ANALYSIS FOR THE ASCORBIC ACID CONTENT **OF THE**

PUMPKIN (CURCUBITA SPP) AND THE
BLOOD OF GUINEA FOWL.

BY

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A THESIS PRESENTED TO THE
AHMADU BELLO UNIVERSITY FOR THE DFGREE OF
MASTER OF SCIENCE (ANALYTICAL CHEMISTRY)

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JANUARY, 1977

ACKNOWLEDGEMENTS

I sieze this opportunity to express my profound gratitude to DR. U.D. Gomwalk who supervised this work for his guidance and encouragement throughout the period of this investigation. My sincere gratitude also goes to DR. O.U. Eka for his interest and very useful comments.

I would also like to thank MR. Offiong of the National Animal Production Research Institute for his interest and help, the Director of National Veterinary Research Institute Vom, for providing the guinea fowls and DR. S.I. Bello of the Faculty of Veterinary Medicine for allowing us to use his pens, and looking after the health of the guinea fowls.

MRS. R. Egharevba and MR. Mike Odillison identified the pumpkins; I am grateful to them.

Finally, I wish to thank Professor A.R. Mathieson, Head of Chemistry Department for his keen interest in the course.

DECLARATION

This work is solely the work of the author and has not been presented else where for the award of another degree.

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ABSTRACT

Prelimnary investigation was carried out to study the daily variation of ascorbic acid content of pumpkin (Curcubita) using spectrophotometric methods. The total ascorbic acid content of the edible portion remained constant while the amount of dehydroascorbic acid increased markedly in three days, and the L- ascorbic acid content decreased correspondingly. The seeds were found to contain more of the vitamin than the edible portion, while the edible portion containted more than the peeling. More work however is still required to establish these results.

The effect of two types of feeds, the guinea corn and the commercial Pfizer Livestock feed, on the weight and ascorbic acid content of the whole blood of the guinea fowl was studied. The total ascorbic acid content remained constant but there was an apparent increase in weight of the birds fed on guinea corn over those fed with the commercial livestock feed. This can be correlated, rather cautiously, with observed higher ascorbic acid content in the guinea corn. Other growth promoting factors will have to be investigated to establish more meaningful correlations.

C O N T E N T S

CHAPTER I

I N T R O D U C T I O N

Ascorbic acid has been known as cevitamic acid, the antiscorbutic vitamin, hexuronic acid scorbutamin, and redoxon. Redoxon, because of its structural relationship to reductone, the enol of hydroxymethylglyoxal. It is commonly known as vitamin C. It is one of the water soluble vitamins and its deficiency in the diet causes a diseased condition known as scurvy.

Symptons of scurvy have been known quite as early as the fourteenth century and the fact that evergreens prevented scurvy has been evidenced by the reports of sailors notably Captain Lancaster and Captain James Cook in those early days when man's ambition was a conquest of the world through travel.(1)

During the deficiency stages defective intercellular material is formed in the collective tissue while in the bones, the osteoid is replaced by a collageneous material and in the teeth the dentin is supplanted by a substitute substance called osteodentin. Further impairment of the teeth causes lessions of the gums which become swollen and bleed and which eventually fall off. The anatomical effects of human scurvy gradually give rise to weakness, rheumatic pains in the leg, lessions, bleeding gums and hemorrhages. Patients may become pale, anaemic looking, and experience swelling of the ankles. The advanced form is accompanied by severe fragmentation and

degeneration of the muscles, bloody tumors of eyes and eyelids discolouration and lessions of the skin, fever, capillary weakness, brawny haematomata in the thighs and optic prolapse.

1.1 SOURCES AND USES OF ASCORBIC ACID

The natural vitamin is widely distributed in animals and plants where it is probably in equilibrium with dehydroascorbic acid. It occurs in large amounts in the adrenal cortex, suprarenal medulla, eyelens and abundantly in vegetables such as paparika, cabbage, spinach, peas, tomatoes, turnips, beets, carrots, celery, lettuce and in fruits such as grapefruit oranges, tangerines, strawberries peaphes, pineapple and apples. Fresh tea leaves are a rich source of ascorbic acid and for practical purposes citrus fruits offer the best source of the vitamin.

In mammalian tissues ascorbic acid has a unique pattern of distribution. Summerwell and Sealock[2] have shown that bound ascorbic acid does exist in the liver tissue, by the isolation and identification of ascorbic acid from the bound fraction. A method of determining the amount of bound ascorbic acid was presented and it involved the extraction of the ascorbic acid from the sample with 95% ethyl alcohol and the liberation of the bound ascorbic acid by acid hydrolysis. Of the samples analysed 15 - 20% of the total ascorbic acid was present in the bound form. The significance of this bound ascorbic acid is not known but its existence as such may serve to protect it against degradation by liver enzymes.

Several tissues[1] have ascorbic acid concentrations several hundred times that of plasma while a large number of tissues have concentrations 50 - 100 times the plasma level.

Sargent[3] has studied the normal distribution of ascorbic acid between red cells and plasma of human blood and found that the following relationship exists between ascorbic acid concentration of blood cells or plasma A'p= 1-43A'c - 0.72 when the ascorbic acid concentration is expressed as mg/100ml of blood. He also found that when the concentration is expressed in mg/100ml of H_2O, the relationship between Ac and Ap is approximately Ap= Ac- 0.45 and that the ratio of the concentration of ascorbic acid in the cells to that in the plasma depends upon the concentration of ascorbic acid in the whole blood. This relationship was found to be approximately $Ac: \frac{Ap}{Awb} = \frac{Awb + 0.28}{Awb - 0.17}$ Where A is expressed in mg/100ml of water.

Ac = Ascorbic acid in cells i.e. total (white + red)

Ap = ascorbic acid in plasma

Aw = ascorbic acid in whole blood.

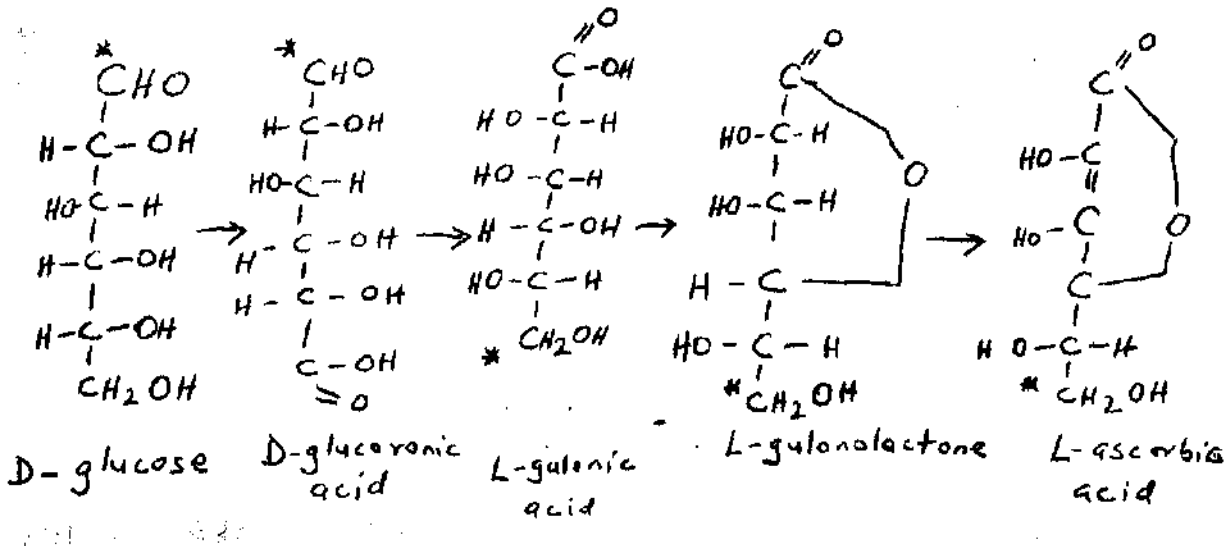
The primary use of ascorbic acid is in the cure of scurvy. Nutritionists feel that 60 - 100mg per day is needed by an adult for saturation of the tissues.[1]

Apart from the antiscorbutic properties, ascorbic acid is important in the prevention of megaloblastic anemia of infancy, and may also be useful in other types of macrocytic anaemia. Ascorbic acid in large quantities increases the absorption of iron from the intestinal tract.

The vitamin is necessary for the normal healing process and is often administered in larger than normal amounts following trauma and infections.

1.2 SYNTHESIS OF ASCORBIC ACID

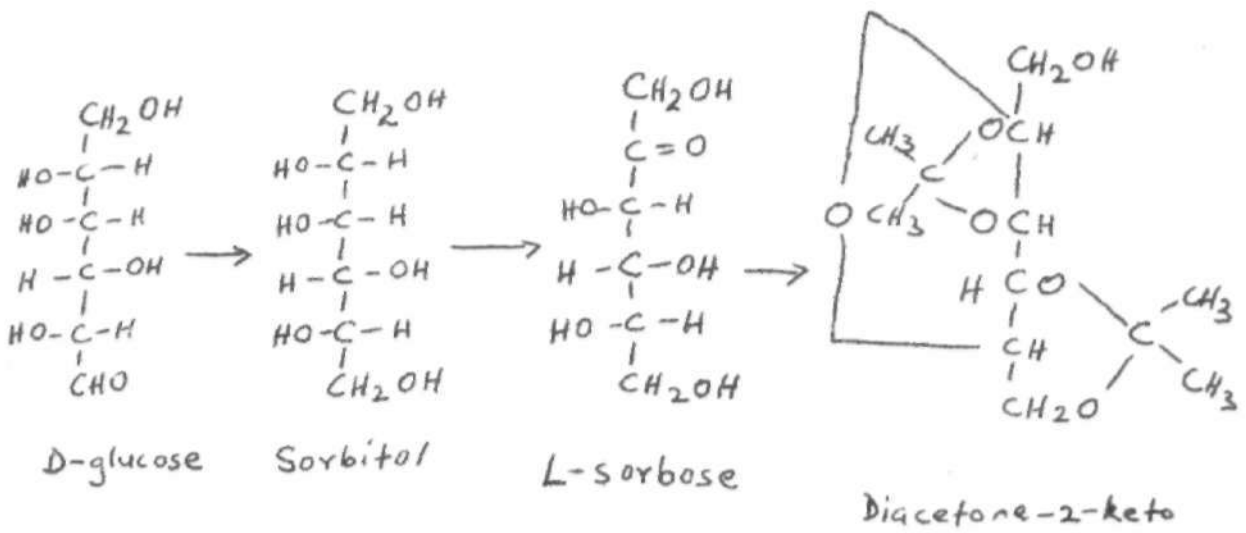
Apart from man, other primates, and the guinea pig, all other animals have been known to synthesize L-ascorbic acid. It has also been known that rats synthesize ascorbic acid. Horowitz, Deerschuk and King[4] provided evidence that glucose was a precursor of ascorbic acid, through radioactive tracer studies. They found that intraperitoneal administration of D-glucose-1-C^{14} to chloretone stimulated rats resulted in the urinary excretion of ascorbic acid containing C^{14} chiefly in the 6-position. Horowitz and King[5] also showed the route to the conversion of glucose-1-C^{14} to ascorbic acid in the albino rat retained the same amount of radioactivity in the 6-position. The biosynthetic pathway was then formulated as



Isherwood, Chen, Mapson[5] also working on rats reported that the administration of D-glucoronolactone and L-gulonolactone produced an increase in the urinary excretion of L-ascorbic acid. Tracer studies further established the conversion of D-glucoronlactone and L-gulonolactone to L-ascorbic acid.

In plants however, it has been shown that the vitamin is synthesized from D-glucose by a fairly direct route. In this case D-glucose-1-C* is converted to L-ascorbic acid labelled predominantly in the 6-position and D-glucose-6C* is converted to L-ascorbic acid labelled in the 6-position. Isherwoodetal[6] demonstrated that L-gulonolactone and D-glucoronolactone are converted to L-ascorbic acid in crest seedlings, thus showing that reactions of the animal biosynthetic pathway must also occur to certain extent in plants.

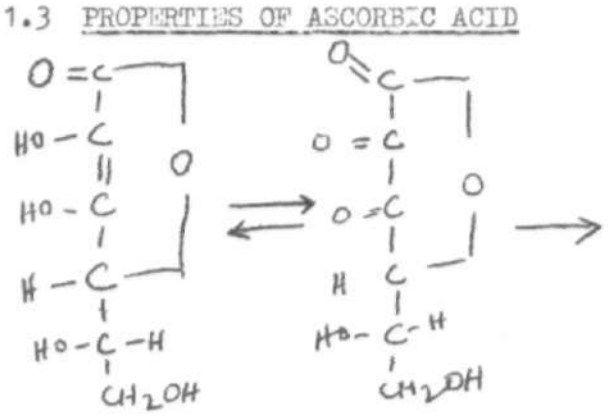
The commercial synthesis of ascorbic acid was first achieved in 1936 when synthetic vitamin C was produced in cyrstalline form. It largely follows the Reichstein (L- sorbose method). This method appears to be the principal and most economically feasible process starting from D-glucose.



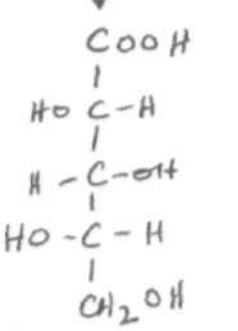
THE REICHSTEIN (L-SORBOSE)

SYNTHESIS OF L-ASCORBIC ACID

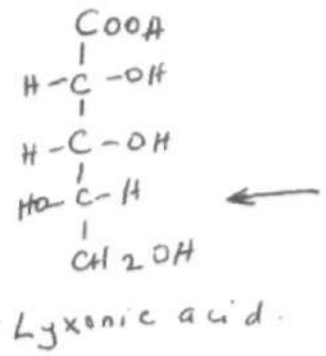
1.3 PROPERTIES OF ASCORBIC ACID



L-ascorbic acid Dehydroascorbic acid 2,3-diketogulonic acid.



L-xylonic acid



L-Lyxonic acid

AN OUTLINE OF THE METABOLISM OF ASCORBIC ACID.

Ascorbic acid has a molecular weight of 176 and its molecular formular is $C_6 H_8 . O_6$. A look at the structural formular would reveal the presence of a dienolic function and this is responsible for its oxidation-reduction properties. It's redox potential Eo' is 0.166V at pH4 and temperature of 35°C.

It is soluble in water, methyl alcohol and ethyl alcohol. It is insoluble in either, xylene, benzene, chloroform or petroleum ether. It has a specific rotation of +23° in water at 20°C and +48° in methyl alcohol at 23°C. The first acid dissociation constant pk_1 is 4.2 and its pk_2 is 11.6. It absorbs maximally at 265nm and shows a small band between 350 and 400nm. and has melting point of 192°C.

Dry crystals of ascorbic acid are stable on exposure to air and daylight at ordinary room temperature for long periods of time. In aqueous solutions below pH 7.6, ascorbic aicd is not oxidised on exposure to air unless traces of copper or other materials that catalyse the reaction are present. In the presence of air and suitable catalyst ascorbic is readily oxidised to dehydro ascorbic acid and is fairly stable. Above pH4 dehydroascorbic acid readily undergoes an irreversible rearrangement to biologically in active 2,3 diketogulonic acid.

Dehydro ascorbic acid has considerable antiscorbutic potency because it is readily reduced to ascorbic acid in the animal body and it is one of the objectives of this project to find out the amount of dehydroascorbic acid present in the pumpkin at the time of harvesting and on subsequent days.

1.4 METHODS OF ANALYSIS FOR ASCORBIC ACID

Most of the chemical methods described so far have utilized the redox properties of ascorbic acid. Hitherto, bioassay methods have been used and a reasonable bioassay method has been attributed to Sherman, Lamer and Campell[7] who determined the minimum quantity of product required to protect a guinea pig from scurvy. Bioassays are too expensive and they consume a lot of time and leave much to be desired in terms of precision. Their advantage, however, is that they measure the summation of chemical entities that possess vitamin C activity but exclude materials devoid of vitamin C activity.

At present they are only used in comparative studies to establish the specificity of chemical methods.

It would appear that the method of choice for any analysis depends on the type of material to be asayed. It would depend on whether ascorbic acid is being determined in fresh or frozen or dehydrated foods or pharmaceuticals.

The analysis of ascorbic acid and its derivatives may be divided into two groups namely, the determination of the reduced form and that of total ascorbic acid.

In the analysis for the reduced forms, the methods are based upon the redocx properties of ascorbic acid. An acid extract of the vitamin is prepared and the reducing capacity of the extract is measured by treatment with any of the following oxidising agents, 2, 6 dichloro phenol indophenol iodine, ferricyanide or methylene blue.

The use of the dye 2, 6 dichlorophenol indophenol has found extensive use. It involves the titration of ascorbic acid in the extract or vitamin source with a dilute solution of the dye. A pink colour that persists for about 15 secs after the addition of one drop of the indophenol reagent marks the end point of the titration. In using this method it is assumed that ascorbic acid is the principal if not the only substance in the acid extract that reduces the dye at pH 1-4. In that case, the capacity of the extract to reduce the dye is proportional to the ascorbic acid content.

Interference in this procedure can be due to reducing substances like phenols, sulphydryl compounds, thiosulphate, Fe(II), Sn (II) and Cu(II) ions. At pH values below 4 interference from phenols can be eliminated as they do not reduce the dye. Correction is usually made for sulphydryl compounds whose reduction of the dye is so slow.

Photometric methods which also involve the use of the dye 2,6 dichlorophenol indopehenol have been developed by Evelyn . , Malley and Rosen[8], Bessey[9], Mindline and Butler[10], Loeffler and Ponting[11] Hochberg, Melnick, Oser.[12] In each of the modifications, the basic principle has been the measurement of the photometric density of a solution of 2,6 dichlorophenol indophenol before and after adding ascorbic acid solution. The decrease therefore in the absorbance of the dye solution by the addition of ascorbic acid is then a measure of the amount of ascorbic acid present.

The advantages which this modification claims over the visual titration method are (a) the elimination of the inaccuracy due to difficulties of judging the end point (b) interference due to turbidity or colour in the unknown extract is overcome by adjusting the colorimeter to compensate for absorption by extraneous materials and (c) correction for those substances that reduce indophenol more slowly than ascorbic acid is made by taking successively timed readings of the colorimeter and extrapolating back to zero. By careful control of the pH at 3-5 with sodium acetate buffer the stability of the dye is assured and interference due to non ascorbic acid reducing substances is eliminated.

In an attempt to adopt the photometric indophenol technique to highly coloured or turbid extracts Stotz[13] developed a method of quantitative extraction of the unreduced 2,6 dichlorophenol indophenol from an aqueous solution by xylene, in the analysis of blood andurine. This is of particular advantage in the analysis of most vegetable extracts which are coloured, since these coloured substances are water soluble and cannot pass into the xylene layer and in any case correction can be made for any extraneous colour in the xylene layer. Pepkowitz[14] found the Stotz method better because much greater amounts of dye are reduced for any amount of ascorbic acid, thus producing a greater defection of the galvanometer, in addition to the fact that a simple correction can be made for extraneous colour such as chlorophyl and carotenoids which may enter the xylene layer. Besides, the dye need not be standardised or a calibration curve be constructed and more dye can always be added, if the initial amount is not sufficient, for the quantity of ascorbic acid contained in the aliqout taken, without decreasing the accuracy of the method. He found the procedure applicable to all plant materials whether fresh, frozen ordehydrated and suitable for routine analysis as 60-70 determinations can be made in a working day.

In the final analysis, the value of the 2,6 dichlorophenol indophenol reagent is limited by the presence of reducing substances such as Fe(II) sulphite, sulphydryl compounds, glucoseamine type of compounds, reductic acid and reductones. Several other procedures have been devised to minimize the interference from these substances. Levy[15]

described a procedure which involved the treatment of the extract with peroxide to eliminate interference from sulphite Fe(II or Sn(II). Robinson and Stolz[16] have presented procedures which can be used to eliminate interence from reductones and related compounds by the addition of formaldehyde to the extract.

Macro and semimacro amounts of ascorbic acid have been determined by iodometric methods. Stevens[17] described the estimation of ascorbic acid in citrus juices. The important consideration was the specificity of the reagent, since plant materials contain many reducing substances which may titrate along with ascorbic acid in the many titrimetric methods discussed above. Other factors which he considered were standardization of the reagents employed, stability, ease and rapidity of the titration procedure, sharpness of the end point, accuracy and reproducibility of the results and extensive routine testing including the cost of the reagents. Accordingly, he felt that the iodine method has several advantages and its short comings eliminated by adjusting the acidity of the titrating medium thereby lessening interference of nonreducing vitamins and improving the end point. The end point can be improved by employing a double-back titration by adding excess iodine, then excess thiosulphate and titrating the excess thiosulphate. At the onset, the excess iodine oxidises the non vitamin reducing substances and excess thiosulphate reacts with excess iodine quickly and quantitatively. The excess thiosulphate is back titrated with more iodine to the end point. This appears to be suitable because in the presence of large amounts of coloured substances the end point can be better detected by the appearance of blue colour rather than by its disappearance. The effect of temperature was found not

to be important within the range 18-30°C.

However, the shortcomings of the method become apparent in the estimation of ascorbic acid in canned foods where the interference of Fe(II) and Sn(II) will be encountered. Besides, this method cannot be used to determine dehydro ascorbic acid whose antiscorbutic potency is about that of ascorbic acid.

Evered[18] described a macromethod for the determination of ascorbic acid in highly coloured solutions with N-bromosuccinimide. The method describes a modification of the original N-bromosuccinimide method in which excess N-bromosuccinimide is detected by liberation of iodine from potassuim iodide in acid solution and the formation of blue colour with starch. In this procedure tested with intensely pigmented material blackcurrant juice which has an absorption maximum at 520nm, the liberated iodine can be conveniently detected by an organic solvent when the ascorbic acid has been preferentially oxidised. It has a distinct advantage over the 2,6-dichlorophenol indophenol visual or spectrophotometric because of the presence of pigments especially those that absorb light at about the same wavelength as 2,6-dichlorophenol indophenol. In a modification of the indophenol procedure described an organic sovent can be used to trap the excess dye during titration and hence indicating the end point, but if mixing is inadequate the dye may enter the organic phase without having reacted with ascorbic acid in the aqueous layer, giving falsely low results. Another advantage of the reagent unlike 2,6 dichloroindophenol is that it is unaffected by reductones, reductic acid and Fe(II) salts.

Leekum-tatt and Leony[19] described a method for assaying ascorbic acid also in black currant and other coloured fruit juices. The quantitative reduction of mercuric chloride to mercurous chloride by ascorbic acid investigated by Suryanarayana Rao etal[20] was adapted as the basis for the estimation of ascorbic acid in fruit juices. The method involved the separation of insoluble mercurous chloride, by centrifugation, dissolved in standard iodine solution and the excess titrated with sodium thiosulphate using starch as indicator. In an analysis of commercial samples of various fruit juices by the proposed method and the potentiometric method of Liebman and Aynes[21] results obtained were in good agreement. Good percentage recovery was obtained when known amounts of ascorbic acid was added to $3cm^3$ of the syrup.

Interferences from citric acid, glucose, and sucrose were absent. No interference was observed when about 16 mg of sulphate, five times the amount normally used as preservative and Fe(II) or lead did not interfere. This method does not, however, take into account, of the presence of other substances that may be present in old samples, which might reduce mercuric chloride.

Another type of reaction described bySchmall etal[22] reports the use of diazonium salts fordetermination of ascorbic acid. They have found the method highly specific for the determination of ascorbic acid in the presence of dehydro ascorbic acid, even when present in quantities twice that of ascorbic acid, and all other vitamins normally found in pharmaceutical preparations. In the described procedure, ascorbic acid is made to react with diazotised 4 methoxy -2 nitroaniline to form an adduct which gives an intense blue colour upon addition of alkali.

Because of the dienolic character of ascorbic acid, the reaction was found to be highly specific for this vitamin in the presence of all other known vitamins. Reducing agents such as Fe(II) Sn(II) as well as sulphur dioxide did not interfere when present in quantities normally encountered. They reported a sensitivity which permits the determination of quantities down to 0.5mg with a low limit of 10µg/ml. They also reported good precision with conventional methods.

Senello and Argoudelis[23] described the use of a fast technique for the determination of ascorbic acid in the presence of other vitamins notably Pyridoxine and nicotinamide. This was achieved by converting the vitamins to BSA(NO-bis(trimethy (setyl) acetamide derivative using phenanthrene as an internal standard. In the report, two brands of vitamin capsules were purchased and their nicotinamide, Pyridoxine and ascorbic acid contents determined using the 2,6 dichlorophenol indophenol technique. They reported comparable results with the GLC method and the GLC method was considereably faster. It appears, however, that this method is limited to pharmaceuticals.

Recently Stookey[24] reported a sensitive method for the determination of Iron(II) using ferrozine- 3(2- Pyridyl) 5, 6 bis(4 phenyl sulphonic acid 1,2,4 triazine disodium salt, to form a stable chelate. Jaselkis et al [25] adapted this to the determination of ascorbic acid in citrus fruits. In this procedure Iron(III) is added to the sample and a coloured chelate complex formed between Fe(II), the redox product and Ferrozine and the absorbance measured at 562 nm after 2-3 mins.

In order to avoid interference due to hydroxy acids like citric and tartaric acids aluminium is added. The aluminium forms chelates with the acids thus leaving Iron(III) available for the reaction

These workers also reported absence of interference from sucrose, glucose, mannose and formaldehyde. The presence of oxalic acid in amounts as high as 10 times the Fe(III) concentration did not produce any interference, neither did phosphate interfere at amounts 100 times. However, Co(II), Cu(II), Nc(II) form chelates with ferrozine and therefore a large excess of the ferrozine was used. In samples which contain Fe(II) the workers suggested the removal by cation exchange resin before analysing the eluent for ascorbic acid.

The basis of another group of versatile methods for the determination of ascorbic acid is the reaction of certain derivatives of ascorbic acid with 2,4, dinitrophenyl hydrazine. Roe and Kuether[26] first developed this method for the determination of ascorbic acid in whole blood and urine. After extraction the sample is oxidised by one of a variety of oxidising agents notably bromine and norit to form dehydroascorbic acid or its transformation product which is coupled to form an insoluble red osazone. In the method first reported by Roe and Kuether[27], the dehydroascorbic acid osazone is separated by centrifugation and washed before treatment with 85% sulphuric acid to produce a reddish coloured product which absorbs maximally at 500 - 550nm and 350-380nm. In the procedure described, the ascorbic acid in blood or urine is extracted with trichloroacetic acid and oxidised with norit. The norit filterate is treated with 2,4, dinitrophenylhydrazine

and thiourea for 3 hrs at 37°C. Thiourea was found necessary to produce a mildly reducing medium as oxidising agents like Fe(II) and H_2O_2 produce an interfering colour with 2,4, dinitrophenylhydrazine.

The accuracy and precision of the method was excellent as shown by recovery experiments. The colour prepared from blood filterate was quite stable and showed no change in forty minutes.and had a maximum fading of 2.25 in the galvanomether in 18 hrs on standing in an open tube.

A mechanism for the reaction was postulated.. It was concluded that the red product obtained was a new compound for the simple reasons that the 2,4dinitrophenylhydrazine derivative of dehydroascorbic acid in alcohol glacial acetic acid and dilute sulphurid acid are brown in colour. When the concentration of sulphuric acid is raised from 60–85% a deep red colour is formed. On the addition of excess water, a brownish compound having the same appearanceand absorption spectrum as the original derivative was formed. It was concluded that the mechanism is dehydration and the reaction Reversible because on the addition of 85% sulphuric acid a red solution was obtained.

Roe and Osterling[28] adapted this method to the determination of dehydroascorbic acid in the presence of ascorbic acid in plant materials. The steps were essentially the same except for the omission of treatment of the acid extract with norit or any other oxidising agent. In comparing the results obtained in this method with that of the indophenol method as modified by Bessey[9] they found that the Bessey method is only reliable for the estimation of ascorbic acid in most plant tissues while the 2,4 dinitrophenyl hydrazine method is satisfactory for the determination of both ascorbic acid and dehydroascorbic acid.

In a further application of the Roe and Kuether method, Roe et al[29] determined diketo gulonic acid, dehydroascorbic acid and ascorbic acid in the same plant tissue extract. Their approach involved three steps, the first step consisting of the determination of the sum of the three compounds after oxidation while the second step comprised the determination of dehydroascorbic acid and diketogulonic acid. The third step consists of the determination of diketogulonic acid alone after the reduction of dehydroascorbic acid. Hydrogen sulphide and stannous chloride were used as the reducing agents. Schaffert and Kingsley [30] carried out an investigation of the Roe and Kuether total ascorbic acid method with a view to shorten the three hour incubation period and to improve the specificity of the measurement of L-ascorbic acid in the presence of diketogulonic acid. This method will be discussed in full in a later section of this work as it was the method adopted. They obtained excellent results by shortening the incubation period for fruits, vegetables and urine to 10 mins and five minutes for blood. Results of several analysis with both the modified and the original Roe and Kuether method compared very well. The accuracy was also confirmed by recovery experiments.

The application of the Roe and Kuether method for the analysis of materials which have a low ascorbic acid potency eg. chicken feeds have not met with considerable success because of the fact that the relative concentration of interfering substances might be quite high. Modifications which involve separation of intefering substances by column chromatography or thin layer chromatography or both have been reported.[31]

CHAPTER 2

ANALYSIS OF ASCORBIC ACID CONTENT OF PUMPKIN (CURCUBITASPP)

2.1 AIM and SCOPE:- The aim of this project is to determine the ascorbic acid contentof some local materials. From the foregoing account, it would appear that techniques and methods for determining ascorbic acid have been adequately well developed, and a number of workers have applied some of these for determining ascorbic acid content of some local materials. Oyenuga[32] has analysed for the mineral and vitamin content of many tropical foods and feeding stuffs including cassava, pawpaw, groundnut seeds, cocoyam etc. In his own work Oke[33] determined the ascorbic acid content of some Nigerian foodstuffs notably yam, yamflour, plantain before and after cooking to determine the extent of loss of the vitamin due to the method of preparation. He also determined the ascorbic acid content of Nigerian vegetables[34] and reported that they were rich sources of ascorbic acid but the method of their preparation into sauces may result in much of this vitamin being destroyed. This project has thus undertaken to carry out some prelimnary work on ascorbic acid content of the species of pumpkin available in this part of the country. The pumpkin serves as food in some parts of the Northern states of Nigeria. One fruit of the pumpkin is not normally consumed in a meal by an average sized family usually about six and thus the interest in studying the variation in the ascorbic acid content of the pumpkin for a period

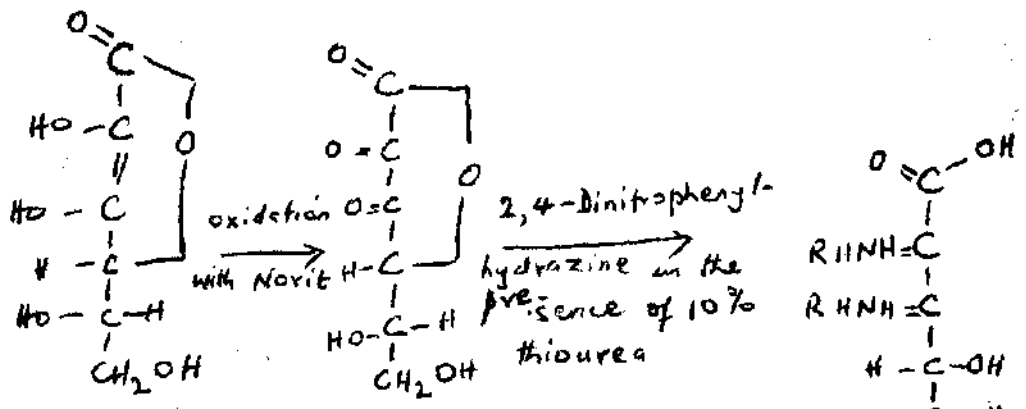
of one week. One week was chosen because most of the local population do not have storagefacilities and an average sized pumpkin about two kilogrammes or more in weight normally starts to decay at the end of seven days at roomtemperature.

In some parts of the northern states, the seeds are not included in the diet. It is the intention of this project to analyse the total ascorbic acid content of the seeds and see how it compared with the content of the generally accepted edible portion comprising the yellow fluffy portion minus the greenish yellow peeling which is also thrown away especially if the pumpkin is fully ripen. An analysis of the peeling would serve to give anindication of the gradient of ascorbic acid content in the three portions namely the peeling, the yellowfluffy portion and the seeds.

Part of the programme (although this was not done) was to study the relationship, if any between the state of maturity or ripeness of the pumpking and the ascorbic acid content in an attempt to establish a correlation between age and the vitamin C content. It was also thought that the leaves could be analysed for the ascorbic acid since some parts of the north depend on it as a source of vegetable for sauces..

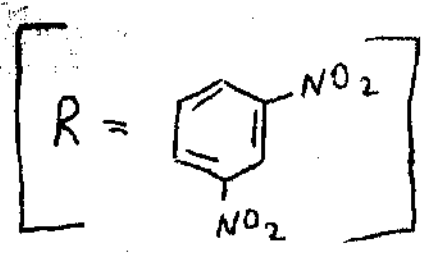
2.2 Materials and Methods

The determination of ascorbic acid and dehydroascorbic acid was done by the method of Schaffert and Kingsley.[30] In this method, the ascorbic acid is oxidised to dehydroascorbic acid. The dehyroascorbic acid is coupled with 2,4 dinitrophenly hydrazine and incubated at a temperature of 100°C for the coupling reaction to reach completion. The reaction reaches completion in 10 minutes. The insoluble red coloured osazone formed is dissolved in 85% sulphuric acid and the transmittance of the complex is measured at a wavelength of 515nm.



L-ascorbic acid

bis 2,4-dinitrophenyl hydrazone



Equipment

Mettler balance

Top loading balance

2 weighing bottles

Waterbath at 100°C

15 volumetric flasks ($100cm^3$)

2 volumetric flasks ($500cm^3$)

3 volumetric flasks ($1000cm^3$)

1 volumetric flask ($2000cm^3$)

2 Beakers $2000cm^3$

2 testube racks with 8 openings

Test tubes

Funnels

Erlenmeyer flasks

1 burrette graduated to $0.1cm^3$

Assorted volumetric pipettes 1,2,5,10,20,$25cm^3$

Filter paper Whatman No 42, 12.5cm diameter

Bausch and Lomb spectronic 20

Medicine dropper

Buchner funnel 7cm diameter

Buchner flask $500cm^3$

Kenwood MFG (Woking)Ltd.

Havant (Hants) blender Model A956A.

Reagents

(1) Metaphosphoric acid sticks (Laboratory reagent (BDH)

(2) Glacial Acetic acid (AnalaR BDH)

A 5% metaphosphoric acid - 10% acetic acid solution was prepared by dissolving 50 grams of the metaphosphoric acid in about 800cm^3 of distilled water. 100ml of glacial acetic acid was added and made up to 1000cm^3 with distilled water in a volumetric flask.

This solution was stored in the refrigerator and a fresh solution prepared weekly.

(3) Thiourea (Laboratory Reagent B.D.H.) 10% w/v solution of this reagent was made by dissolving 10 grams in 500cm^3 of absolute ethanol in a 100cm^{3}volumetric flask and diluting to the mark with distilled water.

(4) Concentrated Sulphuric Acid (AnalaR BDH). Specific gravity 1.84

A 4.5M solution was prepared by adding 300cm^3 of the concentrated acid to 900cm^3 of distilled water. 85% v/v Sulphuric acid was also prepared by adding 900cm^3 of the acid to 100cm^3 of distilled water.

(5) 2,4 dinitrophenyl hydrazine (AnalaR, B.D.H.) A 2% w/v solution was made by dissolving 2 grams of the reagent in 4.5M H_2So_4 and diluting to the 100cm^3 mark in a volumetric flask.

(6) Hydrochloric Acid (Laboratory Chemicals, M & B) Specific gravity 1.180. 10% v/v solution was prepared by adding 464cm^3 of the acid to distilled water in a beaker and colled. On cooling it was transferred to a 2000cm^3 volumetric flask and made up to the mark with distilled water.

(7) Acid - Washed Norit 200 grams of charcoal was put in a large flask and one cubic decimetre of 10% hydrochloric acid was added. The mixture was heated to boiling and then filtered with suction pump. The cake of norit was removed and put in a large beaker. The mixture was stirred with one cubic decimetre of distilled water and then filtered. The process was repeated until the filterate was free from Fe(III) ions.

(8) (L - ascorbic Acid (AnalaR B.D.H.)

(i) Experimental

(a) Preparation of Calibration Curve

The calibration curve was prepared with the same reagents and instrument that were used in the analysis. The method was described by Roe.(35)

100 mg of ascorbic acid reagent was dissolved in 100cm^3 of 5% metaphosphoric acid - 10% acetic acid mixture and made up to the mark in a volumetric flask with the same acid solution. The solution was then oxidised by adding 1 gram of norit per 50cm^3 of solution and shaken for about two minutes. 10cm^3 of the oxidised solution was then made up to 500cm^3 in a volumetric flask with the acetic acid - metaphosphoric acid solution. 1 cm^3 of solution contained 20 ug of dehydroascorbic acid.

A series of standard solutions were made by pipetting 5,10,20,30,50 and 60cm^3 of the diluted solution into 100cm^3 flasks. The final concentration of dehydroascorbic acid in the flasks were then 1,2,4,6,8,10, and

12 micrograms per cm^3 of solution.

$4cm^3$ each of the standard solutions was placed in three test tubes. One of the tubes served as a blank. $1cm^3$ of 2% 3,4 dinitrophenylhydrazine was added to two of the tubes followed by addition of one drop 10% thiourea to the three tubes. The tubes were incubated at a temperature of 100°C for ten minutes. At the end of the ten minutes, the tubes were placed in a large beaker of ice water containing generous quantities of ice blocks. $5cm^3$ of 85% sulphuric acid was added slowly to all the three tubes, a process that was accomplished within one minute for each tube. $1cm^3$ of 2,4 dinitrophenyldrazine solution was added to the blank tube. The three test tubes were shaken while still in the ice water, to ensure thorough mixing. The complex formed were then transferred to matched colorimeter tubes. The tubes were left on the rack for ten minutes.

(b) Measurement of colour

The Bausch and Lomb Spectronic 20 was switched on at least thirty minutes before the measurements were made to ensure that the instrument was stabilized. A wavelength of 515nm was selected and the instrument set to 0% transmittance. With the blank tube in place, the instrument was set to read 100% transmittance. The percent transmittance of the standard solutions were then read. A plot of percent transmittance against concentration of dehydroascorbic acid in micrograms per cm^3 was constructed on semilogarithmic paper. The curve is shown in figure 1. The transmittance data for the seven standard concentrations are tabulated in appendix I.

Determination of Ascorbic acid in pumpkin-
Calibration curve using a Bausch and Lomb Spectronic 20
at 515nm.

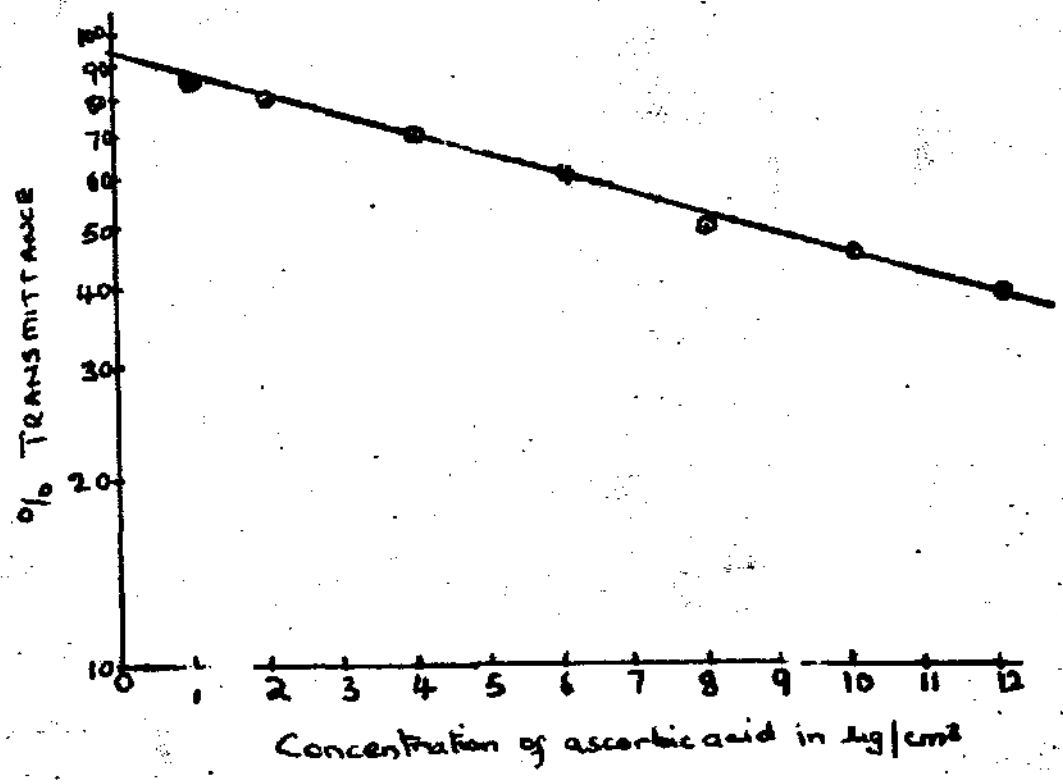


Fig. 1

(ii) Determinations

(a) Extraction

In order to extract the ascorbic acid into solution, several extracting agents are recommended. Oxalic acid, metaphosphoric acid trichloroacetic acid, acetic acid are among the extracting agents used. Ponting[36] studied the stability of ascorbic acid in extracts prepared from plant tissues by thirteen different acids. Oxalic acid and metaphosphoric acid were found to be far superior to other acids and about equal in their capacity to preserve ascorbic acid. Metaphosphoric acid, although far more expensive than oxalic acid, is a protein precipitant and thus has a more universal applicability than oxalic acid. It is also preffered to oxalic acid because of its effectiveness in protecting ascorbic acid against catalytic oxidation by iron. In this investigation, a mixture of metaphosphoric acid and acetic acid was used because ascorbic acid is not oxidised quantitatively by norit unless the extracting solution contains acetic or trichloroacetic acid, which are preferentially adsorbed on the Norit, eluting active oxygen in quantities that are sufficient for rapid oxidation.[37]

A representative sample of the tissue was weighed carefully and macerated in a Kenwood blender for about three minutes. Through out the work, between twenty and twenty five grams of the sample was blended in the presence of 900cm^3 of the extracting agents, for the edible portion.

This assured adilution ratio of not less than 1:30.[38] Although smaller weights were taken in the case of the seeds and peelings, the volume of extracting agent used, was calculated accordingly to give a dilution ratio within the limits stated above.

At the end of the extraction, one aliquot of the extract was shaken vigorously with Norit, about 1gram per 50cm^3 of extract, and then filtered through a fluted Whatman No 42 filter paper.

(b) Treatment of filterate 4cm^3 each of the filterate were placed in three test tubes and treated exactly in the same way as described in sections (i(a)) and i(b)) of this chapter.

Three parallel determinations were run from each extract for all portions of the pumpkin analysed. For each analysis not more than twenty seven test tubes were run simultaneously.

The above procedure refers to the determination of total ascorbic acid. The steps are essentially the same for the determination of dehydroascorbic acid except for the omission of treating the extract with norit.

In the second method the instrument constant K was calculated with the aid of the data obtained for the calibration curve (appendix1) and the Beer-Lambert equation (ii)

$$2 - \log T = \epsilon cl \quad \text{(ii)}$$

where

T = % transmittance

ϵ = extinction coefficient

l = path length

If K is ϵl,

therefore equation(ii) becomes

$$2 - \log T = KC \text{.....(iii)}$$

$$K = \frac{2 - \log T}{C} \text{.....(iv)}$$

With (iv), K is calculated for each standard solution, the values of which are shown in appendix II.

Substituting for K and T in (iv) the concentration C in ug per cm^3 can be calculated for all the unknown samples. This value is then substituted in equation(i) to obtain the amount of ascorbic acid in mg/100g in the sample.

Results of both methods were in good agreement.

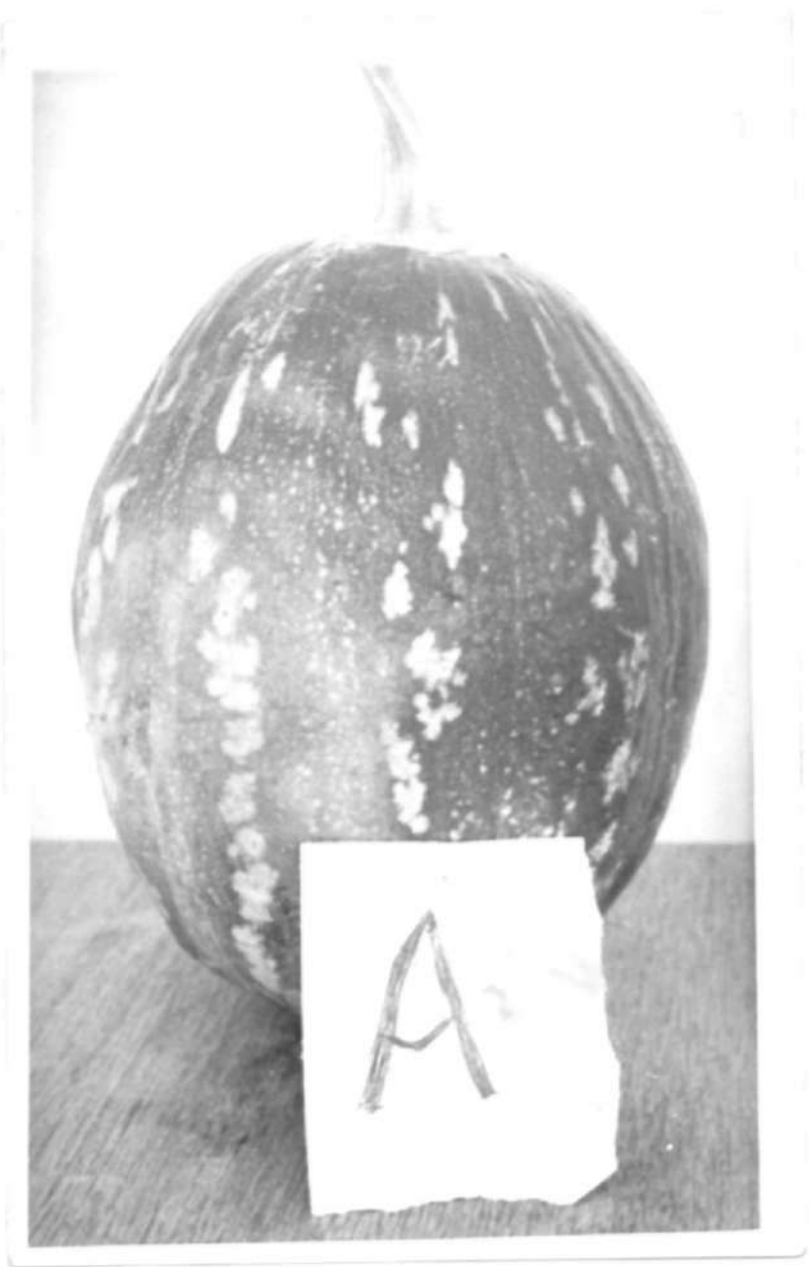
(d) Identification of the Pumpkins The photographs of the pumpkin were taken and later identified as follows by Egharevba[39]

Specimen	Botanical name
A	Curcubita moschata
B	Curcubita melo-pepo
C	Curcubita maxima (squash gourd)
D	Curcubita pepo (vegetable marrow)
E	Benincasa cerifera var ovifera.

Prelimnary determinations were carried out on specimens A,B,C, while specimens D and E were used for the actual work. Analysis was started on both specimens on the day they were harvested.

The results for A, B and C are tabulated in Appendix III,IV and V

PLATE A



Curcubita moschata

PLATE B

Curcubita melo-pepo

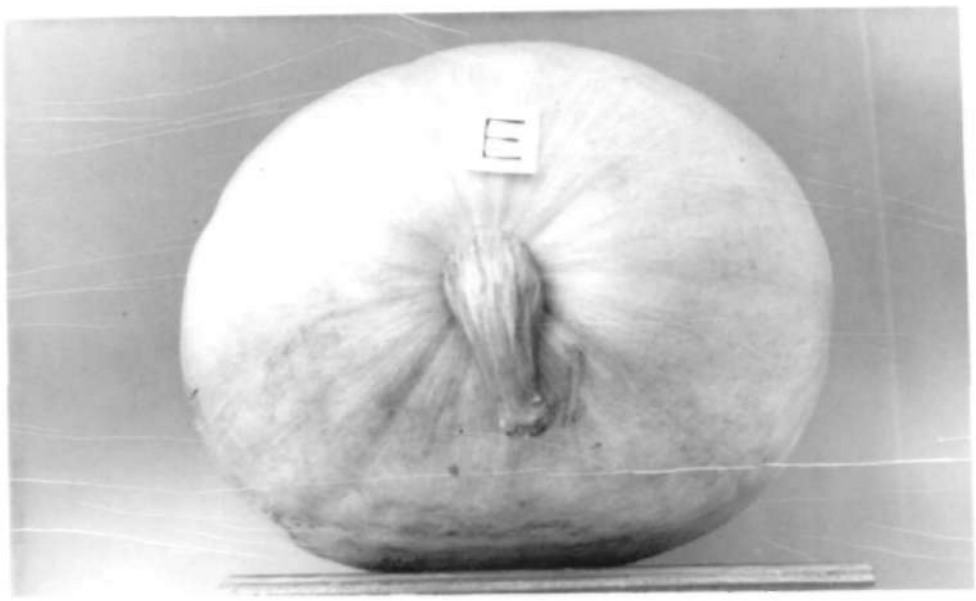
PLATE C

Curcubita maxima

PLATE D

Curcubita pepo

PLATE E



Benincasa cerifera var ovifera

2.3 Results and Discussion

The results in tables I - V represent the values of total ascorbic acid and dehydroascorbic acid in the two species of pumpkin studied. The difference of these two values gives the amount of L - ascorbic acid at the time of analysis. Each value represents a mean of three determinations on the same slurry. The triplicate values were in good agreement.

The reliability of the results can be discussed from the point of view of (a) specificity of the method (b) presence of interfering substances and (c) accuracy of the method as determined by recovery experiments. These are discussed in the next chapter.

Table I shows that the total ascorbic acid content remained constant while the amount of dehydroascorbic acid increased from 37.8% on the first day of analysis to 66.9% on the third day. Similar trends were also observed in Table III where the total ascorbic acid remained fairly constant and the dehydroascorbic acid values increased from 38.3% to 64.7% on the third day. In both cases the L-ascorbic acid values decreases markedly until the third day. The fact that the amount of dehydroascorbic acid on the first day of analysis are quite close in both species is significant but more work is still required to be able to establish any correlation between species and the L-ascorbic acid or dehydroascorbic acid content.

The results of the total ascorbic acid for specimen D agree with literature values[40] where a range of 6 - 15mg/100g, for analysis of sixteen samples of the same species was reported.

Tables II and IV show the mean values for the total ascorbic acid, dehydroascorbic acid as well as the L-ascorbic acid content of the seeds in both species of the pumpking. When both tables are compared with the corressponding tables of values for the edible portion, it is found that the seeds contain more of the antiscorbutic material than the edible portion.

Comparing the mean values of the peeling shown in table V with the L-ascorbic acid value shown in the first horizontal line of Table III, it would appear that the edible portion contains more of the L-ascorbic acid than the peeling, although there is no significant difference in the values for the total ascorbic acid content.

CONCLUSION Although the values obtained represent a fair amount of ascorbic acid in the edible portion and a high amount in the seeds, the results represent the L-ascorbic acid and dehydroascorbic acid content in the raw state. A substantial amount of ascorbic acid might be lost as a result of cooking.[34] The effect of cooking on the amount of the vitamin in the pumpkin was not investigated.

It can be said that analysis was carried out on one sample of each species, however, it appears that the total ascorbic acid content of the edible portion remains constant while the amounts of dehydro-ascorbic acid increases rapidly in three days. During this period also the L-ascorbic acid content decreases. This does not affect the antiscorbutic potency of the pumpkin since dehydroascorbic acid has about the same potency as L-ascorbic acid.[41]

The values for both the total and dehydroascorbic acid content may well include diketogulonic acid. The determination of each of the three components of vitamin C was not carried out.

Suggestions for further work:

More analysis is still required in terms of both number of samples and individual species to establish the variation in the L-ascorbic acid content outlined above. As a follow up, further work can be done to study the relationship between state of maturity and the vitamin C content within the same species. Since the pumpkin is a seasonal crop, it would also be interesting to study the variation of the ascorbic acid content with season. This will have to be an extended investigation as the pumpkin is most abundant during the rains, May to September of each year. The ascorbic acid content of the leaves can also be analysed since it is used as a source of vegetable for sauces.

T A B L E I

Variation in the daily ascorbic acid content of the edible portion of Pumpkin D (Curcubita pepo).

Day	Total AsA[a] mg/100g	Dehydro AsA[a] mg/100g	Dehydro AsA / Total x100	Ascorbic Acid mg/100g
1st	14.3	5.4	37.8	8.9
2nd	13.8	7.5	54.4	6.1
3rd	15.4	10.3	66.9	5.0
4th	13.2	6.0	45.1	7.3

[a] Total AsA = Total Ascorbic acid

Dehydro AsA = Dehydro Ascorbic Acid.

T A B L E I I

Analysis of Ascorbic Acid in the seeds of Pumpkin D (Curcubita pepo).

Determination	Total AsA[a] mg/100g	Dehydro AsA[a] mg/100g	Ascorbic acid ~~Difference~~ mg/100g
1st	24.9	10.2	14.7
2nd	23.6	10.6	13.0
3rd	26.8	12.8	14.0
4th	24.7	13.8	10.9
Mean	25.0	11.9	13.2

[a] Total AsA = Total ascorbic acid

[b] Dehydro ~~AsA~~ AA = Dehydroascorbic acid.

T A B L E I I I

Variation in the daily ascorbic acid content of the edible portion of Pumpkin II (Benincasa cerifera var ovifera)

Day	Total AsA[a] mg/100g	Dehydro AsA[a] mg/100g	$\frac{\text{Dehydro AsA}}{\text{Total}} \times 100$	Ascorbic acid ~~Difference~~ mg/100g
1st	18.3	7.0	38.3	11.3
2nd	17.6	6.9	39.2	10.7
3rd	18.1	9.2	50.8	8.9
4th	18.6	12.1	64.7	6.5

[a] Total AsA = Total Ascorbic Acid

Dehydro AsA = Dehydroascorbic acid.

T A B L E I V

Analysis of the ascorbic Acid content of the seeds of pumpkin E (Beninc sa cerifera var ovifera)

Determination	Total AsA[a] mg/100g	Dehydro AsA[a] mg/100g	Ascorbic Acid Difference mg/100g
1st	23.7	10.5	13.2
2nd	24.0	15.3	8.9
3rd	19.1	9.8	9.3
Mean	22.3	11.9	10.5

[a]Total AsA = Total ascorbic acid

Dehydro AsA = Dehydroascorbic acid.

T A B L E V

Analysis of the ascorbic acid content of the peelings (2mm thick) of pumpkin E (Benincasa cerifera var oblifera).

Determination	Total AsA[a] mg/100g	Dehydro AsA[a] mg/100g	Ascorbic Acid ~~Difference~~ mg/100g
1st	18.6	9.7	8.9
2nd	15.0	7.9	7.1
3rd	18.1	10.8	7.3
Mean	17.2	9.5	7.7

[a] Total AsA = Total ascorbic acid

Dehydro AsA = Dehydroascorbic acid.

CHAPTER 3

ANALYSIS OF ASCORBIC ACID CONTENT OF THE BLOOD OF GUINEA FOWL

3.1 AIM AND BACKROUND:- The aim of this investigation is to compare the effect on weight and whole blood ascorbic acid content of the guinea fowl fed on a commercial feed and a local feed.

It has been generally accepted that birds synthesize their own ascorbic acid and as such many feeds manufacturers have not been including the vitamin in their preparations. Analyses of the vitamin C content of a commercial livestock growers mash from Pfizer and also the ascorbic acid content of the guinea corn bought from Samaru market, on which local poultry farmers depend as a source of food for their livestock, were made.

Hart, Stenbock Halpin and Lepkovsky[42] found out in their work that scurvy does not develop in chicks fed ration of grains and heated skimmilk, presumably free or at least verylow in vitamin C content. However, their livers contained a lot of vitamin C as guinea pigs were cured of scurvy when fed with liver of these chicks at a level of 3 grams daily. They obtained, similar results when the ration consisted of purified food materials - dextrin, casein salts etc. Their conclusion was as follows "Apparently the chicken like the rat does not need vitamin C preformed in the diet in the same sense as does the guinea pig but its presence in large amounts in the livers of chickens even on synthetic diets, indicates that it plays some role

in its metabolism. Whether the chicken synthesizes this vitamin from aninactive precursor or some other organic complex cannot at present be stated".

Carrick and Hauge[43] gave as much as 20cm^3 of egg white or 15cm^3 of yolk daily and found that it did not cure or prevent the occurrence of scurvy in guinea pigs being fed with diets which were deficient in the antiscorbutic vitamin. Chicks however grew normally on scorbutic rations and the livers and kidneys contained abundant supplies of the antiscorbutic substance. They thus interpreted this to indicate that the chick requires antiscorbutic vitamin within its own body.

During the course of 1950-51 Australian National Antartic Research Expedition to Macquarié Island, a study was made of levels of ascorbic acid in the plasma of members of the expedition. At the sametime the oppurtunity wes taken of determining the ascorbic acid levels in plasma of a number of birds and mammals from the island.

Kalnenas[44] found that the birds have approximately, twice the ascorbic acid of mammals including man. It was concluded that probably the higher plasma ascorbic acid levels of birds is related to the higher metabolism of avian tissues compared with those of mammals, because ascorbic acid is an indispensable hydrogen carrier in the metabolism of animals.

Despite these claims one still feels that other additives to the diet might contain enough ascorbic acid to cater for the metabolic need and dietary need, if any, of birds.

Recent research emphasis has been to monitor the changes in ascorbic acid levels in certain tissues and organs as an indicator of response to some kind of pathological stress.

Bell, Satterfield, Cook[45] in an experiment to ascertain the influence of injected ascorbic acid, on the ascorbic acid content of blood, eggs, faeces and certain organs of laying hens, observed that hens fed with ascorbic acid free diet developed a muscular weakness and loss of action of one or both legs, anappetence. Somnolence, inanition with loss of weight, definite faecal changes and depression of the reproductive system. There was dramatic recovery of the hens on injection of ascorbic acid. Their egg production also increased. These observations gave the indication that certain adverse conditions of nutritional and or pathological stress may cause the need for an external source of vitamin C by chickens.

Briggs, Lucky, Elvehjem and Hart[39] investigated the growth promoting activity of ascorbic acid when added to certain highly purified chick rations and reported increased growth in chicks when ascorbic acid was added to purified diets. They arrived at the conclusion that the vitamin may not be synthesized fast enough by the chicks on highly purified ration, such as has been used in their experiments, to give maximum rate of growth. From the data it was difficult to determine just how ascorbic acid stimulated growth of chicks and they postulated that it might act by means of a true vitamin mechanism.

A second possibility they considered was that ascorbic acid stimulates growth by an indirect action. That is, it may act to alter the conditions within the intestinal tract causing a more effective utilization of neccessary nutrients or causing greater synthesis of unknown vitamins by intestinal organisms.

It was not possible that ascorbic acid was acting as a detoxifying agent because even when amounts of solubulized liver or any of the crystalline vitamin were doubled the growth rate was not suppressed. The solubilized liver supplied the vitamin B(6) Vitamin B_1 and folic acid which are lacking in the basal ration used.

March and Biely[47] also investigated the effect of ascorbic acid on the growth rate of chicks. Chicks were given diets of natural ingredients, adequate or low in folic acid, with or without added ascorbic acid. Some chicks were given antibiotics or - P - aminobenzoic acid or both. They found that when the basal diet was low in folic acid, ascorbic acid increased growth, though to a less extent than folic acid. It appeared that the growth stimulating effect of ascorbic acid depended on the composition of the diet. When the diet contained no added fat or when herring oil was included a slight response to ascorbic acid was observed. On the other hand, there was greater response when cotton seed oil was included. Penicillin when added to diets adequate or low in folic acid increased growth. A combination of penicillin, P-aminobenzoic acid and ascorbic acid either separately or together was found to have stimulated growth more than any of the supplements alone.

When on a diet which was considered to be adequate, aureomycin was added, alone or with p-aminobenzoic acid, there was no increase in growth rate, but when aureomycin was given with ascorbic acid growth was slightly but consistently increased.

Ascorbic acid reductions in blood and other tissues have been reported in many animals infected with protozoa. Satterfield, Moseley, Ganger, Holmes, Tripp(48) attempted to correlate avian diseases with ascorbic acid content of the blood. They reported no consitent variation in the ascorbic acid content of blood plasma, either to type of disease, nutrition, age, or sex from that of similar birds not so afflicted.

Hill and Garren(49) reported that plasma ascorbic acid values of chicks with fowl typhoid dropped from 4.56ug/ml to 1.7ug/ml and that supplementation of the diet with 0.1% ascorbic acid resulted in increased plasma ascorbic acid levels, through out the experimental period.

Josephson, Taylor, Greenberg, Nadel(50) in their study of ascorbic acid content of the adrenal glands of chicks infected with plasmodium gallinaceum, reported no change in the adrenal vitamin C content of the infected chickens. However, Chalfey(51) in his investigation of the effect of cecal coccidiosis infections and experimental haemorrhage upon the adrenal ascorbic acid levels in the chicken reported a marked change in the adrenal ascorbic acid content of growing chickens during the course of Eimeria tenella infection. He reported a slight but statiscally significant decrease on the third day of infection followed

by a pronounced increase reaching a maximum at the end of the fifth day. Then he observed a decreasing trend which ~~neared~~ approximated to the controls towards the eighth day. However, this observation of elevation of adrenal ascorbic acid during the period of maximum stress was unexpected in the light of previous observation, of depletion as a characteristic response to stress in mammals and a general lack of response which has been found in birds as reported by Josephson et al.(50)

The observation that experimental haemorrhage brings about adrenal ascorbic acid elevation indicates that the observed changes in infected birds may be due to blood loss and not necessarily due to toxic effects of the parasite and that hemorrhage may not be an appropiate non specific stress. It is possible that the blood loss or one of its attendant effects namely anoxia, hemodilution, hemopoetic stimulation has a specific effect upon adrenal ascorbic acid metabolism, which appears not to be the case with the rat where there is typical adrenal ascorbic acid depletion in response to massive hemorrhage.Long(52)

It would appear therefore that the net gain in ascorbic acid observed may be due to repletion occurring simultaneously and at a greater rate than the usual stress induced depletion. If this was the case, then it could be concluded that ascorbic acid was playing a fundamentally identical role in situations where either depletion or elevation is the net effect of stress.

3.2 Materials and Methods

(a) Whole blood

The determination of total ascorbic acid in whole blood was carried out by the method of Schaffert and Kingsley,[30] the principle of which was discussed in chapter 2.2. The difference, however, is in the period of incubation. In determinations involving whole blood, the norit filterate is incubated for five minutes while the norit filterate for vegetables and fruits are incubated at the same temperature ($100^{o}C$) but for ten minutes.

Equipment

In addition to the equipments listed in Chapter 2.2 with the exception of the Kenwood blender, the following materials were used.

(1) Cotton wool

(2) Disposable Terumo syringes and needles with the following dimersions 216x1½ and 0.80x38mm.

Reagents:-

The following reagents were used in this determination in addition to those listed previously.

(1) Trichloroacetic Acid (AnalaR, B.D.H.). 6% w/v solution was made by dissolving 60 grams of trichloroacetic acid in distilled water and making up to $1000cm^3$ in a volumetric flask.

(2) Potassium Oxalate (AnalaR, B.D.H). 10%w/v solution was made by dissolving 10 grams in distilled water and making up to the mark in a $100cm^3$volumetric flask.

EXPERIMENTAL

(i) Preparation of Calibration Curve.

The calibration curve was prepared with the same reagents and instrument that were going to be used in the analysis. The method described by Roe[35] was adopted with slight modifications. The original method as described by the author involved the use of $4cm^3$ of the norit filterate and a period of incubation of three hours at 37^oC, $3cm^3$ of the norit filterate and ten minutes period of incubation at 100^oC was used.

The percent transmittance of the standard solutions versus concentration of dehydroascorbic acid in microgram per cm^3 was plotted on semilogarithmic paper. The curve is shown in figure 2. The data for the transmittance values are tabulated in appendix VI

Determinations.

Before the commencement of the determinations, the twenty birds purported to be growers were not fed for twenty four hours and randomly divided into two groups of ten each. Each bird was given an identification mark. These marks ranged from 1-20. The birds in the group 1-10 were being fed with the local guinea corn while those that bore identification marks 11-20 were fed with the commercial growers marsh from Pfizer Livestock feeds Ltd.

At the onset of the experiment all the birds were weighed and the ascorbic acid content of the blood was analysed. Subsequently, all birds were weighed once a week and ascorbic acid content of the blood, for a set of five in each group, was determined daily for five weeks.

Method of Bleeding: To obtain $2cm^3$ of blood which was used for the analysis, the neck of each bird being bled on a particular day, was first sterilized with cotton wool socked in ethly alcohol. $2cm^3$ of blood was withdrawn from the prominent jugular vein[53] with a syringe

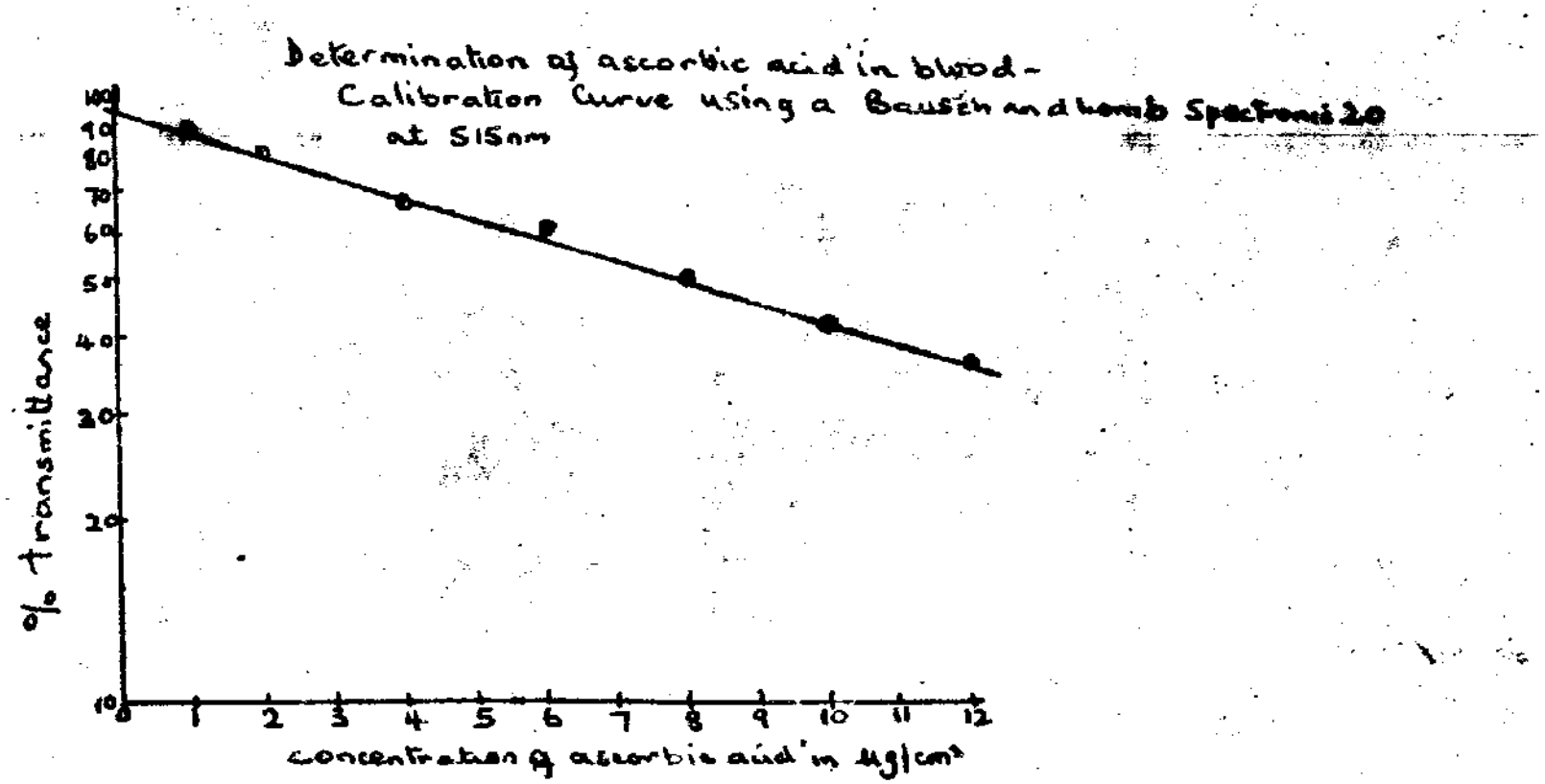

Determination of ascorbic acid in blood -
Calibration Curve using a Bausch and Lomb Spectronic 20
at 515nm
% transmittance
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
concentration of ascorbic acid in µg/cm³

Fig. 2

previously flushed with 10% potassuim oxalate solution.(54) This flushing was done to prevent the clothing of the blood in the needle during the process of withdrawing the blood sample.

Treatment of blood sample

The 2cm^3 of blood samples in the syringes were immediately emptied into 50cm^3 Erlenmeyer flasks containing 10cm^3 of 6% trichloroacetic acid which was used as the extracting agent. Trichloroacetic acid in addition to being an extracting agent is also a protein precipitant. The flasks were shaken vigorously and left for five minutes for extraction to take place. Approximately 0.5g of norit was added to each flask and shaken for about one minute. The mixture was then filtered through a fluted 12.5cm Whatman No42 filter paper.

Development of Colour. 3cm^3 of the filtrates obtained above were withdrawn into each of two tubes. One test tube served as a blank. 1cm^3 2% 2,4 dinitrophenyl hydrazine was added to the sample test tubes and a drop of 10% thiourea was added to both the blank and the sample test tubes. The sample tubes including the blank were then incubated at a temperature of 100°C for five minutes exactly. At the end of this time all the tubes were placed in ice water containing generous quantities of ice. 5cm^3 of 85% sulphuric acid was added slowly to each tube from a 50cm^3 burette and 1cm^3 of the 2, 4 dinitrophenylhydrazine was added to the blank tubes. Finally, all the test tubes were removed from the icebath after thorough shaking and left on the rack for 10 minutes. Before the transmittance values were read at a wavelength of 515nm, the osazone formed were transferred to matched colorimeter tubes.

One colorimeter tube was used for the blank while another was used for the samples throughout the period of the analysis.

The analysis was carried out simultaneously for all the samples, that is ten samples were analysed daily. The amount of ascorbic acid was then read off from the calibration curve previously prepared.

Calculations

In calculating the total ascorbic acid content of the blood per $100cm^3$ two methods were adopted. Firstly, amount of ascorbic acid per cm^3 was read off from the calibration curve and then substituted in the following equation:

$$\frac{\text{ug per } cm^3 \text{ ascorbic acid from curve} \times \text{Vol. of Filterate} \times 100}{1000 \times \text{volume of sample}} \quad \ldots\ldots(iii)$$

Secondly, the instrument constant K was calculated with the aid of the data obtained for standard solutions of ascorbic acid and the Beer - Lambert equation(ii)

The instrument constant K takes into account the path length of the colorimeter tubes, the particular instrument used, the extinction coefficient and the reagents used.

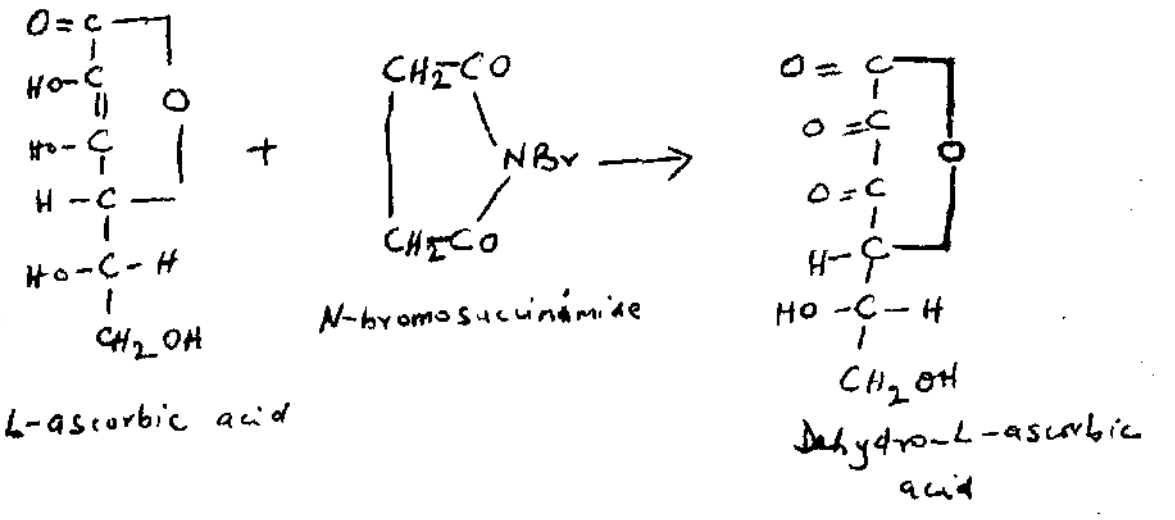
The values for the instrument constant are tabulated in Table Appendix VII

The results obtained from these two methods were in good agreement.

(b) FEEDS

The ascorbic acid contents of the local guinea corn from S maru market and the commercial growers mash concentrate were determine by

the N-bromosuccinimide method of Evered.[18] This method depends on the oxidation of ascorbic acid by N-bromosuccinimide. The ascorbic acid solution is titrated with N-bromosuccinimide and the excess N-bromosuccinimide is detected by the liberation of iodine from potassuim iodide to an upper organic layer.



Equipment and Apparatus

1. 100cm^3 volumetric flasks
2. 25 x 150mm test tubes
3. Assorted pipettes
4. 10cm^3 microburette
5. Test tube racks
6. Filter papers Whatman No 1 25.0cm diameter.

Reagents

1. N-bromosuccinimide:- Laboratory Reagent, BDH A stock solution of this reagent was prepared by dissolving 200mg of the reagent in warm water, cooling and diluting to 100cm^3. The solution was stored in the refrigerator for two days. Before the stock solution was used, it was diluted 1cm^3 to 100cm^3 with water. One cm^3 of this solution is approximately equivalent to 0.02mg of ascorbic acid.

2. Standard Ascorbic Acid:- (AnalaR B.D.H.) A fresh solution of this was prepared by dissolving 200mg in 100cm^3 of 1%v/v acetic acid.
3. Glacial Acetic Acid:- (AnalaR BDH.) 1% v/v solution prepared by diluting 10cm^3 of the acid with distilled water in a 1000cm^{3}volumetric flask.
4. Potassium Iodide((AnalaR, B.P.H.)- A 1% w/v solution was prepared by dissolving 1grams in water and making up to the mark in a 100cm^3 volumetric flask.
5. Diethylether (AnalaR B.D.H.)

Sampling:- In order to obtain a representative sample of the feed in a whole bag of the commercial livestock feed, the quartering technique was applied. The contents of a whole bag were poured on a large cloth, in this case a torn empty bag, and mixed by rolling. One corner of the cloth was pulled over the top of the material in the first instance and then the process was repeated with the other corner. As a result of this, the material was worked up to the centre. These processes were repeated until thoroughmixing was attained. The pile of material was then flattened and divided into quarters. Two opposite quarters were discarded and the remaining two were mixed by rolling on the cloth. The quartering and mixing processes were repeated until the quantity of the material was reduced to a small size. A small amount of the material was then weighed for analysis.

For the guinea corn, the final quantity obtained after quartering and mixing was ground to fine powder using a pestle and mortar before a small amount was weighed for analysis.

Procedure:- Two determinations were carried out for each of the feeds. In each case about 5grams of the material obtained from the sampling technique was transferred into a 100cm^3 volumetric flask with 1%v/v aqueous acetic acid and diluted to the mark with the same reagent. The mixture was shaken in a mechanical agitator for 30 minutes to extract the ascorbic acid into the solution, and then filtered through a fluted Whatman No 1 filter paper.

5cm^3 of the extract was transferred to a 25 x 150mm test tube, 1cm^3 of the glacial acetic acid and 5cm^3 of the potassium iodide solution were added with mixing after each addition. 3cm^3 of diethylether was added and the mixture titrated with N-bromosuccinimide solution added from a 10cm^3 microburette. The test tube was shakened vigorously after each addition of the titrant and the organic solvent layer allowed to separate. The end point was indicated by the first appearance of the brown colour of liberated iodine in the upper ether layer. In order to establish the end point, comparison against an untitrated "dummy" mixiture was made. The titration was done in triplicate.

Another titration, a blank was carried out using 5cm^3 of water. The blank titration gave the volume of N-bromosuccinimide solution necessary to impart a definite brown colour to the ether layer.

The N-bromosuccinimide was standardized using the same procedure by titrating against $5cm^3$ aliquots of the standard ascorbic acid solution.

ABBREVIATIONS

AnalaR - Analytical Reagent.

B.D.H. - British Drug Houses, Laboratory Chemicals Division.

M & B - May and Baker.

3.3 RESULTS AND DISCUSSION

Although literature values giving the normal range of either the total or reduced ascorbic acid content of the blood of the guinea fowl are not immediately available, the reliability of the results in Tables VII and VIII can be evaluated from the point of view of (a) the specificity of the method (b) the presence of interfering substances (c) accuracy of the method as determined by recovery experiments.

SPECIFICTY Roe and Kuether[26] have demonstrated the specificity of the method by analysing the blood and urine of guinea pigs with acute scurvy. They obtained whole blood values ranging from 0.04mg to 0.13mg per $100cm^3$ and urine values of 1.0 to 3.0mg per cubic decimetre. In one urine sample, which they collected post mortem from the bladder of a guinea pig, that died of scurvy, completely negative values were obtained. They argued that in as much as complete disappearance of vitamin C may not be expected from red cells, white cells and platelets of the blood or from the urine of guinea pigs at the onset of acute symptons of scurvy, their findings could be interpreted as good evidence of the specificity of the method.

Interferences Substances likely to interfere in the determination of ascorbic acid content of whole blood are pyruvic acid and acetoacetic acid as they readily couple with 2,4 dinitrophenylhydrazine. Their derivatives, however, do not react with sulphuric acid under the conditions of the experiment and besides sulphuric acid solutions of

these derivatives do not absorb at the 540nm region in which the colour obtained with the ascorbic acid derivative is read. Roe and Kuether[26] have also shown the absorption spectra of sulphuric acid solutions of derivatives of pentoses, hexoses and glucoronic acid which are also likely to be present in the blood. They concluded that the concentration at which these substances yield interference are greater than those found in blood and urine filterates used. The possibility of interference therefore becomes less likely in whole blood filterates of dilution 1:6 adopted in this project when compared with 1:4 used by Roe and his worker.

Recovery Experiments:- Although the accuracy and precision of the method as evaluated by recovery experiments was not carried out, Roe and Kuether[26] obtained recoveries ranging from 96 to 104 percent, showing that the precision of the method is excellent. In an analysis compri ing ten determinations from the same filterate, by Roe and Kuether[26], the mean value obtained was 0.7mg per $100cm^3$, the standard deviation was ±0.01 and the probable maximum error of a single determination was ± 0.007 and the maximum deviation was 0.04mg per $100cm^3$.

It could be argued that the above discussion cannot apply because the Schaffert and Kingsley[30] modification was adopted. However, there is no difference in terms of reagents except for the fact that the incubation period was (10 minutes) shorter but at a higher temperature of 100°C when compared with 37°C and three hours adopted by Roe and Kuether[26].

In fact a comparison of the modified method with the method of Roe and Kuether[26] was carried out on whole blood by Schaffert and Kingsley[30] and they reported good agreement by the two methods.

The results for the analysis of the feeds are tabulated in Table VI. The results indicate that the guinea corn contains more ascorbic acid than the Pfizer Livestock feeds. In this analysis, two separate determinations comprising three titrations each were carried out by the N-bromosuccinimide method of Evered[18]. The standard deviation of the titre values in each set of triplicate titrations, in the analysis of the guinea corn, was 0.03cm^3. While the standard deviation for the titre values, in the analysis of the Pfizer Livestock feeds was 0.02cm^3 and 0.01cm^3 for the first and second determinations. The accuracy and precision of the method has also been verified by recovery experiments where Evered[18] obtained 100 percent recovery of ascorbic acid added to black-currant juices.

A careful study of Tables VII and VIII reveals that it is difficult to say in what way each type of feed has contributed to the ascorbic acid content of the whole blood of the guinea fowls in each of the two groups, as the analysis show a fairly constant value of total vitamin C content.

Tables IX and X show the weekly weights of the birds in both groups. The first column of each table shows the initial weight of the birds. In both tables the differences in weight are not significant

enough to justify that there is either an increase or decrease in weight of the birds. It appears that the birds appear fully grown and cannot grow further. Comparing Tables VII, VIII, IX and X it is difficult to correlate the weight changes with the ascorbic acid content of the whole blood. However, it appears that there is an increase in the average wieght of the birds fed on the guinea corn over the average weights of the birds fed on the Pfizer livestock feeds. This could as well be correlated with the presence of observed higher content of ascorbic acid in the guinea corn when compared with the Pfizer feeds. More work is required to establish any possible correlation.

Conclusion From the available evidence the total ascorbic acid in the blood could be said to represent the tissue concentration, since ascorbic acid is water soluble. It is, however, possible and likely that some tissues may contain higher concentration than is found in the blood. It is difficult to conclude from this investigation that this concentration level is a result of direct intake from the feeds or through synthesis of the vitamin by the bird. However, since there is little difference in the total ascorbic acid content as shown in tables VII and VIII one could conclude rather cautiously that the bird synthesizes its own ascorbic acid to maintain its concentration level requirement. On the other hand, it could be argued that the amount of ascorbic acid present in the feeds are too low, one would have to accept the claim that birds do synthesize vitamin C to meet their requirements, since no symptons of scurvy were observed in the birds through out the experimental period.

The observation that there is a slight increase in average weight of the birds fed on guinea corn over those fed on the commercial feed, should be attributed to the higher ascorbic acid content of the former, with some caution until other growth promoting factors are fully investigated and taken into account.

It should be noted that the value of the vitamin C obtained for the guinea corn represents that used in this experimet as there is no available information as to when it was harvested. This value will definitely bedifferent, if it were analysed fresh.

This investigation has further confirmed the claim that commercial livestock feeds have a low vitamin C content.

Suggestions for further work

Although it has been established and reported in literature that birds synthesize their own ascorbic acid, it isstill necessary to ascertain that the guinea fowl does, and as such more specific investigations are required. One of such investigations could be to analyse for the antiscorbutic vitamin in various tissues of guinea fowl fed on scorbutic diets. Any of the tissues that contain the vitamin can then be used to study its effect on scurvy patients.

Once this fact is conculsively proved, a more precise relationship between dietary intake of ascorbic acid and tissue store can be carried out by increasing the quantity of the vitamin in the diet and monitoring it by analysis of either the whole blood or the liver.

Younger birds can be used to obtain more precise information about the effect of added vitamin in diets on rate of growth and egg production.

T A B L E V I

Ascorbic Acid Content of Guinea Corn and Livestock Feeds in mg/100g.

No. of Analysis	Ascorbic acid content of guinea corn, g/100g	Ascorbic acid content of pfizer feeds in mg/100g
1st	6.29 ± 0.38	4.64 ± 0.33
2nd	5.32 ± 0.49	4.01 ± 0.32
Mean	5.81	4.33

TABLE VII

Total Ascorbic acid mg per $100cm^3$ of blood analysed over a period of six weeks.

These birds were fed on the guinea corn.

Identification Mark	WEEK NO.													
	1	2		3			4		5				6	
1	2.9	-	3.6	-	3.3	-	-	1.7	-	1.8	-	2.1	-	2.0
2	2.9	3.4	-	2.9	-	2.3	2.7	-	2.0	-	2.1	-	1.6	-
3	1.9	2.3	-	-	3.3	-	-	1.5	-	1.9	-	1.5	-	1.9
4	2.0	4.8	-	-	3.7	-	-	2.2	-	2.4	-	2.1	-	2.4
5	2.5	3.4	-	2.7	-	3.1	2.4	-	2.4	-	2.3	-	2.6	-
6	3.0	-	4.1	3.1	-	2.8	2.8	-	3.0	-	2.3	-	2.7	-
7	3.0	-	3.1	-	3.0	-	-	2.3	-	2.2	-	2.2	-	1.9
8	2.1	2.9	-	2.0	-	2.7	2.1	-	2.0	-	2.1	-	1.9	-
9	2.3	-	2.9	1.9	-	2.0	2.5	-	2.3	-	2.2	-	2.3	-
10	2.8	-	3.3	-	3.3	-	-	2.6	-	2.4	-	2.4	-	2.1
* MEAN	2.5	3.4	3.4	2.5	3.3	2.6	2.5	2.1	2.7	2.1	2.3	2.1	2.2	2.1

* Second column represents the mean values for 10 birds
Subsequent columns represent the mean values for 5 birds

TABLE VIII

Total Ascorbic acid in mg 100 cm^3 of whole blood in guinea fowls fed on the Pfizer Livestock feeds for 6 weeks.

Identification Mark	WEEK NO. 1	2		3			4		5				6	
11	3.4	3.9	-	1.9	-	2.6	2.1	-	2.0	-	2.5	-	2.5	-
12	2.4	2.6	-	-	2.0	-	-	1.3	-	1.5	-	1.3	-	1.5
13	2.1	-	2.5	2.1	-	2.4	1.6	-	2.1	-	1.5	-	2.0	-
14	2.6	-	3.2	2.9	-	2.3	2.1	-	2.1	-	2.1	-	1.5	-
15	2.8	-	3.3	2.1	-	2.4	2.0	-	1.6	-	1.5	-	2.8	-
16	2.3	-	3.6	-	3.1	-	-	2.4	-	2.1	-	2.0	-	2.7
17	2.4	4.3	-	-	2.1	-	-	2.4	-	1.5	-	2.0	-	2.2
18	2.4	4.2	-	-	2.7	-	-	2.7	-	2.0	-	2.1	-	2.4
19	2.4	-	2.7	3.3	-	3.2	2.1	-	2.0	-	2.0	-	2.1	-
20	3.0	3.6	-	-	2.6	-	-	2.9	-	2.0	-	1.8	-	2.3
* MEAN	2.6	3.7	3.1	2.5	2.5	2.6	2.0	2.3	2.0	1.8	1.9	1.8	2.2	2.2

* Second column represents mean for 10 birds

Subsequent columns represent mean for 5 birds.

T A B L E I X

Weekly weights (grams) of birds fed on Guinea Corn for five weeks.

Identi-tation Mark	W E E K N U M B E R				
	1	2	3	4	5
1	1550	1550	1500	1500	1475
2	1325	1275	1300	1250	1300
3	1600	1625	1700	1700	1725
4	1575	1600	1600	1600	1600
5	1500	1475	1450	1450	1475
6	1425	1425	1425	1425	1400
7	1475	1475	1450	1500	1500
8	1450	1450	1425	1400	1400
9	1700	1700	1725	1750	1700
10	1650	1650	1650	1675	1700
Mean	1525	1523	1523	1525	1528

T A B L E X

Weekly weight in grams of birds feed on Pfizer Livestock feeds.

Identification Mark	WEEK NUMBER 1	2	3	4	5
11	1625	1525	1600	1525	1525
12	1350	1400	1450	1325	1325
13	1550	1525	1400	1325	1250
14	1700	1725	1725	1675	1650
15	1450	1425	1450	1400	1425
16	1450	1400	1450	1350	1400
17	1625	1500	1550	1375	1400
18	1375	1375	1450	1400	1500
19	1550	1475	1500	1400	1375
20 20	1450	1475	1525	1425	1400
Mean	1512	1483	1510	1420	1273

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A P P E N D I X _ I

% Transmittance values for standard concentrations of ascorbic acid in 4cm^3 of 5% metaphosphoric acid 10% acetic acid mixture at 515nm using a Beusch and Lomb Spectronic 20 colorimter.

Concentration in ug/cm^3	Transmittance (1)	(2)	Mean
1	83.0	85.5	84.3
2	79.9	79.9	79.9
4	70.0	70.2	70.1
6	60.0	60.1	60.1
8	51.0	49.9	50.5
10	44.5	47.0	45.8
12	39.9	39.0	39.5

A P P E N D I X I I

Instrument constant values for seven standard solutions of ascorbic acid in 5% metaphosphoric acid-10% acetic acid mixture using a Bausch and Lomb Spectronic[20] at 515nm.

Concentration in micrograms per cm^3 of ascorbic acid	Instrument constant K *
1	0.07
2	0.05
4	0.04
6	0.04
8	0.04
10	0.03
12	0.03
Mean	0.04

* Instrument constal K takes into account the path length of the colorimeter tubes, the particular instrumet, the extinction coefficient and the reagents.

A P P E N D I X I I I

RESULTS FOR TOTAL ASCORBIC ACID CONTENT OF SPECIMEN A

(Curcubita moschata).

No of determinations	Total Ascorbic Acid mg/100g		Mean
	10ml dilution	5ml dilution	
1	13.0	13.7	13.4
2	14.6	13.9	14.2
3	12.9	12.3	12.6

A P P E N D I X I V

RESULTS FOR THE TOTAL ASCORBIC ACID AND DEHYDROASCORBIC ACID CONTENT OF SPECIMEN B (Curcubita melo-pepo)

No of determinations	Total Ascorbic Acid mg/100g	Dehydroascorbic Acid mg/100g
1	9.36	9.09
2	9.63	8.56
3	9.89	8.56
4	9.36	8.82
Mean	9.56	8.76

A P P E N D I X V

RESULTS FOR THE TOTAL ASCORBIC ACID AND DEHYDROASCORBIC ACID CONTENT OF SPECIMEN C (Curcubita maxima)

No of determinations	Total Ascorbic Acid mg/100g	Dehydroascorbic Acid mg/100g
1	7.6	5.8
2	6.9	5.4
3	6.5	5.1
Mean	7.00	5.4

A P P E N D I X VI

% transmittance values for standard concentrations of ascorbic acid in $3cm^3$ of 6% trichloroacetic acid solution at 515nm using a Bausch and Lomb spectronic 20 colorimeter.

Concentration in ug/cm^3	% transmittance		Mean
	1	2	
1	88.1	87.9	88.0
2	81.0	81.5	81.3
4	67.2	67.2	67.2
6	61.5	61.5	61.5
8	50.1	50.1	50.1
10	40.5	43.0	41.8
12	36.0	37.0	36.5

A P P E N D I X VII

Instrument constant values for seven standard solutions of ascorbic acid using a Bausch and Lomb Spectronic 20 colorimeter at 515nm.

Concentration in cirograms per cm^3 of ascorbic acid	Instrument constant K*
1	0.06
2	0.05
4	0.04
6	0.04
8	0.04
10	0.04
12	0.04
Mean	0.4

*Instrument constant K takes into account the path length of the colorimeter tubes, the particular instrument, the extinction coefficient and the reagents.