ESTIMATION OF BETA-CAROTENE, IRON AND OXALIC ACID CONTENT IN GREEN LEAFY VEGETABLES.

By

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DECLARATION

The work described in this thesis was carried out by me at Food Research Unit, Gannoruwa and Food Science laboratory at Faculty of Applied Science Buttala under the supervision of Mr.T.D.W.Siriwardane and Mr. M.A.Jagath Wansapala report on this has not been submitted to any other university for another degree.

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AFFECTIONALLY DEDICATED

TO MY EVERLOVING

PARENTS AND TEACHERS.

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Abstract

Vitamin A and iron deficiency is observed mainly among the population in rural, dry and arid zones. Oxalic acid may course nutrient deficiencies and also contribute to the formation of kidney stones. It is probably due to the non availability and poor knowledge of vitamin A ,iron and oxalic acid rich foods. Although vitamin A and iron are most bioavailable in foods of animal origin, the high cost of these foods make them less accessible to most people in Sri Lanka.

This project was aimed to identify cheap sources rich in provitamin A, iron and oxalic acid and to evaluate the concentrations of different green leafy vegetables. Further bioavailability of beta-carotene was calculated in terms of retinol equivalent, iron and oxalic acid in terms of mg/100g of edible portion.

All plant pigments were extracted with acetone and running through a open column chromatography to separate beta-carotene. The eluted was determined by spectrophotometry. In the iron estimation measure the absorbance of complex form, between iron (II) and 1,10-phenanthrolene, and determined amount by using spectrophotometry. Oxalic acid converted into form of insoluble calcium oxalate and separating it by centrifugation. Again it was converting back to oxalic acid by using conc.H_2SO_4, and quantified by titrametically with $KMnO_4$ solution of standard normality.

Of the green leafy vegetables, retinol equivalent was highest in kathurumurunga (22.94µg/g) closely followed by murunga (16.79µg/g) and kura-thampala (15.55µg/g). Hin-gotukola (53.98mg) pochchi gotukola (42.95mg) and rathu-thampala (40.43mg) were the highest amounts of iron content found in 100g of edible portion and the murunga (165.25mg), kathurumurunga (122.01mg) and nivithi (78.98mg) leaves are the leafy vegetables that contain highest amount of oxalic acid in 100g of edible portion.

CONTENTS

List of figures

List of tables

CHAPTER 1

1. INTRODUCTION.

1.1 Micronutrient malnutrition

Micronutrient malnutrition is a term commonly used to refer to vitamin and mineral nutritional deficiency diseases. Diets which lack adequate amounts of essential vitamins and minerals lead to such diseases. Vitamin A deficiency and iron deficiency disorders are among the most common form of micronutrient malnutrition.

1.1.1 Vitamin A deficiency

In developing countries vitamin A deficiency is the most important cause of childhood blindness, as a complication of xerophthalmia. It is also a severe public health problem in Sri Lanka as indicated by 36% (against cut off level of 20%) of children with serum vitamin A concentration below 20 micrograms per deciliter (MRI(Medical Research Institute),1998). Increased ill health and mortality have long been associated with vitamin A deficiency.

1.1.2 Iron deficiency

Iron deficiency anaemia affects over 2000 million people worldwide (WHO (World health Organization),1992). Over 90% of affected individuals live in developing countries (ACC/SCN,1997). The group most affected by anaemia are adolescent girls, women of childbearing age and pre-school age children. Iron is most important in synthesizing hemoglobin and myoglobin, in supporting immune function, and in energy metabolism. An iron deficiency can cause decreased red blood cell synthesis, which can lead to anaemia. Severe anaemia may be a contributory factor in up to 50% of maternal deaths, and is the main cause of up to 20% of maternal deaths in developing countries (ACC/SCN,1991).

These are happen mainly due to inadequate intake, poor understanding of dietary sources rich in provitamin A (beta carotene) or vitamin A and iron in addition to other factors.

1.2 Oxalic acid as a disease causing agent.

Oxalic acid is completely different from the above micronutrients. It is occur naturally in quite a large number of plants. Oxalic acid binds with important nutrients, such as calcium, iron, sodium, magnesium and making them inaccessible to the body. Regular consumption of large amounts of foods high in oxalic acid over a period of week to months may result in nutrient deficiencies, most notably of calcium.

Oxalic acid is a strong acid, and is irritating to tissue all by itself. Extremely high doses are fatal. Oxalate on the other hand, form tiny little insoluble crystals with sharp edges, which are also irritating to tissues. So high levels of oxalic acid/oxalate in the diet lead to irritation of the digestive system, and particularly of the stomach and kidneys. They may also contribute to the formation of kidney stones. The presence of oxalic acid or oxalate in plant material has been responsible for a number of ill effects in man as well as in animals. So identification of plants that contain high amounts of oxalic acid and quantification are very important.

1.3 Availability of micronutrients in low-cost food items.

Pre formed vitamin A and iron present only in animal foods. The high cost of animal foods make them less accessible to most people in Sri Lanka. So the cost is the prime constrain on the purchase of animal sources of vitamin A and iron. Commonly available, low cost foods such as green leafy vegetables and certain vegetables are rich in provitamin A/ beta-carotene (beta-carotene is the most widely distributed form in plant sources and more effectively converted into vitamin A), iron and oxalic acid. Market surveys have found that dark green leafy vegetables are the least costly sources of vitamin A, iron in many countries (FAO,1997).

Therefore important to identify rich sources of beta-carotene (provitamin A), iron and oxalic acid among the green leafy vegetables, that could prevent vitamin A, iron and other deficiencies, and contribute towards the dietary vitamin A and iron requirements. Therefore this study is carried out with the following objectives.

1.4 Objectives

- To evaluate the content of beta-carotene, iron and oxalic acid present in green leafy vegetables.
- To identify sources rich in beta-carotene, iron and oxalic acid among green leafy vegetables.
- To identify the best maturity stage which contain high amount of beta-carotene, iron and oxalic acid found in green leafy vegetables.
- To make recommendations for micronutrient deficiencies in Sri Lanka, on the above parameters.

CHAPTER 2

2. REVIEW OF LITERATURE

2.1 Green Leafy Vegetables

2.1.1 Introduction for Green Leafy Vegetables

Traditional food varieties can be effectively used as low cost food sources for low income groups of under developed countries such as Sri Lanka. In this context, a variety of leaves are used. These plant varieties have some distinct advantages due to simple reason that they are common, popular and still contribute significantly to the dietary needs of the rural poor. They adopt well to unfavourable environmental conditions and some of them can even be grown in marginal lands, which have good resistance to pest and diseases. Therefore they require low attention and little or no inputs. They are nourishing and have a value similar to socially accepted food varieties (Rajapaksha,1998).

Gathering wild plants is still the main source of leafy vegetables in rural areas. Modern urban man lacks this knowledge and his food intake is influence by commercial sales methods and agricultural technology. The main source of leafy vegetables in densely populated areas is the home garden. Leaf production could be added to by planting hedges of perennial vegetables or creepers growing along fences. Those who live in high-rise buildings could grow leafy vegetables in plots (Wikramanayake,1996).

Water spinach (*Ipomoea aquatica*. Sin. Kankun), an important creeper with pink flowers, related to the sweet potato. Harvest begins within 6 weeks of planting cuttings. It is a green leafy vegetable that grow in swamps, fish ponds, water courses and rice fields.
The following plants grow on dry ground and could be cultivated in home gardens and pots:
Amaranthus (*Amaranthus viridis*. Sin. Thampala), an important short-lived herb, several species and many varieties of which are found in the tropics, the common ones being *Amaranthus viridis* and *A. spinosus*. Amaranth seeds have been used as grains by some races. The seed formed the staple of the Aztec diet as well as an integral part of their

religious rites. Both the grains and the leaf are sources of high quality protein, rich in lysine. The grain can be cooked into a gruel, parched and milled to produce a flour, or popped. Popped amaranth combine with molasses forms a confection that is called "algeria" by Maxicans. The varieties found in Sri Lanka have very small seeds that are seldom eaten. The plants is harvested before seeds appear and the leaves and tops are cooked in a variety of ways. Amaranths leaves have always been an item of the Sri Lankan menu. The red variety is popular with children (Wikramanayake,1996).

The Spinach (*Basella alba, B. rubra*, Sin. Nivithi) is an important annual or perennial herbaceous climber with thick, fleshy leaves. The young shoots and leaves are eaten. Harvesting is 6 weeks after sowing, less after planting cuttings. It is a very productive leafy vegetable which shows remarkable resistance to pest and disease.

Horse purslane (*Trianthema decandra*, Sin. Sarana) is a shrub that could be cultivated in home gardens or pots. It is cooked in the same manner as the local spinach.

Three popular green leafy vegetables are the Mukunuwenna (*Alternanthera sessilis*), the Indian pennywort (*Centella asiatica*, Sin. Hin-gotukola) and Agathi (*Sesbania grandiflora*, Sin. Kathurumurunga). Mukunuwenna and Hin-gotukola grow very close to the ground and need shade. Gotukola is prepared into a salad, with onions and grated coconut. As well as cooked with coconut milk.

Other leafy vegetables that could be cultivated in home gardens but are usually found growing as weed or wild plants are the *Dregia volubilis* (Sin. anguna), *Cardiospermum helicacabrum* and *C. microcarpum* (Sin. Valpenela), *Acrostichum aureum*, a fern (Sin. Karankoku), *Oxalis corniculata* (Sin. Ambul ambeliya), *Murraya koenigii L.* (Sin. Karapincha) (Wikramanayake,1996).

2.1.2 Importance of Green Leafy Vegetables.

A great variety of dark green leafy vegetables are available and it is possible to find a number of them in any part of Sri Lanka, in the hill- country and the lowlands, in the wet,

dry or arid zones. As the Sri Lankan rice diet tends to be deficient in vitamin A on account of a low intake of animal fat, the consumption of at least 50g of dark green leaves per day become essential.

Choosing of leaves for consumption must be done with care. Intravascular hemolysis may be induced by Kuppamenia (*Acalypha indica L.*) in those with glucose-6-phosphate dehydrogenase deficiency. Fatal poisoning due to misidentification of *Cassia bicapsularis* to *Cassia auriculata* (Sin. Ranawara) has been reported (Wikramanayake,1996).

In general dark green leafy vegetables are rich in carotene and ascorbic acid as well as in riboflavin, alpha-linolenic acid, vitamin K and folic acid. Leaf proteins are rich in lysine which can supplement a deficiency in cereal proteins. Leaves also add calcium and iron to the diet, and add to the content of fibre in the diet. Wilting of green leaves at room temperature reduces the vitamin C content by 30 to 80% during a period of 4 to 24 hours. Leaves should be harvested just before the plant flowers, when their iron content is high. Leaves are always eaten with a source of fat, a commendable practice, because fat increases the absorption of carotene (Wikramanayake,1996).

2.2 Beta-carotene.

2.2.1 Carotenoids

The carotenoids are a group of yellow, orange and orange red fat-soluble pigments widely distributed in nature. And also carotenoids are a class of nearly 400 known naturally occurring pigments found in certain flowers, vegetables and fruits. The red of the ripe tomato and the orange of a carrot are produced by their carotenoids. In green leaves, they occur in the chloroplasts. In leaves, the green colour of the chlorophyll masked the yellow to red colour of the carotenes except in very young leaves in which the chlorophyll content is less. The carotenoids are either hydrocarbons or derivatives of hydrocarbon and are composed of isoprene units. Some of the carotenoides are important in nutrition as precursors for the synthesis of vitamin A (Ranganna,1986).

2.2.2 Beta-carotene (Provitamin A)

Carotene is the precursor of vitamin A in the body and is therefore known as ' Provitamin A'. It is abundant in green and yellow vegetables as well as in yellow fruits, but it is never produce in animals. Provitamin A is the original source of all vitamin A in nature (Gordon and Paul,1993). Carotene has alpha, beta and gamma isomers. The yellow orange pigment beta-carotene is the most common form of provitamin A. Other important carotenoides include alpha-carotene, lutein, lycopene, zeaxanthin and beta-cryptoxanthin. Beta-carotene contains two beta-ionone rings and is capable of splitting into two molecules of vitamin A where as the others possess only one beta-ionone ring and, therefore, have less activity. Beta-carotene is the most widely distributed form in food and more effectively converted into vitamin A (Ranganna,1986).

Beta-carotene is converted into vitamin A (retinol) in the gut. This conversion requires adequate levels of protein, thyroid hormones, bile, zinc and vitamin C. The conversion is also affected by the age and overall health of the individual. Inadequate beta-carotene intake may be a factor in the development of cancers of the skin. Beta-carotene has a dual role, as a precursor to vitamin A and an antioxidant in its own right. Hence, it is mainly used against cancer, eye and skin diseases and various forms of diseases involving free radicals.

2.2.3 Chemistry of Vitamin A

Vitamin A may be a misleading term because it sounds as if only one chemical compound has vitamin A activity. Actually, several forms of vitamin A exist, with each possessing different degrees of activity.

There are two forms of vitamin A as such, retinol and dehydroretinol. Preformed vitamin A is present in animal foods as retinol, the alcohol form (Fig. 2.1), and retinyl esters – compounds that have a fatty acid attach to the alcohol group of retinol. All these forms of vitamin A are derived only from animal foods or nutrient supplements. In the body, retinol is converted to retinal, an aldehyde form, and retinoic acid (Fig. 2.2). Retinyl esters, which don't exhibit vitamin A activity as such, are broken down to yield retinol or

retinoic acid. The three active forms of vitamin A – retinol, retinal and retinoic acid – are collectively called retinoids (Gordon and Paul ,1993).

Dehydroretinol, or vitamin A differs from retinol in that (1) it has an extra double bond, and (2) it has about 40 percent the biological value (activity). It is found only in freshwater fish and in birds that eat these fish; hence, it is of limited interest.

Today, the general term vitamin A is used for both retinol and dehydroretinol. Although foods of plant contain no preformed vitamin A, they contain 400 or so pigments called carotenoids, some of which are convertible to vitamin A. At least 10 of the carotenoids found in plants can be converted with varying efficiencies into vitamin A. Four of these carotenoids – alpha-carotene, beta-carotene, gamma-carotene and cryptoxanthine. Out of the four, beta-carotene (Fig. 2.3) has the highest vitamin A activity and provides about two-thirds of the vitamin A necessary for human nutrition.

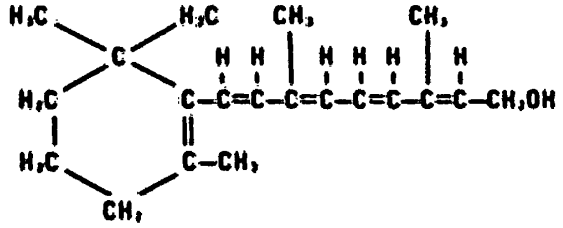


Fig. 2.1 The structure of vitamin A (retinol).

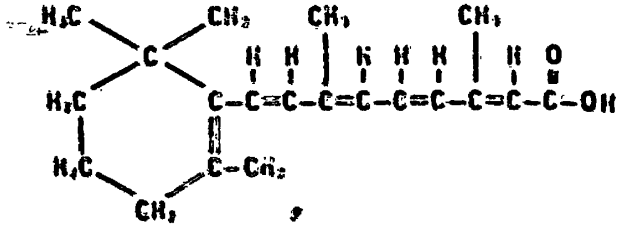


Fig. 2.2 The structure of Retinoic acid.

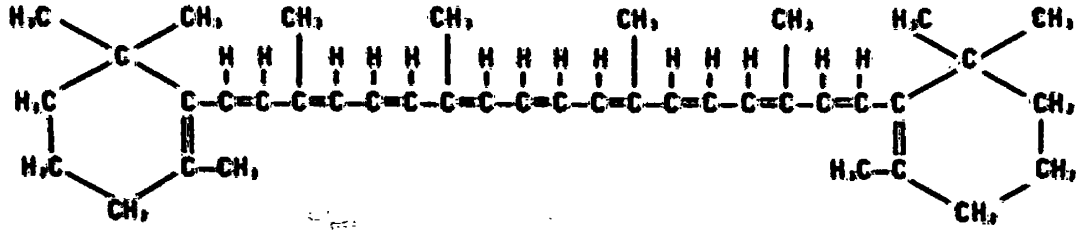


Fig. 2.3 The structure of Beta-carotene.

2.2.4 Digestion, absorption and excretion.

2.2.4.1 Digestion and absorption

In food the vitamin exists as esters, which are hydrolysed in the duodenum, a process that is assisted by bile salts. Both retinol and the carotene (yellow plant pigments), being highly unsaturated compounds, tend to be oxidised readily and such oxidation is prevented by the tocopherols present in foods. Absorption of retinol and the carotene is facilitated by the presence of fat in the diet, being associated with micelles formed during fat digestion. Utilization of both retinol and beta-carotene is also enhanced by dietary protein and zinc (Ersminger et al.,1994).

About 90 % of dietary retinol is absorbed beta-carotene forms micelles less readily and only 60% to 70% is absorbed,less with large doses (Wikramanayake,1996).

2.2.4.2 Storage

Vitamin A is almost entirely stored in the liver in the form of retinyl esters. A well nourished, healthy person stores 60-100 micrograms (0.21-0.35 μ mol) of retinol per g of liver (Antia,1989).

2.2.4.3 Excretion

Unlike water-soluble vitamins, vitamin A is not excreted through the urine unless there is proteinuria stools contain smallamounts of carotene and vitamin A (Antia,1989).

2.2.5 Units of beta-carotene (Vitamin A)

For vitamin A, the current unit of measurement is the retinol equivalent (RE), which is basically 1 microgram (μg) of retinol. In this system, it is assumed that 6μg of beta-carotene yield 1μg of vitamin A activity and that 12μg of other carotenoids yield 1μg of vitamin A activity. The correction factors of 6 and 12 are estimates, based on incomplete knowledge, and primarily compensate for the poorer absorption of beta-carotene and other carotenoids compared with preformed vitamin A, as well as the incomplete

conversion to the active form. The total RE value for a food is calculated by adding the actual weight of retinol and the adjusted equivalent weights of the provitamin- A carotenoids present in the food. Table 2.1 is a handy tool for converting amounts of vitamin A and carotene expressed in one unit of measure into another unit of measure.

Table 2.1 Conversion values for vitamin A

Compound with vitamin A activity	µg =	RE =	IU
Retinol	1	1	3.3
Beta-carotene	6	1	10
Other carotenoids (alpha-,delta-,etc.)	12	1	10
Mixture of both preformed and provitamin	--	1	5

Source : Perspectives in Nutrition,1993.

2.2.6 Nutritional consideration of beta-carotene (Vitamin A).

2.2.6.1 Functions.

Vitamin A is essential for a number of physiological processes;

Vision – The best understood function of vitamin A is related to the maintenance of normal vision in dim light – the prevention of night blindness. Fig. 2.4 shows, in simplified form, the role of vitamin A in vision.

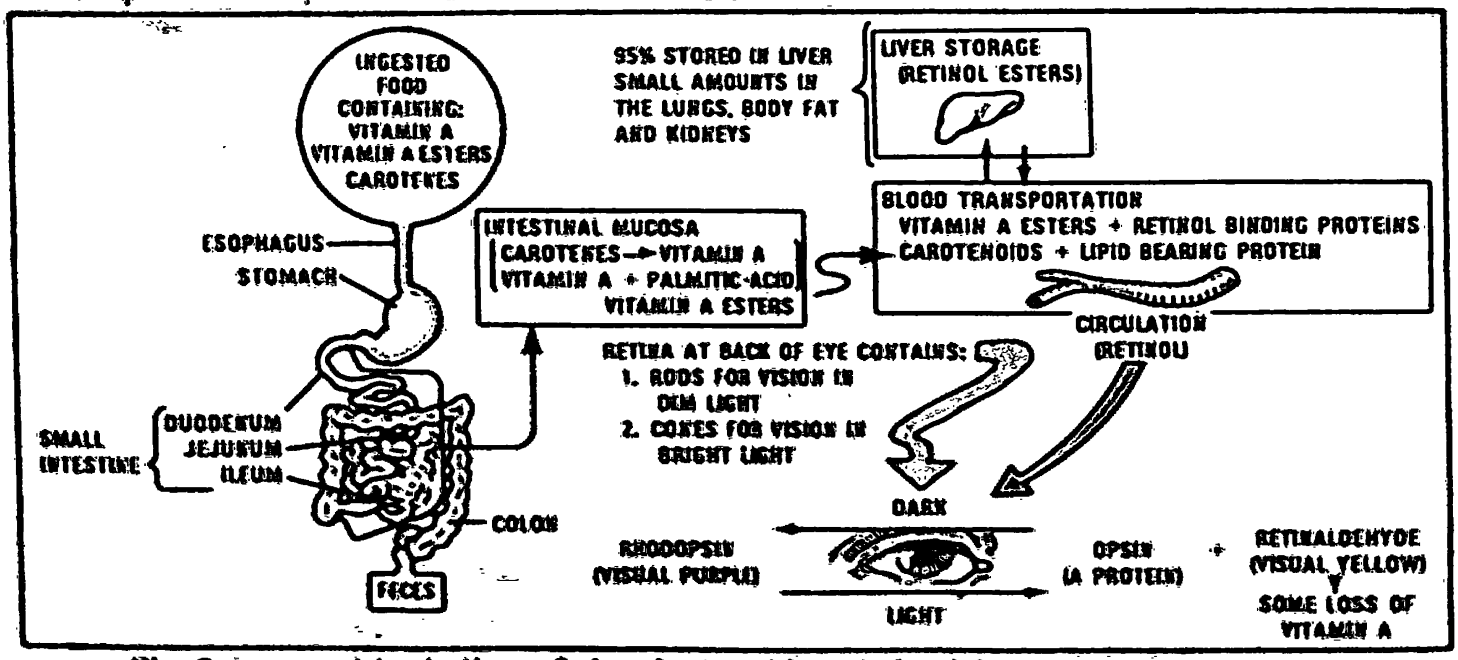


Fig. 2.4 Metabolism of vitamin A and its role in vision in dim light.

- Growth – Vitamin A aids in the building and growth of body cells; hence, it is essential for body growth. Also, it has long been known that vitamin A is essential for the growth of children and for the normal development of babies before birth.
- Bone development – Vitamin A is especially needed for bone growth. If the intake is not sufficient, the bone will stop growth before the soft tissues are affected.
- Tooth development – Vitamin A is essential for normal tooth development. Like other epithelia, the enamel forming cells are affected by lack of vitamin A; instead of an even protective layer of enamel, fissures and pits will be present and the teeth will tend to decay.
- Maintenance of healthy epithelial tissues – Without vitamin A ,the epithelial cells become dry and flat, and gradually harden to form scales that sloughs off. This process is known as keratinization. The skin may become rough, dry and scaly. Whenever these tissue changes occur, the natural mechanism for protection against bacterial invasion is impaired and the tissue may become infected easily.
- Protective effects against cancer – Vitamin A, either as retinol or carotene, appears to play an important nutritional role in keeping the body free of certain kind of cancer, especially cancers of epithelial tissue.
- Reproduction – In most animals, the absence of vitamin A in the diet will dramatically reduce reproductive ability. Lack of vitamin A in the diet of pregnant women during the first trimester (the first 3 months) may cause miscarriage.

2.2.6.2 Vitamin A Deficiency

Vitamin A Deficiency (VAD) is a severe public health problem in Sri Lanka as indicated by 36 percent (against cut off level of 20 percent) of children with serum vitamin A concentration below 20 micrograms per deciliter (MRI,1998). VAD, often in association with protein-energy malnutrition, principally affects preschool children. It is estimated that almost 250 million children in developing countries are at risk, of whom at least 2.8 - 3 million are clinically deficient. VAD causes night blindness and may lead to xerophthalmia and eventually total blindness. Every year 250,000-500,000 children lose their sight as a result of VAD; two-third of these children are likely to die (FAO,1997). Commonly available, low-cost foods such as green leafy vegetables, and certain yellow

fruits and vegetables are rich in provitamin A; regular consumption of these foods in adequate amounts could prevent VAD. Table 2.2 shows distribution of Bitot spots and Night blindness (Vitamin A Deficiency) by health service district of Sri Lanka.

Table 2.2 Percent of Rural Sri Lankan children with Bitot's spots or with a history of Night Blindness, by health service district.

Health Service District	Number children examined (6-71 mos.)	Percentage with Bitot's spots (6-71 mos.)	Number children examined (24-71 mos.)	Percentage Night blindness (24-71 mos.)
Kegalle	896	2.3	625	2.6
Matara	897	2.2	646	2.3
Ratnapura	896	2.0	637	0.9
Galle	897	1.4	663	1.1
Badulla	895	1.0	664	0.8
Kandy	893	1.0	629	1.1
Kurunegala	896	1.0	628	0.5
Kalutara	899	0.9	617	0.3
Batticoloa	894	0.7	642	1.3
Anuradhapura	900	0.7	648	0.5
Matale	899	0.7	638	0.8
Vavuniya	896	0.7	623	0.5
Jaffna	893	0.4	626	0.6
Puttalam	900	0.3	672	0.6
Colombo	899	0	664	1.2
Weighted Average	13450	1.1	9622	1.0

Source : Ministry of Food and Nutrition Policy Planning Division 1982.

2.2.6.3 Deficiency symptoms

A deficiency of vitamin A may be due to a dietary lack of vitamin A and/or provitamin A, or to poor absorption. A diet that contains an insufficiency of vitamin A activity will, in due time, cause deficiency disorders. Common deficiency symptoms follows;

- Xerophthalmia – Xerophthalmia is a serious problem throughout S-E Asia. Approximately 500,000 new cases with active corneal involvement occur annually in India, Bangladesh, Indonesia and the Philippines, and half these cases are likely to lead to blindness(Wikramanayake,1996). Xerophthalmia is the comprehensive term now used by the world health organization to denote all vitamin A deficiency manifestations affecting structure or function of the eyes, including conjunctiva, cornea and retina.

The following are included under xerophthalmia;

(1) Night blindness – In dim light visual purple (rhodopsin) in the rods is converted into chemical energy. With vitamin A deficiency, rhodopsin is not formed and vision in dim light is not possible.- the so called 'night blindness'. (2) Conjuctival xerosis – In this condition the conjunctiva becomes dry and lustreless. (3) Bitot's spots – These are localized elevated areas frequently bilateral on the temporal and sometimes on the nasal side of the conjunctiva.(4) Corneal xerosis – The anterior portion of the corneal surface is rough and lack-luster in appearance. The cornea is insensitive to tough (Antia,1989).

Stunted growth – A deficiency of vitamin A result in stunted growth of children.

Slowed bone growth – When young animals are deprived of vitamin A, the bones fail to lengthen, and the remodeling processes necessary for the formation of compact bone cease to operate. Abnormalities in bone shape result.

Sound teeth – If a child gets too little vitamin A when his teeth are developing, the enamel forming cells become abnormal and pits are formed. Such pits may harbor food deposits, which may ferment and form acids that etch the enamel and lead to decay (Ersminger et al.,1994).

2.2.6.4 Toxicity (Hypervitaminosis A)

Signs and symotoms of toxicity from excessive vitamin A – called hypervitaminosis A – can appear with long – term supplement use at just six to ten times the RDA (Gordon and Paul, 1993). Three kinds of vitamin A toxicity exit: acute, chronic, and teratogenic.

2.2.6.4.1 Acute toxicity

Acute toxicity is caused by the ingestion of one very large doses taken over several days (about 100,000RE). The effects of acute toxicity are largely gastrointestinal upset, headache, blurred vision, and muscular incoordination. Once the dosing is stopped, these signs disappear. Extraordinarily large doses, about 12g (12,000,000RE), however, can be fatal.

2.2.6.4.2 Chronic toxicity

Chronic toxicity, which is much more common than acute toxicity, results from ingesting excessive doses of vitamin A on a regular basis during a period of weeks to years. In infants and adults, there is a wide range of signs and symptoms: bone and muscle pain, loss of appetite, various skin disorders, headache, dry skin, hair loss, increased liver size, and vomiting. Permanent damage to the liver, bones, and eyes, as well as recurrent joint and muscle pain, however, can occur with chronic ingestion of excessive amounts of the vitamin (Gordon and Paul,1993).

2.2.6.4.3 Teratogenic toxicity

The teratogenic effect of the vitamin is well documented. Women taking large doses of retinol during the early months of pregnancy have given birth to deformed children. (Gordon and Paul,1993). Fig. 2.5 shows a summery of diseases associated with excess and inadequate intake of vitamin A.

Consuming carotenoid in huge amounts from foods does not readily result in toxicity in most people. There rate of conversion into vitamin A (retinol), when possible, is relatively slow. In addition, the deficiency of carotenoid absorption from the small

intestine decreases markedly as the oral intake increases. Thus nature protect us from any serious toxic effects from dietary carotenoids.

If some one consume large amounts of carrots (in the form of carrot juice, for example) or if an infant eats a lot of winter squash, the resulting high carotenoid concentrations in the body can turn skin a yellow-orange colour. The result is termed hypercarotenemia, or just carotenemia. The person appears to have jaundice but, unlike a true jaundice, the sclera (whites of the eyes) are white (rather than yellow) and the liver is not enlarged. This carotenemia is generally thought to be harmless (Antia,1989).

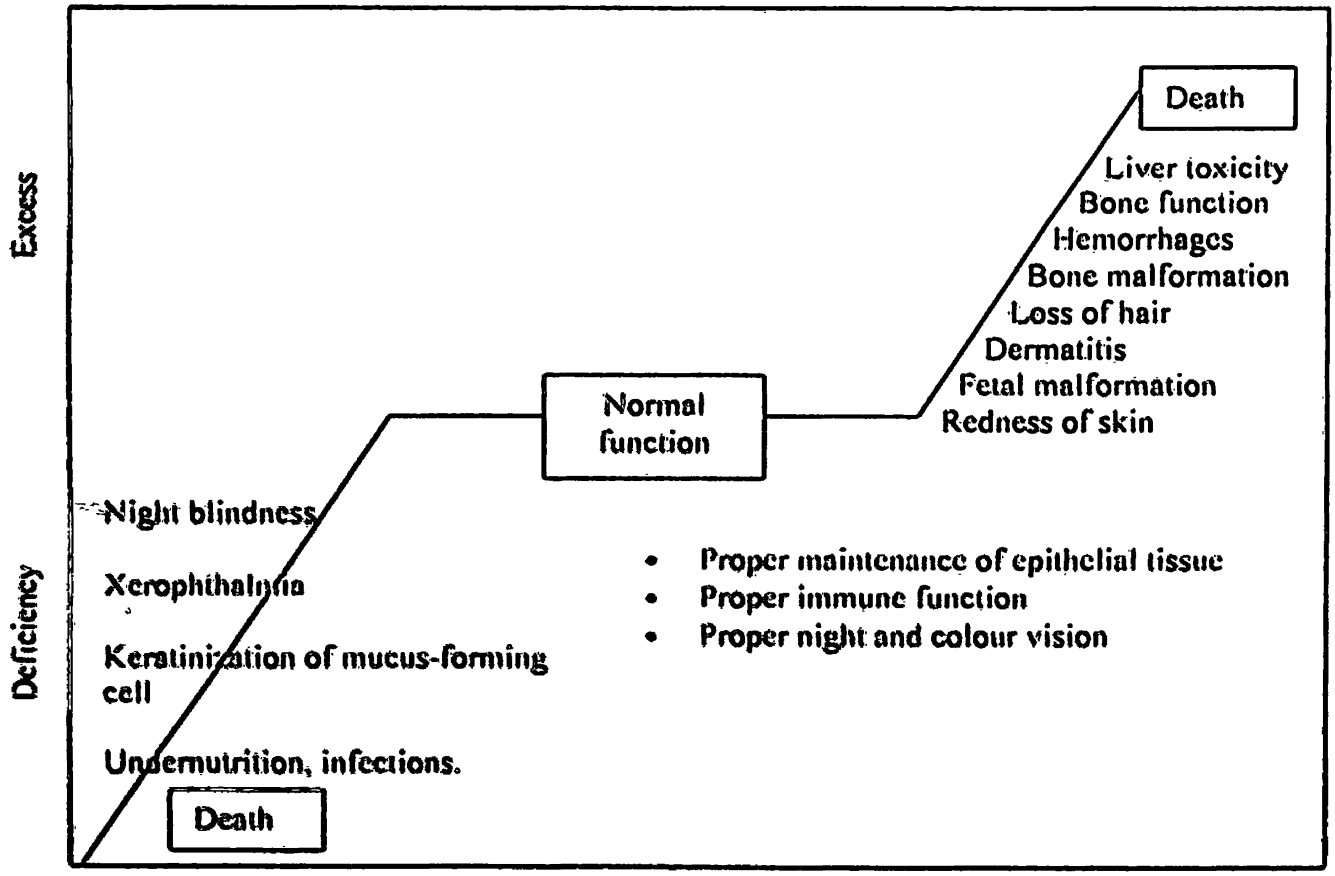


Fig. 2.5 Symptoms associated with vitamin A

2.2.7 Recommended Daily Allowance (RDA)

The estimation of vitamin A requirement can be varied with the criteria used (Beal,1980). Thus RDA values can vary from country to country. The food and Nutrition Board (FNB) of the National Research Council (NRC) recommended daily allowances of vitamin A are given in Table 2.3. At present, there is no separate RDA for beta-carotene or any of the other provitamin A carotenoids.

Table 2.3 Recommended Daily Vitamin A Allowances

Group	Age	Weight		Height		RDA	
	(years)	(lb)	(kg)	(in.)	(cm)	(mcg RE)	(IU)
Infants	0.0 – 0.5	13	6	24	60	375	1250
	0.5 – 1.0	20	9	28	71	375	1250
Children	1 – 3	29	13	35	90	400	1333
	4 – 6	44	20	44	112	500	1667
	7 – 10	62	28	52	132	700	2333
Males	11 – 14	99	45	62	157	1000	3333
	15 – 18	145	66	69	176	1000	3333
	19 – 24	160	72	70	177	1000	3333
	25 – 50	174	79	70	176	1000	3333
	51+	170	77	62	173	1000	3333
Females	11 – 14	101	46	64	157	800	2667
	15 – 18	120	55	64	163	800	2667
	19 – 24	128	58	65	164	800	2667
	25 – 50	138	63	64	163	800	2667
	51+	143	65	63	160	800	2667
Pregnant						800	2667
Lactating						1200	4000

Source : RDA (Recommended Dietary Allowances),1989.

2.2.8 Food sources of vitamin A

The dietary sources of vitamin A fall into two categories: Vitamin A or all-trans retinol, also referred to as preformed vitamin A, and provitamin A, primarily beta-carotene and other carotenoids which are converted to retinol in the body.

Foods rich in retinol :Colostrum, breast milk, liver (chicken liver may be least costly),eggs, whole milk.

Tropical and subtropical plant foods rich in beta-carotene:

Leafy greens (1800 - 6000 IU/100g)

Fruits (1000 - 4700 IU/100g)

Mango, Pirie and Haden varities (*Mangifera indica*) (darker varieties contain higher levels)

Passion fruit (*Passiflora edulis*)

Papaya (*Carica papaya*) (darker, hybrid varieties contain higher levels)

Avocado (*Persea americana*, and *P. drymifolia*)

Tubers (6400IU/100g)

Sweet potato (*Ipomoea batatas*) (orange varities)

Flowers (4800IU/100g)

Pumpkin flower (*Cucurbita pepo*)

Other plant sources

- Carrot (*Daucus carota*)

Pumpkin (*Cucurbita pepo*)

Red pepper (Capsicum frutescens)

2.2.9 Vitamin A losses during processing, cooking and storage.

The carotene losses of vegetables following harvest tend to parallel the degree of wilting. In order to conserve their maximum carotene value, they should be stored at low temperatures or be quick frozen. Freezing and freeze-drying causes little loss. But drying of eggs, vegetables or fruits, with exposure air, sunlight or high temperatures, may causes serious loss of vitamin A value. Because vitamin A and the carotene are insoluble in water and stable to heat at ordinary cooking temperatures, it was once thought that little vitamin A activity was lost from foods in cooking and processing, unless they were

exposed to air. However, cooking or canning vegetables produces a rearrangement of the atoms in the carotene molecule, resulting in carotene of substantially lower vitamin A value. It is estimated that, on the average, the vitamin A vale of cooked green vegetables is decreased by 15 to 20 % and the value of yellow vegetables by 30 to 35 % (Ersminger et al., 1994).

Both vitamin A and carotenoids are easily oxidized and rapidly destroyed on exposure to ultraviolet light, with the rate of destruction influenced by the associated substances and temperature and moisture conditions. Yellow corn has been reported to lose as much as 60% of its carotene in 7 month's storage. Animal fats should be kept in a cold, dark place; and fish liver oils should be protected from light by being kept in dark bottles. Rancid fat destroys both vitamin A and carotene. Also, minerals such as iron oxide, charcoal, and iodine contribute to the destruction of vitamin A in foods.

2.3 Iron

2.3.1 Introduction

Iron is a vital component of haemoglobin which transport oxygen to the various tissues of the body. Life and iron are inseparable. Total body content is about 5g (about 1 teaspoon). The important of the iron for maintenance of health has been recognized for centuries. In 4000 BC, the Persian physician Melampus gave iron supplement to sailors to compensate for the iron lost from breeding during battles. Today, iron-deficiency anemia is common worldwide (Gorden and Paul,1993). With the sole exception of lactic acid bacteria, all living organisms require iron as an essential element for growth and multiplication.

In most developing nations about half of all children and women of child bearing age are estimated to suffer from iron-deficiency; many of them have the more severe form of the disorder, iron-deficiency anemia.

2.3.2 Distribution in body

Body iron may be divided into 4 functional groups:

Haemoglobin iron

Tissue iron – myoglobin and respiratory enzymes.

Storage iron – ferritin, haemosiderin

Transport iron – transferrin and serum ferritin

The amount of each category varies widely, both in health and disease, that in the storage compartment being subjected to the greatest functions. The amount of haemoglobin (Hb) iron varies with the Hb level and with blood volume. The amount of myoglobin (Mb) and enzyme iron are also variable.

Table 2.4 Total iron content of body, in mg.

	In 70kg man	In 50kg women	In 3kg neonate
Haemoglobin iron	2400	1750	156
Tissue iron	350	300	21
Storage iron	1000	400	25
Transferrin	3 to 4	2 to 4	1
Serum ferritin	0.3	0.1	

Source : Food and Nutrition, 1996

2.3.3 Digestion , Absorption and Excretion

2.3.3.1 Digestion and absorption

The greatest absorption of iron occurs in the upper part of the small intestine – in the duodenum and jejunum, although a small amount of absorption takes place from the stomach and throughout the whole of the small intestine. Only 10% of the iron present in cereals, vegetables, and pulses, excluding soybeans, is absorbed. Absorption of iron from

other food is slightly higher – for example, 30% from meat, 20% from soybeans, and 15% from fish. For estimating the absorption of iron in diets, a value of 10% is usually taken as the percentage of iron absorbed from mixed foods (Gorden and Paul,1993).

It is noteworthy that there are two forms of food iron – heme (organic) and nonheme (inorganic). Of the two, heme is absorbed from blood more efficiently than inorganic iron and is independent of vitamin C or iron binding chelating agents. Although the propotion of heme iron in animal tissues varies, it amount to about one-third of the total iron in all animal tissues – including meat, liver, poultry, and fish. The remaining two-thirds of the iron in animal tissues and all the iron of vegetable products are treated as non-heme iron. Haemoglobin iron is absorbed intact as heme, even at neutral pH, and is not affected by the dietary phosphate or phytate. The iron of meat and liver is better absorbed than of eggs and leafy vegetables. Animal protein in beef, pork, chicken or fish (but not egg and fish) increases absorption of non-heme iron from vegetable sources (Antia,1989).

2.3.3.2 Storage

The total body iron is 3 – 5g (54-90mmol), the bulk of which is in haemoglobin. The sites of storage are the liver, spleen and bone marrow as ferritin or hemosiderin.

Ferritin is a sperical storage iron protein which binds up to 4000 atoms of iron per molecule. It has 24 sub units arranged in a cluster like a raspberry and contains 20% iron. Ferritin estimation helps in diagnosing iron deficiency or overload. The normal values range from 12 to 250 micrograms per litre. Values less than 10 microgram ferritin per litre denote iron deficiency. Storage iron is about 980 mg (17.5mmol) in normal males and 450 mg (8mmol) in females (Antia,1989).

Hemosiderrin structure is not well understood but it is believed to be a degradation product of ferritin. Hemosiderrin iron is not readily released.

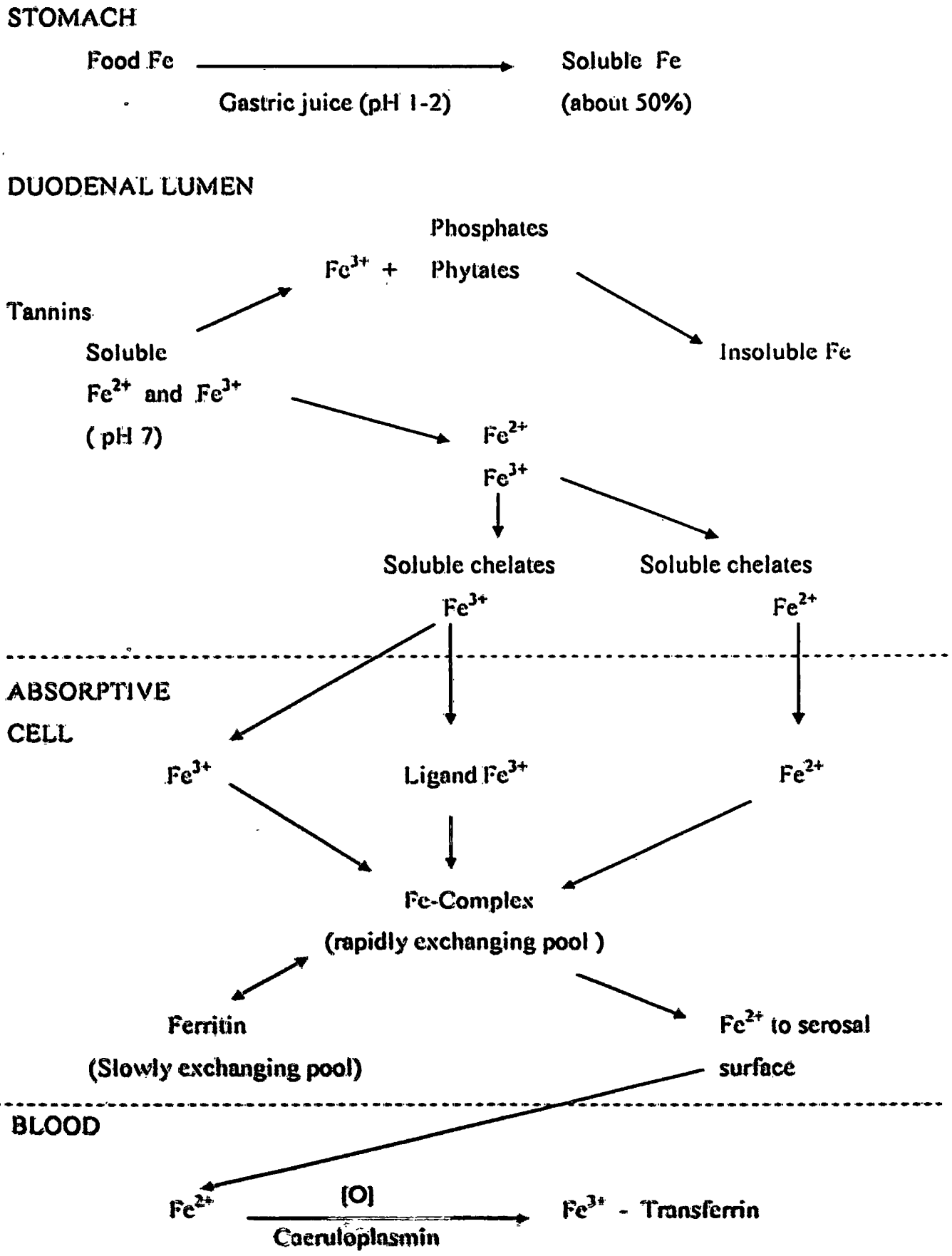


Fig. 2.6 Absorption of dietary iron.

2.3.3.3 Excretion

Absorbed iron is lost only by desquamation (shedding or peeling off the cells) from the alimentary, urinary, and respiratory tracts, and by skin and hair losses. The bulk of ingested iron (about 90%) is excreted in the feces. Only negligible amounts of iron are excreted in the urine. The body conserves and reuses iron once it has been absorbed.

The combined losses of iron by all routes are of the order of about 1 mg per day for a healthy adult man, and about 1.5mg per day for a women during the reproductive period. Added losses of iron of importance occur from blood donation and pathological bleeding (hook worm infections, bleeding ulcer, etc.), and in case of kidney diseases (Ersminger et al.,1994).

2.3.4 Enhancers and Inhibitors of Iron

Heme iron found in animal products (meat, fish, poultry, liver, blood) is better absorbed by the body. Non-heme or plant sources of iron (legumes, vegetables, cereals) are much less bioavailable (WHO,1992).

2.3.4.1 Enhancers of iron absorption

- Animal foods : fish , Meat, Shellfish, Breast milk
- Vitamin C rich foods : Breast milk (from mothers with good vitamin C status)
- Dark green leafy and other vegetables

  Amaranths (Amaranthus spp.), Beet greens (Beta vulgaris),
  Lotus roots (Nelumbium nelumbo), Spinach (Spinacia oleracea),
  Cabbage (Brassica oleracea), Broccoli (Brassica oleracea,var.Italica).
- Fruits :

  Guava (Psidium spp.), Mango, ripe (Mangifera indica),
  Papaya (Carica papaya), Pine apple (Ananas comosus),
  Citrus fruits.
- Root crops : Potatoes, Sweet potatoes may contain small but usefull amounts after cooking.
- Fermented foods: Fermented vegetables such as sauerkraut.
  Soy sauce.

2.3.4.2 Inhibitors of non-heme iron absorption.

- Tannins (found in tea, coffee, eggplant, legumes, many dark green leafy vegetables)
- Phytates (found in whole grains and high extraction flours)
- Oxalates
- Calcium phosphate (found in milk)
- Protein Soybean products, milk, foods containing bovine casein.

2.3.5 Nutritional consideration of iron

2.3.5.1 Functions

Irons combine with protein to make haemoglobin for red blood cells. *Heme* means iron; globin means protein. A small amount of iron (heme) combines with a large protein (globin) to make haemoglobin, the iron-containing compound in red blood cells.

Haemoglobin, myoglobin and cytochromes, which are components of the electron-transport chain in mitochondria. Iron also functions as a co-factor for some enzymes, including those involved in the synthesis of collagen and of various neuro-transmitters.

In addition, iron is needed for proper immune function and plays a role in drugs detoxification pathways. Haemoglobin molecules in red blood cells transport oxygen from the lungs to all cells and assist in the transport of some carbondioxide (CO_2) from cells to the lungs for excretion (Gorden and Paul, 1993).

2.3.5.2 Deficiency symptoms

If neither the diet nor body stores can supply the iron needed for haemoglobin synthesis, red blood cells synthesis is reduced. Eventually, the number of red blood cells falls so low that the amount of oxygen carried in the blood is decreased. Such a person exhibit anemia, which is characterized by a decreased oxygen carrying capacity of the blood. Although there are many types of anemia, the major type found in world wide is, Iron-deficiency anemia (Gorden and Paul, 1993).

2.3.5.2.1 Iron-deficiency anemia.

Iron-deficiency (nutritional) anemia, clinically characterized by a decreased in the amount of haemoglobin and by small, pale red blood cells, depleted iron stores, and a plasma iron content of less than 40 be reduced, but not as markedly as the haemoglobin content. Iron-deficiency anemia is a medical and public health problem of primary importance, causing few deaths but contributing serious to the weakness, ill health and substandard performance of million of people (Ersminger et al.,1994).

The symptoms of anemia are : paleness of skin and mucous membranes, fatigue, dizziness, sensitivity to cold, shortness of breath, rapid heartbeats, and tingling in the fingers and toes. An inadequate dietary intake of iron by growing children, by adolescent girls, or by women especially during pregnancy and in lactation, will produce nutritional anemia (Ersminger et al.,1994).

If a pregnant women has an insufficient intake of iron, the new born infant, in turn, will have relatively low store of iron, causing anemia in developed early in the first year of the life. Anemia during infancy, a frequent occurrence, is closely related to the body stores of iron at birth. It is especially common in premature infants and twins, because in such circumstances the body reserved of iron cannot be built up to desirable levels (Ersminger et al.,1994).

2.3.5.2.2 Other causes of iron deficiency anemia

In addition to the causes list above, Iron-deficiency anemia can be caused by chronic blood loss from heavy menses, ulcers, hemorrhoids, and colon cancer. Iron-deficiency anemia in men is usually linked to ulcers, colon cancer, or hemorrhoids (Gorden and Paul,1993).

In many developing countries a primary cause of Iron-deficiency anemia is the efficient absorption of iron from vegetable foods. The diets in these countries are largely vegetarian beeause meat is too expensive for most people to afford. Iron-deficiency and

anemia eventually affects the majority of individuals in such populations (Gorden and Paul,1993).

2.3.5.3 Toxicity of iron

Although not as common as Iron-deficiency, iron overload can be serious because it can easily leads to toxic symptoms. Even a large single dose of iron of 60mg can be life-threatening to a one-year-old. Approximately 2,000 cases of iron poisoning occur each year in the United States, mainly in young children who ingest the medicinal iron supplements of their parents (Ersminger et al.,1994).

In addition, iron toxicity accompanies the genetic disease called hereditary hemochromatosis. People with hemochromatosis over absorb iron. In those with this disease, the amount of iron in the body eventually build up to dangerous amounts, specially in the blood and liver. Some iron is deposited in the muscles, pancreas, and heart. If not treated, the excess iron deposits contribute to severe organ damage, especially in the liver and heart. Diabetes, liver cirrhosis, and a bronze skin pigmentation are possible complecations. The major causes of death from hemochromatosis are cirrhosis and liver cancer (Gorden and Paul,1993).

2.3.6 Recommended daily allowance of iron

The daily requirement of iron is determined by the type of food taken. Since animal food iron is better absorbed than iron from vegetable foods, a predominantly meat diets decreases, while a predominantly cereal diet increases the iron requirement (Ersminger et al.,1994). Recommended daily iron allowances are given in table 2.5.

2.3.7 Food sources of iron

Milk and milk products are poor sources of iron. Heme iron found in animal products (meat,fish, poultry, liver, blood sausage, egg yolk, shrimps etc.) is better absorbed by the body. Non-heme or plant sources of iron (cereals, beans, lentils, vegetable leaves, spinach, water-cress, cauliflower, radish) are much less bioavailable. Through the iron

content of spinach is high (12mg percent, 214.8 μmol), it is not easily absorbed (Antia,1989).

Table 2.5 Recommended Daily Iron Allowances

Group	Age (years)	Weight (lb)	Weight (kg)	Height (in.)	Height (cm)	Iron (mg)
Infants	1.0 – 0.5	13	6	24	60	6
	0.5 – 1.0	20	9	28	71	10
Children	1 – 3	29	13	35	90	10
	4 – 6	44	20	44	112	10
	7 – 10	62	28	52	132	10
Males	11 – 14	99	45	62	157	12
	15 – 18	145	66	69	176	12
	19 – 24	160	72	70	177	10
	25 – 50	174	79	70	176	10
	51+	170	77	62	173	10
Females	11 – 14	101	46	64	157	15
	15 – 18	120	55	64	163	15
	19 – 24	128	58	65	164	15
	25 – 50	138	63	64	163	15
	51+	143	65	63	160	10
Pregnant						30
Lactating						15

Source : RDA (Recommended Dietary Allowances),1989.

2.3.8 Iron losses during processing and cooking.

The size of the pieces, the amount of water used, the cooking method, and the length of cooking time affect the extent of mineral loss. When food is cooked in iron utensils, some iron is added from the utensils. When acidic foods, such as tomato sauce, are cooked in

iron cookware, some iron from the pan is taken up by the food. The replacement of iron cookware with stainless steel and aluminium cookware in recent times likely has increased the risk for iron deficiency (Wikramanayake,1996).

2.4 Oxalic acid

2.4.1 Introduction

Although oxalate have long been known to present in nearly all kinds of plant matter, it is a matter of interest to note that certain families and species of plant carry relatively high amounts of the substance may be in the form of soluble salts (e.g. Sodium, potassium oxalate) or insoluble salts (calcium oxalate). Plants which are group under the families of Chenapodiaceae, Oxalidaceae are known to have high amounts of oxalic acids (oxalate); , carambola (*Averrhoa carabbola* L), Bilimbi (*Averrhoa bilimbi* L.) and some other plants like spinach, rhubarb etc.

The carliest interest in the toxicity of oxalate arose because of the instances of severe or fatal human poisoning following the ingestion of large amounts of leaves of certain plants known to contain relatively large amounts of oxalate. Accidental ingestion of oxalic acid as pure chemical was also known to produce severe corrosive and other toxic effects; hence the conclusion was drawn that the acute poisonous effect of ingesting high oxalate content plants were probably related to that property (David, 1973).

Identification of plants that contain high amounts of oxalic acid and quantification are very important. Because of the fact that higher concentration may cause serious health problem to human as well as domestic animals.

2.4.2 Chemistry and Structure of Oxalate in plant tissues.

Pure oxalic acid is a colourless rhombic crystalline material, soluble in water, absolute alcohol and ether, with a density of 1.653. Calcium oxalate is the most prominently represented among crystals in plants. They appear as raphides – bundles of needles

(leaves of grape vine impatiens, Arum; stem of *Tradescantia*); styloides – elongated columnar crystals (leaf of *Eichhornia crassipes*); prisms – rectangular or pyramidal (leaves of begonia, *Hyoscyamus niger*, *Vicia sativa*); druess – spheroidal aggregates of prismatic crystals (rhizomes of Rhubarb, root of *Ipomea betides*, leaves of *Datura stramonium*, *Sambucus nigra*, leaf of *Atropa belladonna*).

Appearance and location of crystals may be specific and useful in taxonomic classification (David, 1973).

Prismatic Raphides Columnar

Fig. 2.7 Different types of oxalate crystals commonly found in plant kingdom

2.4.3 Analytical methods for oxalic acid.

Oxalic acid, HOOC-COOH is the simplest of the carboxylic acids. It is a crystalline, white solid, soluble in water to about 10% at 20^0C. Oxalic is a strong acid with a PK1 of 1.46 and PK2 of 4.40 and commonly exists as a hydrate. The neutral salts such as potassium and sodium are readily soluble in water, while alkaline metals are less soluble. Heating of oxalic acid causes loss of carbon dioxide and the formation of formic acid and carbon monoxide.

A variety of analytical methods is available for oxalate, most of which are based on isolation of the oxalic acid as calcium salt followed by oxidation. In a detail study on conditions of precipitation of calcium oxalate from urine, it was found that water saturated with the bactericide pentachlorophenol and with calcium oxalate, helped to prevent losses during washing of the precipitate (David,1973).

Several lengthy methods for determining oxalic acid in food have been reported. Many of these methods involve some basic steps summarized by Zarembski and Hodgkinson (1962); extraction of oxalic acid from plant materials with hydrochloric acid or with sodium carbonate and overnight precipitation of calcium oxalate, followed by treatment of the precipitate with dilute sulphuric acid to form a solution of oxalic acid. The oxalic acid was then quantified by one of the following methods: potentiometric with a quinhydrone electrode (Pucher et.al.,1934), titration with standard potassium permanganate (Baker,1952) or colourimetric methods by conversion of oxalic acid to glycolic acid and derivatization with 3,6-dihydroxy naphthalene-2,7-disulphonic acid (Zarembski and Hodgkinson,1962).

2.4.4 Occurrence of oxalic acid in plants.

Generally the highest level of oxalic acid are found in the following families.

(1) Polyonaceae (*Rheum,Rumex*)

(2) Chenapodiaceae (*Spinacia, Beta, Atriplex, Halogeton glomeratus*)

(3) Portulacaceae (*Portulaca*)

(4) Ficoidaceae (*Tetragonia*)

One of the plant with the highest known oxalate is *Halongenton glomeratus* which grows in USA. This plant contain as much as sodium oxalate. Another plant is *Oxalis cernua* which causes injuries in sheep and cattle.

The distribution oxalate varies with families but generally the leaves are rich in oxalate than stalks. The ratio of oxalate to calcium content also varies widely; it is possible to class plants in three groups as follows.

Oxalate / calcium ratio	Plants
2:7	Spinach, beet, rhubarb,cocoa
	Potatoes, gooseberries
	Lettuce, cabbage, peas

Apart from these plants species mentioned, many kinds of mushrooms and melds also produce oxalate.

2.4.5 Biosynthesis of oxalate in flora.

It is evident that oxalate are an end product and tend to accumulate in plants. Generally, oxalate tend to increase during the life of the plant, although there may be a decrease as the plant edges (David,1973).

Oxalate almost certainly arises from some parts of carbohydrate metabolism eg. From formation of glycolic acid and subsequent oxidation to glyoxylic and oxalic acid. Another possibility is the splitting of oxaloacetic acid; small amounts may be formed from purine or ascorbic acid metabolism (David,1973).

2.4.6 Oxalate metabolism in man and animals.

The absorption of oxalate from human gut is only 1-6% if the oxalate is given with meals. But under fasting conditions 30-50 % of the tracer dose may be absorbed. In mammals including humans, oxalate that are ingested are not broken down within the system and pass unchanged with faces or urine.

The extraction is now known to vary in children and adults from about 6-45 mg/day, with a mean of about 20mg/day, expressed as oxalic acid. About 1/3 of urinary oxalate is derived from ascorbic acid and similar amounts are derived from glycine. The rest is thought to be formed from dietary sources. Normal oxalic acid content in the body is as follows.

Serum	450 microgram/100ml plasma
Brain	60 microgram/100g(wet weight)
Kidney	400 microgram/100g

The other system may contain oxalic content between 60 and 40 micrograms.

Two types of oxalate can be found inside body. One is exogenous (coming from dietary sources) and the other is endogenous (made from ascorbic acid or from glycine). A metabolic, genetically determined disorder occurs in which much larger amounts of

endogenous oxalate occur producing renal zones and deposits of calcium oxalate in tissues. Studies of plants with urinary stones show that most excrete normal quantities of oxalic acid; some increase may be noticed when urine also contains more calcium. The effect of extragenous oxalate in the formation of renal stones seems doubtful in most cases.

2.4.7 Oxalic poisoning.

2.4.7.1 Acute toxic effects.

In history there are many case reports about acute poisoning from ingestion of oxalate containing plant materials. Symptoms are said to be similar to those noted in human being ingesting pure oxalic acid. General symptoms are corrosive effects on mouth and internal tract, gastric haemorrhage, renal colic or hemacturia and sometimes convulsions. Several reports are available on acute poisoning of oxalic acid following the ingestion of rhubarb; a case report published in journal of the American Medical Association in 1919 takes a case of abortion of 6 weeks old foetus after development of cramp like abdominal pain shock vomiting of brownish fluid following the ingestion of rhubarb containing diet. A more recent case is reported by Tallquist and Vananen of the death of a child from oxalic acid poisoning caused by eating rhubarb (Rheum rhaponticum L.) stalks with leaves (Shaw and Wilson,1981).

An increase in blood potassium concentration has been noted and at the autopsy the patient has shown the typical picture of a liver nephrosis but no oxalic has been found. These cases have generally been named as "Rhubarb poisoning" in which case the actual toxicant is oxalic acid.Although some authors have expressed doubt as to the amount of these materials ingested at a time enough to cause fatal conditions in human beings, there are many more incidence of poisoning recorded in history (Hodgkinson,1977).

2.4.7.2 Chronic effect

Chronic effects are the effects that are developing over a long period of time. One of the chronic effect of oxalic acid containing foods are reducing the absorption of calcium

irons. The same problem is raised with phosphate and phytate containing foods.The most important disease conditions that are caused due to chronic oxalate poisoning are triggering affections of the bone system (stunted growth in growing individuals, osteodystrophia, fibrosa, osteoporosis); of kidneys (chronic nephritis uraemia caused by blockage of tubules with calcium oxalate, urethral and bladder stones (urinary calculi); disorders of central nervous system caused by deposition of calcium oxalate on it; rheumatic disorders (Hodgkinson,1977).

2.4.8 Prevention of Oxalate poisoning.

Oxalate is a common constituent of many plants including food plants. Quite a variety of food plant spinach, rhubarb, celery, tomatoes, parsley, beetroot, cashew nuts, almonds, tea, cocoa contain appreciable amounts of oxalate which are probably increased significantly by the injudicious use of nitrogen fertilizers in order to minimize the possible poisoning precautions can be followed.

In the species of plant, different varieties contain different amounts of oxalic acid. Varieties with less content of oxalate can be used for consumption. Injudicious application of nitrogenous fertilizers could be prevented. Even in the same cultivar different plant parts may contain various amounts of oxalic acid. Therefore the parts with high acid content can be avoided. Since young individuals are more prone to the problem of poisoning, fair attention to be focused on them.

Avoidance of ingestion of large amounts of oxalic acid containing foods with in a short period of time, is a very important measure. Always a mixed diet to be followed. The damage of chronic oxalate poisoning in human, especially in children, should be given due consideration as a large number of foods commonly eaten contain significant amounts of oxalate. Formation of urinary calculi is aggravated by high concentration of fluoride in water. Therefore individuals in areas where high fluoride water is present, should be more careful (Shaw and Wilson.1981).

CHAPTER 3

3. MATERIALS AND METHODS.

3.1 Sample collection

Ten varieties of leafy vegetables were grown on plots by giving same environmental and soil conditions. Random plots of leafy vegetables were prepared according to the statistical procedure by using random numbers (Appendix 1). Edible portion of each green leafy vegetables, in their raw, uncooked state were analyzed using five replicates for their beta-carotene, Iron and oxalic acid content and mean were calculated. The food samples were washed throughly with distilled water and dried between filter paper before analysis. Analitycal grade chemicals were used in all experiments. Statistical analysis was done using one-way ANOVA and T-Test.

3.2 Beta-carotene estimation

3.2.1 Materials

3.2.1.1 Reagents

- Acetone
- Petroleum ether (b.p. 65 – 70 °C)
- Anhydrous sodium sulphate (Na_2SO_4), granular
- Adsorbent : One part by weight of magnesium oxide (MgO) was mixed intimately with three parts of supercel.
- Eluent : 3% acetone in petroleum ether.
- potassium dichromate ($K_2Cr_2O_7$)

3.2.1.2 Equipments

- Chromotographic column : Absorbent tube with 250mm length and 19mm inside diameter was taken which was made out of borosilicate. This was constructed at one end to attached 3mm glass tubing and was taken to pack the Chromotographic column.

- Suction pump : D 21 CA Electronic suction pump was used to make a pressure in order to generate a vaccum in the column.
- Spectrophotometer : Milton Roy Spectronic 21 D UV visible Spectrophotometer was taken to check the absorbency. Samples were checked at 452nm wave length.
- Cuvette : 1cm inside diameter glass cuvettes were used in spectrophotometric analysis to record the absorbence.
- Miscellaneous items : Mortar and pestle, glass funnels, seperating funnels, conical flask were used in the extraction procedure. Buchner flask, Plunger, Rubber bushes, Rubber tubes, Pipette, volumetric flask were used.

3.2.2 Method

Beta-carotene content of leafy vegetables were analyzed using open-column chromotography, following the proceduer as described by Ranganna (1986).

3.2.2.1 Sample preparation

All the green leafy vegetables were cleaned under running tap water and then washed with distilled water. Fresh edible portion was used for the estimation of beta-carotene. Before the extraction, the plant material was cut into small pieces to facilitate complete extraction. A representative sample was weighed (2 to 5g).

3.2.2.2 Extraction

Sample was ground in a pestle and mortar with acetone. Pure sand was used, to assist grinding. After 2 or 3 extractions, the residue was formed a resinous mass and cannot be ground. In such cases, 1 or 2 ml of distilled water was added to enables further grinding. Extract was filtered through a wad of cotton into a conical flask. Continue extraction and filtration till the residue was colourless. The filtrate was transfered to a separating funnel 10 to 15ml of petroleum ether was added. The pigments were transferred into the petroleum ether phase by diluting the acetone with distilled water or water containing 5% sodium sulphate. The extraction of the acetone phase was repeated with small volumes of petroleum ether, until no more colour was extracted. Petroleum ether extract was filtered

through anhydrous Na_2SO_4 . Petroleum ether extract was concentrated and was made upto a known volume (25ml) (Ranganna,1986).

3.2.2.3 Chromatographic separation

3.2.2.3.1 Preparation of column

Adsorption tube was attached to a buchner flask and vaccum was applied and enough adsorbent was added to make the column 2 – 2.5cm in length. The adsorbent was pressed down once or twice with a plunger The surface of the adsorbent around the edges was loosened with a thin edged spatula.More adsorbent was added and repeated the steps until the column was approximately 10cm in length. 1cm of Na_2SO_4 was placed over the top of the column (Ranganna,1986).

3.2.2.3.2 Adsorption and elution

The column was wetted by washing with 25 to 50ml of petroleum ether. While the last ml of the petroleum ether was stilled above the Na_2SO_4 ,the vaccum was disconnected and the adsorption column was transferred to a clean dry buchner flask. An aliquot (5 to 10) was pipette out of the extract, and was put onto the column and suction was applied. The column was washed continuously with eluent. Successive portion of the eluent was added, when the preceding one was just barely visible above the Na_2SO_4. Beta-carotene was moved off the column prior to all other pigments. Washing was continued until the beta-carotene pigments were moved off the column and the eluent was get colourless. The contents of the flask was transferred to a volumetric flask and dilute to volume with eluent. The intensity of the colour was measured at 452nm using 3% acetone in petroleum ether as a blank. The concentration of beta-carotene was read in µg per ml in the solution from the standard curve (Ranganna,1986).

3.2.2.4 Calculation

$$\mu g \text{ of beta-carotene per } 100g = \frac{\text{Concentration of beta-carotene in solution as read from standard curve } (\mu g/ml) \times \text{Final volume} \times \text{dilution} \times 100}{\text{Weight of the sample.}}$$

The concentration of beta-carotene was determined by reading the concentration from the standard curve and was multiplied by a factor of 0.006 (Seaber, 1940).

3.2.2.5 Standard curve.

2.5g of potassium dichromate ($K_2Cr_2O_7$) was weighed accurately, and dissolved in 500ml of distilled water. 1,2,3,4,5 and 6ml of this stock solution was pipette out to separate 100ml volumetric flasks, and diluted with distilled water to the mark. The concentrations of the samples were 50,100,150,200,250,300 and 350 µg per ml. Colour intensity was measured at 452nm. Distilled water was used as blank sample. The graph was plotted, absorbance against concentration.

3.3 Iron estimation

3.3.1 Materials

3.3.1.1 Reagents

- Concentrated sulphuric acid
- Concentrated nitric acid
- 1,10 – phenanthroline solution : 0.1g of phenanthroline monohydrate was dissolved in 100ml of distilled water, warming to effect solution if necessary. The solution was stored in a plastic bottle.
- Hydroxylammonium chloride solution : 10g of Hydroxylammonium chloride was dissolved in 100ml of distilled water.
- Sodium acetate solution : 10g of sodium acetate was dissolved in 100ml of distilled water.
- Standard Iron (II) solution : 0.0702g ferrous ammonium sulphate , $Fe(NH_4)_2(SO_4)_2.6H_2O$ was weighed to prepared a standard iron (II) solution. Weighted sample was quantitatively transferred to a 1L volumetric flask and sufficient water was added to dissolved the salt. 2.5ml of conc. Sulphuric acid was added, dilute exactly to the mark with distilled water, and mixed thoroughly. This solution was contained 10.0mg iron per liter (10ppm).

3.3.1.2 Equipments

- Electrical burner : was used to burn volatile compound of the organic matter contain in green leaves.
- Oven : Memmert type oven was maintained at 105^{o}C to find moisture content of the leaves.
- Muffle furnance : Model MR – 120 moving coil (0-1200 ^{o}C) muffle furner was maintained 450-500 ^{o}C to ashed the leafy vegetables.
- Spectrophotometer : Milton Roy Spectronic 21 D UV visible Spectrophotometer was taken to check the absorbency. Samples were checked at 510nm wave length.
- Cuvette : 1cm inside diameter glass cuvettes were used in spectrophotometric analysis to record the absorbence.
- Miscellaneous items : Silica dishes, watch glass, pipettes No.44 Whatman filter papers, volumetric flask.

3.3.2 Method

Iron content of leafy vegetables were analyzed using spectrophotometry following the procedure as described by Ranganna (1986) and AOAC (1984) methods.

3.3.2.1 Sample preparation

All the green leafy vegetables were cleaned under running tap water and then washed with distilled water. Fresh edible portion was used for the estimation of iron. The sample that used for the determination of moisture was taken to ashing. Before ashing, the plant material was cut into small pieces and suitable quantity (10g) of the well mixed sample was weighed acurately into a porcelain dish.

3.3.2.2 Dry Ashing

Sample was heated first on a electrical burner to volatalize as much of the organic matter (until no more of smoke was given out by the material). Then the dish was transferred to a temperature controlled muffle furnace. Ashing was done at 450^{o}C. The time required at this temperature wasl depend on the nature of the material to be ashed. Most of

vegetables or their products were required generally 5 to 7 hr to ash. Sample was removed from the muffle furnace, allowed to cool and note the weight of the ash. The process was repeated until it was given constant weight, to the ash. Then the dish was covered with watch glass and 5ml of dilute HCl (10% HCl) was added gently with the help of a pipette. The watch glass was used to prevent spattering. The dish was heated over a water bath for 30min, remove the cover and rinse. No.44 Whatman filter paper was used to filter ash solution into a 50ml volumetric flask. The residue was washed in the dish once or twice using dilute HCl. The residue on the filter paper was washed with HCl and Made up to volume with water (Ranganna,1986).

3.3.2.3 Spectrophotometric determination

This solution was transferred to a 25ml volumetric flask. 1ml of the hydroxylammonium chloride solution, 5ml of the 1,10-phenanthroline solution and 8ml of the sodium acetate solution were added to produce the red colour of the sample solution prepared. Distilled water was added up to mark. After adding the reagents it was allowed at least 25 minutes before making absorbance measurements, so that the colour of the complex can fully developed. Once developed, the colour was stable for hours. Then the absorbance was measured at 510nm. The concentration of Iron was read by using a standand curve.

3.3.2.4 Calculation

See Appendix 2 for calculation

3.3.2.5 Standard curve

1.0, 2.0, 5.0, 10.0,15.0, 20.0 and 25.0 portions of Standard Iron (II) solution was pipette out into separate 100ml volumetric flasks. To each flasks 1ml of Hydroxylammonium chloride solution , 5ml of the 1,10 – phenanthroline solution and 8ml of the Sodium acetate solution were added. All the solutions were diluted to the 100mlmark and allowed them to stand for 10 minutes. A blank solution was prepared by the addition of all the above solutions except iron (II) solution to distilled water. The absorbance was measured of each of these solutions at 510nm against the blank prepared. Absorbance Vs. concentration of the standards was plotted in a graph.

3.4 Oxalic acid estimation

3.4.1 Materials

3.4.1.1 Reagents

- 0.01N Potassium permanganate solution : 0.35g of potassium permanganate was dissolved in 1L of hot distilled water.
- 0.01N Standard oxalic acid solution : 0.63g of oxalic acid was accurately and dissolved in 1L of distilled water.
- 5N sulphuric acid : 136ml of conc. Sulphuric acid was diluted upto 1L by adding distilled water.
- Saturated calcium chloride solution : Calsium chloride was added to minimum amount of water until it get saturated.
- Buffer solutions : (1) 0.75M Ammonium hydroxide
  (2) Acetic acid solution.

3.4.1.2 Equipments

- High speed blender : Sophia 2001type blender was used to extract the oxalic acid content in green leaves to distilled water.
- Centrifuge : Jenway model centrifuge was used to collect the precipitate of calcium oxalate.
- Oven : Memmert oven was used to maintained constant temperature at 60^{o}C.
- Miscellaneous items : Volumetric flasks, conical flask, Muslin cloth, filter papers, beakers are require for extraction process.

3.4.2 Method

The AOAC (1984) method adopted for the determination of Oxalic acid. It was unable to be directly applied for the determination of Oxalic acid as some of the analytical reagents were not available. Therefore it was simplified and adopted for the purpose with several slight alterations.

3.4.2.1 Sample preparation

All the green leafy vegetables were cleaned under running tap water and then washed with distilled water. Fresh edible portion was used for the estimation of oxalic acid. Before the extraction, the plant material was cut into small pieces to facilitate complete extraction. Nearly 20g of leafy vegetable sample was taken, weighed out accurately.

3.4.2.2 Extraction

The sample was mixed in a high speed blender with nearly 100ml of 3N HCl and blended for 1minute; cooled to room temperature. The well homogenized mixture was filtered through Muslin cloth and the filtrate was transferred into a 200ml volumetric flask and made up to the mark. 30ml aliquots were taken from the sample extract into 100ml conical flask and 10ml of saturated Calcium chloride solution was added. Mixture was well shaked, resulted solution was adjusted to pH 7.0 with buffer solution by using a pH meter and kept at 60^{o}C for over night in a hot air oven. Then the mixture was centrifuge at 1700rpm for 30 minutes to compact the precipitate and the supernatant solution was decanted cautiously with one smooth inversion of the centrifuge upside down in to a filter paper on a funnel.

The precipitate was transferred into clear beaker. 10ml of concentrated H_2SO_4 (1+9) was added to dissolve calcium oxalate and left for 15min followed by filtration through a No.1 Whatman filter paper. The precipitate was washed thoroughly with distilled water and the filtrate was collected into 200ml conical flask. (AOAC,1984 and Wilson et al., 1982).

3.4.2.3 Standardization of $KMnO_4$ solution

Prepared $KMnO_4$ solution was filled into a burette, as usual after rinsing it. The zero mark with upper miniscus was adjusted. 20ml of standard Oxalic acid was pipetted out and transfer inner clean beaker. About 10 to 15ml of diluteH_2SO_4 solution was added. The content was heated to about 60 to 80°C (do not allow to boil because oxalic acid decomposed on boiling). The content was titrated while hot carefully with $KMnO_4$

solution was added drop by drop. Each successive drop was added only after the colour caused by the previous drop was disappeared. Titration was continued until faint pink end point was reached (colour persists for 1 to 2 min). Burette readings (B.R) was recorded and the repeated experiment until two concordant reading were obtained (Gupta et al.,1993).

$$\text{Normality of } KMnO_4 = \frac{\text{Normality of Oxalic acid} \times \text{Volume of Oxalic acid}}{\text{Volume of } KMnO_4 \text{ (B.R)}}$$

$$\text{Strength } (KMnO_4 \text{ g/lit}) = \text{Normality of } KMnO_4 \times \text{Equivalent weight of } KMnO_4$$

3.4.2.4 Titration

The filtrate was taken for the titration process. The conical flask with filtrate was heated in a water bath and the hot content (60-80^{O}C) was titrated against 0.01N standardize $KMnO_4$ solution from a burette while shaking the flask smoothly. The end point was reached when the pink colour of permanganate persisted for 30 seconds. The burette reading was recorded.

3.4.2.5 Calculation

$$\text{Oxalic acid percentage (100g of edible portion)} = \frac{N \times V \times 0.06303 \times 100}{W}$$

where,

N= Normality of potassium permanganate solution.

V= Volume of potassium permanganate solution (B.R.)

W= Weight of the sample

0.06303= conversion factor for Oxalic acid (M.W. = 126)

CHAPTER 4

4. RESULTS AND DISCUSSION

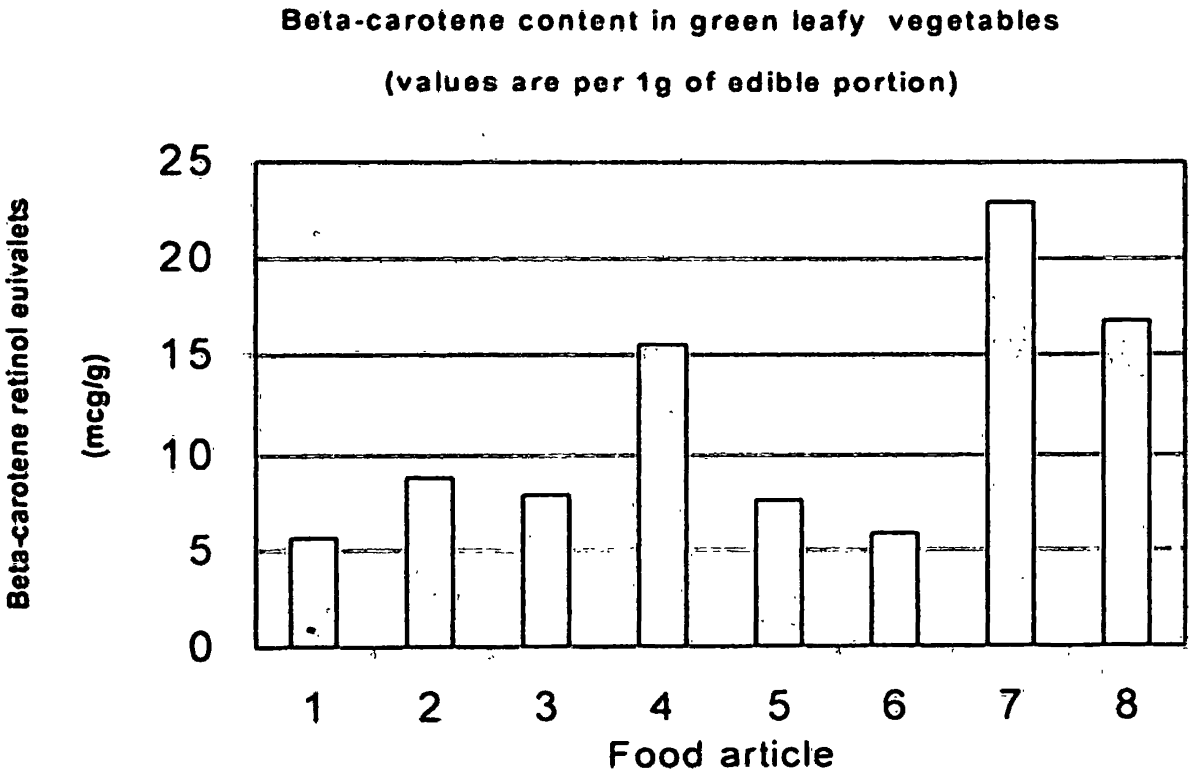
4.1 Results for the Beta-caratene analysis of selected leafy vegetables

Table 4.1 Beta-carotene content in green leafy vegetables

(Values are per 1g of edible portion)

Food Article	Beta-carotene Retinol equivalents (μ g/g)	RDA* (g)
1. Pochchi Gotukoka (*Hydrocotyle javanica*)	5.66	132.5
2. Hin-Gotukola (*Centella asiatica*)	8.83	84.9
3. Mukunuwenna (*Alternanthera sessilis*)	7.89	95.1
4. Kura thampala (*Amaranthus viridis*)	15.55	48.2
5. Sarana (*Trianthema decandra*)	7.60	98.7
6. Nivithi (*Basella alba*)	5.83	128.6
7. Kathurumurunga (*Sesbania grandiflora*)	22.94	32.7
8. Murunga (*Moringa oleifera*)	16.79	44.7

RDA – Recommended Daily Allowance



1 - Pochchi Gotukola
2 - Hin-Gotukola
3 – Mukunuwenna
4 - Kura thampala
5 – Sarana
6 - Nivithi
7 – Kathurumurunga
8 - Murunga

Fig. 4.1 Beta-carotene content of selected leafy vegetables

Table 4.2 Readings of the beta-carotene standard curve

Sample No.	1	2	3	4	5	6	7
Concentration (µg/ml)	50	100	150	200	250	300	350
Absorbance	0.063	0.135	0.195	0.276	0.341	0.410	0.476

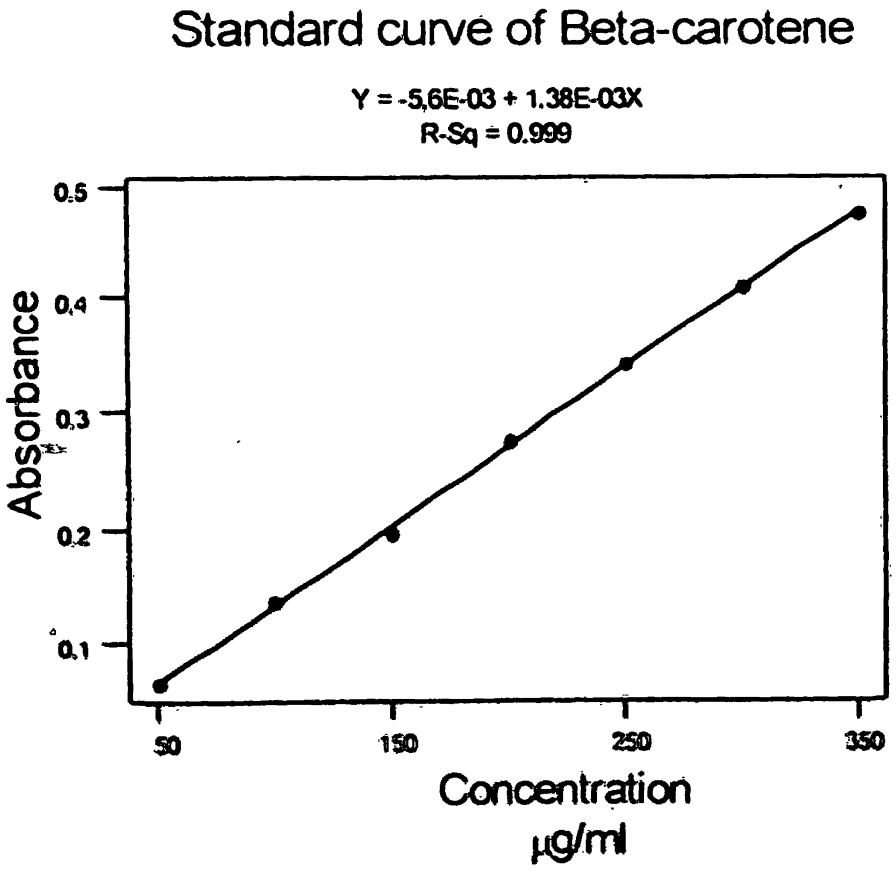


Fig. 4.2 Standard curve for beta-carotene estimation.

Many analytical methods for carotenoids were aimed at measurement of the provitamin A carotenoids for determination of their nutritional value. In my study, Open–column chromatography on MgO-huflo Super Cell (3:1 by weight) was the method used for investigating carotinoids.

The carotinoids were eluted with solvents of increasing polarity. Because most carotinoid extracts consist of a mixture of nonpolar carotenes and more polar xanthophylls, a carotene fraction elutes early from the column in a chromatographic elution. The major concern about using the AOAC procedure was its failure to distinguish between coeluting carotenes; no separation occurs between alpha and beta carotene as well as other hydrocarbon carotenoids. The method also calculates all carotenes as beta carotene, which possesses the highest provitamin A activity of all the carotenes. Thus, if the provitamin A activity was to be determined, the results for vitamin A content could be severely overestimated.

The carotene present in the extraction, can be largely destroyed by photochemical oxidation and the presence of chlorophyll makes carotene even more light sensitive. Therefore neither the initial extraction nor the chromatographic separation should be carried out in direct sunlight. Excessive aeration during extraction and subsequent washing of the extracts should be avoided. The extract should be stored in the refrigerator when not being used. Also chromatographic separation and spectrophotometric determination of carotene should be carried out as soon as possible.

The carotenoids are unstable to many processing procedures because of their conjugated systems of double bonds. Drying and extraction cooking are particularly destructive processing steps which promote oxidation. The loss in carotene content in dehydrated products may be attributed to oxidative degradation of beta-carotene through a free radical process, especially when the samples are stored prior to analysis.

The carotenoids from leafy vegetables were expressed as µg of beta, carotinoids per gram of the edible portion (Table 4.1 & Fig. 4.1). These were then collectively expressed in terms of µg of retinol equivalents per gram of the edible portion (Table 4.1). The previously published results of carotenoids in local foods were those for beta-carotene content and could not be expressed method available retinol equivalent of the food (Atukorala,1985). Table 4.1 also describe the amounts of green leafy vegetables that should be eaten to provide the daily requirement of vitamin A for an adult (Gordon and Paul,1993).

According to the results table 4.1, the SAS system was given the probability value of variance as 0.0001 (Appendix 3). It was less than 0.05 at 5% level of significant. As a result null hypothesis was rejected and alternative hypothesis was accepted. i.e. the beta-carotene levels each and every variety of leafy vegetable was different from each other. To obtain the most significant different leafy vegetable variety, the data was further analyzed according Least significant Difference (LSD) method.

According to LSD (Appendix 4), highest significant different mean value 22.94 was obtained to Kathurumurunga. i.e. beta carotene retinol equivalent highest in Kathurumurunga. According to the results Kura Thampala and Murunga have 16.79µg/g and 15.55µg/g of beta carotene retinol equivalent respectively. Consuming even small quantities of these Kathurumurunga, Kura Thampala and Murunga can obtain the recommended dietary allowances very easily. Pochchi gotukola had the lowest (5.66µg/g), with moderate values ranging from 8.83µg/g to 5.83µg/g in Hin-gotukola, Mukunuwenna, Sarana and Nivithi leaves.

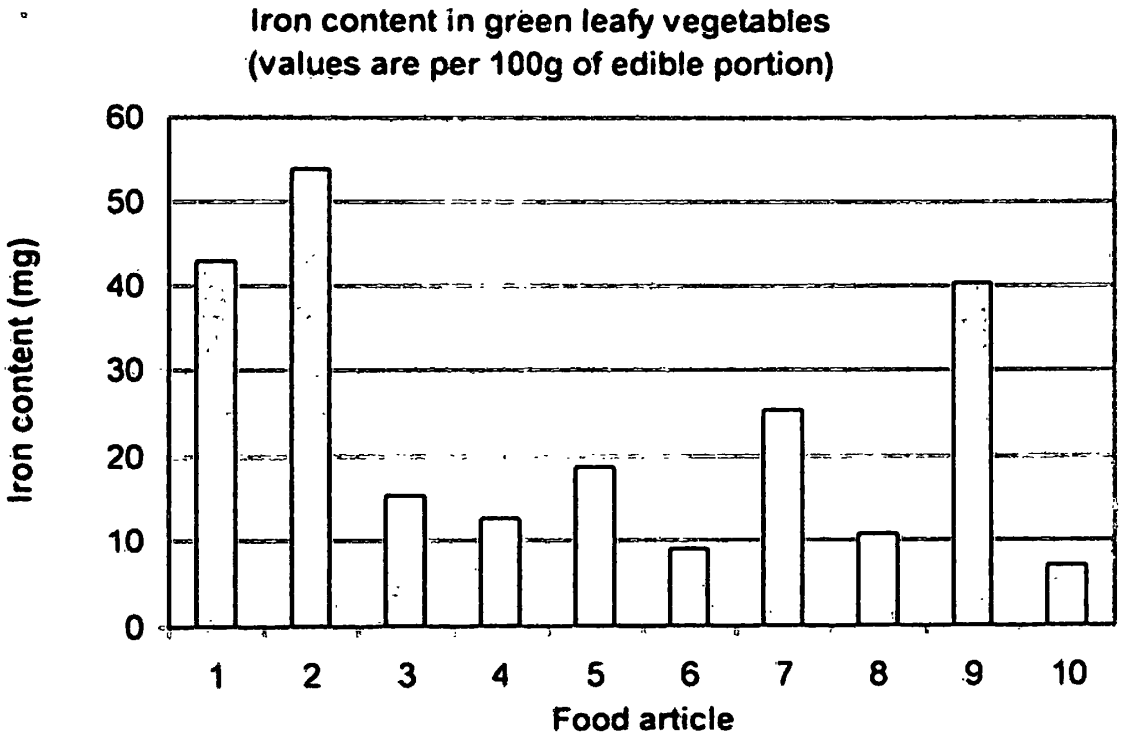
According to the results of the T-test that used to find out which Gotukola variety has highest amount of beta-carotene. It was given the proberbility value of varience as 0.0000 (Appendix 4). It was less than 0.05 at 5% level of significant. As a result null hypothesis was rejected and alternative hypothesis was accepted. i.e. the variety of Hin-gotukola beta-carotene amount was higher than variety of Pochchi gotukola.

4.2 Results for the Iron analysis of selected leafy vegetables

Table 4.3 Iron content in green leafy vegetables
(Values are per 100g of edible portion)

Food Article	Iron (mg)	RDA* (g)
1. Pochchi Gotukoka (*Hydrocotyle javanica*)	42.95	23.3
2. Hin-Gotukola (*Centella asiatica*)	53.98	18.5
3. Mukunuwenna (*Alternanthera sessilis*)	15.38	65.0
4. Kura thampala (*Amaranthus viridis*)	12.57	79.6
5. Sarana (*Trianthema decandra*)	18.72	53.4
6. Nivithi (stem) (*Basella alba*)	9.04	110.6
7. Nivithi (leaves) (*Basella alba*)	25.27	39.6
8. Weda mukunuwenna (*Alternanthera* spp.)	10.78	92.8
9. Rathu thampala (*Amaranthus spinosus*)	40.43	24.7
10. Kankun (*Ipomoea aquatica*)	6.96	143.7

RDA* – Recommended Daily Allowance



1 - Pochchi Gotukola
2 - Hin-Gotukola
3 - Mukunuwenna
4 - Kura thampala
5 - Sarana
6 - Nivithi (Stem)
7 - Nivithi (Leaves)
8 - Weda mukunuwenna
9 - Rathu thampala
10 - Kankun

Fig. 4.3 Iron content of selected leafy vegetables

Table 4.4 Readings of the Iron standard curve

Sample No.	1	2	3	4	5	6	7
Concentration (mg/ l)	0.1	0.2	0.5	1.0	1.5	2.0	2.5
Absorbance	0.015	0.024	0.065	0.128	0.191	0.225	0.361

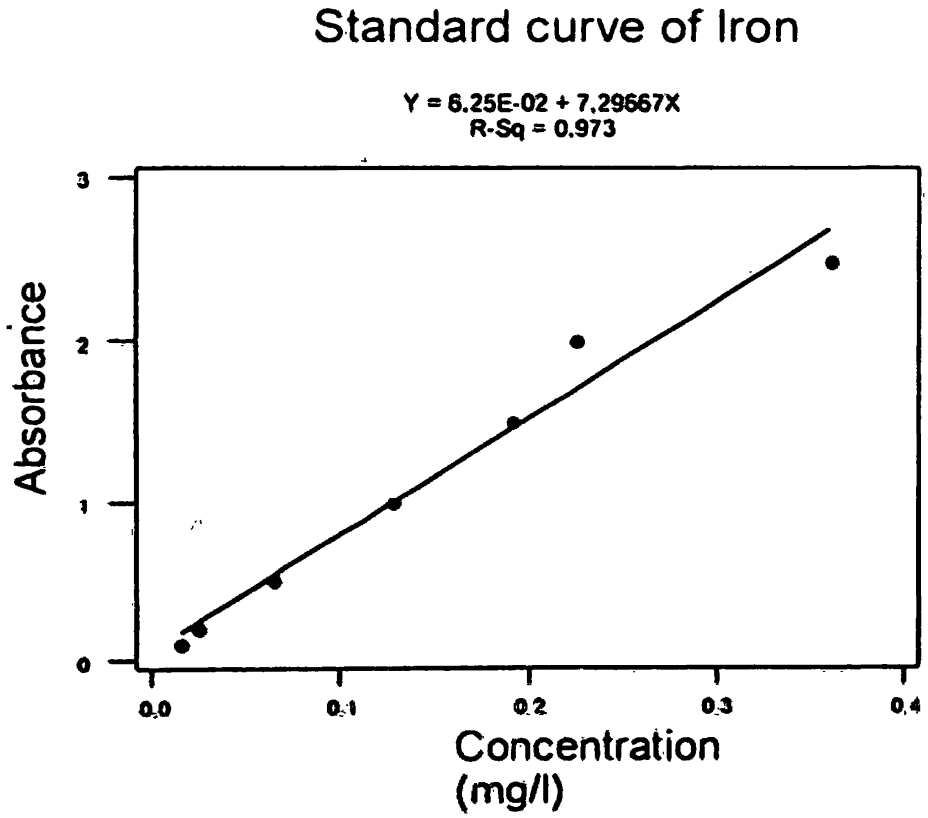
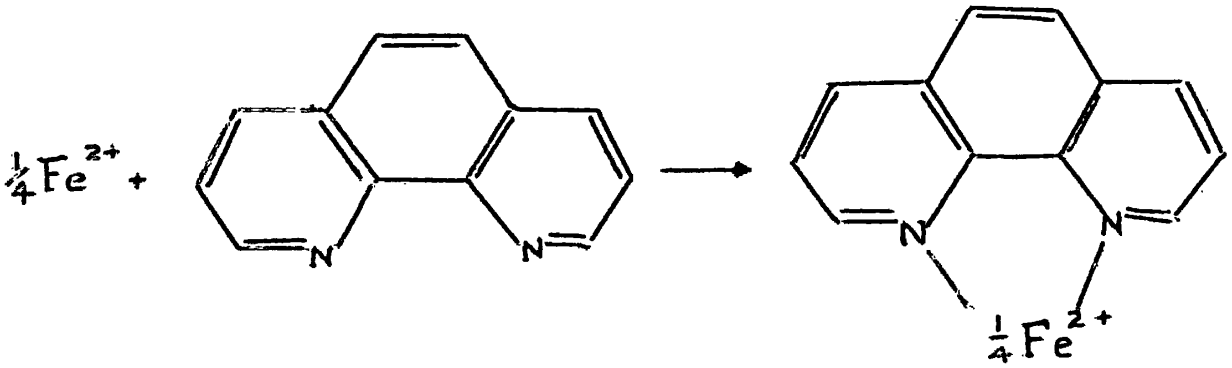


Fig. 4.4 Standard curve for Iron estimation

Organic matter present in leafy vegetables must be destroyed prior to the estimation of iron in the leafy vegetables. Dry ashing was carried out to destruct the organic matter in the leafy vegetable sample. It is more convenient than wet ashing because large amount of matter can be handled. Iron in digested sample was dertermined by using the red complex $Fe(C_{12}H_8N_2)_3^{2+}$ formed between ferrous iron and 1,10-phenanthroline and the absorbance of this coloured solution was measured with a spectrophotometer.

$$4Fe^{2+} + 2NH_2OH + 2OH^- \longrightarrow 4Fe^{2+} + N_2 + 4H_2O$$



1,10- phenanthroline tris (1,10- phenanthroline) iron (II)

The iron must be present in the ferrous state. Hence the reducing agent was added to convert iron (III) to iron (II) before the colour was developed. Hydroxyl ammonium chloride was added for this purpose.

$$2Fe^{3+} + 2NH_2OH + 2OH^- \rightleftharpoons 4Fe^{2+} + N_2 + 4H_2O$$

To maintain the maximum colour development pH was adjusted to a value between 6 to 9 by the addition of sodium acetate. The temperature of the muffle furner was difficult to maintain at constant level at all the time. As a result change in weight after ashing was cause some errors.

According to the result table 4.3 the SAS system was given, the probability value of analysis of variance as 0.0001 (Appendix 5). It was less than 0.05 at 5% level of significant. As a result null hypothesis was rejected and alternative hypothesis was accepted. i.e. the iron content of each and every variety of leafy vegetable was different

from each other. To obtain the most significant different leafy vegetable the data was further analyzed according to LSD (Appendix 5).

The highest mean value was 53.98 and it was given the variety of Hin-gotukola. i.e. the highest content of iron was found in Hin-gotukola (53.98mg/100g). According to the result rathu thampala and pochchi gotukola also containing 40.43mg and 42.95mg of iron in 100g of edible portion. Consumption of even small quantities of these leafy vegetables specially Hin-gotukola, Pochchi gotukola and Rathu thampala, can get the recommended daily allowance of iron easily. For example 18.5g of Hin-gotukola fulfill the RDA of iron in adult male (Table 4.3).

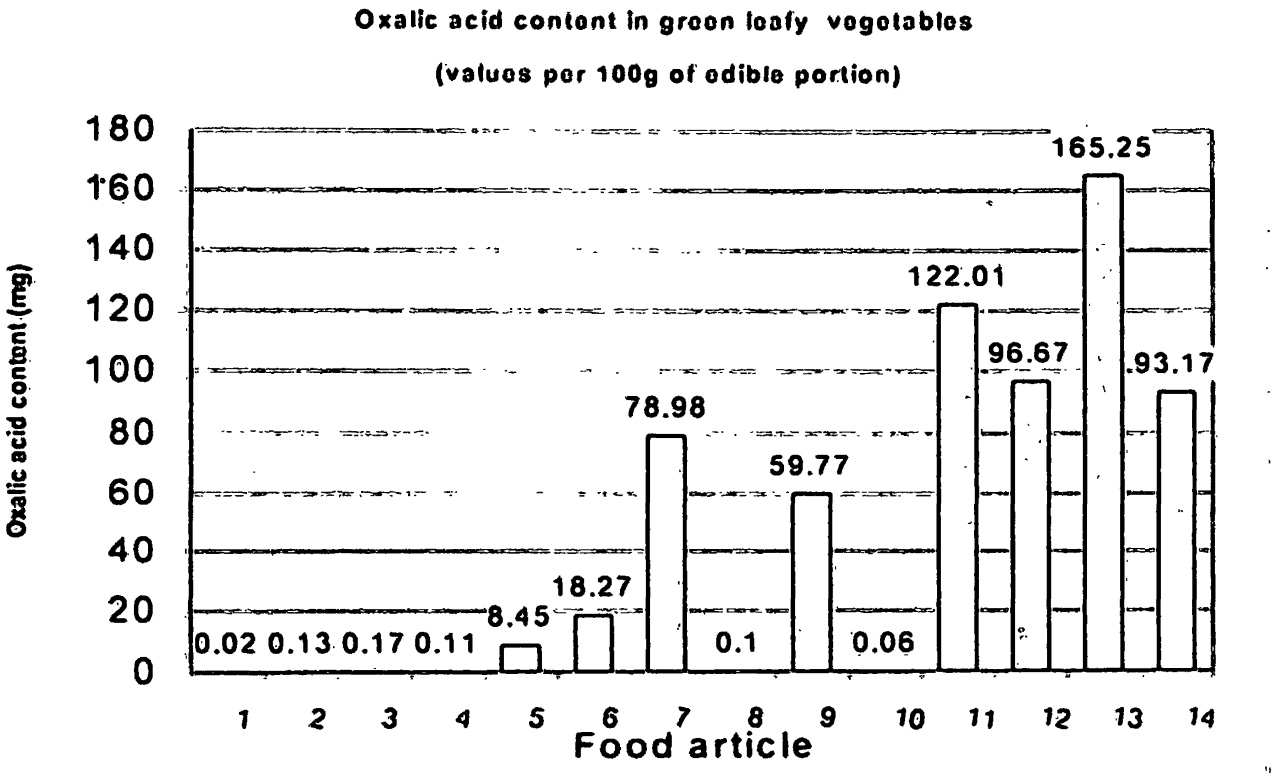
Kankun had the lowest amount of iron (6.96mg /100g) and moderate values ranging from 9.04mg/100g to 25.27mg/100mg in Nivithi stems and leaves, Kura-thampala, Weda-mukunuwenna, Mukunuwenna, and Sarana. According to the result of the T-test that carried on to find Iron contents comparing two varieties was given overall probability value as 0.5847,0.5450 and 0.8065 (Appendix 5 & 6) for Mukunuwanna, Thampala and Nivithi. It was higher than 0.05 at 5% significant level. As a result null hypothesis was not rejected. So equal probability value was accepted. It was less than 0.05 at 5% significant. i.e. Mukunuwenna, Rathu-thampala and Nivithi leaves had high amount of Iron than Wedamukunuwenna, Kura-thampala and Nivithi stem respectively.

Result of the overall probability of two Gotukola varieties (0.0072) (Appendix 6) were less than 0.05 at 5% level of significant and reject null hypothesis. Accepted unequal probability value 0.0001. It also less than 0.05 at 5% level of significant. Reject null hypothesis and accepted alternative hypothesis. So the result was high Iron content given by variety of Hin-gotukola than Pochchi-gotukola.

4.3 Results for the Oxalic acid analysis of selected leafy vegetables

Table 4.5 Oxalic acid content in green leafy vegetables (Values are per 100g of edible portion)

Food Article	Oxalic acid(mg)
1. Pochchi Gotukoka (*Hydrocotyle javanica*)	0.02
2 Mukunuwenna (*Alternanthera sessilis*)	0.13
3. Kura thampala (*Amaranthus viridis*)	0.17
4. Sarana (*Trianthema decandra*)	0.11
5. Nivithi (stem) (*Basella alba*)	8.45
6. Nivithi (tender leaves) (*Basella alba*)	18.27
7. Nivithi (mature leaves) (*Basella alba*)	78.98
8. Weda mukunuwenna (*Alternanthera* spp.)	0.10
9. Red thampala (*Amaranthus spinosus*)	59.77
10. Kankun (*Ipomoea aquatica*)	0.06
11. Kathurumurunga (mature leaves) (*Sesbania grandiflora*)	122.01
12. Kathurumurunga (tender leaves) (*Sesbania grandiflora*)	96.67
13. Murunga (mature leaves) (*Moringa oleifera*)	165.25
14. Murunga (tender leaves) (*Moringa oleifera*)	93.17



1 - Pochchi Gotukola
2 - Mukunuwenna
3 - Kura thampala
4 - Sarana
5 - Nivithi (Stem)
6 - Nivithi leaves (tender)
7 - Nivithi leaves (mature)
8 - Weda mukunuwenna
9 - Rathu thampala
10 - Kankun
11- Kathurumurunga leaves (mature)
12 - Kathurumurunga leaves (tender)
13- Murunga leaves (mature)
14 - Murunga leaves (tender)

Fig. 4.5 Oxalic acid content of selected leafy vegetables

Plant material contains the water-soluble and insoluble oxalates. Some of the important water-soluble oxalates are the ammonium, sodium and potassium oxalates, where as calcium oxalate represents as the main fraction of insoluble oxalate.

Several lengthy methods for determining oxalic acid in foods have been reported. Extraction of oxalic acid from the plant material with hydrochloric acid (HCl) or sodium carbonate and overnight precipitation of calcium oxalate, followed by treatment of the precipitate with dilute sulfuric acid to form a solution of oxalic acid. The oxalic acid was then quantified by titration with standard potassium permanganate (Baker,1952) or colourimetric method (Zarembski et al.,1962). And some methods proposed by various researchers: e.g. High Performance Liquid Chromatography method by Wilson W.C. et al.,(1982) avoids the classical lengthy procedures of testing ; Micro determination of oxalate through enzymatic methods proposed by Turner N.A. (1980) required sophisticated equipment and expensive chemicals.

The AOAC-official methods of analysis (AOAC,1984) happens to be the most readily available technique for oxalate analysis in foods in most laboratories of developing countries, it required some scarce analytical reagents. So this was the chief problem encountered in the course of determination of oxalic acid content in leafy vegetables.

Oxalic acid was extracted by HCl, therefore careful adjustment of the extract above pH 3.0 was necessary to precipitation of calcium oxalate. Calcium oxalate solubility decreases rapidly above pH 3.5 and at is almost insoluble at pH 4.0. So when done overnight precipitation pH should be adjusted at 7.0.

Quantitative recovery of oxalic acid from non-acidic foods such as leafy vegetables probably requires extraction with at least 3N HCl as noted by Zarembski and Hodgkinson (1962). Acidic food products, such as fruits, probably do not require extraction with HCl for quantitative recovery of oxalic acid. The problem associated with this methods are those of loss during precipitation, low sensitivity and an indistinct end-point through the presence of co-precipitated organic materials such as pectins.

According to the results table 4.5 (& Fig.4.5) the SAS system was given the probability value of analysis of variance as 0.0001 (Appendix 7). It is less than 0.05 at 5% level of significant as a result, null hypothesis was rejected and the alternative hypothesis was accepted, i.e. the Oxalic acid content of each and every variety of leafy vegetable was different from each other. To obtain the most significant different leafy vegetable variety,

the data was further analyzed according to LSD. According to LSD highest mean value was 165.25 and it gave the variety Murunga. i.e. highest oxalic acid content exist in Murunga leaves.

According to the result Pochchi-gotukola (0.02mg/100g) has the lowest content of oxalic acid. Kura-thampala, Sarana, Kankun ,Mukunuwenna, Weda-mukunuwenna also contained low level of oxalic acid content. Kathurumurunga, Rathu-thampala, Nivithi was contained high level of oxalic acid ranging from 122.02 -78.98mg/100g.

To find the which maturity stage has high content of oxalic acid performed a T-test for mature and tender Kathurumurunga,Murunga as well as Nivithi leaves. According to that T-test result (Appendix 7 & 8) reject null hypothesis at 5% significant level and therefore accepted alternative one. So maturity stages of leafy vegetables contain high amount of oxalic acid content than tender stages.

The T-test carried out for the Nivithi stems and leaves the overall hypothesis 0.6920 (Appendix 8) greater than 0.05 at 5% significant level. As a result do not reject null hypothesis and consider equal probability value (0.0000). It was less than 0.05 at 5% significant level and null hypothesis was relected.i.e.Nivithi leaves has high oxalic acid content than Nivithi stem.

Micronutrient deficiencies occur in developing countries because of poor demand for and inadequate consumption of available foods. Cost was a prime constraint on the purchase of animal sources of vitamin A and Iron. But the leafy vegetables are inexpensive food items which was contained high amounts of above micronutrients. According to the results of the present study (Fig.4.1,4.3 and 4.5) has proved above statement.

The value that were calculated (Table 4.1,4.2 and 4.3) given slightly different from Sri Lanka Standard food composition table values. Colourimetry errors, volumetric errors, spectrophotometry and chromotography errors, the area from where the sample was obtained, can influence the final results. Therefore slight variation from actual value was inevitabled.

CHAPTER 5

5. CONCLUSIONS

It is believed that micronutrient such as beta-carotene , iron are rich in green leafy vegetables. The result of the present study conclude that all the green leafy vegetables do not contain higher amount of beta-carotene and iron.

The leaves of kathurumurunga, kura-thampala, murunga and hin-gotukola had highest retinol equivalent level of beta-carotene ranging from 22.94 – 8.83µg/g. All other leaves had low retinol equivalent levels ranging from 7.89 – 5.66µg/g. The recommended dietary allowance (RDA) of retinol for an adult male is 750µg (Gordon et al.,1993). This could be obtained by eating daily 50g of murunga leaves or kura-thampala or 95g of hin-gotukola or mukunuwenna. Pochchi-gotukola, sarana and nivithi leaves contained less retinol but could added variety to the total diet and some contribution to the daily requirement. The leaves of hin-gotukola, pochchi gotukola, rathu thampala and nivithi leaves had highest level of iron ranging from 53.98 – 25.27mg/100g. Mukunuwenna, weda- mukunuwenna, kura-thampala and sarana has moderate value range between 10.78-18.72mg/100g. All other leaves had low mg levels ranging 9.04 - 6.96mg/100g.
The RDA of iron for an adult male is 10mg per day (Gordon et al.,1993). This could be obtained by eating daily; 20g of hin-gotukola or 25g of pochchi-gotukola or rathu-thampala or 40g of nivithi leaves. Other varieties also contribute to overcome dietary deficiencies by consuming adequate amounts.

Oxalic acid content in green leafy vegetables also very much different. Murunga, kathurumurunga, nivithi ,rathu-thampala had highest amount of oxalic acid ranging between 165.25 – 59.77mg/100g. Levels of oxalic acid found in other varieties are ranging from 0.17-0.02mg/100g of edible portion.

When maturing green leaves oxalic acid content is increased. Tender leaves have low oxalic acid content than mature leaves. And also some leafy vegetables have much more nutritional benefits than what the people thought about it quality of nutrition and vise

versa. These low cost leafy vegetables varieties are nourishing and have a value similar to socially accepted food varieties.

5.1 Recommendations for further studies

Due to the limitation of time in here I studied only estimation of beta-carotene, iron and oxalic acid content in fresh edible portions of green leafy vegetables. But most varieties of green leafy vegetables we consumption after cooking. So it is important to studying what are the effects of cooking, on beta-carotene, iron and oxalic acid content in green leaves.

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Appendices

Appendix 1

Random Numbers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	10	09	73	25	33	76	52	01	35	86	34	67	35	48	76	80	95	90	91	17	39	29	27	49	45
2	37	54	20	48	05	64	89	47	42	96	24	80	52	40	37	20	63	61	04	02	00	82	29	16	65
3	08	42	26	89	53	19	64	50	93	03	23	20	90	25	60	15	95	33	47	64	35	08	03	36	06
4	99	01	90	25	29	09	37	67	07	15	38	31	13	11	65	88	67	67	43	97	04	43	62	76	59
5	12	80	79	99	70	80	15	73	61	47	64	03	23	66	53	98	95	11	68	77	12	17	17	68	33
6	66	06	57	47	17	34	07	27	68	50	36	69	73	61	70	65	81	33	98	85	11	19	92	91	70
7	31	06	01	08	05	45	57	18	24	06	35	30	34	26	14	86	79	90	74	39	23	40	30	97	32
8	85	26	97	76	02	02	05	16	56	92	68	66	57	48	18	73	05	38	52	47	18	62	38	85	79
9	63	57	33	21	35	05	32	54	70	48	90	55	35	75	48	28	46	82	87	09	83	49	12	56	24
10	73	79	64	57	53	03	52	96	47	78	35	80	83	42	82	60	93	52	03	44	35	27	38	84	35
11	98	52	01	77	67	14	90	56	86	07	22	10	94	05	58	60	97	09	34	33	50	50	07	39	98
12	11	80	50	54	31	39	80	82	77	32	50	72	56	82	48	29	40	52	42	01	52	77	56	78	51
13	83	45	29	96	34	06	28	89	80	83	13	74	67	00	78	18	47	54	06	10	68	71	17	78	17
14	88	68	54	02	00	86	50	75	84	01	36	76	66	79	51	90	36	47	64	93	29	60	91	10	62
15	99	59	46	73	48	87	51	76	49	69	91	82	60	89	28	93	78	56	13	68	23	47	83	41	13
16	65	48	11	76	74	17	46	85	09	50	58	04	77	69	74	73	03	95	71	86	40	21	81	65	44
17	80	12	43	56	35	17	72	70	80	15	45	31	82	23	74	21	11	57	82	53	14	38	55	37	63
18	74	35	09	98	17	77	40	27	72	14	43	23	60	02	10	45	52	16	42	37	96	28	60	26	55
19	69	91	62	68	03	66	25	22	91	48	36	93	68	72	03	76	62	11	39	90	94	40	05	64	18
20	09	89	32	05	05	14	22	56	85	14	46	42	75	67	88	96	29	77	88	22	54	38	21	45	98
21	91	49	91	45	23	68	47	92	76	86	46	16	28	35	54	91	75	08	99	23	37	08	92	00	48
22	80	33	69	45	98	26	94	03	68	58	70	29	73	41	35	53	14	03	33	40	42	05	08	23	41
23	44	10	48	19	49	85	15	74	79	54	32	97	92	65	75	57	60	04	08	81	22	22	20	64	13
24	12	55	07	37	42	11	10	80	75	60	43	62	29	56	62	99	64	43	94	39	38	70	72	58	15
25	63	60	64	93	29	16	50	53	44	66	02	21	36	98	25	99	65	17	70	82	07	20	73	17	90
26	61	19	69	04	46	26	45	74	77	74	51	92	43	37	29	65	39	45	95	93	42	58	26	05	27
27	15	47	44	52	66	95	27	07	99	19	94	48	31	59	66	81	39	61	01	18	33	21	15	94	66
28	94	55	72	85	73	67	89	75	43	87	54	62	73	11	79	91	19	01	25	92	92	92	74	59	73
29	42	48	11	62	13	97	34	40	87	21	16	86	84	87	67	03	07	11	20	59	25	70	14	66	70
30	23	52	37	83	17	73	20	88	39	97	79	07	15	06	74	29	25	22	96	63	05	52	28	25	62
31	04	49	35	24	94	75	24	63	38	24	45	86	25	10	25	61	96	27	93	35	65	33	71	24	72
32	00	54	99	76	54	64	05	18	81	90	96	38	96	23	70	84	69	28	23	91	23	28	72	95	29
33	35	96	31	53	07	26	89	80	93	54	33	35	05	96	62	77	97	45	00	24	90	10	33	93	33
34	59	80	80	83	91	45	42	72	68	42	83	60	94	97	00	13	02	12	48	92	78	56	52	01	06
35	46	05	88	52	36	01	39	09	22	86	77	28	14	40	77	93	91	08	36	47	70	61	74	29	41
36	32	17	90	05	97	87	37	92	52	01	83	29	99	12	49	96	72	31	71	57	85	39	41	18	38
37	69	23	46	14	06	20	11	74	43	79	01	77	09	61	87	76	37	90	20	96	52	26	95	69	65
38	19	56	54	14	30	01	75	87	53	79	40	41	92	15	85	66	67	43	68	06	84	96	28	52	07
39	45	15	51	49	38	19	47	60	72	46	43	66	79	45	43	59	04	79	00	33	20	82	66	95	41
40	94	86	43	19	94	36	16	81	08	51	34	88	88	15	53	01	54	03	54	56	05	01	45	11	76

Appendix 2

Sample weight = wt. g

If y.0 ml aliquot of the sample solution were used to developed colour and the concentration corresponding to the absorbance value of that solution was C mg/l (from the calibration curve), then:

$$\text{Concentration of the iron in the sample solution} = \frac{C \times 25}{1000 \times y} \text{ mg}$$

$$\text{Amount of iron in 50 ml} = \frac{C \times 25 \times 50}{1000 \times y} \text{ mg}$$

$$\text{mg of iron in 100g of edible portion} = \frac{C \times 25 \times 50 \times 100}{1000 \times y \times \text{wt.}} \text{ mg}$$

Appendix 3

The SAS system for analysis of beta-carotene content in green leafy vegetables

The SAS System 22

09:45 Saturday, February 08, 2003

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
VARIETY	8	hg kt m mu nl pg s sa

Number of observations in data set = 40

Analysis of Variance Procedure

Dependent Variable: AMOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1383.422878	197.631840	55.31	0.0001
Error	32	114.351120	3.573473		
Corrected Total	39	1497.773998			

R-Square	C.V.	Root MSE	AMOUNT Mean
0.923653	16.60361	1.890363	11.38525

Analysis of Variance Procedure

Dependent Variable: AMOUNT

Source	DF	Anova SS	Mean Square	F Value	Pr > F
VARIETY	7	1383.422878	197.631840	55.31	0.0001

Appendix 4

The SAS System 25
09:45 Saturday, February 08, 2003
Analysis of Variance Procedure
T tests (LSD) for variable: AMOUNT

NOTE: This test controls the type I comparison wise error rate not the experiment wise error rate.
Alpha= 0.05 df= 32 MSE= 3.573473
Critical Value of T= 2.04
Least Significant Difference= 2.4353
Means with the same letter are not significantly different.

T Grouping	Mean	N	VARIETY
A	22.936	5	s
B	16.792	5	m
B			
B	15.546	5	kt
C	8.826	5	hg
C			
D C	7.892	5	mu
D C			
D C	7.600	5	sa
D			
D	5.830	5	nl
D			
D	5.660	5	pg

T-test procedure for comparing beta-carotene in two Gotukola varities.
(Pochchi gotukola and Hin-gotukola.)

The SAS System 37
09:45 Saturday, February 08, 2003
TTEST PROCEDURE
Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
hg	5	8.82600000	0.52870597	0.23644450	8.06000000	9.41000000
pg	5	5.66000000	0.52516664	0.23486166	5.20000000	6.41000000

Variances	T	DF	Prob>|T|
Unequal	9.4999	8.0	0.0001
Equal	9.4999	8.0	0.0000

For H0: Variances are equal, F = 1.01 DF = (4,4) Prob>F = 0.9899

Appendix 5

The SAS system for analysis of Iron content in green leafy vegetables

The SAS System 16
09:45 Saturday, February 08, 2003

Analysis of Variance Procedure
Class Level Information

Class	Levels	Values
VARIETY	10	hg ka kt mu nl ns pg rt sa wm

Number of observations in data set = 50

The SAS System 17
09:45 Saturday, February 08, 2003

Analysis of Variance Procedure
Dependent Variable: AMOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	12269.93872	1363.32652	1878.73	0.0001
Error	40	29.02648	0.72566		
Corrected Total	49	12298.96520			

R-Square	C.V.	Root MSE	AMOUNT Mean
0.997640	3.611312	0.851858	23.58860

The SAS System 18
09:45 Saturday, February 08, 2003

Analysis of Variance Procedure
Dependent Variable: AMOUNT

Source	DF	Anova SS	Mean Square	F Value	Pr > F
VARIETY	9	12269.93872	1363.32652	1878.73	0.0001

The SAS System 19
09:45 Saturday, February 08, 2003

Analysis of Variance Procedure
T tests (LSD) for variable: AMOUNT

NOTE: This test controls the type I comparison wise error rate not the experiment wise error rate.

Alpha= 0.05 df= 40 MSE= 0.725662
Critical Value of T= 2.02
Least Significant Difference= 1.0889

Means with the same letter are not significantly different.

T Grouping	Mean	N	VARIETY
A	53.9780	5	hg
B	42.9540	5	pg
C	40.4280	5	rt
D	25.2720	5	nl
E	18.7160	5	sa
F	15.3820	5	mu
G	12.3740	5	kt
H	10.7840	5	wm
I	9.0400	5	ns
J	6.9580	5	ka

Ttest procedure for compairing iron content in two Mukunuwenna varieties. (Wedamukunuwenna and Mukunuwenna)

The SAS System 33
09:45 Saturday, February 08, 2003

TTEST PROCEDURE

Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
mu	5	15.38200000	0.60816938	0.27198162	14.61000000	16.12000000
wm	5	10.78400000	0.81488036	0.36442558	9.68000000	11.83000000

Variances	T	DF	Prob>|T|
Unequal	10.1115	7.4	0.0001
Equal	10.1115	8.0	0.0000

For H0: Variances are equal, F = 1.80 DF = (4,4) Prob>F = 0.5847

T-test procedure for comparing iron content in Nivithi Leaves and Stem.

The SAS System 36
09:45 Wednesday, June 11, 1997

TTEST PROCEDURE

Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
nl	5	25.27200000	0.67210118	0.30057279	24.68000000	26.42000000
ns	5	9.04000000	0.48584977	0.21727862	8.64000000	9.80000000

Variances	T	DF	Prob>|T|
Unequal	43.7659	7.3	0.0001
Equal	43.7659	8.0	0.0000

For H0: Variances are equal, F = 1.91 DF = (4,4) Prob>F = 0.5450

Appendix 6

Ttest procedure for compairing iron content in two Thampala varieties. (Red-thampala and Kura-thampala)

The SAS System 35

09:45 Saturday, February 08, 2003

TTEST PROCEDURE

Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
kt	5	12.57400000	1.07588568	0.48115070	11.15000000	13.68000000
rt	5	40.42800000	1.22579362	0.54819157	38.25000000	41.16000000

Variances	T	DF	Prob>|T|
Unequal	-38.1877	7.9	0.0001
Equal	-38.1877	8.0	0.0000

For H0: Variances are equal, F = 1.30 DF = (4,4) Prob>F = 0.8065

T-test procedure for compairing iron content in two Gotukola varieties. (Pochchi-gotukola and Hin-gotukola)

The SAS System 34

09:45 Saturday, February 08, 2003

TTEST PROCEDURE

Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
hg	5	53.97800000	0.28172682	0.12599206	53.67000000	54.25000000
pg	5	42.95400000	1.48048303	0.66209214	40.68000000	44.75000000

Variances	T	DF	Prob>|T|
Unequal	16.3567	4.3	0.0001
Equal	16.3567	8.0	0.0000

For H0: Variances are equal, F = 27.62 DF = (4,4) Prob>F = 0.0072

Appendix 7

The SAS system for analysis of Oxalic acid content in green leafy vegetables

The SAS System 4

09:45 Saturday, Feb 08, 2003

Analysis of Variance Procedure

Class Level Information

Class	Levels	Values
VARIETY	14	k ka m mm mu n nn ns p r s sa ss wm

Number of observations in data set = 70

The SAS System 5

09:45 Saturday, Feb 08, 2003

Analysis of Variance Procedure

Dependent Variable: AMOUNT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	204453.0456	15727.1574	1191.84	0.0001
Error	56	738.9619	13.1957		
Corrected Total	69	205192.0075			

R-Square	C.V.	Root MSE	AMOUNT Mean
0.996399	7.907186	3.632595	45.94043

The SAS System 6

09:45 Saturday, Feb 08, 2003

Analysis of Variance Procedure

Dependent Variable: AMOUNT

Source	DF	Anova SS	Mean Square	F Value	Pr > F
VARIETY	13	204453.0456	15727.1574	1191.84	0.0001

The SAS System 7

09:45 Saturday, Feb 08, 2003

Analysis of Variance Procedure

T tests (LSD) for variable: AMOUNT

NOTE: This test controls the type I comparison wise error rate not the experiment wise error rate.

Alpha= 0.05 df= 56 MSE= 13.19575

Critical Value of T= 2.00

Least Significant Difference= 4.6024

Means with the same letter are not significantly different.

T Grouping	Mean	N	VARIETY
A	165.254	5	mm
B	122.018	5	ss
C	96.670	5	s

C			
C	93.166	5	m
D	78.978	5	nn
E	59.772	5	r
F	18.274	5	n
G	8.452	5	ns
H	0.166	5	k
H	0.128	5	mu
H			
H	0.110	5	su
H			
H	0.100	5	wm
H			
H	0.056	5	kn
H	0.022	5	p

T-test procedure for oxalic acid in kathurumurunga maturity stages
The SAS System 27
09:45 Saturday, February 08, 2003

TTEST PROCEDURE
Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
ma	5	122.0180000	3.76772080	1.68497596	118.4500000	128.3100000
te	5	96.6700000	2.98933939	1.33687322	92.0300000	99.2000000

Variances	T	DF	Prob>|T|
Unequal	11.7848	7.6	0.0001
Equal	11.7848	8.0	0.0000

For H0: Variances are equal, F = 1.59 DF = (4,4) Prob>F = 0.6648

Ttest procedure for oxalic acid in Murunga maturity stages
The SAS System 28
09:45 Saturday, February 08, 2003

TTEST PROCEDURE
Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
ma	5	165.2540000	6.89234575	3.08235073	155.6200000	173.4500000
te	5	93.1660000	1.93453612	0.86515085	90.3100000	95.1500000

Variances	T	DF	Prob>|T|
Unequal	22.5172	4.6	0.0001
Equal	22.5172	8.0	0.0000

For H0: Variances are equal, F = 12.69 DF = (4,4) Prob>F = 0.0304

Appendix 8

Ttest procedure for oxalic acid in Nivithi leaves maturity stages

The SAS System 29

09:45 Saturday, February 08, 2003

TTEST PROCEDURE

Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
ma	5	78.97800000	2.40986929	1.07772631	75.23000000	81.31000000
te	5	18.27400000	1.11131904	0.49699698	16.72000000	19.41000000

Variances	T	DF	Prob>|T|
Unequal	51.1492	5.6	0.0001
Equal	51.1492	8.0	0.0000

For H0: Variances are equal, F = 4.70 DF = (4,4) Prob>F = 0.1630

Ttest procedure for oxalic acid in Nivithi leaves and stems.

The SAS System 30

09:45 Saturday, February 08, 2003

TTEST PROCEDURE

Variable: AMOUNT

STAGE	N	Mean	Std Dev	Std Error	Minimum	Maximum
L	5	18.27400000	1.11131904	0.49699698	16.72000000	19.41000000
S	5	8.45200000	0.89953877	0.40228597	7.46000000	9.45000000

Variances	T	DF	Prob>|T|
Unequal	15.3611	7.7	0.0001
Equal	15.3611	8.0	0.0000

For H0: Variances are equal, F = 1.53 DF = (4,4) Prob>F = 0.6920

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