MODIFIED TRADITIONAL COWPEA LEAF VEGETABLE PREPARATION METHODS FOR ENHANCING CAROTENOID RETENTION AND IRON BIOAVAILABILITY

BY

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ABSTRACT

Consumption of vegetables is the most sustainable way of reducing and controlling micronutrient deficiencies in resource-poor communities. However, not much has been documented about standard recipes that are of high nutritional quality in terms of minerals and vitamins. The aim of this study was to modify traditional cowpea leaf vegetable preparation methods to enhance carotenoid retention and iron bioavailability. Modification principles included reducing cooking time, addition of yoghurt, addition of oil and use of oven drying. Other objectives were to measure nutrient composition of two varieties of cowpea leaf (Dakawa and Ex-Iseke) found in rural Tanzania and to compare carotenoid retention and iron bioavailability of cowpea leaves with those of selected leafy vegetables (cowpea, pumpkin, amaranth, kangkong and sweet potato). Carotenoids were analyzed using High Performance Liquid Chromatography and iron bioavailability by *in vitro* method. The two cowpea leaf varieties were found to be good sources of carotenoids 44.43±0.03mg (Dakawa) and 41.54±0.01mg (Ex-Iseke), vitamin C 86±0.02mg and 94±0.00mg, calcium 165±0.03mg and 142±0.01mg, phenols 575±0.02mg and 558±0.01mg, flavonoids 604.47±0.03mg and 723.36±0.02mg per 100g edible portion and antioxidants 2596±0.01µmoleTE and 2416±0.01µmoleTE respectively. However, very high amounts of anti-nutrient oxalate 418±0.00mg and 348±0.03mg were observed. Traditional cowpea leaf dish cooked with oil, onion, tomatoes and coconut milk for 30 minutes had significantly ($P<0.05$) the highest beta-carotene (40.83%±7.00) and lutein (34.60%±3.30) retention compared to other traditional recipes. The highest iron bioavailability (10.04%±0.49) was observed in traditional recipe which involved

boiling fresh cowpea leaves for 15 minutes. Modifying traditional preparation methods did not significantly improve carotenoid retention and iron bioavailability ($p>0.05$). All selected vegetables had promising carotenoid retention with lutein and beta-carotene having more than 50% retention. Iron bioavailability increased with cooking except for sweet potato leaves. Based on the results, it is concluded that not all principles used for modifying traditional vegetable recipes for enhanced carotenoid retention and iron bioavailability works for every vegetable.

DECLARATION

I, IRENE MDUMA, do hereby declare to the Senate of the Sokoine University of Agriculture that this thesis is my own original work and has neither been submitted nor being concurrently submitted for a degree award in any other University

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The above signature is confirmed

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Date

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LIST OF ABBREVIATIONS

AIDS – Acquired immunodeficiency syndrome

ALV – African indigenous leafy vegetables

AOA	–	Antioxidant activity
AVRDC	–	Asian Vegetable Research Development Centre
HIV	–	Human immunodeficiency virus
HPLC	–	High Performance Liquid Chromatography
IVs	–	Indigenous vegetables
MNM	–	Micronutrient malnutrition
TE	–	Trolox equivalents

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Vitamin and mineral deficiencies have a significant impact on human welfare and on the economic development of poor countries. These deficiencies can lead to serious health problems, including blindness, mental retardation and reduced resistance to infectious diseases, and in some cases to death. Among the debilitating consequences of these dietary deficiencies is loss of human capital and worker productivity (FAO, 1992). More than 2 billion people have been reported to be micronutrient deficient in the world whereby the deficiencies of greatest public health significance are those of vitamin A, iron and iodine (FAO, 1997). In Tanzania, 45% of children 0-59 months suffer from iron deficiency (UNICEF, 2004) while the prevalence of Vitamin A deficiency in children of 6 month to 6 years stands at 24% (TFNC, 1997).

Consumption of vegetables, legumes, and fruits is the most sustainable way of reducing and controlling micronutrient deficiencies in resource-poor communities (Aphane *et al.*, 2003). Studies on traditional vegetable processing methods in Tanzania found significant nutrients losses (Mosha *et al*., 1997; Lyimo *et al*., 2003; Mulokozi *et al*., 2004). However, not much has been documented about standard recipes that are of high nutritional quality in terms of minerals and vitamins. Hence, a gap exists in our knowledge regarding carotenoids retention and iron bioavailability of vegetables under varying cooking methods in Tanzania. In this study the effects of traditional and modified preparation methods on carotenoids retention and in-vitro

iron bioavailability were examined by conducting laboratory analysis on vegetable samples.

Cowpea is cultivated in many tropical countries and is widely popular for its enormous economic importance particularly for resource poor farmers of sub-Sahara Africa (Fatokun *et al*., 1997). Leaf and pod are used as vegetable; seed is used for various meal preparations; haulms are used for animal fodder while the plant fixes nitrogen and improves soil fertility. Because of its multipurpose uses and its vast ecological adaptability, cowpea is researched to exploit its potential contribution to solve problems of malnutrition (Tefera-Tolera, 2006). In Tanzania, cowpea leaves are highly important as a relish since eating rice or ugali (maize porridge) alone would be too dry or unpalatable (Keller, 2006). In general, a typical meal consists of rice or ugali with a relish made from green leafy vegetables plus beans or meat if one could afford. In spite of wide use of cowpea as a leafy vegetable, it has been addressed to a limited extent in research and development (Keller *et al*., 2006).

The frequency of growing indigenous vegetables (IVs) in home gardens differs across countries and even region. The vegetables most frequently grown in Tanzania are; amaranth (67% of all households), okra and pumpkin leaves (each 49%), sweet potato leaves (46%) and cowpea (39%). In addition, 68.8% of households in Kongwa and 35.5% in Muheza are engaged in cultivating cowpea leaves (Weinberger and Msuya, 2004). Keller (2006) found out that among many leafy vegetables cultivated, cowpea was one of the top five as expressed and rated by participants in focus group meetings in all ecologically contrasting districts of Kongwa and Muheza in Tanzania.

1.2 Problem statement and justification

Many locally and regionally important food crops exist and can help to solve the problem of malnutrition, food shortage, and chronic starvation (Maundu *et al*., 1999). Various products (young shoots, young leaves, young pods, immature seeds, mature seeds, and sprouts) of different legume species and cultivars are consumed in diverse ways (Tefera-Tolera, 2006). Despite their significant role in food provision, many of these locally and regionally important crops such as cowpea leaves are neglected in research and development (Padulosi *et al*., 2002).

A number of studies in East Africa have focused on nutritive value of uncooked vegetables (Mwajumwa *et al*., 1991; Kinabo *et al*., 2004). However, information about carotenoids and iron bioavailability in cooked vegetables is insufficient (Marcela and Rodriguez- Amaya, 2004). Some studies reported changes in physical and chemical composition in vegetables due to cooking and other processing methods (Reddy and Love, 1999; Turkmen *et al.*, 2005) but very little has been done on the effects of traditional preparation processes on carotenoids retention and iron bioavailability. This might be associated with high costs and analytical difficulties involved in the assessment of cooked vegetables (Marcela and Rodriguez-Amaya, 2004).

The nutritional value of legume leaves, such as those from cowpeas, has been largely discounted due to their high water content and the difficulty of documenting their production and consumption (Bittenbender *et al*., 1992). Recently, the nutrient composition of cowpea leaves for human consumption was extensively reviewed

(Rensburg *et al*., 2004). Processing of cowpea leaves is generally not common. This is because most consumers prefer fresh vegetables. But cowpea leaves are seasonal, despite the plant's resistance to dry conditions, which calls for a need to process them for use during shortages. There is therefore a challenge on how to develop processed and/or value-added products that are more appealing to consumers. Also, developing products that appeal to specific market segments or population groups (e.g. the youth and children) could be critical. The success of this study will contribute to the efforts on alleviation of micronutrient deficiencies in Rural Tanzania. The research questions addressed in the current study were:

(i) Are the two cowpea varieties (Dakawa and Ex-Iseke) similar in carotenoid retention and iron bioavailability?

(ii) How much carotenoids and iron are left in cowpea vegetable dishes prepared according to traditional recipes of rural Tanzania?

(iii) Will modified preparation methods such as oven drying, reduction of cooking time, addition of oil, and/or addition of yoghurt enhance carotenoids retention and iron bioavailability?

1.3 Objectives

1.3.1 General objective

The general objective of this study was to investigate and identify feasible and acceptable preparation methods of cowpea vegetable dishes that can increase carotenoid retention and iron bioavailability in comparison with traditional methods used by rural households in Tanzania.

1.3.2 Specific objectives

(i) To measure nutrient composition of two cowpea leaf varieties found in rural Tanzania.

(ii) To determine and evaluate the carotenoid retention and *in vitro* iron bioavailability of cowpea leaf dishes prepared according to traditional recipes of rural Tanzania (Muheza and Kongwa).

(iii) To modify the traditional recipes of fresh cowpea leaves and validate the modification for increased carotenoids retention and iron bioavailability.

(iv) To measure carotenoid retention and iron bioavailability of raw and cooked cowpea leaves and compare with those of common consumed vegetables (amaranth, kangkong, sweet potato and pumpkin).

1.4 Hypothesis

H_o: Modified preparation methods of cowpea leafy vegetables result in higher carotenoid retention and iron bioavailability than the traditional preparation methods.

H_1: Modified preparation methods of cowpea leafy vegetables do not result in higher carotenoid retention and iron bioavailability than the traditional preparation methods.

The findings of this study were expected to:

- Guide households to make use of locally available cowpea vegetable dishes to improve their micronutrient nutrition status.
- Guide households to choose proper preparation methods to improve carotenoid retention and iron bioavailability.
- Contribute to scientific knowledge of improving food and nutrition security by using indigenous vegetables.
- Help policy makers put emphasis on importance of indigenous vegetables in alleviation of micronutrient deficiencies in Tanzanian communities.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Strategies to Reduce Micronutrient Malnutrition

The control of vitamin and mineral deficiencies is an essential part of the overall effort to fight hunger and malnutrition. Different strategies to fight micronutrient malnutrition especially of iron and vitamin A deficiencies have been attempted and found to be feasible or rather effective (WHO/FAO, 2006). The aims of these strategies have been to increase the population's access to micronutrient-rich foods, as well as to increase the consumption and the amount of micronutrients that can be absorbed and utilized by the body (Ruel, 2001). Strategies include food-based strategies (WHO, 2003) such as dietary diversification and food fortification, as well as nutrition education, public health and food safety measures, and finally supplementation. These approaches should be regarded as complementary, with their relative importance depending on local conditions and the specific mix of local needs (WHO/FAO, 2006).

2.1.1 Increasing the diversity of foods consumed

Increasing dietary diversity means increasing both the quantity and the range of micronutrient-rich foods consumed. In practice, this requires the implementation of programmes that improve the availability and consumption of, and access to, different types of micronutrient-rich foods (such as animal products, fruits and vegetables) in adequate quantities, especially among those who are at risk for, or vulnerable to micronutrient malnutrition (MNM) (WHO/FAO, 2006; Karim *et al.*, 2005). However, as a strategy for combating MNM, increasing dietary

diversity is not without its limitations, the main one being the need for behaviour change and for education about how certain foods provide essential micronutrients and other nutritive substances. Lack of resources for producing and purchasing higher quality foods resulting to food insecurity can sometimes present a barrier to achieving greater dietary diversity, especially in the case of poor populations (WHO/FAO, 2006).

2.1.2 Food fortification

Food fortification refers to the addition of micronutrients to processed foods. In many situations, this strategy can lead to relatively rapid improvements in the micronutrient status of a population, and at a very reasonable cost, especially if advantage can be taken of existing technology and local distribution networks (WHO/FAO, 2006). Fortification of food, although a cost-effective way to increase nutrient availability to large population groups, requires effective management that includes advocacy, communications, regulation and quality control, along with monitoring and evaluation (FAO, 1997). The main barriers to successful iron fortification as observed by Hurrel (2002) are the following: finding an iron compound that is adequately absorbed but causes no sensory changes to the food vehicle; and overcoming the inhibitory effect on iron absorption of dietary components such as phytic acid, phenolic compounds and calcium. These barriers have been successfully overcome with some food vehicles but not with others. Iron-fortified fish sauce, soy sauce, curry powder, sugar, dried milk, infant formula and

cereal based complementary foods have been demonstrated to improve iron status in targeted populations (Hurrel, 2002).

2.1.3 Supplementation

Micronutrient supplements often provide the fastest improvement in the micronutrient status of individuals or targeted population. In developing countries, supplementation programmes have been widely used to provide iron and folic acid to pregnant women, and vitamin A to infants, children under 5 years of age and postpartum women (WHO/FAO, 2006). While donors in the past have favoured supplementation and fortification strategies (Ruel, 2001), these approaches are beset with many problems. Supplementation of iron, for example, is difficult to supervise, particularly in regions where reliable infrastructures are missing (ACC/SCN, 2000; Karim *et al*., 2005).

2.2 Role of Indigenous Vegetables in Food Security and in Fulfilling Dietary Requirements

Indigenous vegetables (IVs) provide an important contribution to the diet, particularly so in the rainy season, when they are readily available. Nearly 80% of households in Kongwa, Muheza, Singida and Arumeru were reported to collect IVs during the rainy season (Weinberger and Msuya, 2004). In addition, African indigenous vegetables are considered valuable because of their ability to fit into year-round production systems and their nutritional value (Keller, 2006).

African indigenous vegetables play a highly significant role in food security of the under-privileged in both urban and rural settings (Schippers, 1997). They can serve

as primary foods or secondary condiments to dishes prepared from domesticated varieties. They are also valuable sources of energy and micronutrients in the diets of isolated communities. Vegetables provide supplementary protein, vitamins and minerals to meet basic nutritional requirements. Leafy vegetables are rich sources of vitamins, minerals and fibre, and provide diversity in the diet. Green leaves are good sources of lysine, although deficient in sulphur amino-acid content (Grivetti and Ogle, 2000).

The contribution of IVs to fulfilment of the overall requirements of the household is shown by Weinberger and Msuya (2004) in a study conducted in Tanzania. Results showed that poor households rely on the consumption of IVs to fulfil their daily requirements of micronutrients, particularly vitamin A and iron. In poor households, approximately 50% of all vitamin A requirements and slightly less than one-third of iron requirements are consumed through IVs. The share is much lower for exotic vegetables. On average, only 1.5% of all iron requirements and 3% of all ß-carotene requirements are fulfilled (Table 1). Thus there is a need to promote consumption of indigenous vegetables. IVs are certainly not a panacea for the elimination of micronutrient deficiencies but do have an important role to play for maintaining adequate levels of micronutrient consumption. In fact, many of the deficiencies observed may relate to decreasing importance of IVs in the diet (Weinberger and Msuya, 2004). A study conducted by Phillips *et al.* (2003) in Anambra State of Nigeria demonstrated that an increase in cowpea consumption might improve 50% of children malnutrition, though cowpeas were already prevalent in the local diet.

Table 1: Contribution (percentage) of IVs to fulfilment of daily Iron, Zinc and B- carotene requirements of households

Wealth parameters	Iron %	Zinc %	B-carotene %
0 (Poorest)	29.5	3.8	53.1
1	22.0	2.6	35.1
2	13.2	1.9	23.7
3	14.6	2.0	24.7
4	10.4	2.8	32.8
5 (Richest)	1.7	0.6	1.7
Total	16.6	2.3	29.0
Mean contribution of exotic vegetables	1.5	0.5	3.0

Source: Weinberger and Msuya (2004)

2.3 Role of indigenous vegetables in Health Promotion and Protection

Quite a large number of African indigenous leafy vegetables (ALV's) have long been known and reported to have health protecting properties and uses (Okeno *et al*., 2003; Ayodele, 2005). Several of these indigenous leafy vegetables continue to be used for prophylactic and therapeutic purposes by rural communities (Ayodele, 2005). This indigenous knowledge of the health promoting and protecting attributes of ALVs is clearly linked to their nutritional and non-nutrient bioactive properties. More recent reports have shown that they also contain non-nutrient bioactive phytochemicals that have been linked to protection against cardiovascular and other degenerative diseases (Yang *et al*., 2006) although some phytochemicals were found to pose some toxicity when consumed in large quantities or over a long period of time (Orech *et al*., 2005).

A recent study conducted on *moringa* leaves showed that, *moringa* leaf extract exhibit anti-microbial activity including inhibition of growth of S*taphylococcus*

aureus strains isolated from food and animal intestines (Yang *et al*., 2006). The authors concluded that consumption of *moringa* enhances the immune response and that *moringa* leaves should be promoted for greater consumption to improve nutrition and strengthen immune functions for fighting infectious diseases. In addition, Johns (2004) observed a potential relationship of indigenous vegetables and ability to treat diabetes, gout, hyperlipidemia, gastro-intestinal tract infections, protozoan parasites, amongst others in Kenya and Tanzania. Kimiywe *et al*. (2007) also noted that traditional vegetables have medicinal properties for the management of HIV/AIDS, stomach-related ailments and other diseases according to a study conducted in peri-urban and urban areas of Nairobi (Table 2).

Table 2: Medicinal value of indigenous vegetables as reported by respondents

Indigenous vegetable	Attached value	Medicinal value
Amaranth	Boosts appetite	Malaria, colds, coughs, AIDS, stomach ache, diarrhoea, skin rashes, diabetes, back ache.
Cowpea leaves	Boosts appetite	Digestive problems
Jute mallow	Boosts appetite	Stomach ache, anaemia
Pumpkin leaves	Boosts appetite	Malaria, typhoid, high blood pressure, oedema, constipation, general health, stomach ache
Garlic	Boosts appetite, revitalizes the body	Colds and coughs, high blood pressure, cancer, asthma, TB, stomach ache
Ginger	Nourishing and revitalizing	Colds and coughs, high blood pressure

Source: Kimiywe *et al*. (2007)

2.4 Vegetable processing

In Tanzania, green leafy vegetables are widely grown and consumed throughout the year in areas with favourable weather conditions. However, in semiarid areas, the availability of vegetables is limited during the dry season (Mosha *et al.*, 1997). To overcome this problem, various preservation methods are adopted. Drying is the most commonly used method for enhancing shelf life of vegetables. Sun-drying in the open sun is commonly used in preserving the vegetables for dry-season consumption. Drying in enclosed (cabinet) solar driers, which protects the drying vegetables from direct sunlight is also practiced (Mulokozi *et al.*, 2000). Prior to drying, various traditional preparatory procedures are used on the fresh vegetable. They include, pounding, prolonged boiling followed by squeezing off excess water and rubbing between the palms to form small coils. These are done in order to obtain the desired flavour and texture. Most traditionally treated vegetables are dried without blanching and thus exposing the vegetables to continued enzyme activity (Mulokozi and Svanberg, 2003).

Blanching is a primary step in processing of vegetables and storage as it beneficially inactivates enzymes, such as peroxidase and lipoxygenase that are involved in carotenoid destruction through oxidation (Negi and Roy, 2000). Most processing procedures of

vegetables have a potential impact on the carotenoid structure and content. This is due to the fact that carotenoids have a series of conjugated double bonds making them susceptible to degradation by light, oxygen, heat and acids, as well as to cis/trans isomerization (Thane and Reddy, 1997). Generally, dehydration and powdering of vegetables increase surface area and lead to poor stability of carotenoids, unless the products are protected from air and light (Thane and Reddy, 1997).

2.5 Possible interactions between vitamin A and Iron

Iron metabolism can be described as a closed loop in which the primary processes are the formation and destruction of red blood cells. Small amounts of iron enter this loop via the absorption of dietary iron and in balance an equivalent amount of iron exit the loop as losses from blood and tissues (Hess *et al*., 2005) (Figure 1). Vitamin A has been proposed to influence iron metabolism either via its effect on erythropoiesis, with vitamin A deficiency leading to decreased erythropoiesis with less iron incorporated into red blood cells (Roodenburg *et al*., 2000), or indirectly by its beneficial effects on immune function leading to a decrease in the anaemia of infection (Thurnham, 1993). In addition, as infection is reported to block iron absorption (Semba and Bloem, 2002), the promotion of immune function by vitamin A may remove this blockade of iron absorption by reducing inflammation. However, testing these theories and confirming the effect of vitamin A on iron absorption has proved difficult, and the exact mechanism by which vitamin A interacts with iron metabolism remains obscure (Hess *et al*., 2005). In addition to the effects of vitamin

A deficiency on iron metabolism, it also appears that iron deficiency can impair vitamin A metabolism by decreasing vitamin A mobilization from liver stores and perhaps also by decreasing retinol absorption (Jang *et al*., 2000). Iron (and zinc) supplementation have been reported to improve indicators of vitamin A status in Mexican school children (Munoz *et al*., 2000).

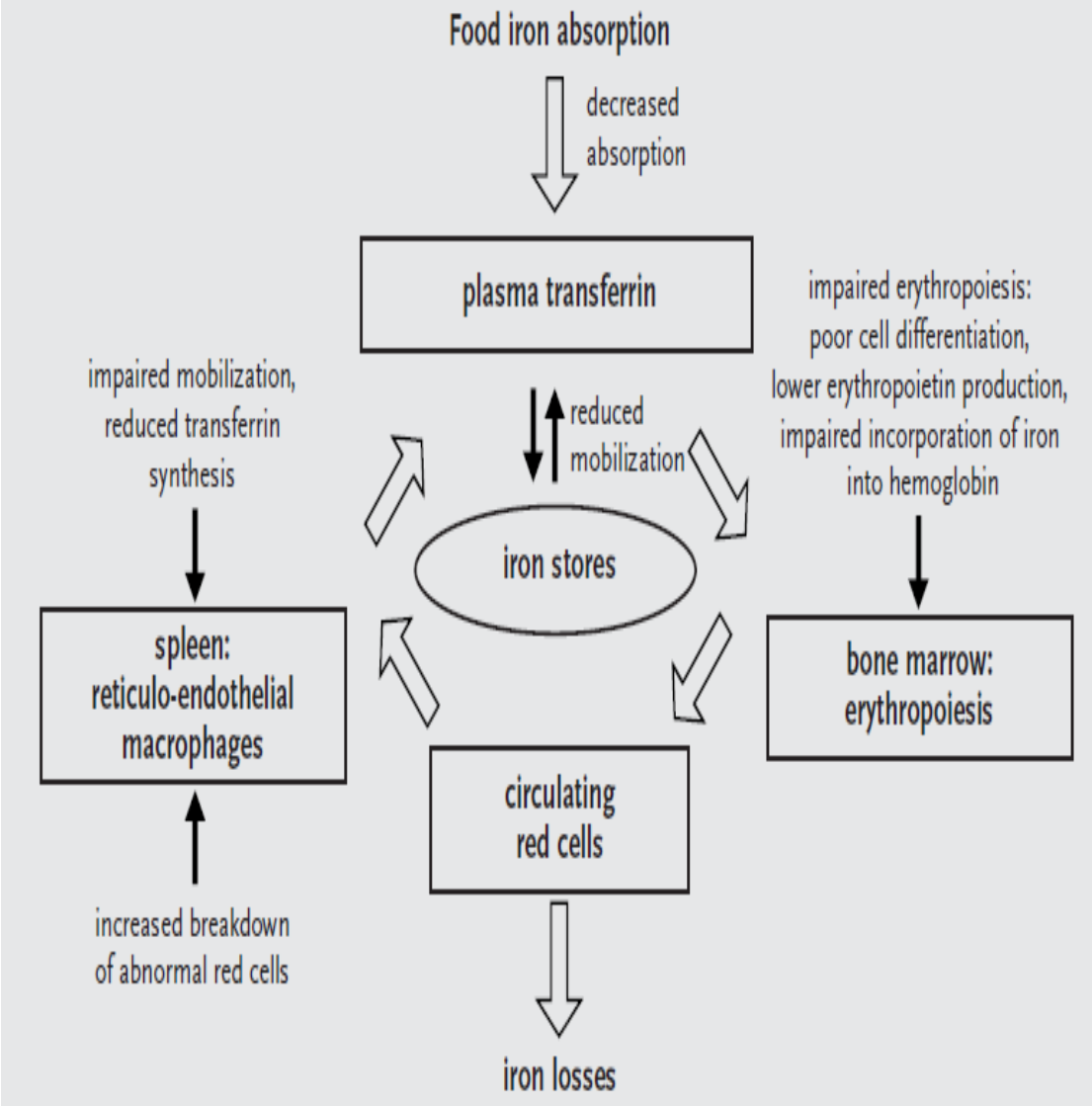

Figure 1: Possible influences of vitamin A deficiency on Iron Metabolism

Source: Hess *et al*. (2005)

2.6 Link between Vitamin A deficiency and Iron status

The existence of a link between vitamin A deficiency and anaemia has been known for many years. However, the stage of iron metabolism at which vitamin A exerts its critical effect remains obscure (Hess *et al*., 2005). Several mechanisms have been proposed (Roodenburg *et al*., 2000) and, in tropical countries, the high prevalence of infectious diseases may also play a role as vitamin A deficiency can decrease immune function, increase infections and, due to a modulation of haematopoiesis (Thurnham, 1993), anaemia increases (Means, 2000).

Cross-sectional studies in developing countries have reported a positive correlation between serum retinol and haemoglobin concentration. This correlation can arise from common risk factors or shared metabolic pathways. The correlation becomes stronger with lower vitamin A status (Fishman *et al*., 2000). Similarly in Tanzania, pregnant women with haemoglobin levels below 90 g/L were found to be 2.2 fold more likely to have low serum retinol concentrations, and anaemia in these women was also found to be associated with elevated serum c- reactive protein (CRP) concentrations (Hinderaker *et al*., 2002). Intervention studies have shown that vitamin A supplements, or foods fortified with vitamin A, improve blood haemoglobin concentrations in children and in pregnant or lactating women (Semba and Bloem, 2002). In addition, studies have shown that dual supplementation with iron and vitamin A has a greater impact on haemoglobin concentrations than iron alone in both children (Mwanri *et al*., 2000) and pregnant women (Suharno *et al*., 1993).

2.7 Effects of β-Carotene on vitamin A status

The efficacy of provitamin A carotenoids in improving vitamin A status was recently found to be much low than had been previously thought (Institute of Medicine, 2001). Individuals with lower vitamin A status appear to have higher absorption and/or bioconversion of carotenoids (Ribaya-Mercado *et al*., 2000). Using the increase in plasma β-carotene concentration to measure absorption is, by definition, guaranteed to underestimate the true absorption and is also unreliable thus precautions should be taken to control for changes in sub-clinical inflammation. The β-carotene that enters the plasma following a meal is the fraction of absorbed β-carotene that was not converted to retinol in the intestinal cells (Hess *et al*., 2005).

In addition, studies in rats have shown that conversion of β-carotene to retinol is higher if the rats are vitamin A deficient and lower if they are protein deficient (Parvin and Sivakumar, 2000). Thus, nutritional status also affects the variability in β-carotene bioavailability from different foods, further complicating any attempt to interpret absorption from plasma β-carotene concentrations. No significant correlation between frequency of vegetable consumption and either plasma retinol or plasma carotenoids was observed in pregnant women in Tanzania (Mulokozi *et al*., 2003). However, although the mean plasma retinol concentrations were low in the Tanzanian women, plasma lutein and β-carotene concentrations were high, respectively, indicating that a large amount of carotene was absorbed. Given that the amount of β-carotene in the plasma represents the fraction not converted to retinol, there was adequate dietary β-carotene to convert to retinol. In other words, other factors were probably depressing the plasma retinol concentration and obscuring the

association between plasma retinol concentrations and the amount of vitamin A consumed (Hess *et al*., 2005). There is some evidence, however, that consumption of high β-carotene containing foods increases vitamin A status, with fresh fruits seemingly being better than vegetables at providing bio available provitamin A carotenoids (de Pee *et al*., 1998).

2.8 Composition of iron and β-Carotene in vegetables

Studies by researchers from the Sokoine University of Agriculture on indigenous vegetables in Iringa and Morogoro regions (Kinabo *et al*., 2004) reported contents of Fe in African nightshade of 6.10 mg per 100 g edible portion. Lyimo *et al*. (2003) have also reported high levels of iron and other minerals in African indigenous vegetables and recorded values of up to 7.7mg/100g of iron in fresh vegetables. In addition, Weinberger and Msuya (2004) found high levels iron of up to 37.05mg per 100g in Amaranth leaves.

A study conducted by Weinberger and Msuya (2004) in four districts of Tanzania reported high contents of ß-carotene (exceeding 5.0 mg per 100 g edible portion) in pumpkin leaves and cassava leaves from Kongwa district, and spider flower plant, bitter lettuce, jute mallow, *Erythrococoa kirkii*, cassava leaves and chili pepper leaves from Muheza district. Cowpea leaves, cassava leaves, puncture vine and chilli pepper leaves from Arumeru district were also in this category. Mwajumwa *et al*. (1991) reported contents of ß-carotene in cowpea leaves collected from three locations in Machakos district in Kenya to be 6.7 ± 1.5 mg per 100 g of edible portion.

2.9 Importance of carotenoids to human health

The principal carotenoids found in foods are: β-carotene, ά-carotene, β-cryptoxanthin, lycopene, lutein and violaxanthin. With the exception of violaxanthin, these carotenoids are also most commonly found in the human plasma and have been, together with zeaxanthin, be the most studied in terms of health promoting effects (Rodriguez-Amaya *et al*., 2008). Structurally, vitamin A (retinol) is essentially one-half of the molecule of β -carotene. This carotenoid is the most potent provitamin A; it is also the most widely distributed carotenoid in foods (Rodriguez-Amaya, 1993). The minimum requirement for a carotenoid to have provitamin A activity is an unsubstituted β -ring with a polyene chain of 11 carbon atoms. Thus, ά -carotene and β-cryptoxanthin have about 50% of the activity of β -carotene (Rodriguez-Amaya *et al*., 2008). Carotenoid provitamins A have been credited with other beneficial effects to human health: enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract and muscular degeneration (Krinsky and Johnson, 2005). The carotenoids' action against diseases has been attributed to antioxidant activity, specifically to their ability to quench singlet oxygen and interact with free radicals (Krinsky, 2001). However, other mechanisms have been reported such as modulation of carcinogen metabolism, regulation of cell growth, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of cell-to-cell gap junctional communication, retinoid-dependent signalling and filtering of blue light (Krinsky and Johnson, 2005; Stahl and Sies, 2005). Lutein, zeaxanthin and astaxanthin have recently been found to protect against DNA damage in human neuroblastoma cells induced by reactive nitrogen species (Santocono *et al*., 2007)

2.10 Effects of food preparation and processing on carotenoids retention

Food preparation and processing can reduce provitamin A carotenoid content of foods by up to 90%. In terms of retaining provitamin A carotenoids, of which β-carotene is the most important for vitamin A nutriture, steaming is better than boiling, and boiling is better than sautéing (or stir-frying). Deep-frying, prolonged cooking, combining several preparation and processing methods, baking, and pickling (with the possible exception of the pickling of olives) all result in substantial losses of provitamin A carotenoids (Rodriguez-Amaya, 2008).

Whatever the processing method chosen, retention of provitamin A carotenoids decreases with longer processing time, higher processing temperatures, and greater cutting/ chopping or maceration of the food (Nestel and Nalubola, 2003). Simple modifications, such as cooking with the lid on, reducing the time between peeling/cutting and cooking/processing (Rodriguez-Amaya, 1997) and shortening the cooking/processing time improve retention significantly (Mulokozi *et al*., 2004). Protection from light, lower storage temperature, and minimum storage time also contribute to better retention (Nestel and Nalubola, 2003).

2.10.1 Effect of drying

Traditional direct sun-drying of fruits such as mangoes and of vegetables such as carrots and green leaves causes considerable provitamin A carotenoid destruction, whereas drying in a solar dryer, in which the food is dried covered in shade rather than under direct sunlight, can appreciably reduce losses. Protection of the food from

direct sunlight, the presence of a natural or added antioxidant (e.g., vitamin E), and salt treatment all may reduce provitamin A carotenoid degradation during the preservation and storage of processed foods (Rodriguez- Amaya, 1997). A number of studies have reported significant effect of traditional drying and storage practices on the retention of provitamin A carotenoids (Mosha *et al*., 1997; Koskei, 2006; Svanberg, 2007). Amaranth and cowpea leaves lose more carotenoids when they are open sun-dried than when they are solar dried (Table 3) or blanched (Svanberg, 2007).

Table 3: Comparison of the vitamin A activity of selected solar and direct sun dried foods

Food	Solar dried RE100/⋆g	Sun dried RE100/⋆g
Carrot	6,850	5690
Amaranth	2,170	1,690
Pumpkin leaves	2,900	1,250
Cassava leaves	3,000	3,280
Sweet potato leaves	3,060	1,730

1⋆RE = 6µg ά- carotene, 12 µ g other provitamin A

Source: Linheman (1994)

2.10.2 Effect of cooking

Conventional cooking is reported to cause a significant increase in the concentration of carotenoids in cowpea, peanut and pumpkin leaves. Carotenoid retention increases up to 30 minutes cooking time and there after the retention decreases (Mosha *et al*.,

1997) (Table 4). However, elevated temperature and greater exposure to sunlight increase carotenogenesis in fruits, but may also promote carotenoid photo-degradation (Rodriguez-Amaya, 2008).

Table 4: Beta-carotene contents (mg/100g dry weight) of raw selected leafy vegetables and cooked at different time intervals

Green leaves	Raw	Cooked		
		15 minutes	30 minutes	60 minutes
Amaranth	19.12	14.23	15.85	16.66
Cowpea	14.72	21.06	29.40	22.89
Pumpkin	11.20	20.38	24.86	25.90
Sweet potato	8.99	5.38	4.73	3.78

Source: Mosha *et al*. (1997)

2.11 Other factors affecting carotenoids composition

Leafy vegetables produced in greenhouses or in plots covered with plastic roofing, such as the hydroponic leaves and those used for minimal processing, show higher carotenoid concentrations in the summer. In contrast, carotenoid levels in leafy vegetables cultivated in open fields are significantly lower in the summer, suggesting that photodegradation prevails over heightened carotenogenesis (Rodriguez-Amaya *et al*., 2008). Alteration or loss of carotenoids during processing and storage of foods occurs through physical removal (e.g., peeling), geometric isomerization, and enzymatic or non-enzymatic oxidation (Rodriguez-Amaya, 2002). The major cause of carotenoid loss is enzymatic or non-enzymatic oxidation, which depends on the availability of oxygen and the structure of the carotenoid. It is stimulated by light,

heat, metals, enzymes and peroxides and is inhibited by antioxidants. Degradation is known to increase with the destruction of the food cellular structure, increase in surface area or porosity, length and severity of the processing conditions, length and temperature of storage, use of packaging permeable to oxygen and light (Rodriguez-Amaya *et al*., 2008).

2.12 Iron Bioavailability

Heme iron is highly bio-available 15% - 35% is absorbed, whereas non-heme iron is much less bio-available 2% - 20% (Allen and Ahluwalia, 1997; Gambling, 2006). The absorption of iron is affected by two main groups of factors: physiological and dietary factors (Gibson, 2007). The major factors include the individual's iron status and requirements, the sources and content of iron in the meal, the amount of acid secreted by the stomach during digestion and rate of passage of acid in the digestive tract and the other constituents of the meal (Lynch, 2005). Regarding the two forms of iron present in foods, heme iron has greater availability than non-heme iron. Beside this, non-heme iron availability is conditioned by several dietary factors, such as classic factors (meat, ascorbic acid, fiber, phytic acid, and polyphenols) and new factors (caseinophosphopeptides and fructo-oligosaccharides with prebiotic characteristics) (Lopez and Martos, 2004).

2.13 Factors affecting iron bioavailability

a) Phytates

Phytate (phytic acid) chelates metal ions such as iron and zinc and therefore inhibit their absorption (Zijp, 2000). Recently, vitamin A and ß-carotene have been shown to

enhance non-heme iron absorption by preventing the inhibitory effect of phytates (Manju *et al.*, 2000). Ascorbic acid is a potent enhancer of non-heme iron absorption that can overcome the inhibiting effect of phytic acid when present in high enough quantities (Davidson, 2003).

b) Oxalates

Oxalic acid may form insoluble iron-oxalate complexes, although this may be compensated for, to some extent, by the presence of ascorbic acid. The low availability of iron from some green vegetables, such as spinach, is partly due to their high oxalic acid content. Oxalate may also lead to renal calcium stone formation and it also decreases calcium absorption (Kawazu *et al*., 2003).

c) Polyphenols

Polyphenols, which are present in the form of phenolic acids, flavonoids and their polymerization products in tea, coffee, coke and red wine, can inhibit iron absorption. The compounds form insoluble complexes with iron and may exist as an "iron-tannin" complex and thus inhibit iron absorption (Kannah, 2006).

d) Dietary fibres

Dietary fibre may have a greater effect on iron balance in infants and children compared to adults. But the inhibitory effect depends on the presence of phytates, oxalic acid, minerals and protein in the food. In adults, up to 32 g per day of dietary fibre and 2 g per day of phytic acid may not cause a significant effect. In children, up

to 25 g per day of dietary fibre and 1 g per day of phytic acid is unlikely to have a significant effect on iron bioavailability (Kannah, 2006).

e) Ascorbic acid

Ascorbic acid is a strong enhancer of non-heme iron absorption. It may exert its "enhancing" effect by promoting acid conditions within the stomach so that the dietary iron is efficiently solubilized; by reducing ferric iron to its better absorbed ferrous form; by forming chelates with iron in the stomach; and by maintaining the solubility of non-heme iron when the food enters the alkaline environment of the small intestine which counteracts the inhibitory effect of dietary ligands such as phytates and tannins (Allen and Ahluwalia, 1997). In addition, ascorbic acid is a potent enhancer of non-heme iron absorption that can overcome the inhibiting effect of phytic acid when present in high enough quantities (Davidson, 2003)

2.14 Effects of processing and preparation on iron bioavailability

Food processing and iron chemistry are important factors affecting iron bioavailability. The chemistry of iron, particularly its valence, solubility, and types of chelation, influence its absorption (Yang and Tsou, 2006). When eaten together certain combinations of foods promote non-heme iron bioavailability either by increasing substances that enhance absorption of iron or by decreasing substances that inhibit absorption (Miller, 1998; Gibson and Ferguson, 1998). Food processing methods such as baking, canning, drying, and cooking can have different effects on iron bioavailability and they therefore should be considered (Yang and Tsou, 2006).

Studies by Kapanidis and Lee (1995) indicated that in vitro bioavailability of iron in cruciferous vegetables can be enhanced from 5% to more than 20% through cooking. Also several studies found out that iron bioavailability of certain vegetables and fruits can be enhanced simply by boiling in water (Yang *et al.*, 2002) and that enhancing effect of cooking is not as apparent in fruits as in vegetables (Yang *et al*., 1998). The factors contributing to enhancing effects on iron dialyzability in cooked vegetables may be explained by the fact that, heating denatures the polyphenol oxidases preventing their action, but leaves intact a sufficient amount of ascorbic acid to maintain iron in a soluble form through chelation, even at pH 2 in the stomach and pH 6-7 in the intestine. Thus, more available iron can be absorbed from cooked vegetables than from the raw form (Yang and Tsou, 2006). In addition, cooking eliminates the inhibitors, and then enhancers raise up iron dialyzability of cooked vegetables (AVRDC, 2000). Three conventional household cooking methods; blanching, boiling and stir-frying, were used to evaluate the effects of cooking on Vitamin C, total phytates and tannin of selected Thai vegetables and there was a decrease in the total vitamin C, with losses from 14% to 95%(Somsub *et al*., 2008). The greatest loss was found in boiled bitter cucumber and vitamin C enhances Iron absorption in the gastrointestinal absorption

2.15 Modification of preparation methods for enhanced carotenoids retention and Iron bioavailability

2.15.1 Improved cooking and preparations

Retention can be significantly improved by reducing processing time, lowering cooking temperature and shortening time lag between peeling, cutting and cooking

(Rodriguez-Amaya, 2003). The effects of domestic cooking methods (including boiling, steaming, microwaving, baking and stir-frying) on different nutrients in a range of vegetables were studied by Canet *et al*. (2004). It was concluded that methods involving short cooking times and minimal water should be used for best nutrient retention during cooking. However, Halvorsen *et al*. (2006) reported large increases in antioxidant activity for several vegetables after microwaving, steaming and boiling.

Blanching may provoke carotenoids losses but the inactivation of oxidative enzymes that occurs during this treatment prevents further and greater losses during holding before thermal processing, slow processing and storage. According to Ndawula (2004), blanching cowpea leaves resulted to improved β-carotene and vitamin C retention by 15% and 7.5%. Mulokozi *et al.* (2004) also reported higher carotenoid contents in blanched cowpea leaves than in traditionally prepared and modified cowpea leaves preparation methods. There were significant lower amounts of 9-cis-β-carotene in vegetables cooked by modified method without oil as compared to the amount of the same in blanched vegetables. The modifications were reducing cooking time, the use of lid during cooking and cutting leaves in small pieces did not increase carotenoid contents in vegetables (Mulokozi *et al.*, 2004) (Table 5).

Table 5: Total content of 9-cis- β- carotene in vegetables cooked in modified methods as compared to the blanched samples

Green leaves	Total amount (µg/g dry matter)		
	Blanched	Traditional preparation	Modified preparation
Amaranth	89	96	60
Cowpea	121	93	77
Sweet potato	83	10	73
Pumpkin	83	78	75

Source: Mulokozi *et al.* (2004)

2.15.2 Effect of adding oils

Several studies have found significant carotenoid contents on addition of oils (Mulokozi *et al*., 2004; Hedren *et al*., 2002) but the amount differs with the type of oil. Hedren *et al.* (2002) reported accessibility of 39-94% of β -carotene content from vegetables cooked with sunflower oil or red palm oil. Higher effects of adding red palm oil to vegetables are observed compared to sunflower oil. For example, adding red palm oil to vegetables instead of sunflower oil resulted in about twice as much accessible β-carotene, due to the high accessibility of its β-carotene content. Red palm oil supplementation was also found to significantly improve maternal and neonatal vitamin A status and reduced the prevalence of maternal anaemia (Radhika *et al*., 2003). In addition, a 6 month intervention trial in Tanzania beginning in the third trimester to investigate the effect of red palm oil compared with sunflower oil on serum retinol concentrations showed no difference in maternal plasma retinol concentrations between the two groups, but a significant increase in concentrations of α- and β-carotene in maternal serum and in breast milk in the women receiving the red palm oil (Lietz *et al*., 2001).

2.15.3 Effect of adding vitamin C

Eating acidic foods, especially those rich in ascorbic acid, along with iron containing foods can increase absorption three to seven fold. The acid is more effective when present in high enough quantities (Davidson, 2003; Hurrel, 2004). Fresh fruits like orange, lemons, grapefruit, watermelon, papaya and pineapple are rich in ascorbic acid (Naidu, 2003). In addition, roselle, one of the high value crops contains 12.5-31.33mg ascorbic acid per 100g of roselle on fresh weight basis (Dahiru *et al*., 2003; Fasoyiro *et al*., 2005; Adaniawo and Ajibade, 2006). The limitation of using ascorbic acid as enhancer to iron bioavailability is associated with its instability during processing and unwanted sensory changes (Davidson, 2001; Teucher, 2004). A study by Bergqvist *et al*. (2006) showed that lactic acid fermentation stimulates iron absorption. Lactic acid fermentation enhances Fe absorption because of increase in iron solubility after digestion and improved efficiency of iron uptake due to increased level of soluble $Fe2^{+}$.

2.15.4 Improved drying methods

Dried green leafy vegetables constitute a major dietary source of provitamin A carotenoids for people living in semi-arid areas of Tanzania (Mulokozi and Svanberg, 2003). The use of solar and oven drying are reported to improve nutrient content in dried products and retain higher quantities of carotenoids and other nutrients that are destroyed by traditional methods of drying (Rensburg *et al*., 2004). Consumption of a 100 g portion of solar-dried vegetable relish could provide the recommended daily intake of vitamin A (Mulokozi and Svanberg, 2003). In addition,

there is a high level of nutrient retention in vegetables dried by oven compared to sun-dried vegetables (Badifu, 2001). Blanching prior to drying has also been found to be useful in improving nutrient retention whereby it increases the mineral availability (Yadav and Sehgal, 2003).

In developing countries such as Tanzania, solar drying has been reported to have great potential for social acceptance because it is the simplest and most economical method of dehydration. It protects produce from adverse environmental conditions, and enhances quality, shelf-life, and micronutrient content of the dried products (Ruel and Levin, 2000). Drying in an appropriate solar drier leads to considerable reduction of drying time by up to 50% and a significant improvement in product quality in terms of colour, texture, flavour, and nutrient retention (Bala *et al.*, 2003). Quality characteristics are also affected by moisture content and water activity, temperature, relative humidity, and rate of dehydration (Hatamipour and Mowla, 2002; Chen *et al.*, 2005). Thus, solar drying is currently being investigated as an alternative method to improve the physical, nutritive, and aesthetic quality of dried materials (Mclziniso *et al.*, 2006).

2.16 Intestinal non-heme iron absorption

Dietary iron is absorbed in the proximal intestine by a regulated process that controls body iron homeostasis as iron excretion is not regulated in mammals. Non-heme food iron is released by acid digestion in the stomach and must be reduced to the

ferrous (Fe^{2+}) ion prior to uptake by duodenal enterocytes. Food factors, especially ascorbic acid, can reduce ferric (Fe^{3+}) ions in the intestinal lumen, however an iron regulated ferric reductase protein (Dcytb, Cybrd1) is also present on enterocyte apical membrane (Ganz and Nemeth, 2006; Gunshin *et al*, 2005). Ferrous ions are transported across the enterocyte apical membrane by Divalent metal iron transporter 1 (DMT1), a proton coupled divalent ion transporter. The amount of DMT1 in the apical membrane is regulated by body iron requirements (Gunshin *et al.*, 2005). Iron effluxes from the enterocyte basolateral membrane through ferroportin and is oxidised by a membrane bound ferroxidase, hephaestin, yielding ferric ions that are then bound by plasma transferrin for distribution around the body (Donovan *et al.*, 2005).

Availability of dietary iron for absorption is determined by meal composition and can be affected by loss of stomach function, especially gastric acid production. Iron deficiency is a major nutritional problem. It is usually caused by poor dietary iron availability coupled with high iron requirements due to rapid growth, pregnancy, or high blood losses (usually menstrual or gastrointestinal). The rate of absorption of iron by enterocytes is controlled by the activity of the transporters DMT1 and ferroportin in the appropriate membranes (Donovan *et al.*, 2005; Gunshin *et al.*, 2005). The principle regulatory mechanism involves sensing of high body iron stores or low erythroid iron requirements by the liver which produces an inhibitory peptide, hepcidin that acts on the intestine to decrease iron absorption. When erythroid iron requirements are increased or body iron stores are low, hepcidin production by liver is decreased, leading to increased iron absorption (Ganz and Nemeth, 2006).

Hepcidin is a 25 amino acid peptide that binds to ferroportin and induces its internalization and degradation, thereby reducing iron export from enterocytes. Dcytb and DMT1 levels are also affected, most likely in response to altered iron levels in enterocytes caused by hepcidin's action on iron efflux through ferroportin. It is also possible that hepcidin has some other effect that leads to altered Dcytb and DMT1 (Gunshin *et al*., 2005). Non-heme iron absorption is very tightly controlled by hepcidin (Ganz and Nemeth, 2006).

Human diseases where inappropriate iron absorption occurs include genetic haemochromatosis, where mutations in hepcidin or ferroportin genes affect regulation of iron absorption leading to too much iron in the body (Pietrangelo, 2006). These diseases have shown that production of appropriate levels of hepcidin and its correct interaction with ferroportin are central to normal body iron homeostasis in man (De Domenico *et al*., 2007). Too much iron absorption can also occur when there are high levels of ineffective erythropoiesis as in thalassaemia. Inappropriate decreases in iron absorption are seen in chronic diseases with increased inflammation, as hepcidin levels are increased in these conditions (Ganz and Nemeth, 2006).

2.17 In-vitro estimation of iron bioavailability

In-vitro assays are done by simulating gastrointestinal digestion using a commercially available enzyme and then measurement of soluble iron released by the digestion to dialyzing tubing. In-vitro methods permit the simulation of in-vivo digestion conditions, permit pH control, provision of gradual adjustment with a mild

base. It also distinguishes between low and high molecular weight soluble iron and it accommodates food mixtures (Miller and Schricker, 1982). Three approaches have been established to estimate iron bioavailability in meals. These include two in-vitro assays: measurement of dialyzable iron and Caco-2 cell uptake, both carried out after in-vitro simulated gastric and pancreatic digestion (Lynch, 2005). The third method is the use of algorithms based on the predicted effects of specific meal components of absorption derived from isotopic studies in human volunteers. However, the accuracy of such predictions appears to be much lower when the algorithm is applied to meals eaten by different populations (Lynch, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Approach of the study

In accomplishing the objectives, this study was designed to be carried out in laboratory. The laboratory analysis was done to determine the nutrient composition of two cowpea leaf varieties, the retention of carotenoids and iron bioavailability of cowpea leaf dishes prepared according to traditional and modified preparation methods. The study also determined the retention of carotenoids and iron bioavailability of selected leafy vegetables (cowpea, sweet potato, pumpkin, amaranth and kang kong). The laboratory analyses were carried out in the Nutrition Laboratory at the Asian Vegetable Research Development Centre (AVRDC), Shanhua, Taiwan. The detailed descriptions of the laboratory analyses are presented below.

3.2 Nutritional analysis of cowpea leaves variety

Fresh cowpea leaves were harvested from the AVRDC fields and cleaned to remove dirty materials. Two cowpea leaf varieties namely Dakawa and Ex-Iseke were analyzed for dry matter, fiber, protein, sugar, vitamins (carotenoids, Vitamin E, ascorbic acid, folates), minerals (iron, calcium, zinc), flavonoids, antioxidant activity (ABTS) and anti-nutrient (oxalate). The detailed methods involved for each nutrient analysis are attached as Appendix 1.

3.3 Determination of carotenoid retention and iron bioavailability of cowpea leafy dishes prepared according to traditional preparation methods

Seven traditional recipes were compiled by Keller (2006) but only five recipes were selected for the determination of carotenoid retention and in-vitro iron bioavailability (Appendix 2) in this study because the remaining two recipes had almost similar ingredients and method of preparation as the other five recipes. The five traditional recipes were assigned names i.e. T1, T2, T3, T4 and T5 in order of arrangement. From the five traditional preparation methods only four recipes (T1, T2, T3 and T4) were modified basing on the principles of reducing cooking time (M1), addition of acidic food (yoghurt) (M2), oven drying (M3) and addition of oil (M4) (Appendix 2). Traditional cowpea leaf dishes were prepared at the same time with modified dishes to reduce biasness and the results were generated basing on three replications.

For sun dried dishes, fresh cowpea leaves were dried under direct sunlight for 3 days at varying temperatures of the day ranging from 29˚C at 10:45am, 38.5˚C at 13:00pm and 36˚C at 16:00pm. For oven dried cowpea leafy dishes, fresh cowpea leaves were oven dried at 50˚C for 16 hours in the oven. Carotenoids were determined by using High Performance Liquid Chromatography (HPLC) and iron was determined by *in vitro* method. Before cooking, fresh cowpea leaves were sorted and washed to remove dirty materials and were cut equivalent to one cut per leaf. The analytical procedures involved are attached as appendix 1.

3.4 Determination of carotenoids retention and iron bioavailability in selected leafy vegetables

Selected vegetables namely amaranth (*amaranth spp*), kangkong (*Ipomoea acquatica*), sweet potato (*Ipomea batatas*), pumpkin (*Cucurbita pepo*) and cowpea leaves (*Vigna unguiculata*) were analyzed for carotenoids retention by HPLC and iron bioavailability by *in vitro* method. Fresh vegetables were collected from AVRDC fields. Details of the preparations and analyses are given in Appendix 1 item 8. Results were generated based on two replications.

3.5 Statistical analysis

The results for nutritional analyses of two cowpea varieties were summarized using Excel software and the means separated by t-test at $P<0.05$ using SAS (Statistical Analysis System) program (Version 9.1) for Windows. In order to test the effect of modifications on carotenoid retention and iron bioavailability the results were analyzed for completely randomized block design. To compare recipes (traditional versus modified) the general linear model (GLM) procedure was used and the means were separated using the t-test. Carotenoids retention and iron bioavailability of raw and cooked vegetables were summarized using Excel and the means were tested for statistical significance at $P<0.05$ using SAS program.

CHAPTER FOUR

4.0 RESULTS

4.1 Overview

This chapter presents the results of the study. It is divided into four sections. Section one presents the nutrient composition of two cowpea leaf varieties. The second section describes the carotenoid retention and in-vitro iron bioavailability of cowpea leaves prepared according to traditional preparation methods. The third section delineates the carotenoid retention and in-vitro iron bioavailability of cowpea leaves prepared according to modified method. The fourth section describes the carotenoid retention and in vitro iron bioavailability of selected vegetables (cowpea, amaranth, sweet potato, kang kong and pumpkin leaves).

4.2 Nutrient composition of cowpea leaf varieties

Table 6 presents the summary of the selected nutrient composition of fresh cowpea leaf varieties. Between the two cowpea leaf varieties significant nutrient differences were observed in oxalates, vitamin C, folic acid, calcium, phenolics, antioxidants activity and flavonoids. There were no significant nutrient differences in dry matter, protein, crude fiber, free sugar, carotenoids, iron and zinc. Dakawa variety has shown to have higher contents of folic acid, phenolics, antioxidant activity, flavonoid quercetin and anti-nutrient oxalate while Ex-Iseke variety was observed to have higher contents of vitamin C, flavonoid kaempferol and total flavonoid. Basing on the results, Dakawa variety was found to be more nutritious than Ex-Iseke variety.

Table 6: Selected nutrient composition of cowpea leaf varieties per 100g of edible portion

Nutrient	Dakawa	Ex-Iseke
Dry matter (%)	12.3 ± 0.17[a]	11.6 ± 0.20[a]
Protein (%)	4.37 ± 0.02[a]	4.41 ± 0.01[a]
Crude fiber (g)	1.37 ± 0.02[a]	1.23 ± 0.02[a]
Free sugar (g)	1.16 ± 0.01[a]	1.08 ± 0.01[a]
Anti-nutrient		
Oxalate (mg)	418 ± 0.00[a]	348 ± 0.03[b]
Vitamins		
Violaxanthin (mg)	6.11 ± 0.01[a]	6.67 ± 0.01[a]
Neoxanthin (mg)	6.47 ± 0.01[a]	6.10 ± 0.10[a]
Lutein (mg)	21.84 ± 0.01[a]	19.47 ± 0.02[a]
Beta-Carotene (mg)	10.02 ± 0.01[a]	9.30 ± 0.17[a]
Total carotenoids(mg)	44.43 ± 0.03[a]	41.54 ± 0.01[a]
Vitamin C (mg)	86 ± 0.02[b]	94 ± 0.00[a]
Folic acid (µg)	136 ± 0.00[a]	121 ± 0.01[b]
Gamma-tocopherol (mg)	0.25 ± 0.01[a]	0.28 ± 0.02[a]
Alfa-tocopherol (mg)	3.70 ± 0.10[a]	2.7 ± 0.17[a]
Total tocopherol (mg)	3.90 ± 0.10[a]	2.90 ± 0.10[a]
Minerals		
Calcium (mg)	165 ± 0.03[a]	142 ± 0.01[b]
Iron (mg)	2.25 ± 0.02[a]	1.96 ± 0.02[a]
Zinc (mg)	0.61 ± 0.01[a]	0.51 ± 0.02[a]
Phytochemicals		
Phenolics (mg)	575 ± 0.02[a]	558 ± 0.01[b]
ABTS (µmole TE)	2596 ± 0.01[a]	2416 ± 0.01[b]
Chlorogenic acid (mg)	0.92 ± 0.03[a]	0.72 ± 0.04[a]
Quercetin (mg)	65.48 ±0.01[a]	58.58 ± 0.01[b]
Kaempferol (mg)	6.37 ± 0.03[b]	22.45 ± 0.02[a]
Isohamnetin (mg)	8.29 ± 0.01[a]	11.78 ± 0.02[a]
Total flavonoids (mg)	81.06 ± 0.03[b]	93.53 ± 0.02[a]

Key: -Values with the same letters within same row are not statistically significantly different at $P<0.05$

- Means ± SD based on three replications

4.3 Carotenoid retention and in-vitro iron bioavailability of cowpea leaf dishes prepared according to traditional methods

The cowpea leaf variety which was found to be more nutritious i.e. Dakawa variety was used. Table 7 presents the results for carotenoid retention and in-vitro iron bioavailability of cowpea leaf dishes prepared according to traditional preparation methods. Traditional cowpea leaf dishes cooked with oil, onion, tomatoes and coconut milk for 30 minutes (T1) was significantly ($P < 0.05$) high in beta-carotene (40.83%) and lutein retention (34.60%) compared to other recipes. In iron bioavailability, traditional recipe 4 (T4) which involved cooking fresh cowpea leaves for 15 minutes had the highest bio-available iron (10.04%). The least beta-carotene (9.60%) and lutein (2.83%) retention and bio-available iron (0.41%) were obtained in dishes involving sundried cowpea leaves + oil + tomatoes + groundnuts/coconut milk cooked for 30 minutes (T3 and T5) and fresh cowpea leaves + groundnuts cooked for 30 minutes (T2). Values of total iron content per dish were observed ranging from 9.22 µg/g in traditional recipe T4 to 17.55 µg/g in traditional recipe T3. Similarly, traditional recipes T2 and T3 had very high dry matter contents of 39.33% and 39.30%, respectively.

Table 7: Carotenoid retention and in vitro iron bioavailability of cowpea leaf dishes prepared according to traditional recipes

Nutrient	T1	T2	T3	T4	T5
Beta-carotene retention (%)	40.83±7.0[a]	25.93±5.0[b]	10.17±0.30[c]	26.33±4.4[b]	9.60±1.6[c]
Lutein retention (%)	34.60±3.30[a]	18.13±3.30[b]	2.83±0.40[c]	34.53±3.00[a]	5.27±0.1[c]
Total iron (µg/g)	13.49±1.27[b]	13.33±1.81[b]	17.55±0.00[a]	9.22±0.00[c]	11.39±0.00[c]
Bio-available iron (%)	4.13±0.90[b]	0.41±0.16[c]	1.56±0.55[c]	10.04±0.49[a]	1.94±0.17[c]
Dry matter (%)	9.13	39.33	39.30	5.90	7.9

Key: - Values with the same letters within same row are not statistically significant different at $P<0.05$

- Percentage retention calculated on fresh weight basis compared with amount in raw vegetable dish sample.

- T1: Fresh cowpea leaves + oil + onion + tomato + coconut milk cooked for 30 minutes

- T2: Fresh cowpea leaves + groundnuts cooked for 30 minutes

- T3: Sun-dried cowpea leaves +oil+ onion + tomato + groundnut cooked for 30 minutes

- T4: Fresh cowpea leaves cooked for 15 minutes

- T5: Sun-dried cowpea leaves + oil + onions + tomatoes + coconut milk cooked for 30 minutes

4.4 Beta-carotene and lutein retention and in-vitro iron bioavailability of cowpea leaf dishes prepared according to modified methods

Traditional recipes were modified basing on the principles to enhance carotenoid retention and *in vitro* iron bioavailability. Out of the five traditional recipes only four recipes were modified; T1, T2, T3 and T4. T5 was not modified because it was similar to T4 as it involved sun-dried cowpea leaves. The modifications included reducing cooking time, addition of yoghurt, oven drying and addition of oil respectively.

4.4.1 Method 1: Reduction of cooking time

Table 8 presents the results for carotenoid retention and iron bioavailability when cooking time was reduced from 30 minutes to 15 minutes. Reduction of time did not significantly increase carotenoid retention or iron bioavailability ($P>0.05$).

Table 8: Carotenoid retention and iron bioavailability of method 1

Nutrient	**Traditional** Fresh cowpea leaves + oil +onion + tomato + coconut milk cooked for 30 minutes	**Modified** Fresh cowpea leaves + oil + onion + tomato + coconut milk cooked for **15 minutes**
Beta carotene (%)	40.83 ± 7.0^{a}	41.50 ± 4.4^{a}
Lutein (%)	34.60 ± 3.30^{b}	36.83 ± 0.90^{b}
Bio- available Iron (%)	4.13 ± 0.90^{c}	5.44 ± 1.92^{c}

Key: - Values with the same letters within same row are not statistically significant different at $P<0.05$

- Percentage retention calculated on fresh weight basis compared with amount in raw vegetable dish sample.

4.4.2 Method 2: Addition of yoghurt

The traditional preparation method 2 was modified by addition of yoghurt. Dishes cooked with addition of yoghurt were not statistically significant (P>0.05) different from dishes cooked according to traditional preparation method in terms of carotenoid retention and iron bioavailability (Table 9). However the traditional recipe had very high dry matter content (39.33%). The amounts of lutein retention in the modified dish decreased (16.53%) compared to that of the traditional dish (18.1%).

Table 9: Carotenoid retention and iron bioavailability of method 2

Nutrient	**Traditional** Fresh cowpea leaves +groundnuts+ 30 minutes	**Modified** Fresh cowpea leaves + groundnuts + 30 minutes. Add **yoghurt**
Beta carotene (%)	25.93±5.00[a]	29.03±4.80[a]
Lutein (%)	18.1±3.30[a]	16.53±2.26[b]
Bio-available iron (%)	0.40±0.16[c]	0.50±0.21[c]
Dry matter (%)	39.33	4.70

Key: - Values with the same letters within same row are not statistically significant different at P<0.05
- Percentage retention calculated on fresh weight basis compared with amount in raw vegetable dish sample.

4.4.3 Method 3: Oven drying

The traditional method of sun-drying cowpea leaves was modified by adopting oven drying. Table 10 presents the findings. Oven drying did not significantly increase carotenoid retention and iron bioavailability (P>0.05).

Table 10: Carotenoid retention and iron bioavailability of method 3

Nutrient	**Traditional** Sun-dried cowpea leaves + oil + onion + tomato + groundnut cooked for 30 minutes	**Modified** **Oven dried** cowpea leaves + oil+ onion + tomato + groundnut cooked for 30 minutes
Beta carotene (%)	10.17±0.30[a]	6.70±0.70[a]
Lutein (%)	2.80±0.4[b]	3.43±0.20[b]
Bio-available iron (%)	1.56±0.55[c]	1.47±0.70[c]

Key: - Values with the same letters within each row are not statistically significant different at P <0.05
- Percentage retention calculated on fresh weight basis compared with amount in raw vegetable dish sample.

4.4.4 Method 4: Addition of oil

Table 11 presents the results of Beta-carotene and Lutein retention as well as iron bioavailability in cowpea leaf dishes cooked with traditional method and modified method of adding cooking oil. Dish cooked with oil was not significantly different from that cooked without oil (P>0.05).

Table 11: Carotenoid retention and iron bioavailability of method 3

Nutrient	**Traditional** Fresh cowpea leaves + 15 minutes cooking	**Modified** Fresh cowpea leaves + 15 minutes cooking + **oil**
Beta carotene (%)	26.33±4.4[a]	28.13±0.80[a]
Lutein (%)	34.53±3.0[b]	33.70±3.0[b]
Bio-available iron (%)	10.04±0.49[c]	9.03±2.98[c]

Key: - Values with the same letters within same row are not statistically significant different at $P<0.05$
- Percentage retention calculated on fresh weight basis compared with amount in raw vegetable dish sample.

4.5 Comparisons of Carotenoid retention and iron bioavailability of cowpea leaves with those of selected vegetables

The performance of four selected leafy vegetables was compared with that of cowpea leaves. The four leafy vegetables were namely Kang-kong, Pumpkins, Sweet potato and Amaranth.

4.5.1 Carotenoid retention

Table 12 presents the results of carotenoid retention when selected vegetables were cooked for 10 minutes. The highest carotenoid retention was observed in amaranth leaves (184% beta-carotene, 152% lutein, 95% violaxanthin and 81% neoxanthin) followed by cowpea leaves (140% beta-carotene, 133% lutein, 67% neoxanthin and 31% violaxanthin) with beta-carotene and lutein being highly retained. The least retention was observed in kang kong leaves except for beta-carotene.

Table 12: Results of Carotenoid retention of selected vegetables (cooked for 10 minutes)

Leafy Vegetables	Retention (%)			
	Neoxanthin	Violaxanthin	Lutein	Beta-carotene
Cowpea	67±0.32[b]	31±0.11[c]	133±1.17[b]	140±0.56[b]
Kang kong	22±0.04[c]	7±0.05[d]	66±0.05[e]	106±0.29[c]
Pumpkin	20±0.06[c]	30±0.05[c]	73±0.14[d]	102±0.42[c]
Sweet potato	21±0.12[c]	64±0.08[b]	105±0.45[c]	104±0.10[c]
Amaranth	81±0.03[a]	95±0.08[a]	152±0.42[a]	184±0.06[a]

Key: - Means with the same letters within each column are not statistically significant different at $P<0.05$
- Percentage retention calculated on fresh weight basis compared with amount in raw vegetable dish sample

4.5.2 Iron bioavailability

Figure 2 presents the results for iron bioavailability of selected leafy vegetables. Iron bioavailability increased with cooking except for amaranth and kang kong leaves. In raw vegetables iron bioavailability ranged from 0.21%±0.34 in sweet potato to 19.85%±5.5 in Amaranth while in cooked vegetables it ranged from 0.66% ±0.09 in Kang-kong to 15.40%±0.01 in Amaranth. In cooked vegetables, the highest iron bioavailability was observed in amaranth leaves (15.40% ±0.01) and cowpea leaves (12.01%±2.81). In raw vegetables the highest iron bioavailability was observed in amaranth leaves (19.85%±5.5). The least iron bioavailability in raw and cooked vegetables were respectively observed in sweet potato leaves (0.21%±0.34) and kang kong leaves (0.66% ±0.09).

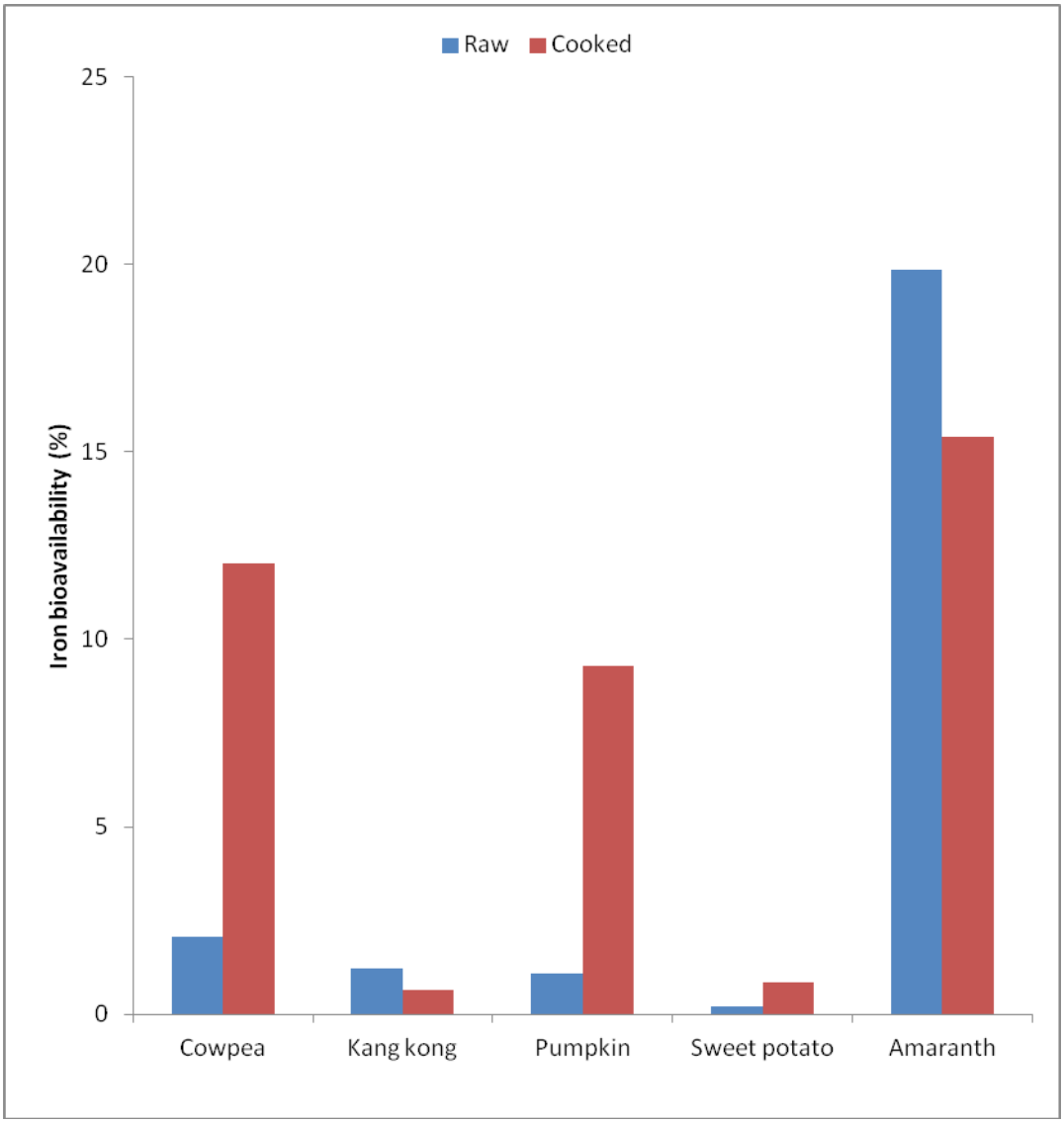

Figure 2: Iron bioavailability of raw and cooked vegetables (±standard error)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Nutritional composition of two raw cowpea leaf varieties

The nutritional value of legume leaves, such as those from cowpeas, has been largely discounted due to their high water content and the difficulty of documenting their production and consumption (Bittenbender, 1992). From the current study cowpea leaves were found to be good source of flavonoids, phenols, calcium, carotenoids (lutein, ά-carotene, neoxanthin and violaxanthin) and antioxidant activity (AOA). However, very high amounts of oxalates were also observed (Table 6). The protein values of this study compares well with 3.2% to 6.7 % observed by Ahenkora *et al*. (1998) on different cowpea leaf varieties grown in Ghana. However higher values of 20.64% to 46.56% were reported by Mosha *et al.* (1995). The dry matter values of Ex-Iseke variety is comparable with 11.4% observed by Mosha *et al*. (1997).

The study found higher amounts of calcium in both varieties than iron and zinc (Table 6). Similar mineral values were observed by Mosha *et al*. (1995) in the range of 83.64 - 229.34mg, 0.96 - 5.90mg and 0.40 - 2.24mg of Calcium, Iron and Zinc respectively per 100g of fresh vegetables. Slightly higher ascorbic acid values were observed compared to 43.78mg - 89.0mg observed by Mosha *et al.* (1995) in cowpea leaves consumed in Tanzania. Lower ascorbic acid values were also reported by Ahenkora *et al*. (1998) who found 8.8mg to 26mg per 100g of fresh cowpea leaves. However, higher ascorbic acid values of 164.3mg/100g on dry matter basis in cowpea leaves were reported by Ndawula *et al*. (2004) and 410mg per 100g of solids by Imungi and Potter (2006). Carotenoid values reported by Mosha *et al*. (1997) of

44.74 ± 0.009mg total carotenoids and 10.26 ± 0.098mg of ά-carotene per 100g dry weight basis compare well with the current study on fresh weight basis. Higher total carotenoid values of 57mg per 100g of solids were reported by Imungi and Potter (2006). The results in Table 6 show that Dakawa variety is more nutritious than Ex-Iseke variety. According to Mosha *et al*. (1997), the variations in the values could be attributed to the variations in the genetic composition of both varieties, season of the year, harvesting and handling practices prior to analysis. The period July-October was very hot with temperatures ranging between 38˚C to 39.5˚C; this might have affected the nutrient content of one or both cowpea leaf varieties in the field.

Phenols, flavonoids and antioxidant activity

This study found very high amounts of phenolics, total flavonoids and antioxidant activity in both types of cowpea varieties (Table 6). Phytochemicals, and in particular, phenolic compounds, present in plants may be partly responsible for health benefits through a variety of mechanisms (Young *et al*., 2005). It is underlined that the existing studies on humans demonstrate a convincing effect of polyphenols on some aspects of health (Kroon and Williamson, 2005). However, phenolic compounds have been identified as potent inhibitors of iron absorption, presumably by forming insoluble complexes with iron ions in the gastro-intestinal lumen and thereby making the iron unavailable for absorption (Brune *et al*., 1991). Natural antioxidants present in the diet increase the resistance toward oxidative damages and they may have a substantial impact on human health. The health benefits of fruits and vegetables are largely due to the antioxidant vitamins supported by the large number of phytochemicals, some with greater antioxidant properties (Dimitrios, 2006).

According to Stanners *et al*. (2004), the antioxidant hypothesis says that as antioxidants can prevent oxidative damages, increased intakes from the diet will also reduce the risks of chronic diseases. Flavonoids are strong antioxidants, scavengers of reactive oxygen, reactive nitrogen species and chelators of transition metals *in vitro* (Brown *et al*., 1998). Some flavonoids including quercetin, catechin, epigallocatechin gallate and epicatechingallate have shown much stronger antioxidant activities than vitamin C and ά-tocopherol in an *in vitro* cupric ion reducing assay (Apak *et al*., 2004). Flavonoids also exhibit a wide range of activities such as anti-carcinogenic, anti-inflammatory and anti-mutagenic activities and these functions frequently have been attributed to their free radical scavenging and antioxidant activities (Min and Ebeler, 2008). Recent studies have also indicated that flavonoids may function intracellularly by interacting with specific proteins involved in the intracellular signal cascade or by regulating gene expressions (Agullo *et al*., 2004; Kong *et al*., 2000).

5.2 The effect of traditional preparation methods on Carotenoid retention and Iron bioavailability of cowpea leaf dishes

5.2.1 Carotenoid retention

According to Table 7, the traditional recipe with higher beta-carotene and lutein retention is recipe T1 with fresh cowpea leaves + oil + onions + tomatoes + coconut milk + 30 minutes cooking time + boil/stir frying. Thus traditional recipe T1 was good for lutein and beta-carotene retention. Domestic processing and cooking methods are probable one of the most important factors affecting the daily intake of

carotenoids (van den Berg *et al*., 2000). Cutting of vegetables allows exposure of inner tissues to oxygen and light. Acid and light have been reported to cause isomerization of carotenoids from the *all-trans* form to the *cis* form which is biologically less active (Gayathri *et al*., 2004). The carotenoid content in dishes depends on the ingredient chemical nature, even when they are submitted to similar cooking conditions their values might change depending on the matrix in which they are involved (high/low fat content, antioxidants, metals, ripening status, etc) (Ruiz-Rodriguez *et al*., 2008). This may be one of the reasons which explain the differences and even different results from those found in this study (Tables 7).

Carotenoids are highly unsaturated structures which make them sensitive to light, oxygen and heat, therefore they are extremely susceptible to degradation. The degradation level is dependent on the cooking methodology utilised including temperature and time conditions (Ruiz-Rodriguez *et al*., 2008). Conditions during stir-frying might be more drastic than those of boiling, thus reducing the carotenoid levels in a higher percentage than boiling (Ruiz-Rodriguez *et al*., 2008). According to de Sa´ and Rodriguez-Amaya, (2004) boiled broccoli (5 min) retained approximately 7% more carotenoids (neoxanthin, lutein) than stir-fried broccoli (4 min). However, stir frying might also facilitate carotenoid intake as the process improves their bioavailability for pumpkins, carrots, amaranth leaves and fenugreek leaves respectively according to an in vitro digestion test (Veda *et al*., 2006).

Significant losses of carotenoids (Table 7) were observed in dishes where sun-drying was involved. This is because traditional sun-drying exposes vegetables into direct

sunlight and oxygen which results into chemical changes. Geometric isomerization (a change of geometry about a double bond) during sun-drying is mainly prompted by heat and light. Isomerization changes the usual configuration *trans*-forms of carotenoids into *cis*-forms (Gayathri *et al*., 2004). However, the level of oxidation depends on the extent of exposure to light, packaging material and presence of oxygen, storage materials, metals, enzymes, unsaturated lipids, antioxidants and physical state of the carotenoids (Rodriguez-Amaya, 2003). Generally, dehydration and powdering of vegetables increases surface area and leads to poor stability of carotenoids, unless the products are protected from air and light (Ruiz-Rodriguez *et al*., 2008). The results of this study (Table 7) compares well with other researchers who found reduction of carotenoids and other nutrients as a result of sun-drying (Mosha *et al*., 1997; Mulokozi and Svanberg, 2003). Svanberg (2007) found that amaranth and cowpea leaves lost more carotenoids when they were open sun-dried than when they were solar dried. In addition, open sun drying method caused 58% loss of β-carotene in cowpea leaves (Ndawula *et al*., 2004).

5.2.2 Iron bioavailability

In the current study the in-vitro iron was highest in traditional recipe 4 (T4) which involved cooking cowpea leaves for 15 minutes. The bioavailability in traditional recipes ranged from 0.41% to 10.04% (Table 7) and according to Allen and Ahluwalia (1997) the iron bioavailability for non-heme iron ranges between 2% to 20%. Thus traditional recipes T4 and T1 are good for enhanced iron absorption since they provide 10% and 4.13% non-heme iron. Food processing and chemistry of the iron are important factors affecting iron bioavailability. The chemistry of iron,

particularly its valence, solubility, and types of chelation, influence its absorption (Yang *et al*., 2006). The traditional preparations including prolonged cooking and exposure to sun during drying could significantly reduce the content of ascorbic acid in the dishes and the degradation of ascorbic acid as an enhancer of iron bioavailability could facilitate the inhibition of iron bioavailability (Naidu, 2003). In addition, ascorbic acid is highly unstable during processing thus high levels of the acid are lost during drying especially in open sun (Teucher, 2004). The losses of ascorbic acid during drying could be attributed to increased effects of iron bioavailability inhibitors such as polyphenols present in vegetables due to increased concentration of the inhibitors (SCN, 1993). Furthermore, dry matter content of the cowpea dishes can be another reason for decreasing iron bioavailability. During in-vitro determination of iron bioavailability only 5% to 7% of the sample's dry matter content is required for effective dialysis procedure. The more vicious the sample (dry matter weight) is the difficulty the pepsin digestion and pancreatic-bile suspension thus poor dialysis hence less iron bioavailability (Miller and Schricker, 1982). In this study, it was observed that traditional recipe T4 which had 5.90% dry matter content had the highest iron bioavailability (10.04%) and other recipes with dry matter contents of 39.33% and 39.30% had the lowest bioavailability suggesting that dry matter weight may have negative effect on iron bioavailability.

5.3 Effects of cooking or preparation modifications on carotenoid retention and iron bioavailability

5.3.1 Reduction of cooking time

Optimizing traditional processing is among the most common ways of reducing loss of nutrients in vegetables (WHO, 2001). According to Rodriguez-Amaya (2003) and Herman and Muhilal (1995) retention of carotenoids can be significantly improved by reducing cooking time. However, reduction of time did not increase carotenoid retention in this study (Table 8). This may be contributed by two things; the fact that cowpea leaves are more fibrous in nature thus requiring more time for cooking and for the nutrients to be released or the leaf matrix protect carotenoids against heat effect. Similar findings were observed by Mosha *et al*. (1997) who observed that cooking cowpea, pumpkin and peanut leaves for up to 30 minutes caused significant increase in the concentration of carotenoids. Reducing cooking time reduces exposure of vegetables to heat responsible for thermal degradation and isomerization (Mulokozi *et al*., 2004).

5.3.2 Oven drying

The current study findings show that carotenoid retention of cowpea leaves dried by sun is not significantly different from cowpea leaves dried by oven (Table 10). The results suggest that oven drying of cowpea leaves does not improve carotenoid retention that is destroyed by traditional method of sun-drying. The negative results observed in oven drying may be due to the extended time used during oven drying, where cowpea leaves were oven dried at 50°C for two days thus exposing carotenoids to heat and oxygen. Previous researches on vegetables reported that

modern ways of drying vegetables such as the use of solar and oven driers improve the nutrient content in dried products and retain higher quantities of carotenoids and other nutrients that are destroyed by the traditional methods of drying (FAO, 1997; Rensburg *et al*., 2004). The principle employed in oven drying is to protect the food from direct sunlight, which may reduce provitamin A carotenoid degradation during the preservation and storage of processed foods (Rodriguez- Amaya, 1997).

5.3.3 Addition of oil

The addition of oil has been found not to increase the contents and bioavailability of carotenoids (Table 11). However previous researches on vegetables found increasing carotenoids retention in vegetables cooked with oil. For instance, Mulokozi *et al*. (2004) found that vegetables cooked with oil had 2-5 times higher amount of in-vitro accessible all-trans-β-carotene and β-carotene than vegetables cooked without oil with the rates differing with the type of oil used. Other researchers (Laswai, 2006; Koskei, 2006) have also reported increasing levels of carotenoid retention when vegetables are cooked in modified method with oil than vegetables cooked without oil. However, the result of this study concurs well with those of Hedren *et al*. (2002) who found decreasing amounts of beta-carotene in cowpea leaves cooked with oil from 1475 (µg/100g) to 1418 (µg/100g) but significantly increased total beta-carotenoids in amaranth, sweet potato, pumpkin and cassava leaves. The effect of adding oil in vegetables is presumably due to its lipophilic nature of oil and the extraction of carotenoids into the lipophilic phase during cooking (Gartner *et al*., 1997). However factors affecting the retention of carotenoids include: species of carotenoids, molecular linkage and the matrix in which the carotenoid is incorporated

(Castenmiller and West, 1998; de Pee and West, 1996). This may explain the negative results obtained in this study.

5.4 Effect of reducing cooking time and addition of yoghurt on Iron bioavailability

Iron absorption from the diet can be improved by increasing the iron content or by increasing the bioavailability (Chiplonkar *et al*., 1999). Hallberg e*t al*. (1992) have reviewed interaction of dietary factors influencing iron absorption and concluded that modifying meal composition has a great potential for improving iron status in a population. Tables 8 and 9 show that reducing cooking time and addition of yoghurt did not significantly improve iron bioavailability. Yoghurt promotes non-heme iron absorption due to the ascorbic acid present which is soluble with high pH in the duodenum and small intestine (SCN, 1993). According to Naidu (2003), eating acidic foods especially those rich in ascorbic acid, along with iron containing foods can increase absorption three to seven fold. The non significant increase in bio-available iron (Tables 8 and 9) could be attributed by the fact that the promotion of iron absorption in the presence of ascorbic acid is determined by the amount of inhibitors in the meals. Meals containing low to medium levels of inhibitors require less amount of ascorbic acid as compared to meals with high levels of phytic acid (Teucher, 2004). According to the current findings (Table 6) cowpea leaves (Dakawa variety) contain 348 mg/100g of oxalates and 558mg/100g of phenolics. Oxalates, phytates, phosphates or food fibers perhaps decrease iron bioavailability (Kumari *et al*., 2004). The inhibitory effects of fibre to iron absorption are generally dependent on the presence of other factors such as phytate, oxalic acid, minerals and protein in

the foods (Zijp, 2000). In addition, the limitation of using ascorbic acid as enhancer to iron bioavailability is associated with its instability during processing and unwanted sensory changes (Davidsson, 2001).

5.5 Carotenoid retention of selected vegetables

Provitamin A carotenoids from green leafy vegetables constitute a major dietary source of vitamin A in Tanzania (Mulokozi *et al.*, 2004). Changes in carotenoids retention in food processing is caused by physical removal and chemical changes i.e. geometric isomerization, enzymatic and non-enzymatic oxidation (Rodriguez-Amaya, 2004). Geometric isomerization (a change of geometry about double bond) is mainly prompted by heat and light. Isomerization changes the usual configuration trans-forms of carotenoids into cis-forms (Kopsell, 2007). Oxidation is the main cause of carotenoids losses in vegetables. The level of oxidation depends on the extent of exposure to light, packaging material and presence of oxygen, storage materials, metals, enzymes, unsaturated lipids, antioxidants and physical state of the carotenoids (Rodriguez-Amaya, 2003). Food preparation practices at home have been associated with lowered retention of carotenoids, though information among studies is difficult to compare due to different methods used in the studies (Booth *et al*., 1992).

In this study all selected vegetables were observed to have high carotenoid retention with beta-carotene and lutein being highly retained more than 50% (Table 12). The highest retention was observed in amaranth, cowpea and sweet potato leaves. pumpkin and kangkong leaves had the lowest retention. Since the vegetables that

were found to have high carotenoid retentions are among the top ten leafy vegetables cultivated in ecological contrasting districts of Tanzania (Keller, 2006), efforts to promote their consumption is important following higher rates of Vitamin A deficiencies. Vegetables with low carotenoid retention (pumpkin and kangkong) should also be promoted for consumption as they also offer better carotenoid retention and when consumed together with Vitamin A rich foods requirements can be met.

In addition, the fact that cooking selected vegetables for 10 minutes without adding other ingredients led to more than 50% lutein and beta-carotene retention suggests that vegetables can be good source of carotenoids but the question which remains to be answered is how to make the retained carotenoid bio-available[1] and bio-accessible[2] to the body. Bioavailability of *β*-carotene from vegetables has been found to be lower than that from the pure compound (Castenmiller *et al*., 1999). There are various factors that may affect the bio-accessibility of carotenoids from its food sources including the food matrix, particle size of the food, presence of fat, and low bile acid secretion (Erdman *et al*., 1993). For green leafy vegetables, the leaf matrix seems to be an important factor that affects the accessibility of carotenoids, as it may hinder the release of the carotenoids during digestion. But cooking and reduction of particle size has been

[1] Bioavailability has been redefined as meaning the fraction of an ingested nutrient available for utilization in normal physiological functions and storage.

[2] Bioaccessibility is the amount of an ingested nutrient that is available for absorption in the gut after digestion

reported to reduce the matrix effect in green leafy vegetables (Erdman *et al.*, 1993).

5.6 Iron bioavailability in raw and cooked vegetables

Food processing and iron chemistry are important factors affecting iron bioavailability. The chemistry of iron, particularly its valence, solubility, and types of chelation, influence its absorption (Yang *et al*., 2006). In the current study, three of the five vegetables had improved iron bioavailability after ten minutes of cooking in boiling water (Fig. 2). The results compare well with those found by Yang *et al*. (2002) with slight variations. Simple cooking method of boiling vegetables in water raises available iron content thus improved iron bioavailability (Yang *et al.*, 1998; Yang, *et al.*, 2002; Yaday and Sehgal, 2003).

According to Yang *et al*. (1998), iron bioavailability of vegetables, legumes and cereals ranged from 0.2% to 25.3% in raw form and 0.8% − 33.8% in cooked items. These values compare well with the current study where iron bioavailability for raw vegetables ranged from 0.18% to 19.9% and 0.75%- 12.39% in cooked vegetables. The Iron bioavailability values for both raw and cooked vegetables are also within the range of 2% to 20% for non-heme iron absorption (Allen and Ahluwaia, 1997). In addition, according to Yang *et al*. (2002) vegetables were categorized into three groups based on their iron bioavailability of raw and cooked form where amaranth was in group one as having low iron bioavailability in raw form but two times or more after cooking. Kang kong in group two as having low bioavailability in raw form and slightly improved by cooking. Sweet potato fell in group three for having relatively high

iron bioavailability (>10%) in raw and cooked forms. However different findings were observed in this study where cowpea and pumpkin leaves were in first category, sweet potato in category two while amaranth fell in group three. Raw kangkong leaves had high bioavailability than cooked kangkong. The variation in the values could be attributed to environmental conditions and season of the year. The period July-October was very hot with temperatures ranging between 38˚C to 39.5˚C. The heat stress may have affected the nutrient composition of the vegetables. In addition, the soil's nutrient content of the fields in AVRDC plots may be another reason for the opposite and varied results. After observing the results obtained, the study went further and measured the amounts of carotenoids and iron bioavailability of sweet potato leaves available in the local market at Shanhua, close to AVRDC and observed higher amounts of carotenoid and iron bioavailability similar to those observed in other literatures.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The following conclusions can be made basing on the findings of this study:

(a) Cowpea leaves are very good sources of carotenoids, vitamin C, calcium, antioxidants, flavonoids and phenols. The nutritional compositions of the two cowpea leaf varieties (Dakawa and Ex-Iseke) suggest that Dakawa is more nutritious than Ex-Iseke in terms of folic acid, calcium, phenolics, antioxidant activity and flavonoid quercetin.

(b) With respect to traditional preparation methods, the recipe involving fresh cowpea leaves + oil + onion + tomatoes + coconut milk cooked for 30 minutes was observed to have the highest carotenoid retention. The recipe involving fresh cowpea leaves cooked for 15 minutes had the highest iron bioavailability.

(c) Addition of oil, reducing cooking time, and addition of yoghurt as modified preparation methods did not improve carotenoid retention and iron bioavailability in cowpea dishes.

(d) Selected vegetables (cowpea, amaranths, pumpkin, sweet potato and kangkong) leave have very high carotenoid retention when cooked for 10 minutes. Raw vegetables have low iron bioavailability compared to cooked vegetables.

Generally, this study found out that not all principles used for modifying vegetable preparation methods to enhance carotenoid retention and iron bioavailability work

for every vegetable. Thus rejecting the hypothesis that modified preparation methods of cowpea leafy vegetables result in higher carotenoid retention and iron bioavailability than the traditional preparation methods. The results suggest that other intrinsic or unknown factors in the given vegetables are responsible for non-responsiveness to the modifications.

6.2 Recommendations

Basing on the results found, the following are recommended:

i. Efforts should be made to promote the cultivation and consumption of Dakawa cowpea leaf variety in our communities due to its high nutritional values. However, the nutritional composition of other cowpea leaf varieties should be explored.

ii. Traditional recipes involving fresh cowpea leaves + oil + onion + tomatoes + coconut milk cooked for 30 minutes and fresh cowpea leaves cooked for 15 minutes are recommended as better traditional preparation methods in Kongwa and Muheza for enhanced carotenoid retention and iron bioavailability.

iii. Further studies to modify traditional cowpea leaf preparation methods for enhanced carotenoid retention and iron bioavailability are necessary considering the dietary importance of cowpea leaves in rural Tanzania.

iv. Further studies are needed to validate the modification principles used for enhanced nutrient retention on other indigenous vegetables as well as studies on how to modify traditional cowpea leaf preparation methods for enhanced nutrient retention.

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APPENDICES

Appendix 1: Determination of dry matter in cowpea leaf varieties

Dry matter was determined according to AOAC (1990). The involved principle is that moisture was evaporated from the sample by oven drying. Total dry matter was determined gravimetrically as residue remaining after drying.

Procedure

The sample surface was cleaned and dried by air. The fresh sample was cut into 2 – 3 cm of length approximately and mixed well into the nylon mesh bag, 150-300g of the sample was weighed (W_S). The sample was then oven dried at 50˚C for 24 hours and the weight of the dry samples recorded (W_D).

Dry matter was calculated in percentage as follows

$$\% \text{ Dry matter} = W_D / W_S \times 100\%$$

Where:

W_S = Weight of fresh sample

W_D = Weight of dry sample

2. Determination of fibre in cowpea leaf varieties

Fibre was determined according to analytical procedures of AOAC (1990). The principle involve is that crude fibre is determined gravimetrically after chemical digestion and solubilization of other materials present. The fibre residue weight is then corrected for ash content after ignition.

Reagents

Sodium hydroxide solution 0.312 N (1.25%) and sulphuric acid solution 0.255 N (1.25%).

Procedure

A sample of about 0.3g was weighed into a sealed handmade filet paper bag. The bag was boiled in a 30ml of 1.25% H_2SO_4 for 30 minutes and there after washed with boiling water followed by 1.25% NAOH solution three times each. When the boiling water from the bag was clear, the bag was placed in a crucible and oven dried for 3 hours at 125˚C. The bag was transferred to the muffle furnace for 4 hours at 600 ˚C. The bag was then cooled in the muffle furnace overnight and transferred to the desiccators and weight recorded.

Fibre was calculated using the following formula:

% Fibre = { [(W_1- W_2) - (Wn - W_B)] ×100 }/ S.Wt

Where:

W1 = bag + dry residue (g)

W2 = bag weight (g)

W_n = crucible + ash (g)

W_B = crucible weight (g)

S.Wt = Weight of dry sample (g)

3. Determination of protein in cowpea leaf varieties

Protein was determined according to AOAC (1990).

Principle:

The product was digested with concentrated sulphuric acid as a catalyst to convert organic nitrogen to ammonium ions. Alkali was added and the liberated ammonia distilled into sulphuric acid solution. The distillate was titrated with sodium hydroxide solution to determine the ammonia absorbed in the sulphuric acid solution.

Reagents

Reagents used were; catalyst: K_2SO_4: $CuSO_4.5H_20$ (30:1), 0.02 N H_2SO_4 (nitrogen free), 50% NaOH which was prepared by dissolving 50g NaOH in 100ml of water, 0.02 N NaOH prepared by dissolving 50g NaOH in 1000ml of water and indicator which was prepared by dissolving bromoresol green 0.3g and Methyl red 0.2g in 400ml 90% alcohol.

Procedure:

A. Digestion

A total of 100mg of the sample was weighed into kjeldah flask with the same amount of catalyst and 5mls of concentrated sulphuric acid added and mixed by gently swirling the liquid. The flask was heated slowly starting from 170 ˚C for 2 hours to 320 ˚C for 2 more hours until foaming ceased and the contents were completely liquefied. In the contents 3-5mls of 30% hydrogen peroxide were added and digested by boiling vigorously at 320 ˚C for 1 hour while rotating the flask occasionally until the liquid was completely clear. The contents were cooled to about 40 ˚C and cautiously diluted to 25ml by hydrogen peroxide.

B. Steam distillation

In a clean distillation apparatus 10ml of 0.02N H_2SO4 and 4 drops of indicator solution were added and mixed. The solution was placed under the condenser of the distillation apparatus so that the outlet of the adapter deepen into the liquid. A 3ml of the sample solution was transferred to the distillation flask. At the inclined neck of the distillation flask 5ml of 50% sodium hydroxide was poured carefully. Steam was passed through alkaline liquid slowly to reduce foaming until it boiled; the liquid was let to boil until 75ml of the distillate was collected. The conical flask was lowered just before terminating the distillation so that the outlet of the adapter was above the liquid level. The outlet of the adapter above the liquid was rinsed with little water.

C. Titration

The content of the conical flask was titrated with 0.02N sodium hydroxide and the volume recorded. Nitrogen was calculated in percentage as:

$N (\%) = [(V_2-V_1) \times 14 \times N \times 25/ A] / \text{Sample weight (g)} \times 100\%$

Where;

V_1 = Volume (ml) of 0.02N NaOH solution required for the test portion

V_2 = Volume (ml) of 0.02N NaOH solution required for the blank portion

N = Normality of 0.02N NaOH

A = Sample volume (ml) of distillation

4. Determination of sugar in cowpea leaf varieties

Sugar in cowpea leaf varieties was determined using Anthrone method AOAC (1970).

Principle

Total sugar content of the vegetable powder was determined by spectrophotometer using the anthrone reagent, which reacts specifically with carbohydrate in concentrated sulphuric acid solution to produce a blue green colour at 630 nm. The final result was expressed as sucrose equivalent.

Reagents

The following reagents were used: 80% ethanol which was prepared by adding 1600ml of 95% ethanol in 300ml of distilled water, 36N concentrated sulphuric acid,

0.2% anthrone (9, 10 – dihydro-9-oxontracene) prepared by dissolving 2g anthrone in 36N concentrated sulphuric acid up to 1000ml and stored at 4˚C for less than two days and 100ppm glucose stock which was prepared by dissolving 50mg of glucose in 500ml distilled water.

Procedures

A powdered sample (W_S) of 0.1g was put into a 35ml centrifuge tube and 10ml of 80% ethanol was added and vortexed to uniform. The tube was covered with a glass lip and heated to 80˚C in water bath for 10 minutes. The sample was cooled to room temperature and centrifuged for 10 minutes at 10,000 rpm. The supernatant was concentrated at 80˚C-85˚C to 3ml on the heat plate. The concentrate was diluted to 250ml with distilled water (V_1) and 2.5 ml (V_2) of the standards were diluted with 250ml distilled water into tubes respectively. The tubes were placed in ice bath with 0.2% anthrone added slowly and mixed gently. The samples were transferred to boiled water bath for 7.5 minutes and cooled down to room temperature with water. The absorbance was detected at 630nm by spectrophotometer

The amount of carbohydrate was calculated in percentage as follows;

% Carbohydrates = $y \times X (V_1/V_2)/W_S \times 100$ %

Where x = the concentrations of the standards or the samples

y = the absorbance detected

W_s = sample weight

5. Determination of Vitamin C (ascorbic acid) in cowpea leaf varieties

Ascorbic acid in cowpea leaf varieties was extracted using the method of Niesperos-Carriedos *et al*. (1992).

Reagents

Extraction solution: was prepared by mixing 45 g of metaphosphoric acid and 120 ml acetic acid in distilled water and diluted to 1500 ml, 2,6- DCPIP solution prepared by dissolving 0.2 g of 2,6- Dichlorophenolindophenol sodium salt in 100 ml hot water, Metaphosphoric acid solution: prepared by mixing 50 g of metaphosphoric acid in 100ml distilled water, Thiourea solution: prepared by dissolving 10g of thiourea in 500ml 5% metaphosphoric acid solution, 9N H_2SO_4, 2, 4-DNPH prepared by mixing 10g of 2,4-Dinitrophenylhydrazine in 9N H_2SO_4 and 85% H_2SO_4.

Procedure

Fresh sample of 20g was mixed with 80 ml extraction solution and blended for 5 minutes. The sample solution was then centrifuged at 7000 rpm for 10 minutes and filtered. From the sample solution 0.3 ml were pipetted into three test tubes (third test tube was for blank). In the test tubes, 1.7ml of the extraction solution was added together with one drop of 2, 6-DCPIP solution and a pink colour appeared. Thiourea solution of 2ml was added in each test tube including the standard and stirred. 2,4 -DNPH of about 1ml was added to two test tubes including the standard and all test tubes were placed in water bath at 37 ˚C for 3 hours. The three test tubes were kept on ice bath for 10 minutes and 5 ml of 85% sulphuric acid added and mixed thoroughly. In the blank test tube 1ml of 2, 4 – DNPH was added and all three test

tubes were let to stand for 30 minutes at room temperature before reading at 520nm atomic absorption spectrophotometer.

6. Determination of folic acid in cowpea leaf varieties

Folic acid was determined according to AOAC (1991).

Reagents

Reagents used for the determination of folates were; 0.1M Phosphate/0.15% AA buffer of pH 6.0, 0.05M phosphate/0.1% AA buffer of pH 6.0, 0.85% Sodium chloride, folic acid stock solution (200 µg/ml), freshly prepared folic acid standard solution (1 µg/ml) and kidney enzyme solution.

Procedures

Fresh cowpea leaves of about 10g were mixed with 0.1M phosphate/AA buffer and the pH adjusted to 6.0. The sample was mixed in a warring blender for 1-2 minutes. The sample was poured to 250ml flask and washed with additional 10ml 0.1M phosphate/AA buffer and pH adjusted to 6.0. The mixture was then autoclaved for 10 minutes and then diluted to 250ml with 0.1M phosphate/AA buffer of pH 6.0. From the diluted solution 10 ml were taken and stored at -20 ˚C for HPLC analysis. Into a 50ml centrifuge tube, 30g was weighed and centrifuged at 16,000 rpm for 10 minutes. From the supernatant 20ml was mixed with 1.6ml enzyme solution and incubated at 37 ˚C with shaker for 2 hours. After incubation the samples were heated in 100 ˚C water bath for 5 minutes. A weight of 5 ml of the incubated sample was diluted to 100-250ml with 0.05M phosphate/AA buffer. Three duplicate

concentrations in tubes were made (6 tubes per sample) and all the tubes were autoclaved for 10 minutes and cooled down to below 40˚ C. In laminar flow 0.040 ml of bacteria solution was added and incubated at 37 ˚C for 15-17 hours (overnight). OD values were read at 640nm and turbidity determined.

7. Determination of flavonoids in cowpea leaf varieties

Flavonoids were determined using High performance Liquid Chromatography (HPLC) a method of Hertog *et al*. (1992).

Reagents

Extract solution: 1% formic acid in 80% methanol, 2.4M hydrochloric acid in 20% methanol which was prepared by mixing 41.38ml hydrochloric acid and 40 ml methanol and diluted to 200ml with water.

Procedure:

A vegetable sample of 100mg was mixed with 5 ml of extract solution (Merck LC grade) and shaken for 30 minutes then centrifuged for 3 minutes (10*1000 rpm). Thereafter 2mls of the resulting supernatant and 2ml of mobile phase A (MPA) were mixed and after 10-15 minutes filtered through a 0.45µm membrane and HPLC auto inject 30µl

Hydrolysis

The procedure for hydrolysis was as follows; 2 ml of the resulting supernatant and 2ml of 2.4M hydrochloric acid in 20% methanol were mixed in 7ml vials and the weight of the vials with sample recorded. The sample was then flashed with nitrogen

screw capped and vortexed for 15-20 seconds and put in water bath of 90˚ C for 2 hours. The vials were then cooled and vortexed for 3-5 seconds and filtered through a 0.45µm membrane and HPLC Auto inject 30µl.

HPLC system

Flavonoid was determined using waters alliance module 2695-996 photo diode array detector. The column used was agilent zorbax ODS (SB-C18), 4.6 x 150MM, 3.5 µm (part no: 863953-902, SN USEG007615, LN B04095, made in USA). Three mobile phases were used; MPA %: 1% Formic acid in acetonitrile/water (1:99), MPB%: 1% Formic acid in acetonitrile/water (1:1) (Merck LC grade) and MPC%: 1% formic acid in acetonitrile. The flow rate was set to 0.7ml/min gradient at 40˚C. The photodiode array detector used was 210-600nm (280nm: 350nm).

Identification

Identification and confirmation was done on the basis of retention times and compared with co – chromatography standards and spectra standard peaks of chlorogenic, caffeic, quercetin, luteolin, apigrnin, kaempferol and isohamnetin (appendix 3, page 113).

8. Determination of Carotenoids Contents by High Performance Liquid Chromatography (HPLC)

Sample preparation and extractions, identification and quantification of carotenoids (β – carotene, violaxanthin, neoxanthin and lutein) were done as explained by Rodriguez – Amaya and Kimura, (2004).

Chemicals

HPLC solvents including; hexane, acetone, acetonitrile, tetrahydrofolate (THF), ether and methanol and nitrogen gas were used.

Sample extraction

Sample extraction was done in order to release all carotenoids from the vegetables and bring them into solution, without altering them. To achieve this, 10g of sample together with 40 ml of acetone were homogenised for 2 minutes by using a polytron homogenizer. The sample was filtered under vacuum with Whatman filter paper no 1 in a funnel. From the extract sample 2mls were transferred to 100 ml bottle and dried by nitrogen gas.

Saponification

Saponification was done on samples from recipes only; fresh samples did not undergo saponification. Saponification was done in order to remove lipids and chlorophylls. On the nitrogen dried samples 100µg of ether were added followed by 2 ml of methanol and 1ml of 15% potassium hydroxide in methanol solution and mixed. The sample was then exposed to nitrogen gas to evaporate ether and the sample was then put in the incubator for 2 hours at 30 ˚C. In the saponified sample, 1.5 ml hexane and 1.5 ml of distilled water were added and mixed for 1 minute. The saponifiable sample was then transferred to a 60 ml separating funnel. The upper layer was extracted three times with 3 ml of hexane. The hexane layers were together washed four times with distilled water. The hexane layers were diluted to 100 ml and the lower layer was drawn off. Carotenoids extract 10 ml were dried by nitrogen and

diluted to 1ml in 2ml HPLC vials with 100µl THF and 2mls CH_3CN:CH_3OH on a 75:25 ratio and together were mixed by a vortex machine. The extract was the filtered using a 0.2µm membrane and 20 µl were injected on HPLC.

HPLC instrumentation and conditions

The analysis was done with a water separation module (model 2695) equipped with an autosampler injector and a waters model UV 996 (450nm) photodiode array detector. The column used were LiChrospher 100, RP – 18e column (5µm) and LiChroCART 125-4 (cat. No 1.50734). The mobile phase was composed of 90% methanol in water, 100% methanol and 100% THF. Flow rate was set at 1.0ml/minute.

Identification

Identification and confirmation was done on the basis of retention times and comparison with co – chromatography standards and spectra standard peaks of β – carotene, lutein, violaxanthin and neoxanthin.

9. Determination of antioxidant activity (ABTS) in cowpea leaf varieties

Antioxidant activity in cowpea leaves variety was determined according to the method of Osawa and Namiki (1991).

Principle

This antioxidant decoloration method was based on the capacity of antioxidant substances in vegetable extracts to scavenge the ABTS radical cation, namely $ABTS^{+}$.

Reagents

The following reagents were used for the determination of ABTS; 50mM sodium phosphate buffer of pH 7.5: which was prepared by dissolving 0.6899 g of NaH_2PO4 in 100 ml of distilled water and dissolving 3.549 g of Na_2HPO4 in 500ml of distilled water. The two were mixed and pH value adjusted and the solution was stored at 4˚C, peroxidase acidic isoenzyme from horseradish, EC 1.11.1.7 (Sigma P-6782): was prepared by dissolving 1.0 mg of lyophilized powder with 1 ml of 50mM sodium phosphate buffer. Then 0.5 ml of stock solution was distributed into micro tube and stored at –20 ˚C, 31% H_2O_2 (Merck 108552) which was freshly prepared by diluting 0.3 ml of 31% H_2O_2 into 100ml with distilled, 2, 2-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt, 98% (ABTS, Sigma A-1888): prepared by dissolving 0.5487 g of ABTS and 0.75 ml of dilute aqueous H_2O_2 in 25 ml of 50mM sodium phosphate buffer and stored at 4˚C, ethanol (Merck 1.11727)**,** Methanol (Merck 1.06007) and 6-Hydroxy-2, 5,7,8-tetramethyl-chroman-2-carboxylic acid, 97% trolox (Aldrich 238813) which was prepared freshly by

dissolving 0.025 g of Trolox in 10 ml of methanol. The stock solution was diluted into following concentrations, 0, 1, 2, 3 and 4nm.

Procedures

Freeze dried sample of 10g in 40ml of methanol were homogenized with a warring blender for 2 minutes and centrifuged at 12,000 rpm for 10 minutes. The supernatant were transferred into vials and stored at 70˚C ready for analysis.

Antioxidant activity determination

A mixture of 10ml of ABTS/50M sodium phosphate buffer with 0.5ml of HRP stock solution and 90 ml of ethanol were centrifuged at 12,000 rpm for 5 minutes and the supernatants collected. Of supernatant sample 0.02 ml were reacted with 2 ml of the reaction mixture and incubated for 5 minutes and the absorbance was determined at 730nm. ABTS was calculated as follows;

$$\% \text{ Inhibition} = (Abs_b - Abs_s) \div Abs_b$$

Abs_b: absorbance of blank

Abs_s: absorbance of standards

The percentage inhibition described above and concentrations of the standards composed the regression equation; y = ax + b. The absorbance of the samples was used in substitution for Abs_s to produce the % inhibition of sample. The % inhibition of sample was used in substitution for y to result the concentration of sample as compared to a standard antioxidant Trolox in a dose response curve.

10. Determination of Calcium, Zinc and Iron in cowpea leaf varieties

Calcium, zinc and Iron were determined according to a method explained by Benton (2001).

Procedure

A vegetable sample of 0.2g was mixed with 5ml of concentrated sulphuric acid to digest the plant tissues. The sample was heated to 300˚C for 3-4 hours and cooled to below 150˚C followed by addition of 2ml of 30% hydrogen peroxide. The mixture was heated to 300˚C for half an hour until the solution was clear and diluted with distilled water to 50ml. Iron and zinc was analyzed using atomic absorption spectrophotometer (Hitachi Z-6000 Polarized Zeeman Atomic Absorption Spectophotometer). Calcium was determined using Lanthanum (III) chloride heptahydrate.

Analytical conditions for Calcium

The light source used was calcium-magnesium hollow cathode lamp of 7.5 mA current set at 422.7nm wavelength. The slit and burner height were set to 2.6nm and 12.5mm respectively. A standard type of burner head was used with oxidant pressure of 1.6 kg/cm^2 and fuel gas pressure of about 0.3 kg/cm^2. Linearity of calibration curve was up to about 2 ppm (ABS 0.14), measurable concentration limit was 0.02ppm for the lower limit and 50ppm for upper limit. Absorption resonance lines were set at 422.7nm and 239.9 nm wavelength while the sensitivity ratio was 1 and 0.004. Air-acetylene flame was used.

Analytical conditions for Zinc

The light source used was zinc hollow cathode lamp of 10 mA current set at 213.8nm wavelength. The slit and burner height were set to 1.3nm and 7.5mm respectively. A standard type of burner head was used with oxidant pressure of 1.6 kg/cm^2 and fuel gas pressure of about 0.2 kg/cm^2. Linearity of calibration curve was up to about 0.5 ppm (ABS 0.35); measurable concentration limit was 0.02 ppm for the lower limit and 4ppm for upper limit. Absorption resonance lines were set at 213.8nm and 307.6nm wavelength while the sensitivity ratio was 1 and 0.001. Air-acetylene flame was used.

Analytical conditions for Iron

The light source used was iron hollow cathode lamp of 12.5 mA current set at 248.3nm wavelength. The slit and burner height were set to 0.2nm and 7.5mm respectively. A standard type of burner head was used with oxidant pressure of 1.6 kg/cm^2 and fuel gas pressure of about 0.3 kg/cm^2. Linearity of calibration curve was up to about 4 ppm (ABS 0.2), measurable concentration limit was 0.2ppm for the lower limit and 20ppm for upper limit. Absorption resonance lines were set at 248.3nm, 248.8nm, 252.7nm, 302.1nm, 305.9nm, 344.1nm, 372.0nm, 373.7nm and 392.0nm wavelength respectively while the sensitivity ratio was 1.0, 0.34, 0.23, 0.24, 0.02, 0.01, 0.16, 0.08 and 0.003 respectively. Air-acetylene flame was used.

11. Determination of Oxalates in cowpea leaf varieties

Oxalate was determined using HPLC method (AOAC, 1990).

Reagents

Oxalic acid standard and 0.01 N sulphuric acid of pH 2.1

Procedures

Oven dried powder sample of about 100mg was defatted with hexane four times. To the defatted sample 100ml of distilled water was added and the sample was shaken for 1 hour. The sample was centrifuged at 1000 rpm for 3 minutes and filtered through a 0.45µm membrane. From the centrifuged sample 30 µl of the sample was auto injected for analysis.

HPLC system

The HPLC system used was Waters 600 controller-717 plus autosampler -410 differential refractometer. The column used was ICSep ICE-ORH-801 (transgenomic). The flow rate was set to 0.8 ml/min at 35˚C and the detector was refractometer.

Identification

Identification was done on the basis of retention times and comparison with co – chromatography standards of oxalates.

12. Determination of Vitamin E (tocopherols) in cowpea leaf varieties

The Saponification and extraction procedures were adapted from Piironen *et al.*, (1985).

Procedures

A powdered vegetable sample of about 5g was mixed with 0.25 ascorbic acid, 10ml water and 30ml of 100% ethanol and homogenized for 2 minutes. The sample was let to stand for 15-20 minutes where 6ml of 50% KOH was added. The sample was flushed with nitrogen screw cap and vortexed for 15-20 seconds. The sample was put in water bath of 80˚C for 1 hour for Saponification. The sample was filtered and rinsed with 30ml of 50% ethanol before it was extracted three times with 30ml of hexane. The sample was washed with distilled water and diluted to 200ml with hexane. The hexane layer was dried by nitrogen and then 1ml of the sample mixed with 1ml of methanol was used for for HPLC analysis.

HPLC instrumentation and conditions

The analysis was done with a water separation module (model 2695) equipped with an autosampler injector and a waters model UV 996 (450nm) photodiode array detector. The column used werePhenomenex prodigy ODS- 2, 2 x 250mm, 5-um particle size P/NO 00G-3300-B0; S/NO 98085-1. The mobile phase was composed of acetonitrile and methanol in a 85:15 ratio (v/v). Flow rate was set at 0.4ml/minute and 30 minutes running time

Identification

Identification was done on the basis of retention times and comparison with co – chromatography standards of δ-tocopherols, γ-tocopherols and α-tocopherols.

13. Iron Bioavailability by *in vitro* Method

An in vitro method for estimating iron availability was used by a modified calorimetric method as described in Miller and Schricker (1982), modified by Kapanidis and Lee (1995).

Chemicals

Chemicals and reagents used were hydrochloric acid (0.01N HCL, 0.1N HCL, 6N HCL) sodium hydroxide (0.5M NaOH), 0.1M sodium bicarbonate ($NaHCO_3$), 0.5M sodium bicarbonate, Iron (Fe) standard solution in 0.1N HCL for iron determination in raw vegetables (AA), Fe standard solution in 0.01N HCL for "Batho" and 2 M sodium acetate (CH_3COONa). Pepsin solution, pancreatin-bile suspension, batho reagent and protein precipitant solution were also used.

Solution preparation

Pepsin solution; was prepared by dissolving 16g of pepsin in 0.1M HCL to 100g. Pancreatin – bile suspension was prepared by suspending 1g of pancreatin (from porcine pancrease) and 6.5g porcine bile extracts in 0.1M $NaHCO_3$ to 250ml. Batho reagent was prepared by dissolving 125g of bathophenanthrolin disulfonic acid disodium salt and 50g of hydroxhylamine hydrochloride in 2M sodium acetate to

500ml. Protein precipitant solution was prepared by dissolving 50g TCA (trace mineral grade) and 50ml concentrated HCL in distilled water (DI) to 500ml.

In-vitro gastro intestinal digestion

100g of the sample in 100g of distilled water was blended in a warring blender to make a creamy consistency. The pH of the sample was adjusted to 2 ± 0.05 with 6N HCL which is about 2ml. About 20g of the sample was weighed in three 125ml flasks. Pepsin 0.75ml solution was added to each flask. All the flasks were covered and incubated at 37^{o}C for two hours with shaking. One flask was taken from the incubators after 1 hour and 30 minutes for analysis of titratable acidity.

Determination of titratable acidity (one flask)

Titratable acidity is defined as the number of equivalents of NaOH required to titrate a 20g aliquot containing 5ml of the pancreatin – bile mixture to pH 7). About 5 ml of pancreatin – bile suspension was added to the flask. Through titration by 0.5 N NaOH, pH was adjusted to 7 ± 0.05. The volume (X ml) NaOH was recorded. For one flask, X ml of 0.5N $NaHCO_3$ was added, then diluted with distilled water to 25ml.

Dialysis procedure (two flasks)

Two dialysis bags tied at both sides containing an amount of 25ml of 0.5N $NaHCO_3$ solution each were put into the remaining two flasks (pepsin digest) after 2 hours of incubation at 37^{o}C with shaking **.** The sample was then incubated at 37^{o}C with shaking for 30 minutes. About 5ml of pancreatin – bile suspension mixture was

added to the digestion flasks outside the dialysis bag and the flasks were incubated at 37^{o}C for 2 hours. The dialysis bags were removed, rinsed thoroughly in distilled water and the contents (dialysate) were transferred to vials. The dialysates were weighed and stored in the refrigerator waiting iron analysis by batho method (bathophenanthroline reactive iron).

Iron determination in the dialyzates (Batho method)

5 ml of dialysate was weighed in 15 ml centrifuge tube. Protein precipitant solution (2.5 ml) was added. The sample was mixed by a votex mixer. The tube was placed in boiling water for 10 minutes, then cooled and centrifuged at 5000rpm for ten 10minutes. To the supernatant 2.5 ml of batho reagent was added, mixed by votex mixer. The sample was let to stand for 10 minutes for development of red colour. Reading was done at OD 535 nM using atomic absorption spectrophotometer (AAS) with a blank 0.01 N HCL. The standards were 0, 0.1, 0.2, 0.3, 0.5, 1, 2 ppm of 1015 ppm Fe.

Calculation of dialysability (bioavailability)

The iron bioavailability was expressed as percent of total non-heme in the original aliquot that was present in the dialysis bag at the end of the digestion, therefore referred as iron dialysability.

Iron dialysability (%) = $[iron]_{dial}$ * wt dial / $[iron]_{recipes}$

Where:

$[Iron]_{dial}$ = iron in dialysate (µg/g)

$[Iron]_{recipes}$ = iron in recipes (µg/g)

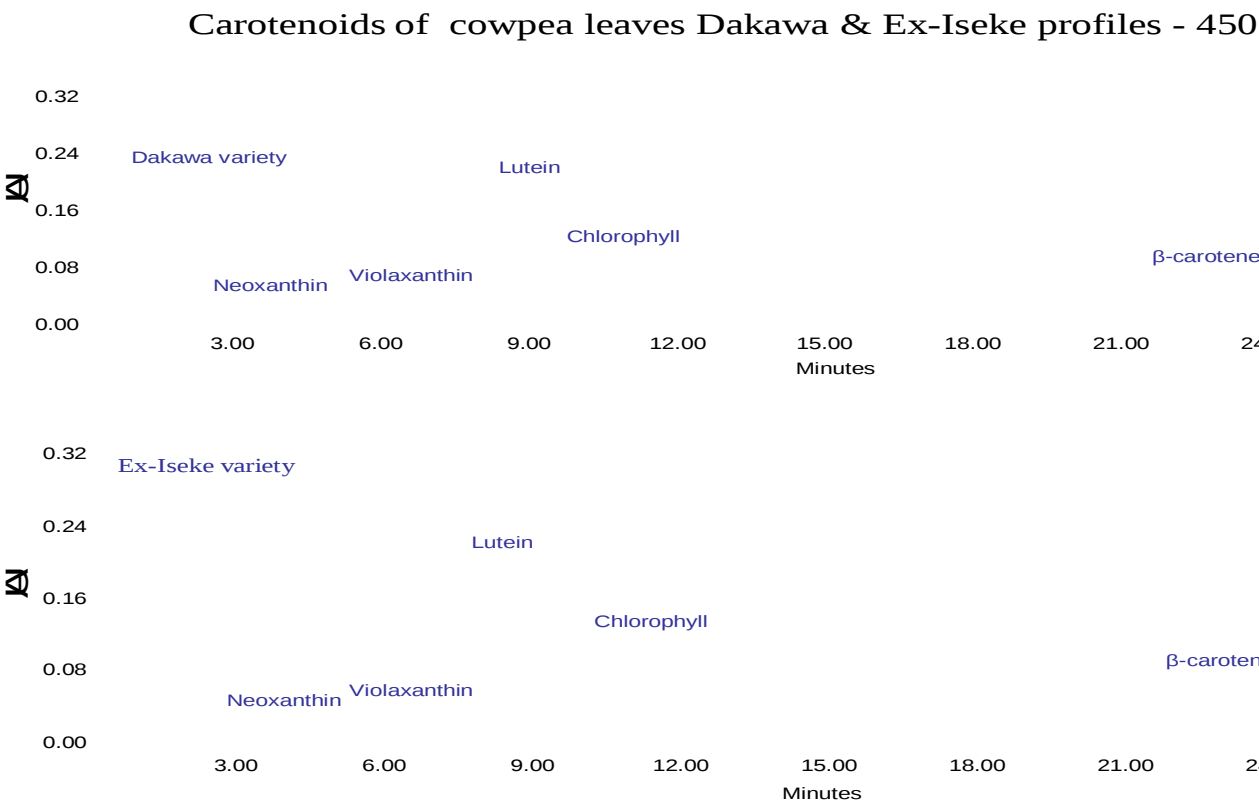
Wt_{dial} = Weight of dialysate after dialysis (g)

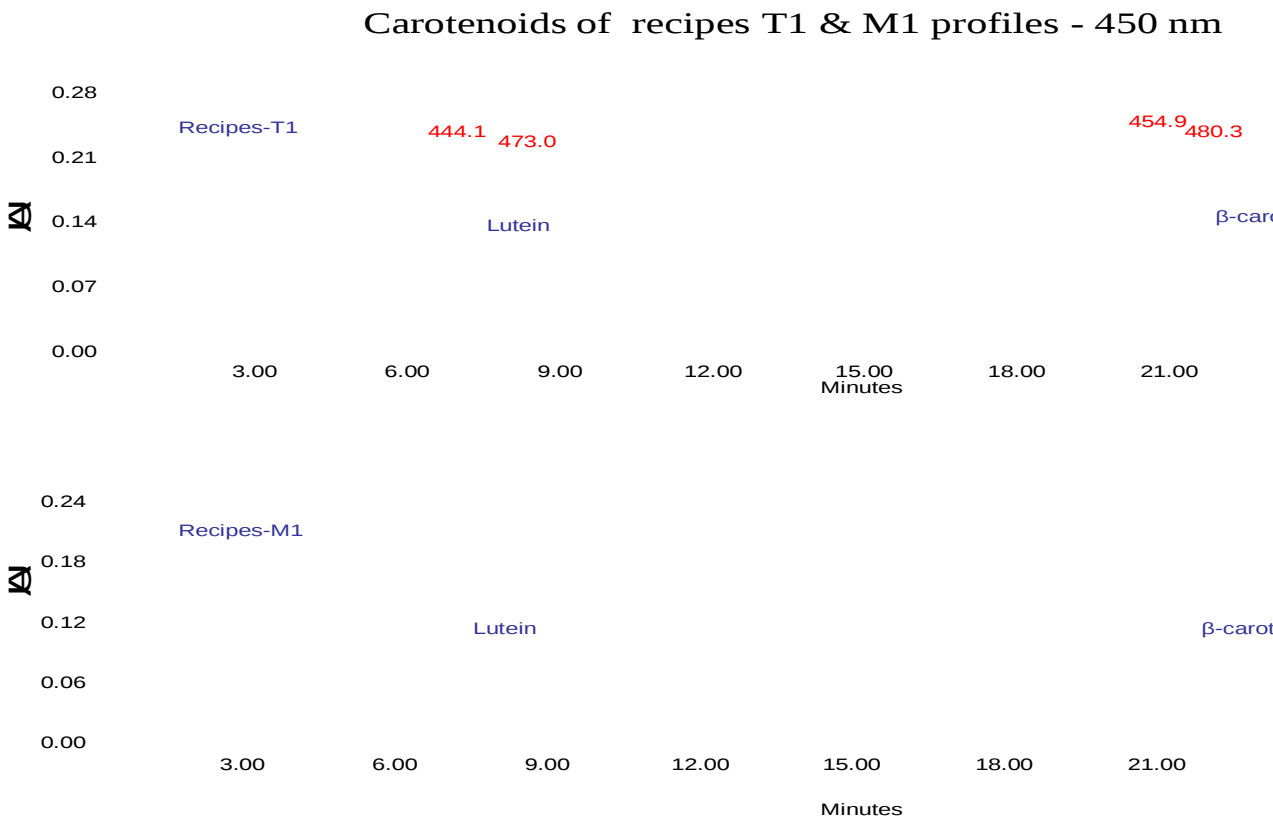
Total iron content (iron recipes) was determined by atomic absorption spectroscopy (AOAC, 1995).

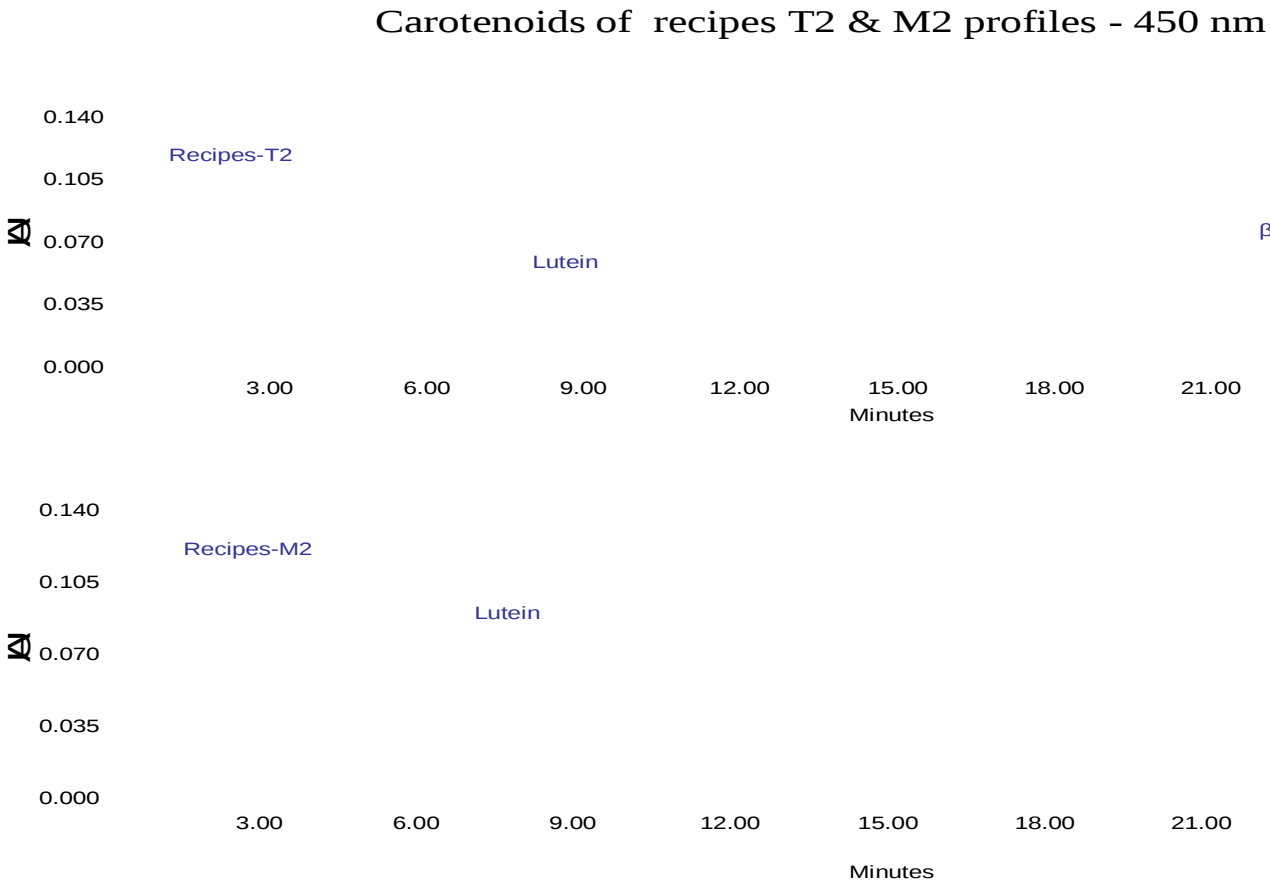
Appendix 2: Traditional and Modified preparation methods of cowpea leaf dishes

RECIPE	Traditional Methods (T)	Modified Methods (M)
1	-Boil 100g fresh cowpea leaves +150g water and boil for 15 minutes. Then fry 5g oil + 10g onions + 50g tomatoes for 5 minutes. Add boiled cowpea leaves then add 100g coconut milk and cook for 10 minutes -Total cooking time 30 minutes	-Boil 100g fresh cowpea leaves +150g water and boil for 5 minutes. Then fry 5g oil + 10g onions + 50g tomatoes for 5 minutes. Add boiled cowpea leaves then add 100g coconut milk and cook for 5 minutes -Total cooking time 15 Minutes
2	- Boil 100g fresh cowpea leaves + 150g water for 15 minutes - Add 50g grounded groundnuts and cook for 15 minutes	-Same procedures as traditional method 2 but **Add 100g yoghurt** after cooking
3	-Boil 10g sun dried leaves + 250g water for 15 minutes - Fry 5g oil + 10g onions + 50g tomatoes for 5 minutes. Add the boiled sundried cowpea leaves and 50g grounded groundnut and cook for 15 minutes	- **Oven dry cowpea leaves at 50C for 16 hours** Then follow same procedure for cooking as traditional recipe 3
4	-Boil 100g fresh cowpea leaves +150g water for 15 minutes	Same procedure as T4 but **Add 5g oil** to boil with fresh leaves
5	-Boil 10g sun dried leaves + 250g water for 15 minutes. - Fry 5g oil + 10g onions + 50g tomatoes for 5 minutes. Then add boiled cowpea leaves and 100g coconut milk and cook for 15 minutes.	

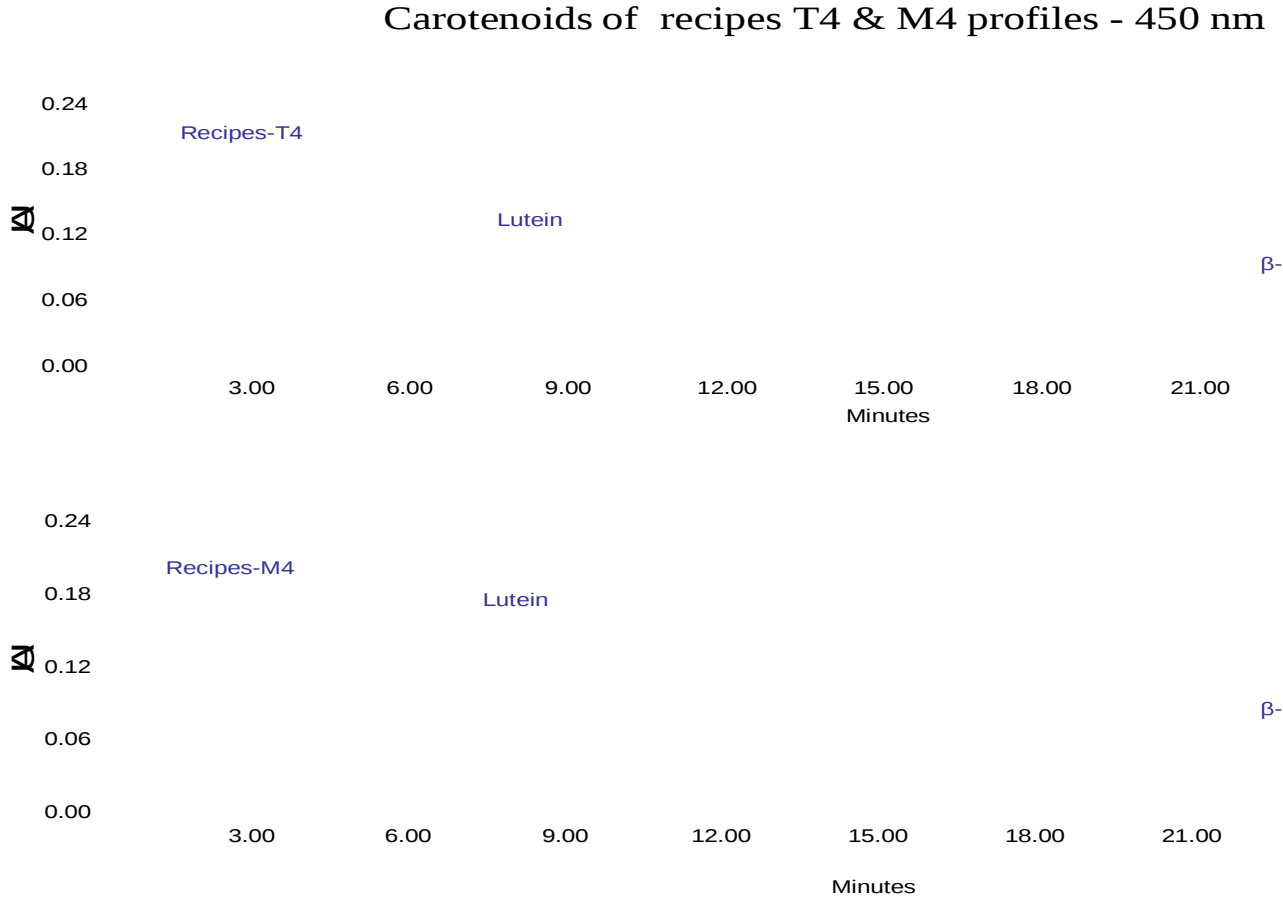
Appendix 3: Co-chromatography and spectra standards of Carotenoids

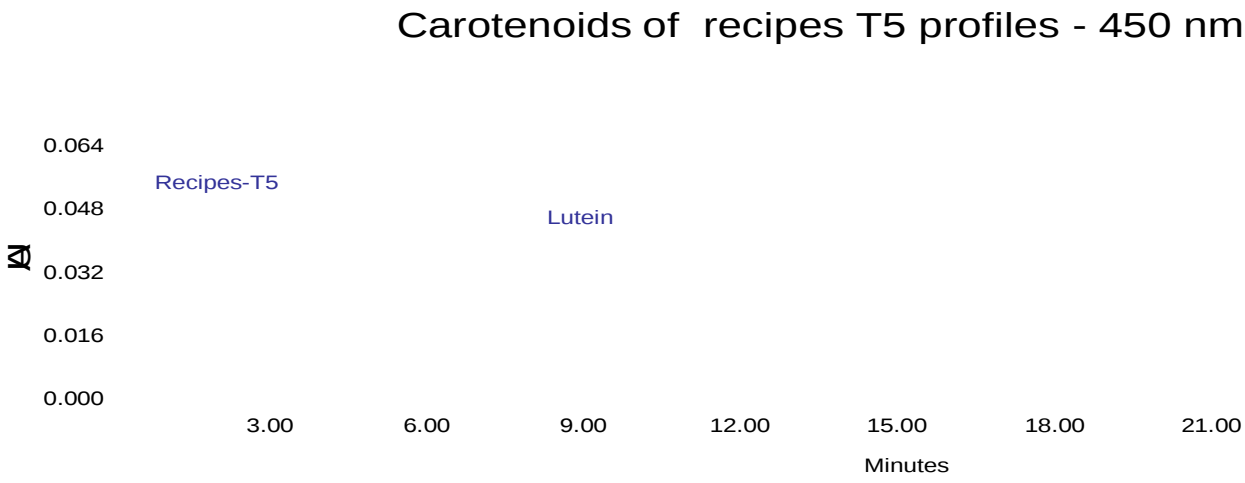

Carotenoids of cowpea leaves Dakawa & Ex-Iseke profiles - 450
0.32
0.24
0.16
0.08
0.00
Dakawa variety
Lutein
Chlorophyll
β-carotene
Neoxanthin
Violaxanthin
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes
0.32
Ex-Iseke variety
0.24
Lutein
0.16
Chlorophyll
0.08
β-caroten
Neoxanthin
Violaxanthin
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes

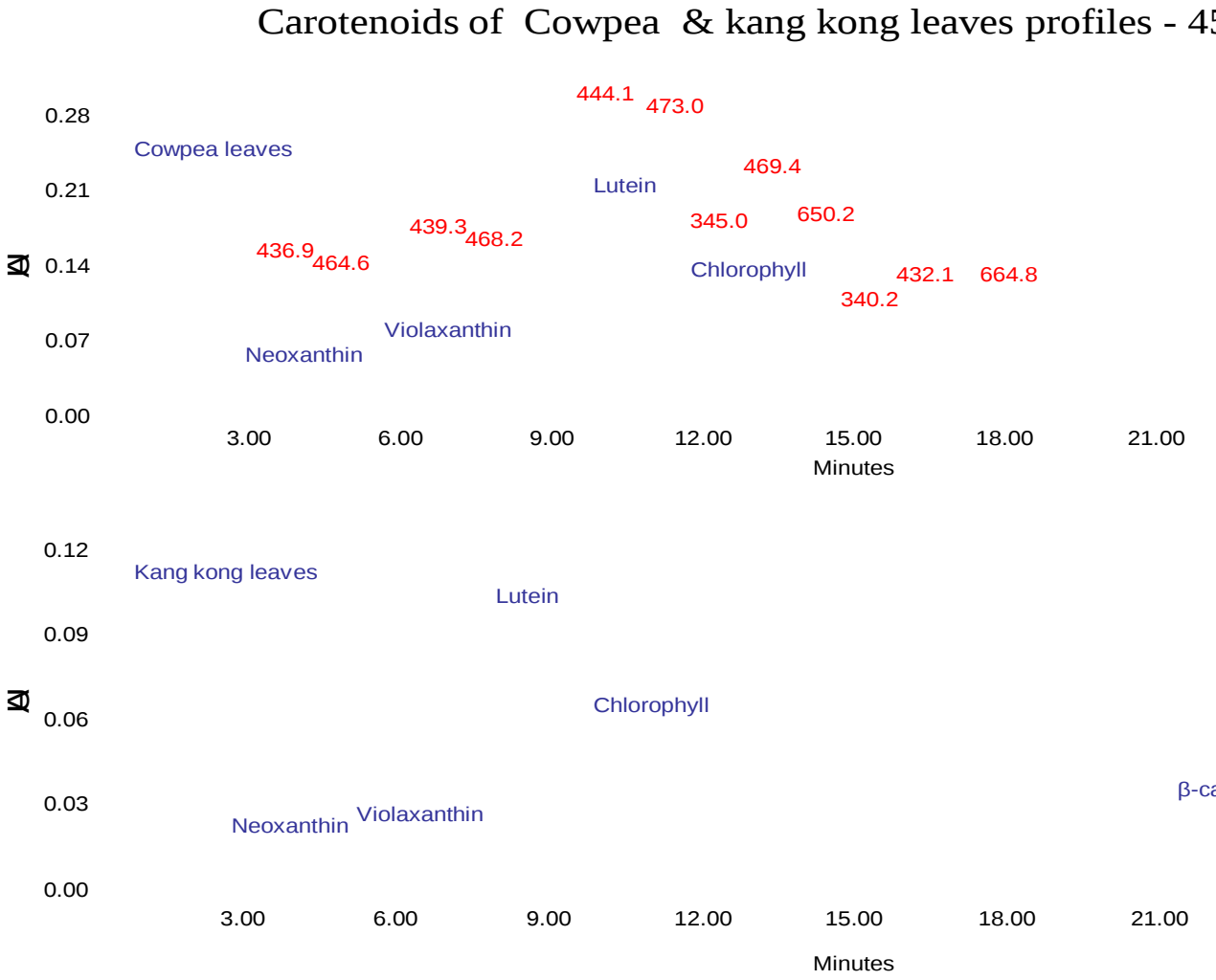

Carotenoids of recipes T1 & M1 profiles - 450 nm
0.28
0.21
0.14
0.07
0.00
Recipes-T1
444.1
473.0
454.9
480.3
Lutein
β-car
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes
0.24
0.18
0.12
0.06
0.00
Recipes-M1
Lutein
β-carot
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes

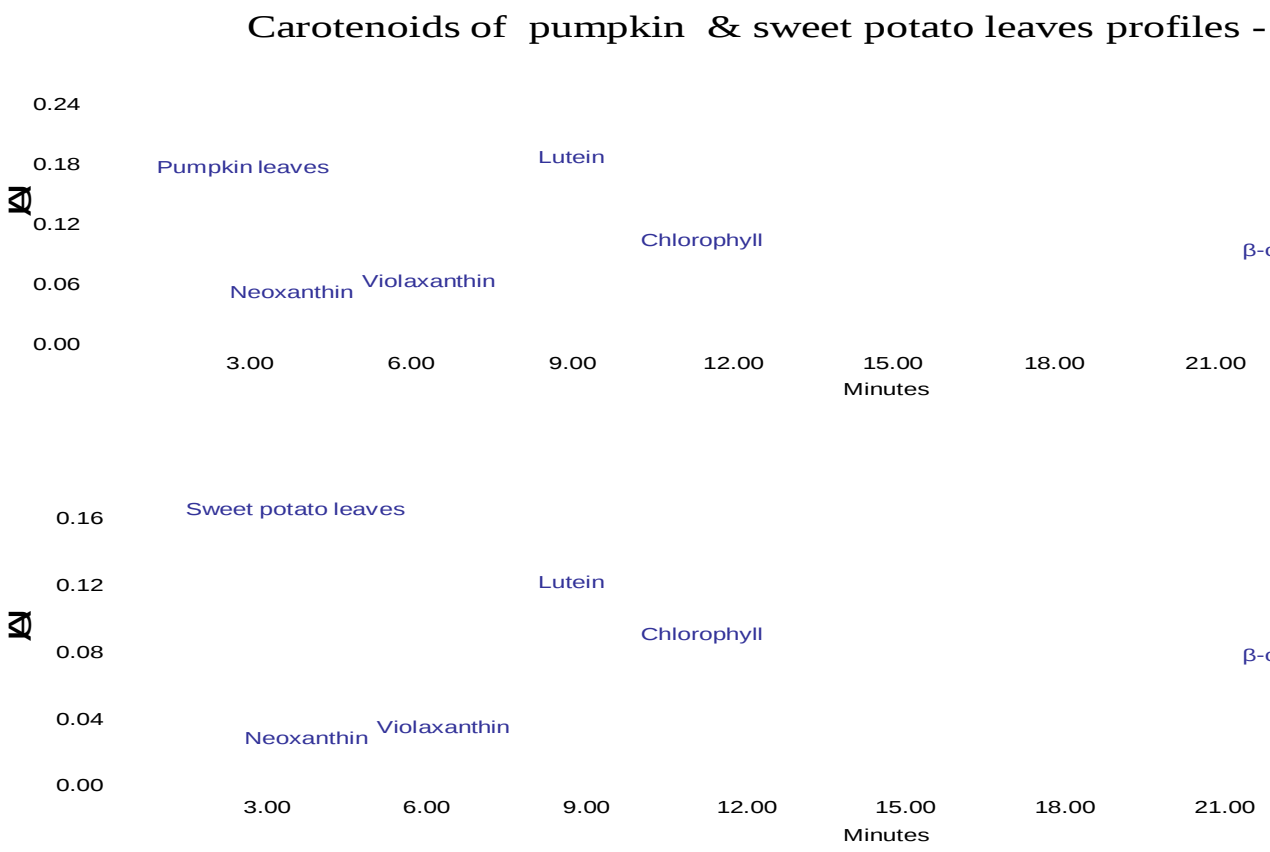

Carotenoids of recipes T2 & M2 profiles - 450 nm
Recipes-T2
Lutein
β
0.140
0.105
0.070
0.035
0.000
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes
Recipes-M2
Lutein
0.140
0.105
0.070
0.035
0.000
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes

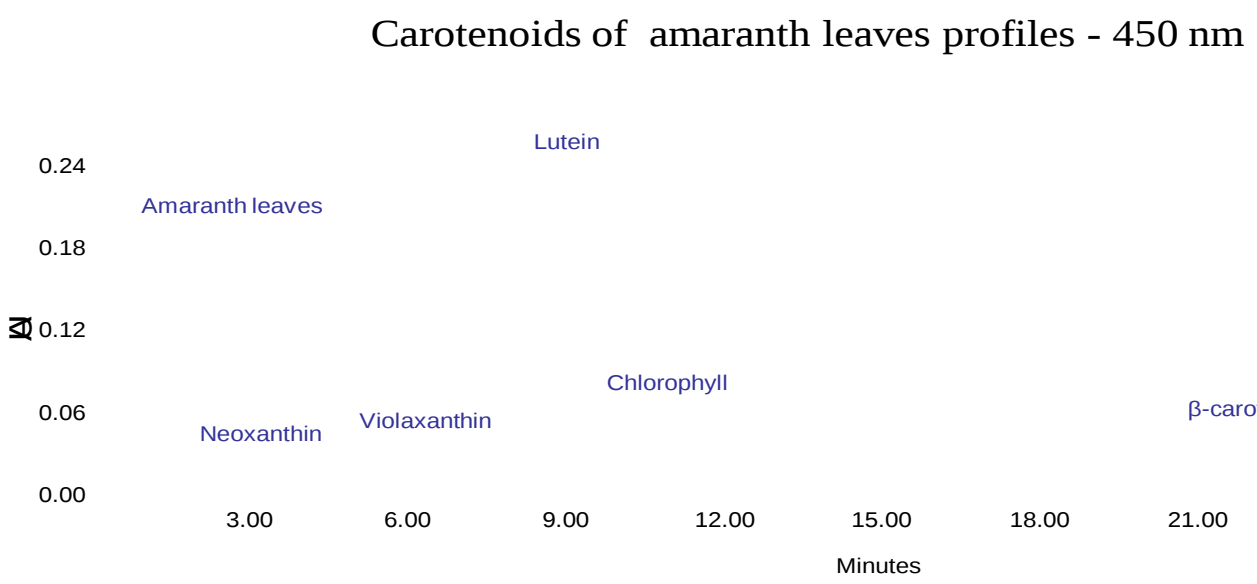
Carotenoids of recipes T3 & M3 profiles - 450 nm


Carotenoids of recipes T4 & M4 profiles - 450 nm
Recipes-T4
Lutein
β-
0.24
0.18
0.12
0.06
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes
Recipes-M4
Lutein
β-
0.24
0.18
0.12
0.06
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes


Carotenoids of recipes T5 profiles - 450 nm
0.064
0.048
0.032
0.016
0.000
AU
Recipes-T5
Lutein
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes


Carotenoids of Cowpea & kang kong leaves profiles - 45
0.28
0.21
0.14
0.07
0.00
AU
Cowpea leaves
444.1
473.0
469.4
Lutein
345.0
650.2
439.3
468.2
436.9
464.6
Chlorophyll
432.1
664.8
340.2
Violaxanthin
Neoxanthin
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes
0.12
0.09
0.06
0.03
0.00
AU
Kang kong leaves
Lutein
Chlorophyll
β-ca
Neoxanthin
Violaxanthin
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes


Carotenoids of pumpkin & sweet potato leaves profiles -
0.24
0.18
0.12
0.06
0.00
Pumpkin leaves
Lutein
Chlorophyll
β-c
Neoxanthin
Violaxanthin
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes
0.16
0.12
0.08
0.04
0.00
Sweet potato leaves
Lutein
Chlorophyll
β-c
Neoxanthin
Violaxanthin
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes


Carotenoids of amaranth leaves profiles - 450 nm
Lutein
0.24
Amaranth leaves
0.18
0.12
Chlorophyll
0.06
Neoxanthin
Violaxanthin
β-caro
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
Minutes