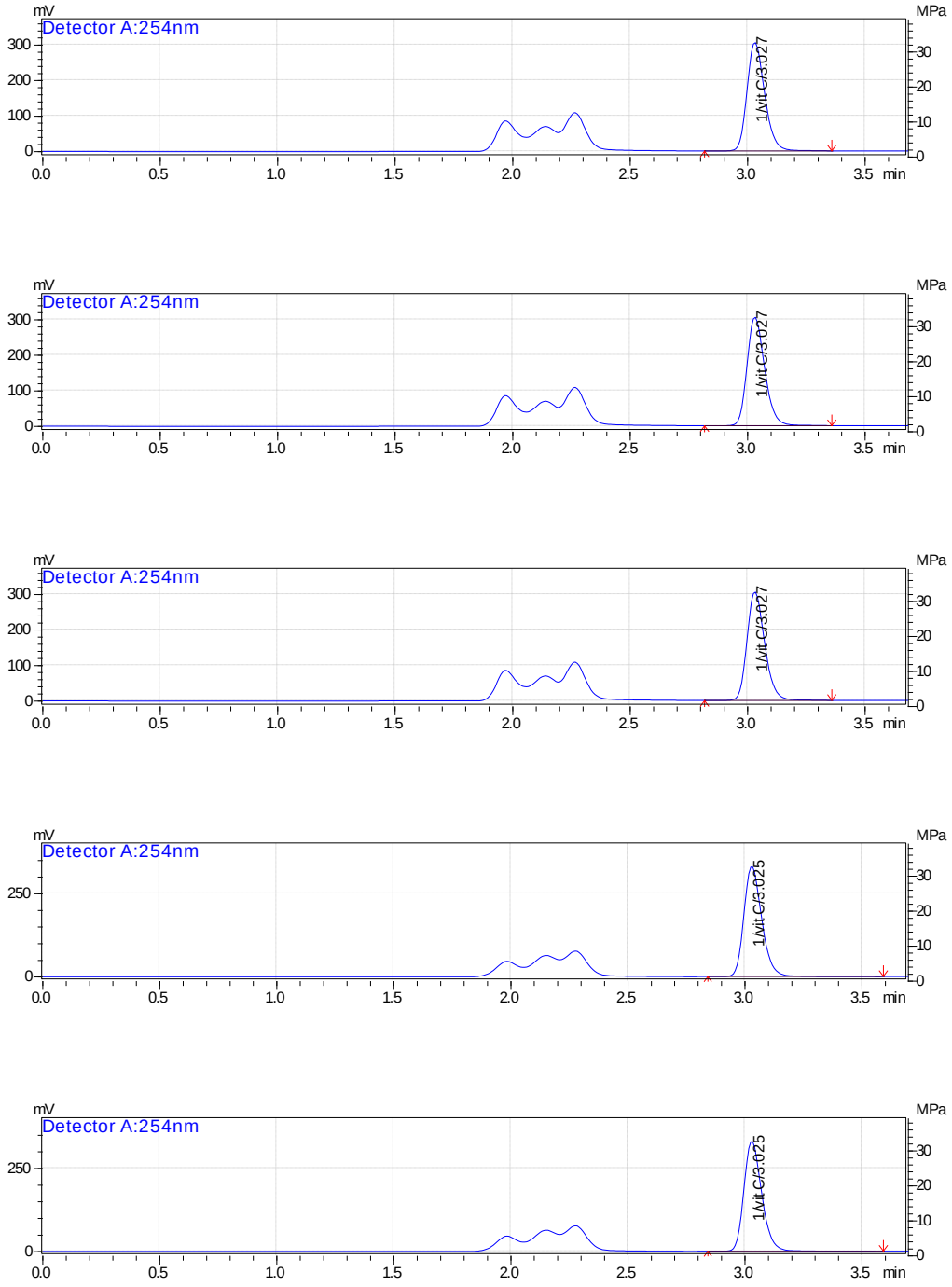
Table 3.14 Ruggedness results of Vitamin C standard at increased flow rate (0.8) cm^3/min

Vitamin C(rug flow rate 0.8)	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Rug injection 1	2.646	1528417	317834	5868.126	1.333	0.000
Rug injection 2	2.628	1550124	323393	5803.646	1.336	0.000
Rug injection 3	2.637	1547522	322748	5861.352	1.336	0.000
Average	2.637	1542021	321325	5844.375	1.335	0.000
%RSD	0.337	0.769	0.946	0.606	0.135	0.000
Maximum	2.646	1550124	323393	5868.126	1.336	0.000
Minimum	2.628	1528417	317834	5803.646	1.333	0.000
Standard Deviation	0.009	11853	3040	35.434	0.002	0.000

Standard vitamin C solution was injected three times after decreasing the flow rate (0.1cm^3/min)of its optimized value (0.7cm^3/min).The chromatogram of the standard solution is shown in Figure 3.34 and the ruggedness of the HPLC method was shown in Table 3.13, standard vitamin C solution was injected three times after increasing the flow rate (0.1cm^3/min) of its optimized value (0.7cm^3/min).The chromatogram of the standard solution is shown in Figure 3.35 and the ruggedness of the HPLC method was shown in Table 3.14.

It was found that small variation in the wavelength or flow rate did not significantly affect the retention time and the tailing factor.

3.4.4Vitamin C standard precision



Figures 3.36 Five chromatic scans of standard vitamin C precision day 1

Table 3.15 Vitamin C precision day 1 results

Vitamin C precision D1	Ret.time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Pre injection 1	3.019	1758964	332239	6377.870	1.317	19.845
Pre injection 2	3.018	1782649	332119	6275.371	1.312	20.112
Pre injection 3	3.025	1755637	329360	6262.064	1.320	19.808
Pre injection 4	3.015	1761675	329271	6258.214	1.320	19.876
Pre injection 5	3.023	1786470	342893	6406.922	1.301	20.156
Pre injection 6	3.021	1787944	338533	6318.437	1.309	20.172
Pre injection 7	3.018	1778230	336812	6304.510	1.315	20.063
Pre injection 8	3.022	1790432	340733	6354.713	1.309	20.200
Pre injection 9	3.010	1794025	340835	6278.507	1.310	20.241
Average	3.019	1777336	335866	6315.179	1.313	20.052
%RSD	0.148	0.827	1.557	0.850	0.475	0.827
Maximum	3.025	1794025	342893	6406.922	1.320	20.241
Minimum	3.010	1755637	329271	6258.214	1.301	19.808
Standard Deviation	0.004	14700	5229	53.654	0.006	0.166

Table 3.16 Vitamin C precision day 2 results

Vitamin C precision D2	Ret.time	Area	Height	Theoretical plate	Tailing factor	Conc.
Pre injection 1	3.013	1780074	340927	6370.926	1.310	20.083
Pre injection 2	3.017	1773574	336724	6315.495	1.316	20.010
Pre injection 3	3.017	1778385	337666	6299.090	1.321	20.064
Pre injection 4	3.007	1783409	340296	6290.174	1.317	20.121
Pre injection 5	3.002	1780228	339658	6329.453	1.317	20.085
Pre injection 6	3.009	1779953	338874	6316.262	1.313	20.082
Pre injection 7	3.000	1775672	337481	6271.692	1.319	20.034
Pre injection 8	3.009	1774366	338286	6372.161	1.315	20.019
Pre injection 9	2.998	1780785	340488	6250.204	1.311	20.091
Average	3.008	1778494	338933	6312.829	1.316	20.066
%RSD	0.228	0.185	0.440	0.651	0.261	0.185
Maximum	3.017	1783409	340927	6372.161	1.321	20.121
Minimum	2.998	1773574	336724	6250.204	1.310	20.010
Standard Deviation	0.007	3284	1491	41.090	0.003	0.037

Table 3.17 Vitamin C precision day 3 results

Vitamin C precision D3	Ret.time	Area	Height	Theoretical plate	Tailing factor	Conc.
Pre injection 1	3.004	1780816	339265	6361.324	1.313	20.092
Pre injection 2	3.006	1777634	339440	6329.047	1.312	20.056
Pre injection 3	3.004	1772987	339070	6347.623	1.311	20.003
Pre injection 4	2.998	1778996	338225	6228.943	1.319	20.071
Pre injection 5	3.006	1776616	338478	6243.959	1.313	20.044
Pre injection 6	3.011	1774677	337835	6337.261	1.313	20.022
Pre injection 7	3.006	1772459	337809	6228.717	1.314	19.997
Pre injection 8	3.007	1768625	335206	6269.179	1.323	19.954
Pre injection 9	3.037	1738960	329590	6523.112	1.299	19.619
Average	3.009	1771308	337213	6318.796	1.313	19.984
%RSD	0.377	0.716	0.927	1.471	0.501	0.716
Maximum	3.037	1780816	339440	6523.112	1.323	20.092
Minimum	2.998	1738960	329590	6228.717	1.299	19.619
Standard Deviation	0.011	12683	3125	92.926	0.007	0.143

The results obtained showed that the HPLC method was precise according to the results shown in Figures 3.36, and Tables 3.15, 3.16 and 3.17 for precision results of vitamin C for three days.

The precision factors were low standard deviation (0.166, 0.037 and 0.143), retention time (3.019, 3.008, and 3.009) and tailing factor (1.313, 1.316 and 1.313), for day 1, 2 and 3, respectively.

Vitamin C recovery for the samples

Recovery of the HPLC method for the determination of vitamin C was carried out by applying standard addition technique (spike method).A different amount of standard solution was added to known concentration of vitamin C (10ppm).

Chromatograms of vitamin C (spike and un spike samples) 1, 2, 3 and 4 are shown in Figures: 3.37 and 3.38, 3.39 and 3.40, 3.41 and 3.42, and 3.43 and 3.44, and their recovery results are shown in Tables 3.18, 3.19, 3.20 and 3.21 respectively.

Vitamin C Recovery for sample 1

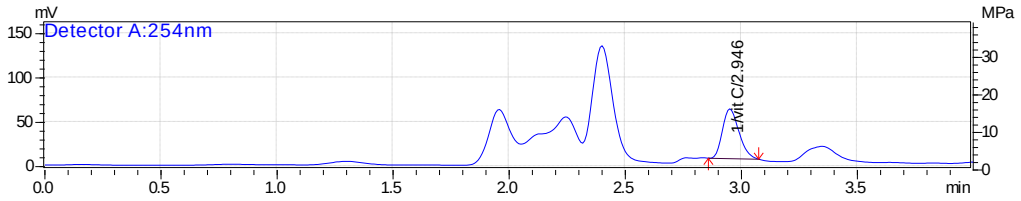


Figure 3.37 Recovery chromatogram of vitamin C un spike sample 1

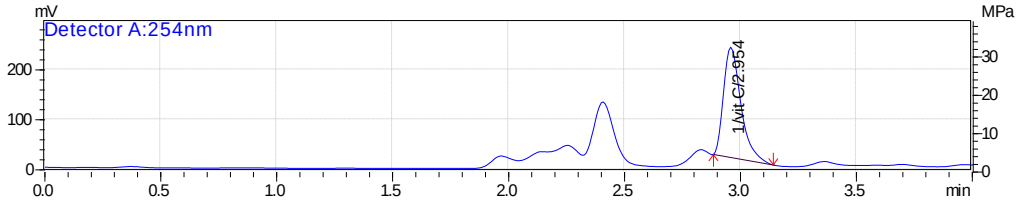


Figure 3.38 Recovery chromatogram of vitamin C spike sample 1

Table 3.18 Recovery results of vitamin C sample 1

Vitamin C (spike ,un spike)sample 1	Ret.time	Area	Height	Theoretical plate	Tailing factor	Conc.
Sample1 un spike1	2.954	277221	56060	6614.091	1.345	3.128
Sample1spike 4	2.948	1070133	208012	6146.643	1.359	12.074
Sample1 un spike2	2.946	276686	56045	6595.470	1.342	3.122
Sample1spike 5	2.948	1068409	207937	6152.097	1.357	12.054
Sample1 un spike3	2.946	283432	56509	6494.012	1.399	3.198
Sample1spike 6	2.951	1072061	208112	6241.670	1.344	12.095
Average	2.949	674657	132113	6373.997	1.358	7.612
%RSD	0.111	64.226	62.941	3.434	1.582	64.226
Maximum	2.954	1072061	208112	6614.091	1.399	12.095
Minimum	2.946	276686	56045	6146.643	1.342	3.122
Standard Deviation	0.003	433305	83153	218.878	0.021	4.889

$$R\% = \frac{(\text{Spike sample} - \text{un spike sample}) \times 100}{\text{known spike con} \times D.F}$$

Dilution factor: D.F. = 0.95

Spike conc = 10ppm

Sample 1 R% = (12.074-3.128)×100/9.5 R%=94.16

Vitamin C recovery for sample 2

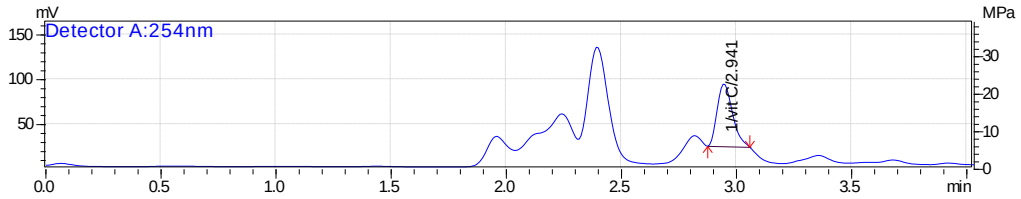


Figure 3.39 Recovery chromatogram of vitamin C un spike sample 2

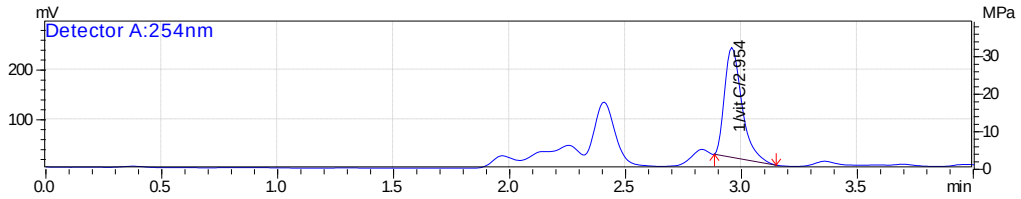


Figure 3.40 Recovery chromatogram of vitamin C spike sample 2

Table 3.19 Recovery results of vitamin C sample 2

Vitamin C(spike ,un spike)sample 2	Ret. time	Area	Height	Theoretical plate	Tailing factor	Conc.
Sample2 un spike1	2.941	327270	72491	7606.753	1.290	3.692
Sample2 spike1	2.955	1134332	218860	6429.780	1.588	12.798
Sample2 un spike2	2.941	326282	69600	7255.331	1.429	3.681
Sample2 spike2	2.954	1141118	220604	6418.891	1.589	12.874
Sample2 un spike3	2.941	315709	68838	7453.670	1.358	3.562
Sample2 spike3	2.954	1142598	220663	6414.541	1.593	12.891
Average	2.948	731218	145176	6929.828	1.475	8.250
%RSD	0.241	61.146	56.499	8.202	9.090	61.146
Maximum	2.955	1142598	220663	7606.753	1.593	12.891
Minimum	2.941	315709	68838	6414.541	1.290	3.562
Standard Deviation	0.007	447112	82024	568.369	0.134	5.044

R%= (Spike sample-un spike sample)×100

known spike con ×D.F.

Dilution factor: D.F.

D.F=0.95

Sample 2 R% = (12.798-3.692)×100/9.5R%=95.85

Vitamin C recovery for the sample 3

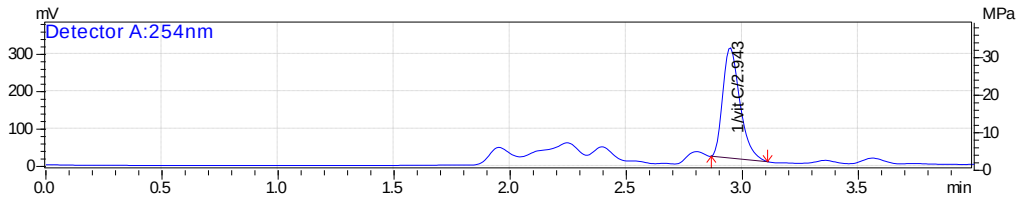


Figure 3.41Recovery chromatogram of vitamin C un spike sample 3

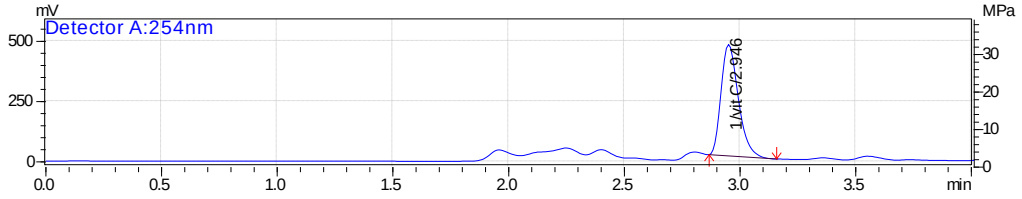


Figure 3.42 Recovery chromatogram of vitamin C spike sample 3

Table 3.20 Recovery results of vitamin C sample 3

Vitamin C (spike ,un spike)sample 3	Ret. Time	Area	Height	Theoretical Plate	Tailing Factor	Conc.
Sample3 un spike 1	2.943	1535766	295404	6196.021	1.484	17.327
Sample3 spike 1	2.946	2385182	462171	6244.673	1.428	26.910
Sample3 un spike2	2.943	1525298	294947	6219.566	1.467	17.209
Sample3 spike 2	2.946	2383688	462159	6244.673	1.428	26.894
Average	2.945	1957484	378670	6226.234	1.452	22.085
%RSD	0.065	25.186	25.461	0.375	1.942	25.186
Maximum	2.946	2385182	462171	6244.673	1.484	26.910
Minimum	2.943	1525298	294947	6196.021	1.428	17.209
StandardDeviation	0.002	493020	96412	23.361	0.028	5.562

$$R\% = \frac{(\text{Spike sample} - \text{un spike sample}) \times 100}{\text{known spike con} \times \text{D.F.}}$$

Dilution factor: D.F.

D.F=0.95

Sample 3 R% = (26.910-17.327)×100/9.5 R% =100.87

Vitamin C recovery for the sample 4

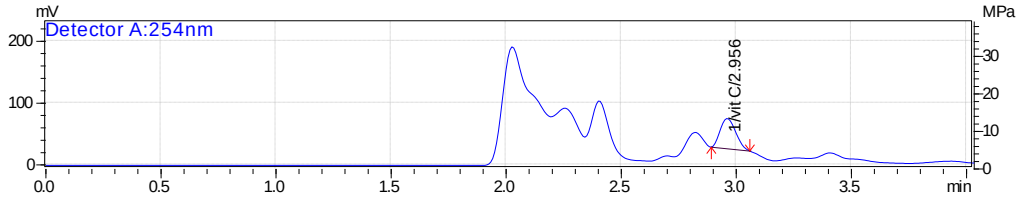


Figure 3.43 Recovery chromatogram of vitamin C un spike sample 4

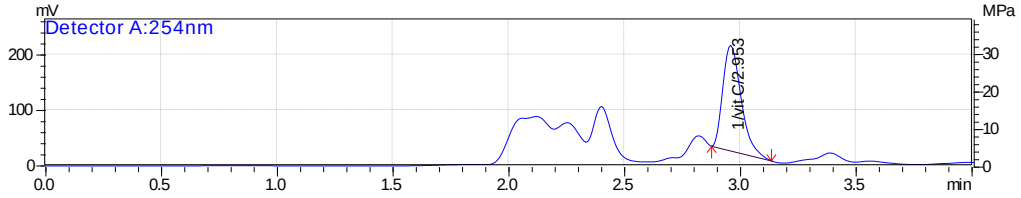


Figure 3.44 Recovery chromatogram of vitamin C spike sample 4

Table 3.21Recovery results of vitamin C sample 4

Vitamin C (spike un spike)sample 3	Ret. time	Area	Height	Theoretical plate	Tailing Factor	Conc.
Sample 4 un spike 1	2.956	223460	48217	7096.912	1.251	2.521
Sample 4 spike 1	2.946	1038754	189352	5641.346	1.503	11.720
Sample 4 un spike2	2.956	229252	48722	6929.708	1.290	2.586
Sample 4 spike 2	2.953	1038607	190083	5719.950	1.512	11.718
Average	2.953	632518	119093	6346.979	1.389	7.136
%RSD	0.166	74.148	68.476	12.181	9.913	74.148
Maximum	2.956	1038754	190083	7096.912	1.512	11.720
Minimum	2.946	223460	48217	5641.346	1.251	2.521
Standard Deviation	0.005	469002	81550	773.101	0.138	5.291

R%= (Spike sample-un spike sample)×100

known spike con ×D.F.

Dilution factor: D.F.

D.F=0.95

Sample 4 R%= (11.718-2.521)×100/9.5 R% =96.83

The accuracy of HPLC method for the determination of vitamin C concentration in the fruits and leaves of *Balanites aegyptiaca* and *Prosopis juliflora* to recovery results of standard samples of vitamin C was shown in Table3.22

Table 3.22 Concentration of vitamin C in *Balanites aegyptiaca* and *Prosopis juliflora*

Samples	Sample1(BF)	Sample2(BL)	Sample3(PF)	Sample4(PL)
Concentration ppm	391	461.5	216.588	315.1

D.F. =125

Concentration = un spike sample×D.F.

Sample1: *Balanites aegyptiaca* fruits Sample2: *Balanites aegyptiaca* leaves

Sample3: *Prosopis juliflora* fruits Sample4: *Prosopis juliflora* leaves

3.5 UV-Spectrophotometeric method for the determination of ceric sulphate

Optimization of the experimental conditions

The optimum conditions for the developed spectrophotometeric method was established by varying the parameters one at time while keeping the others fixed and observing the effect produced on the absorbance of ceric sulphate .In order to

establish the optimum experimental conditions ,the effect of various parameters such as absorption ,wavelength, temperature ,reaction time after preparation and pH were investigated

3.5.1 Determination of absorption maximum (λ_{max}) of ceric sulphate

The absorbance of 20 ppm ceric sulphate in 4M H_2SO_4 was measured at the (nm) 300,323,325,328,332 and 340 nm and the results were recorded in Table 3.23.

Table 3.23 Effect of wavelength on the absorbance of ceric sulphate in 4M H_2SO_4

λ nm / conc.of Ce	300nm	323nm	325nm	328nm	332nm	340nm
20	0.160	0.162	0.160	0.156	0.153	0.141

It was found that ceric (IV) sulphate in 4M H_2SO_4 exhibits a maximum absorption peak (λ_{max}) at 323 nm (0.162).

The absorption of different concentrations of ceric sulphate in 4M H_2SO_4 at 323nm was recorded in Table 3.24

Table 3.24 Absorbance of ceric (IV) sulphate standards in 4M H_2SO_4 at 323nm

Ce conc. ppm	20	40	60	80	100	120	140	150	160
Absorbance at 323nm	0.162	0.338	0.496	0.725	0.936	1.169	1.333	1.457	1.621

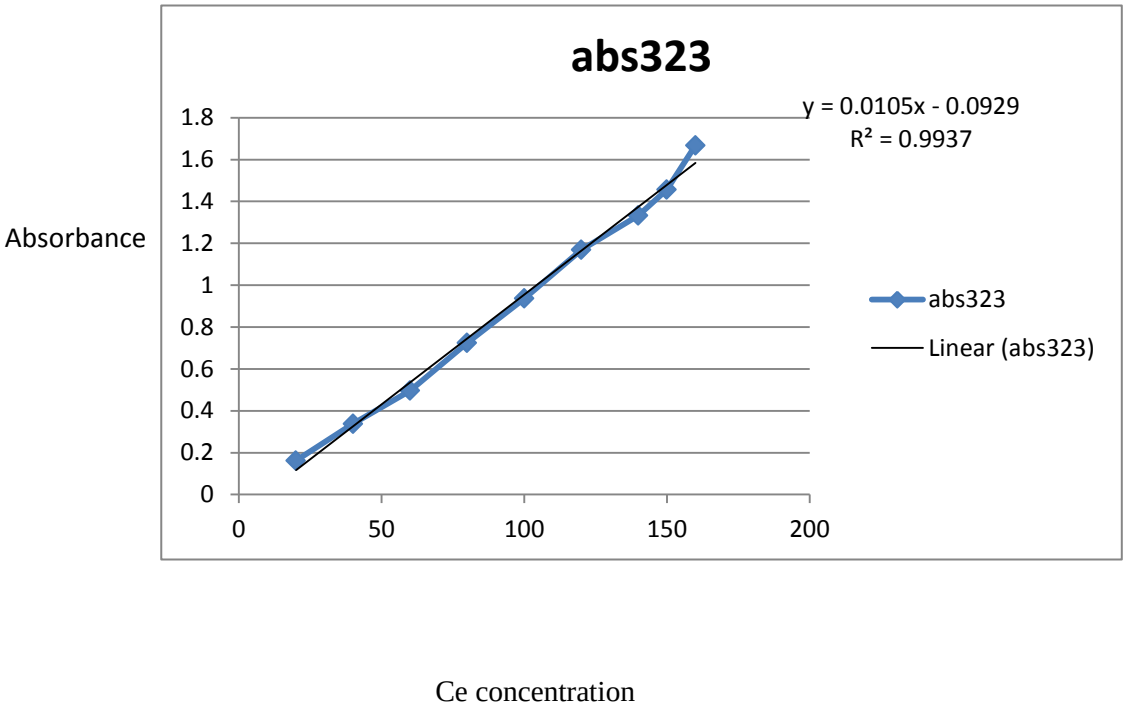


Figure 3.45 Beer's low plot of ceric sulphate in 4M H_2SO_4 on the absorbance at 323nm

It was found that it was possible to determine the spectrophotometrically 4 M Ce $(SO_4)_2$ solutionin 4 M H_2SO_4 at a high concentration up to 1.62 ppm at a wavelength of 323 nm.

The absorbance of 1.62 ppm ceric sulphate in 4M H_2SO_4 at 323nm was recorded in Table 3.25

Table 3.25 Effect of temperature on the absorbance of ceric sulphate in 4M H_2SO_4 at 323 nm

Temp(˚C) / Absorbance	At room temp	30˚C	40˚C
20	0.162	0.158	0.143

It was found that determination of absorbance of Ce $(SO_4)_2$ in 4 M solution at wavelength 323 nm at room temperature was 0.162.The absorbance of 1.62 ppm ceric sulphate in 4M H_2SO_4 at 323nm at room temperature was recorded in Table 3.26

Table3.26 Effect of time on the absorbance of ceric sulphate at 323 nm

Ce con ppm	5 min	10 min	20min	30 min	40 min
20	0.162	0.179	0.227	0.233	0.236

It was found that it was possible to determine the spectrophotometrically Ce $(SO_4)_2$ in 4 M solution at a wavelength of 323 nm, the absorbance after 10 minitues was 0.179.

λ nm : 323 nm Range of Ce concentration ppm: 20-160 ppm

Temp: At room temp Time: After 10 min Absorbance range: 0.162-1.621

Table 3.27 Effect of wavelength on the absorbance of ceric (IV) sulphate in 5 M H_2SO_4

λ nm / conc of Ce	300	323	325	328	332	340
20 ppm	0.189	0.182	0.180	0.179	0.173	0.170

It was found that it was possible to determine the spectrophotometrically Ce $(SO_4)_2$ 5 M solutionat concentration 20 ppm and wavelength 323 nm the absorbance was 0.182

Table 3.28 Absorbance ofceric (IV) sulphate standards in 5M H_2SO_4 at 323nm

Ce con ppm	20	40	60	80	100	120	140	150
Absorbance at 323nm	0.182	0.380	0.592	0.782	0.999	1.231	1.451	1.647

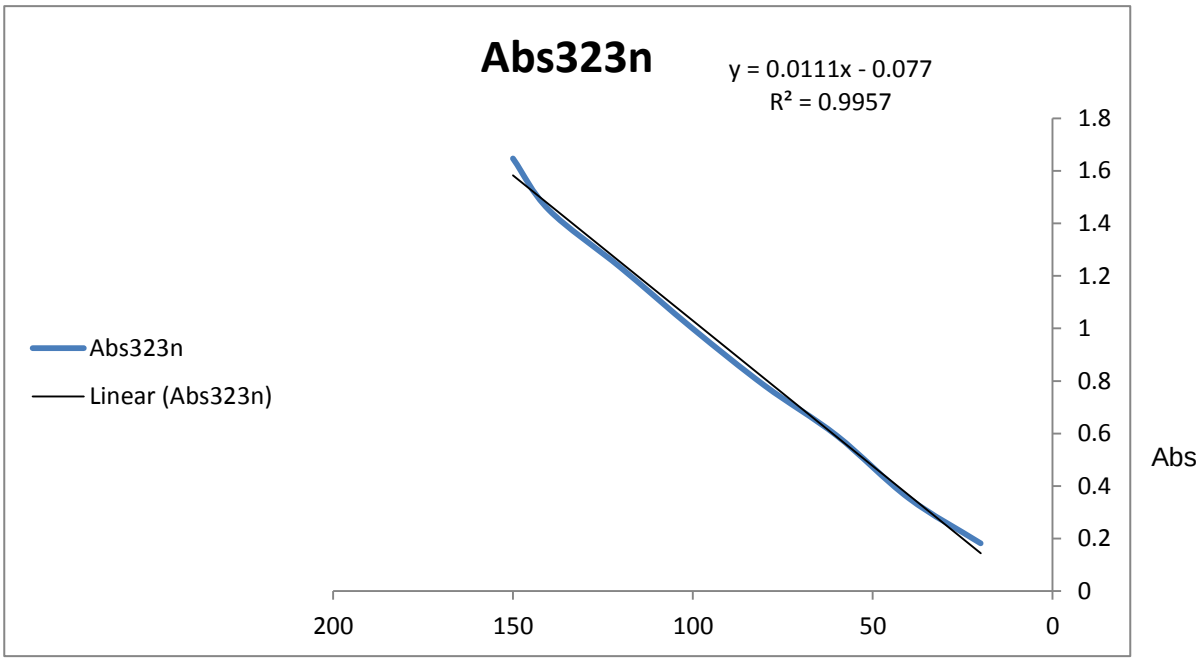


Figure 3.46 Beer's low plot of ceric (IV) sulphate in 5M H_2SO_4 at 323 nm

It was found that spectrophotometrically determination of $CeSO_4$ 5 M solution absorbance was in the range of 0.182 to 1.647 at a wavelength of 323 nm.

Table 3.29 Effect of temperature on the absorbance of ceric (IV) sulphate in 5M H_2SO_4 at 323 nm

Temp(˚C) / Absorbance	At room temp	30 ˚C	40 ˚C
20 ppm	0.182	0.162	0.139

It was found that determination of absorbance of $CeSO_4$ in 5 M solution at wavelength 323 nm at room temperature was 0.182.

Table 3.30 Effect of time on the absorbance of ceric (IV) sulphate in 5M H_2SO_4 at 323 nm

Ce con ppm	5 min	10 min	20 min	30 min	40min
20	0.182	0.195	0.233	0.246	0.252

The determination of absorbance of $CeSO_4$ in 5 M solution at wavelength 323 nm after 10 minutes was 0.195

λ nm : 323 nm Range of Ce concentration ppm: 20-160

Temp: At room temp Time: After 10 min Absorbance range: 0.182-1.647

Table 3.31 Effect of wavelength on the absorbance of ceric sulphate in 6M H_2SO_4

Ce con ppm	300	323	325	328	332	340
20	0.146	0.147	0.145	0.141	0.136	0.125

It was found that it was possible to determine the spectrophotometrically

Ce $(SO_4)_2$ 6 M solution at concentration 20 ppm and wavelength 323 nm the absorbance was 0.147

Table 3.32 Absorbance of ceric (IV) sulphate standards in 6M H_2SO_4 at 323 nm

Ce con	20	40	60	80	100	120	140	150	160
Absorbance at 323nm	0.147	0.332	0.499	0.708	0.844	1.086	1.382	1.461	1.546

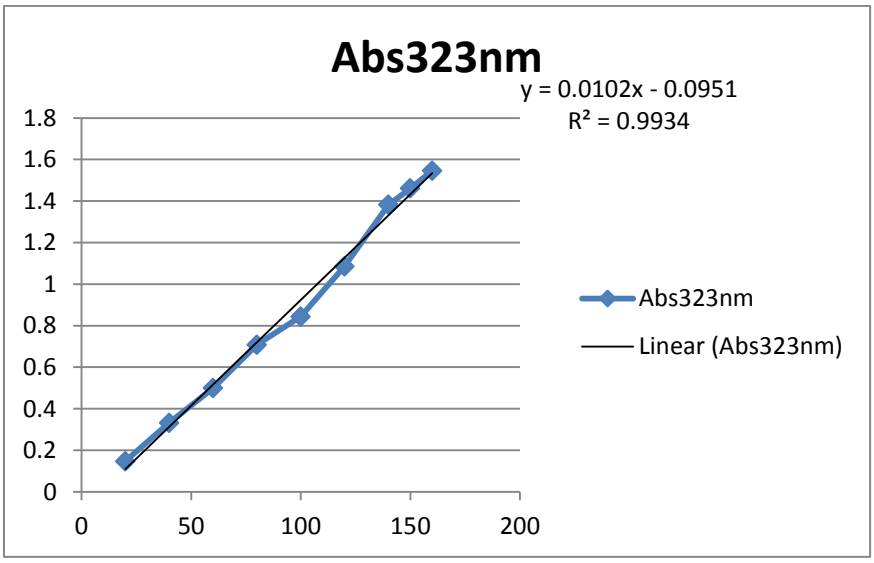


Figure 3.47 Beer's low plot of ceric (IV) sulphate concentration in 6M H_2SO_4 at 323 nm

It was found that spectrophotometrically determination of Ce $(SO_4)_2$in 6 M solution absorbance was in the range of 0.147 to 1.546 at a wavelength of 323 nm.

Tables 3.33 Effect of temperature on the absorbance of ceric (IV) sulphate in 6 M H_2SO_4 at 323 nm

Ce con ppm	At room temp	30 ˚C	40 ˚C
20	0.147	0.139	0.120

It was found that determination of absorbance of $CeSO_4$ in 6 M solution at wavelength 323 nm at room temperature was 0.147.

Table 3.34 Effect of time on the absorbance at 323 nm

Ce con ppm	5 min	10 min	20 min	30 min	40 min
20	0.147	0.179	0.231	0.243	0.280

The determination of absorbance of Ce $(SO_4)_2$ in 6 M solution at wavelength 323 nm after 10 minutes was 0.179

λ nm : 323 nm Range of Ce concentration ppm: 20-160

Temp: At room temp Time: After 10 min Absorbance range: 0.147 to 1.546

Table 3.35 Effect of pH on the absorbance at323 nm

Ce con ppm	4M H_2SO_4	5M H_2SO_4	6M H_2SO_4
20	0.162	0.182	0.147

The absorbance linear range of Ce $(SO_4)_2$in 5M H_2SO_4 (from 0.182-1.647) was greater than that of 4M (from 0.162-1.621) and that of 6M (from 0.147 to 1.546).

It was also found that regression value (R^2) for 5M (0.995) was greater than that for 4M (0.993) and 6M (0.993).

According ly the spectrophotometric determination of Ce $(SO_4)_2$was carried out at $5MH_2SO_4$

3.6 Indirect UV-spectrophotometric determination of ascorbic acid in fruits and leaves of *Balanites aegyptiaca* and *Prosopis juliflora*

Absorbanceof 150 ppm ceric (IV) sulphate in $5MH_2SO_4$ which was not reduced by the addition of varing volumes 100 ppm ascorbic acid are recorded in Table 3.36 and drawn in Fiqure 3.48

Table 3.36 Absorbance of standard ascorbic acid solutions at 323nm

Ascorbic conc.ppm	Absorbance at323nm
0	0.831
50	0.752
100	0.640
150	0.561
200	0.470
250	0.363
300	0.272
350	0.193
400	0.131
450	0.030

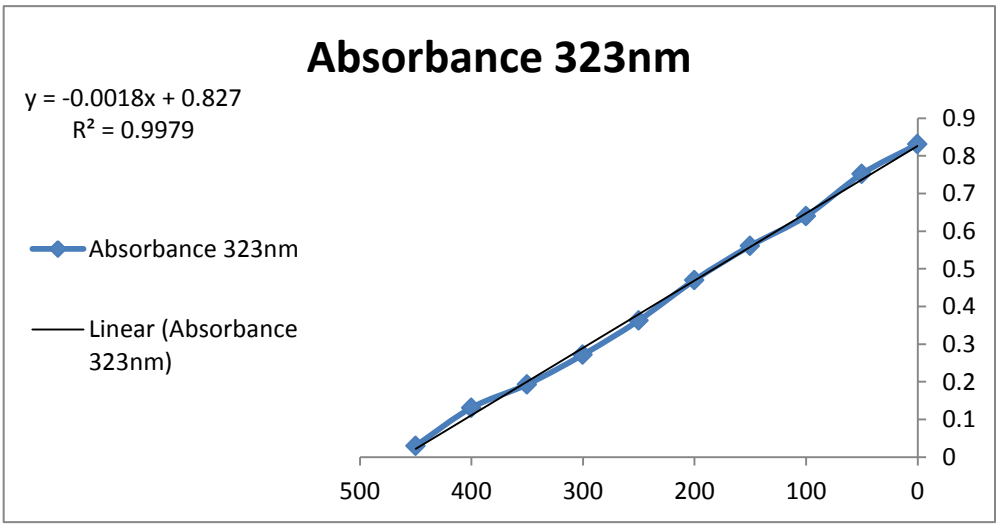


Figure 3.48 Beer's low plot of ascorbic acid solutions at 323 nm

Table 3.37 Concentration results of vitamin C in the samples extracted with distilled water

sample	Abs 323nm	Conc. ppm
BF	0.212	326
BL	0.080	423
PF	0.521	179
PL	0.310	283

Table 3.38 Concentration results of vitamin C in the samples extracted with 4.5% metaphosphoric acid

sample	Abs 323nm	Conc.ppm
BF	0.150	380
BL	0.001	465
PF	0.430	216
PL	0.220	315

Table3.39 Concentration results of vitamin C in the samples extracted with acetic acid

sample	Abs 323nm	Conc.ppm
BF	0.171	361
BL	0.060	441
PF	0.491	198
PL	0.250	306

BF: *Balanites aegyptiaca* fruits BL: *Balanites aegyptiaca* leaves

PF: *Prosopis juliflora* fruits PL: *Prosopis juliflora* leaves

Effect of wavelength

The influence of ultraviolet radiation (300,323,325,328,332 and 340nm) of the absorption of ceric sulphate in different molarization of H_2SO_4 (4M, 5M and 6M) was investigated .In 4M H_2SO_4 the absorbance was increased at 323nm but then decreased at 340nm (Table 3.23).In 5M H_2SO_4, however, the absorbance was also increased at 323nm but then decreased at 340nm (Table 3.27).In 6M H_2SO_4 the absorbance behaved similarly to that in the latter molarity of H_2SO_4 (Table3.31).

Effect of ceric sulphate concentration

The influence of the concentration of ceric sulphate on its absorption of radiation at 323nm, in different molarites of H_2SO_4(Tables 3.24, 3.28 and 3.32) was investigated.It was found that the absorbance increased with increasing concentration from 20ppm to 160 ppm in 4M,5M and 6MH_2SO_4

Effect of temperature

The effect of increasing the temperature from room temperature to 30 ˚C and to 40 ˚C of ceric sulphate in 4M (Table 3.25), 5M (Table 3.29), and 6M (Table 3.33) increased its absorption of radiation at 323nm.

Effect of time

The absorption of radiation at 323nm by ceric sulphate solution in 4M, 5M and 6M H_2SO_4 was measured after periods of 5,10,20,30 and 40 minutes were recorded in Tables 3.26,3.30 and 3.34,respectively.It was found that the absorption of radiation increased with increased period of time before measurement.

Effect of pH

The effects of pH on the reaction of ceric sulphate was examined by varying the pH with 4M H_2SO_4, 5M H_2SO_4 and 6M H_2SO_4, The results revealed that ceric sulphate have to react with H_2SO_4 as showed in Table (3.35) and Figure (3.49).When pH decreased, the readings increased rapidly from 4M to 5M and decreased in 6M.The maximum readings were attained at pH values of 5M H_2SO_4.Therefore pH 5M H_2SO_4 was selected for optimal experimental condition.

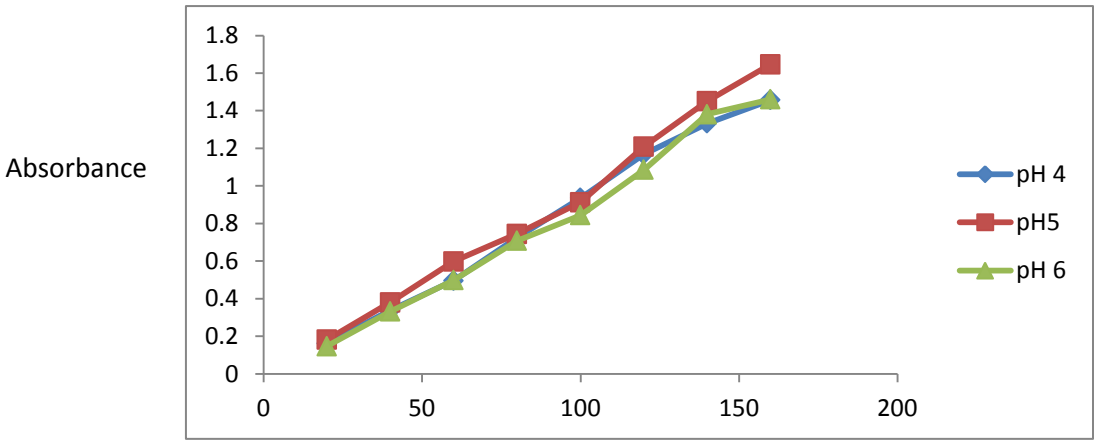


Figure 3.49 The effect of pH on the absorbance

Determination of ascorbic acid using ceric sulphate

The absorbance of ceric sulphate (150ppm) and ascorbic acid (100ppm) at 323 nm, the absorbance decrease with increase of ascorbic acid volume, Figure (3.50)

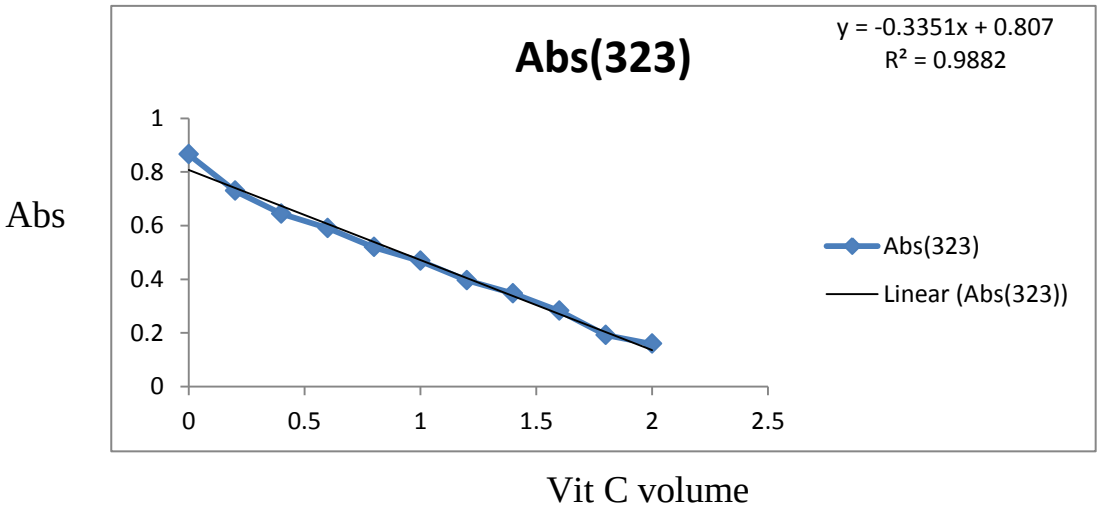


Figure 3.50 The absorbance of ceric sulphate (150ppm) and ascorbic acid (100ppm) volumes at 323 nm

Which shows R^2 =0.988 , Y =0.335 ×+0.807.

In this study 150 ppm ceric(IV) sulphate was used to determined ascorbic acid in fruits, leaves of *Balanites aegyptiaca* (laloub)and *Prosopis juliflora* (mesquite) used different extract solution for the sample (in extract with metaphosphoric acid *Balanites aegyptiaca* leaves had the highest concentration of ascorbic acid (465ppm) followed by its fruits (380ppm) while the fruits of *Prosopis juliflora* was (216 ppm) and the leaves values was (315ppm).when extracted with acetic acid *Balanites aegyptiaca* leaves concentration was (441ppm) f ollowed with it is fruits (361ppm),while *Prosopis juliflora* leaves (306ppm) and fruits of the former was (198ppm).Also it was found that the ascorbic acid cocentration when extracted with distilled water was (423ppm) for *Balanites aegyptiaca* leaves (326ppm) for its fruits, while (283ppm) for *Prosopis juliflora*l eaves and (179ppm) for *Prosopis juliflora* fruits.

3.7 Suggestions for further research work

The success of the indirect UV-spectrophotometric method for the determination of vitamin C in laloub and mesquite lures further investigations to apply the developed method to:

- Apply the method in other regions of the spectrum including visible and infrared spectrophotometry.
- Attempt other indirect instrumental techniques including voltammetry and potentiometry.
- Determine other constituents in other samples including plants and vegetables.

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