OPTIMIZATION OF NUTRITIONAL AND QUALITY PROPERTIES OF AFRICAN COWPEA (*Vigna unguiculata*) LEAVES AND OTHER SELECTED TRADITIONAL AFRICAN VEGETABLES FROM FARM TO FORK

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Optimization of Nutritional and Quality Properties of African Cowpea (*Vigna unguicul*ata) Leaves and Other Selected Traditional African Vegetables from Farm to Fork

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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LIST OF ABBREVIATIONS AND ACRONYMS

AA	-	Ascorbic acid
AAO	-	Ascorbic acid oxidase
L-AA	-	L-ascorbic acid
DHAA	-	Dehydro ascorbic acid
V. unguiculata	-	*Vigna unguiculata* (cowpea)
A. hybridus	-	*Amaranthus hybridus* (pig weed)
S. nigrum	-	*Solanum nigrum* (black night shade)
C. gynandra	-	*Cleome gynandra* (cat's whiskers)
POD	-	Peroxidase
ALVs	-	African leafy vegetables
FAO/WHO	-	United Nations Food and Agriculture Organization/World Health Organization
NHS	-	non heat shock
HS	-	heat shock
$\mathbf{k_t}$	-	inactivation rate constant (min^{-1})
$\mathbf{k_{ref}}$	-	inactivation rate constant (in min^{-1}) at reference temperature
$\mathbf{E_a}$	-	activation energy (in kJ/mol)
R	-	ideal gas constant (0.008314 kJ/mol.K)
WAP	-	weeks after planting
GGH	-	γ-glutamyl hydrolase
DM	-	dry matter
N.D	-	not detected

ABSTRACT

African leafy vegetables (ALVs) (*Vigna unguiculata*, *Amaranthus hybridus*, *Solanum nigrum* and *Cleome gynandra*) are great sources of micro nutrients in the diet of many in Kenya and there is need to improve the the intake of these nutrients from these vegetables. The main objective of the thesis was to monitor the changes of nutrient and quality characteristics of the cowpea leaves at different levels of maturity, handling, thermal processing, storage up to consumption; that is, farm to fork with the aim of optimizing these nutrients' availability.

Cowpeas were grown in a green house where the temperature, light and relative humidity were controlled. The leaves were harvested between 4-9 weeks of maturity. The analysis and quantification of ascorbic acid (AA) content was done by HPLC while the polarographic assay was used for ascorbic acid oxidase (AAO). The AAO activity, total AA content and L-ascorbic acid/dehydro ascorbic acid (L-AA/DHAA) ratio in the leaves increase with increasing maturity. Thermal inactivation of AAO follows first order reaction kinetics. The folates in the cowpea leaves (4-8 weeks of maturity) were assayed through the extraction, conversion, purification, and HPLC quantification processes. The total folate content and the folate poly-γ-glutamate chain length increases with increasing maturity of the leaves. Enzymatic conversion to short chain folate poly-γ-glutamates in crushed leaves indicates the presence of endogenous GGH enzyme. Thermal traetment affects the stability and profile of folates. The mineral content of the cowpea leaves were assayed using the inductively coupled plasma-optical emission spectrophotometry (ICP-OES). There is a general decline (>60%) in the levels of minerals during maturation of the leaves. The effect

of thermal treatment on peroxidise (POD) inactivation in the leaves was studied in the range of 75-100°/120 mins while that of the visual green colour was in the range of 55-80°C/90 mins. The kinetics of the enzyme *in situ* thermal inactivation that of POD and the visual green colour shows a first-order, biphasic and fractional conversion model respectively, however they both follow an Arrhenius model of the temperature sensitivity of the reaction rates.

The other ALVs were purchased from Thika municipal market, in Thika district of central Kenya and after multi stage sampling, samples were used to quantify their nutrient content using standard A.O.A.C methods. The proximate composition, total carotene and mineral content were evaluated. The molar ratio of phytates to the different minerals was also calculated. The thermal degradation of AA follows a first order reaction and the temperature dependence of k values can be described by Arrhenius relation.

In conclusion, the preharvest, harvest and postharvest factors greatly affect the nutrient and quality properties of the cowpea leaves and other ALVs and the eventual availability of these nutrients to the consumer.

CHAPTER ONE

1.0. Introduction

1.1 Background

Cowpeas are part of a group of traditional vegetables consumed in Kenya. These traditional vegetables include cat's whiskers (*Cleome gynandra)*, pig weed (*Amaranthus. hybridus)* and black night shade (*Solanum nigrum)* (Appendix 2). They are popular because they are available throughout the year, are cheap and can be found in virtually every open market within Kenya, particularly in high potential areas. They are easy to cultivate and some even grow wildly. They are not easily attacked by pests and when attacked by pests and diseases, it only leads to a drop in the yield rather than a total loss of the crop as witnessed in other vegetables. They are major sources of essential dietary nutrients such as AA, carotenes and micronutrients like folates and essential minerals (Akindahunsi and Salawu, 2005; Orech et al., 2005).

Cowpea (*Vigna unguiculata*) is an annual plant legume that is produced for food and animal fodder. This plant originated in Africa and it is widely grown in Asia (mainly in the Southeast) and America (Latin America and southern United States). As a food crop, different parts of cowpea can be used at different stages of growth namely, young leaves, immature pods, immature seeds, and mature dried seeds. Fifty-two percent of Africa's production is used for food, 13% as animal feed, 10% for seeds, 9% for other uses, and 16% is wasted (Davis, 1992; Singh et al., 2003).

The leaves are considered as an important food source, prepared by plucking the tender leaves from the stem, chopping into pieces and cooking into a stew. Harvesting of the leaves commences as soon as plants are well established (Schippers, 2000; Vorster et al., 2002; Hart and Vorster, 2006). After flower initiation, (usually at around 10 weeks of plant maturity) the leaves become very fibrous and are no longer harvested for consumption (Schippers, 2000; Vorster et al., 2002; Hart and Vorster, 2006). Immature snapped pods are used in the same way as snap-beans, often being mixed with other foods. Green cowpea seeds are boiled as a fresh vegetable, or may be canned or frozen. Dry mature seeds are also suitable for boiling and canning (Davis, 1992). The stems, leaves, and vines serve as animal feed and are often stored for use during the dry season.

In Kenya, cowpeas are grown mainly by small-scale farmers because they can be cultivated with other crops as they grow and cover the ground quickly, thus preventing erosion. They tolerate shade and drought, perform well in a wide variety of soils, and being a legume they replenish low fertility soils when the roots are left to decay (Davis, 1992; Singh et al., 2003). In a crop rotation programme, they can significantly improve soil nitrogen levels by nitrogen fixation and they can also be incorporated in the soil as a green manure crop.

Previous studies and surveys show a high prevalence in nutrient deficiencies in the population in Kenya (Neumann et al., 1989–1992; The United Nations Children's Fund, [UNICEF], 1994; Government of Kenya, [GoK] 1999; Ngare et al., 2000; Bwibo and Neumann 2003; Siekmann et al., 2003; Central Bureau of Statistics, (CBS), 2004) despite a high consumption of cowpea leaves and other traditional

vegetables. On the other hand, cowpea leaves are an excellent source of essential minerals (like Ca, Mg, Na, K, P, Fe, Zn, Mn and Cu), and vitamins A, C and folates (Imungi and Porter, 1983; Ahenkora et al., 1998; Van Rensburg et al., 2007). Like many leafy vegetables, the stability of the nutrients and quality properties in cowpeas depends on many factors in the preharvest, harvest and postharvest systems (Lee and Kader, 200). The nutrient (vitamins, minerals) and quality losses (colour, texture and flavour) can be brought about by physical, chemical, or biological processes including thermal processes, leaching during washing/cooking or enzymatic activity (ascorbic acid oxidase (AAO), peroxidase (POD), polygalacturonase) among others (Aked, 2002).

For many people in Kenya, cowpeas and other African leafy vegetables (ALVs) provide an important source of micronutrients in the diet (Davis, 1992; Singh et al., 2003). An integrated study on the nutritional and quality properties of the cowpea leaves and their subsequent evolution and stability in the preharvest, harvest and postharvest system will enable us to optimize these nutrient and quality properties up to the point of consumption. In the study, several nutritional (vitamin C (ascorbic acid, AA), folates, minerals) and quality properties (ascorbic acid oxidase (AAO), peroxidase (POD), and visual green colour) were studied. These properties were chosen on the bases of their importance in diet, stability, impact on the shelf life of the vegetables and the role they play in the consumer-decision making process when purchasing or consuming vegetables. Indeed AA and folates are classified as some of the essential micronutrients in the diet. However, they are highly unstable during handling, processing and storage. Thus they can provide an indication of the loss of

other nutrients and organoleptic properties (Esteve et al., 1999). Besides, the degradation kinetics of AA can be used to ascertain optimal thermal processing conditions (Uddin et al., 2002). As for minerals, despite their importance in the diet, most of the data available for minerals in cowpea leaves is generalized data. More information is needed on the concentrations of all the human essential minerals in cowpea leaves, and their changes at different levels of vegetable maturity. The effectiveness of thermal processing in terms of degrading the harmful enzymes can be gauged by the residual POD, since POD is easy to assay and in most cases thermally stable even upto temperatures >70°C (Morales-Blancas et al., 2002). Colour degradation during the thermal processing gives an indication of the degree of thermal degradation of the organoleptic properties since these organoleptic properties form a basis of consumer acceptability of the vegetables.

1.2 Problem statement

Micro-nutrient deficiencies (vitamins like A, C and folates; minerals like Zn, Fe and Ca) are a big problem in Kenya and Africa at large. On the other hand, ALVs, including cowpea leaves are the most commonly consumed foods in the country. Thus, the challenge for food technologists in the country is to tackle these micro-nutrient deficiencies by improving the nutrient intake from these vegetables. This technology and its applications must be cheap, easy to use and readily available since majority of the population are poor. The technology should impart some value in the products so as to attract a higher sale price and, thus, improve income to the farmers and traders of these vegetables.

1.3 Hypotheses

(a) African leafy vegetables (ALVs) like cowpea (*Vigna unguiculata*) leaves, cat's whiskers (*Cleome gynandra),* pig weed (*Amaranthus hybridus)* and black night shade (*Solanum nigrum)* are good sources of micronutrients.

(b) Cowpea leaves maturity has an effect on quantity and quality of AA, folates and minerals in the vegetables.

(c) Thermal processing has an effect on AAO, AA and folates stability in the cowpea leaves.

(d) The vegetable POD and colour can be used to optimize the blanching process of the vegetables.

1.4 Objectives

1.4.1 General objectives

To monitor the changes of nutrient and quality characteristics of the cowpea leaves at different levels of maturity, handling, thermal processing, storage up to consumption; that is, farm to fork with the aim of optimizing these nutrients' availability.

1.4.2 Specific objectives

To investigate;

(a) the thermal stability of ascorbic acid and ascorbic acid oxidase in cowpea (*Vigna unguiculata*) leaves of different maturities

(b) the effect of harvest age and thermal processing on micro nutrients (poly-γ-glutamate folates and minerals) in the cowpea leaves

(c) the thermal inactivation of POD and thermal degradation of the visual green colour properties of cowpea leaves

(d) the nutrient value and stability of AA and minerals during cooking of the cowpea leaves versus 3 selected ALVs namely cat's whiskers (*Cleome gynandra*), pig weed (*Amaranthus hybridus*) and black night shade (*Solanum nigrum*) consumed in Kenya.

1.5 Justification

The high prevalence of micronutrient deficiencies (vitamins like A, C and folates; minerals like Zn, Fe and Ca) in Kenya, even in sections of the population that are food secure, (Neumann et al., 1989–1992; The United Nations Children's Fund, [UNICEF], 1994; Government of Kenya, [GoK] 1999; Ngare et al., 2000; Bwibo and Neumann 2003; Siekmann et al., 2003; Central Bureau Of Statistics, (CBS), 2004) points to a diminished intake of these nutrients. This low intake of the nutrients can be due to a variety of factors including:

(a) Low bioavailability of the nutrients, due to factors like illness, presence of anti nutrients in the food and poor dietary habits of the consumers.

(b) Food scarcity, especially in populations that have a low purchasing power, drought/floods or other environmental factors that make it difficult to grow food for populations that practice subsistence farming.

(c) Low quantity and quality of nutrients in the foods that are being consumed due to preharvest, harvest and postharvest factors.

Therefore, one of the ways to improve the availability of nutrients in the diet of the population is to increase the nutrients in the food both in terms of quality and quantity. This can be achieved by:

(a) Genetic engineering, whereby, through genetic manipulation, high yielding crops, with increased target nutrient content can be developed.

(b) By optimization of processing techniques aiming at maximum nutrient retention.

Due to the cost and time involved in genetic modification of food crops, especially ALVs, nutrient and quality optimization of the existing crops would be the cheap and rapid option to improve the nutrient and quality properties of cowpea leaves and hence it could help improving the nutrient intake in the population. Moreover, nutrient optimization also makes it easy for value addition processes such as blanching to be operationalised, further improving the nutrient and quality stability of the vegetables.

CHAPTER TWO

2.0. Literature review

2.1 Introduction

Leafy vegetables are very popular in the diet of many in Kenya, and they are the main sources of nutrients (including vitamins, phytochemicals, essential minerals and trace elements in the daily diet), Orech et al., 2005). These vegetables include the leaves of cowpeas (*Vigna sp.*), amaranth (*Amaranthus sp*.), pumpkin (*Cucurbita sp.*), black nightshade (*Solanum nigrum*), sunnhemp (*Crotalaria brevidens*), jute plant (*Corchorus olitorius*), pig weed, (*Amaranthus sp.*) and the spider plant (*Cleome gynandra*), collectively known as ALVs.

African leafy vegetables (ALVs) may be defined as plant species which were introduced to that region for long enough to have evolved through natural processes or farmer selection. This also applies to leafy vegetable species that were externally derived but have since been incorporated in the local food culture (Phillips-Howard, 1999; Van Rensburg et al., 2007). Cowpea is a warm-season, annual, herbaceous legume that grows in a wide range of soils (Hugues, 1989; Van Rensburg et al., 2007).

Black night shade, *S. nigrum,* is a species in the *Solanum* genus, and it is the largest and the most variable of the family Solanaceae (comprising of about 84 genera and 3000 species, Yasin, 1985; Van Rensburg et al., 2007). It grows well in moderately light and warm situations (optimal temperatures of 20-30°C), fertile soils with high

nitrogen and phosphorus contents (Van Averbeke and Juma, 2006). It has a yield potential of 20 $t \cdot ha^{-1}$ of cumulative leaf yields (Edmonds and Chweya, 1997). It is an erect, branched annual or biannual herbaceous plant that can reach a height of 1m. The leaves have a characteristic bright green colour with purple pigmentation. Unlike *A. hybridus* and *C. gynandra*, that produce seeds, *S. nigrum* produces small, shiny, black to purple-black berries, that can be used to make jam (Fox and Norwood, 1982). It is harvested by cutting the entire shoot, so as to stimulate re-growth (Van Rensburg et al., 2007). Regular harvesting of the young shoots and de-budding encourages the production of lateral shoots and extends the harvesting period (Van Rensburg et al., 2007). Leaves are harvested until the fruit starts to develop and the leaves become narrow, thin and leathery (Manoko et al., 2007; Van Averbeke and Juma, 2006). Traditionally, *S. nigrum* has been put to a variety of uses including as an analgesic, antiseptic, antinarcotic, anticancer and antiulcer (Saijo et al., 1982; Akhtar and Muhammad, 1989; Schilling et al., 1992; Edmonds and Chweya, 1997; Manoko et al., 2007). This medicinal value is mainly attributed to the alkaloidal contents of the plants. The same alkaloidal content of the vegetables also contributes to their toxicity due to solanine, a neurotoxic glycoalkaloid (Abbas, 1998; Mohy-ud-dint et al., 2010).

Pig weed, *A. hybridus*, belongs to the family Amaranthacae. It tolerates varying soil and climatic conditions but grows optimally under warm tropical conditions (Van den Heever and Coertze, 1996; Schippers, 2000). Amaranth is tolerant to adverse climatic conditions, including dry conditions, albeit with reduced leaf yield and excessive flowering (Tindall, 1983; Schippers, 2000; Palada and Chang, 2003;

Grubben, 2004; Maundu and Grubben, 2004). It is an annual plant, spineless and grows up to 1.5m high. The leaves are green and variable in shape and size. Taproot is long, fleshy red or pink. The seeds are small and lenticellular in shape (Akubugwo et al., 2007).

Cat's whiskers (*C. gynandra*) are a species of the Capparaceae family. The plant grows to about 1m tall with oval-shaped leaves and it produces spherical seeds (Codd and Kers, 1970). One of the major identifying characteristics of the plant is that the stems and leaves are covered with glandular hair and also the stems have a distinct pigmentation varying from green to pink and purple (Van Rensburg et al., 2007). Cleome grows best during warm weather and is sensitive to cold. It prefers well drained medium-textured soils and does not grow well in temperatures below 15°C, shade, in poorly drained or heavy clay soils. It does tolerate a degree of water stress, but prolonged water stress hastens flowering and senescence. It is harvested by uprooting and ratoon harvesting (Chweya and Mnzava, 1997; Schippers, 2000; Schippers et al., 2002; AVRDC, 2003; Mnzava and Chigumira, 2004). Like many ALVs, it is highly nutritious especially in terms of vitamins A and AA) and minerals (calcium and iron) (Van den Heever and Venter, 2007).

As earlier indicated most ALVs can be cultivated in the wild or propagated by the farmers. In case of wild ALVs, a strategy of selective weeding is adopted, that is, the control of weeds with due regard to the weed species concerned. When practicing selective weeding, ALVs, are treated as crops and allowed to grow without being disturbed, whilst other weed species, which are not used as food, are controlled (Van

Rensburg et al., 2007). When selective weeding is used with the intention of raising the natural population of a particular weedy leafy vegetable species, the plants are left in the field to complete their full life cycle, including the release of seed (Van Rensburg et al., 2007).

The above ALVs are prepared and consumed in different forms including as potherbs, crushing to make a concoction, boiling the leaves, drying and grinding them to powder (especially for consumption by babies) for later reconstitution (Van den Heever and Venter, 2007). The cooked leaves can be consumed in a dish with cooked legumes (beans) and maize, ugali, bananas and other starchy dishes. They may be prepared alone or in a combination of different species. Other ingredients, such as tomatoes, onions, peanut flour and spices may be added to enhance their taste. One of the drawbacks of ALVs is that the recipes used to prepare the different leafy vegetables are fairly homogeneous and therefore, their culinary diversity is limited and monotonous (Vorster et al., 2005; Van Rensburg et al., 2007).

Many of the ALVs grow as weeds or in the wild; are seasonal and highly perishable. To extend their availability, different ways of preserving these vegetables have been used. The two main methods are the sun-drying of fresh leaves and the sun-drying of blanched or cooked leaves. Sun drying is popular because it is a cheap and easy technology to adopt and use. Indeed sun drying has been found to be effective in drying of Amaranth seeds, (Shitanda and Wanjala, 2003; Shitanda and Wanjala, 2006; Ronoh et al., 2009). Both these methods transform the leafy vegetables into

dry products that have long shelf lives (Vorster et al., 2005). The dry vegetables can be re-hydrated during moist cooking.

For most ALVs, especially *C. gynandra*, when cooked, the leaves have a characteristic bitter taste, and, therefore, in most recipes they are cooked with other leafy vegetables or they are fermented (by soaking cooked vegetables in fermented milk for several days). Indeed consumer and marketing studies have shown that taste, as opposed to perceived nutrition or health value, is the key influence on food selection (Drewnowski and Gomez-Carneros, 2000).

The bitter taste in vegetables is mainly due to plant-based phenols, flavonoids, isoflavones, terpenes, and glucosinolate (Drewnowski and Gomez-Carneros, 2000). Although potentially beneficial to human health in small doses, many such compounds are, in fact, toxic. Thus, the challenge in food processing is debittering of the vegetables, reduction of the toxicity levels while managing to keep these beneficial compounds in the vegetables.

ALVs are consumed at all stages of growth including young leaves, stems, whole seedlings, seeds or berries (fruits). This makes ALVs a versatile dish in the diet of the consumer and an all year round supply of food.

Consumers increasingly require food products that preserve their nutritional value, retain a natural and fresh colour, flavour and texture, and contain fewer additives such as preservatives. Consumers use physical attributes of vegetables to judge the quality of the vegetables and hence make a decision on whether to purchase/consume

them. These physical attributes include colour, flavour and texture. On the other hand nutrient and quality stability of these vegetables depends on a lot of factors including preharvest, harvest and postharvest factors (Lee and Kader, 2000). Thus, understanding the nutrient and quality properties of these vegetables forms a foundation for their optimization in the diet. In these thesis, several nutritional (vitamin C (ascorbic acid, AA), folates, minerals) and quality properties (ascorbic acid oxidase (AAO), peroxidase (POD), and visual green colour) were studied.

The degradation of nutritional and quality of leafy vegetables in the postharvest system is usually caused by biochemical changes including oxidative damage, free radical injury and cell wall degradation (Wang et al., 1999). These changes are linked to a number of enzymes, eventually leading to processes that alter colour, flavour, aroma, texture and other quality factors in vegetables. Enzymes are biological catalysts, protein in nature, that promote most of the biochemical reactions at the cellular level. In relation to the nutrient and quality properties of fruits and vegetables, the enzymes can be broadly divided into (a) hydrolases (lipase, invertase, tannase, chlorophylase, amylase, cellulase) and (b) oxidoreductases (POD, tyrosinase, catalase, ascorbinase, polyphenoloxidase).

The enzymes polyphenol oxidase (Vaughn et al., 1988; Walker, 1995), POD (Haard, 1977; Burnette, 1977), lipoxygenase (Eskin et al., 1977) and cholorophyllase (Terpstra and Lambers, 1983) affect the green colour of many leafy vegetables and in turn this affects the physical (visual) appearance of the green vegetables. On the other hand, the texture properties of the vegetables are gauged by the firmness,

hardness and softness and this in turn can be affected by the action of several enzymes including β-galactosidase (Wallenfels andWeil, 1972), polygalacturonase (Poretta et al., 1995; Thakur et al., 1996; Lopez et al., 1997) and pectin methyl esterase (Seymour et al., 1991; Giovane et al., 1990) while the toughness and fibrousness can be caused by the action of enzyme cellulase (Wood, 1960). The flavour properties of the vegetables can be due to action of enzymes POD (Gonçlaves et al., 2009), lipoxygenase (Engeseth et al., 1987; Theerakulkait et al., 1995; Savage, et al., 1995), leading to off-flavours and off-odours. The nutritional stability of the different nutrients (ascorbic acid, lipids and glucosinolates) in the vegetables is also affected by enzymes. For example, ascorbic acid is affected by ascorbate oxidase (Munyaka et al., 2010), ascorbate peroxidase (Raven, 2000) and lipoxygenase (Eskin et al., 1977), lipids are affected by lipoxygenase, (Ludikhuyze et al., 2003).

2.2 Ascorbic Acid

2.2.1 Biological role, requirements, and occurrence.

AA consists of two forms, L-ascorbic acid (L-AA, 2-oxo-l-threo-hexono-1,4, lactone-2,3 enediol, **Figure 2.1 (a)**) and dehydroascorbic acid (DHAA, threo-2,3-hexodiulosonic acid-γ-lactone, **Figure 2.1 (b)**) both having equal antiscorbutic activity(Lee and Kader, 2000). However, most published data on fruits and vegetables refers to AA as L-AA. This is because until recently, most analytical methods for determination of AA used a redox titration (Dichlorophenolindophenol method) or a direct treatment with phenylhydrazine to form a derivative detectable by spectrophotometry and these methods can only assay L-AA. Hence, it is crucial

that AA in vegetables (and food at large) be referred to as total AA, that is, L-AA and DHAA and the ratio of L-AA/DHAA also needs to be determined.

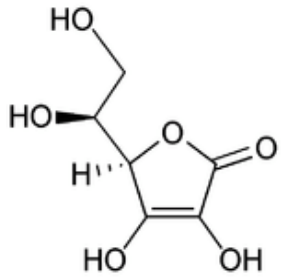


Figure 2.1 (a) L-AA chemical structure

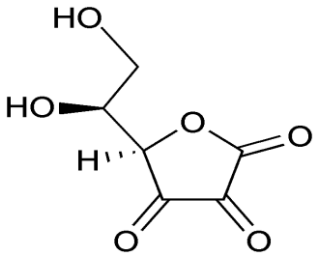


Figure 2.1 (b) DHAA chemical structure

This is because despite the fact that both L-AA and DHAA posses antiscorbutic activity, only L-AA has oxygen scavenging activity. Also the thermal stability of L-AA and DHAA differs (Vieira et al., 2000) and they follow different pathways of degradation with L-AA degradation being reversible, since DHAA can be reduced to L-AA by the ascorbate-glutathione cycle (**Figure 2.2**) (Smirnoff, 1996; Smirnoff, 2000).

The biochemical functions of AA (Smirnoff, 1996; Smirnoff, 2000) can be divided into four main categories.

(a) Antioxidant. It plays a part in the removal of reactive oxygen species (superoxide, singlet oxygen, ozone and hydrogen peroxide) generated during aerobic metabolism and during exposure to some pollutants, herbicides and environmental stress. In the human body, AA (L-AA) is also known to react with singlet oxygen and other free radicals. As an antioxidant, it reduces the risk of

arteriosclerosis, cardiovascular diseases and some forms of cancer (Harris, 1996; FAO/WHO, 2005).

(b) Enzyme cofactor. It is a cofactor in a number of hydroxylase enzymes (e.g. prolyl residues) involved in hydroxyproline synthesis (HP) (Davies et al., 1991; FAO/WHO, 2005). In the human body, it also acts as an electron donor (**Figure 2.2**) for several enzymes; three of these enzymes participate in collagen hydroxylation (Kivirikko and Myllyla, 1985, Peterkofsky, 1991; Prockop and Kivirikko, 1995; FAO/WHO, 2005) and two of them in carnitine biosynthesis (Rebouche, 1991). The consequences of scurvy such as breakdown of connective tissue fibres, (Myllyla, 1978; Oguntibeju, 2008) and muscular weakness (Hulse et al., 1978; Oguntibeju, 2008) are both linked to hydroxylation reactions.

(c) Electron transport. It acts as an *in vitro* electron donor for photosynthetic and mitochondrial electron transport. In the human body AA aids in the absorption of non-heme iron (Hallberg et al., 1989b; Oguntibeju, 2008).

(d) Synthesis of oxalates and tartrate (used as antioxidants, acidity regulators, and emulsifiers in food). L-AA can be cleaved to form oxalate and tartrate (Loewus, 1980; 1988; Saito, 1996; Pallanca and Smirnoff, 2000).

The daily requirements of AA in any given population vary among the different groups. Those that are the most vulnerable to AA deficiencies include pregnant and lactating women, children, the elderly and smokers (FAO/WHO, 2005, **Table 2.1**).

Table 2.1 Recommended nutrient intakes (RNIs) for vitamin C (AA)

Group	RNI mg/day [a]
Infants and children	
0-6 months	25
7-12 months	30 [b]
1-3 years 4-6 years	30 [b] 30 [b]
7-9 years	35 [b]
Adolescents, 10-18 years	40 [b]
Adults	
19-65 years	45
65+ years	45
Pregnancy	55
Lactation	70

[a] Amount required to half saturate body tissues with vitamin C in 97.5 % of the population. Larger amounts may often be required to ensure an adequate absorption of non-haem iron. [b] Arbitrary values.

Vegetables are a big contributor of AA in the diet (Lee and Kader, 2000) especially in populations that consume a lot of leafy green vegetables like the ALVs. In plants, it occurs in the cytosol, chloroplasts (related to its central role in photosynthesis, Foyer, 1993; Pallanca and Smirnoff, 2000), vacuoles, mitochondria and cell wall (Anderson et al., 1983; Rautenkranz et., al., 1994; Pallanca and Smirnoff, 2000). AA is also abundant in many fruits.

Leafy vegetables such as cabbage, broccoli, kale, cauliflower etc and ALVs may be more important sources of AA than fruits since vegetable supply often extends for longer periods during the year than does the fruit supply. In many developing countries, including Kenya, the supply of AA is often determined by seasonal factors such as availability of water, time, and labour for the management of household gardens and the short harvesting season of many fruits (FAO/WHO, 2005).

2.2.2 Quantification of ascorbic acid

Several analytical methods exist for the determination of AA, and they include (a) titrimetry (AOAC, 1990), (b) spectrophotometry (Arya et al., 1998) and (c) electrochemical methods / amperometry (Arya et al., 2000) (d) chromatographic techniques (Munyaka et al., 2010).

The titrimetry is based on the titration of ascorbic acid with 2,6-dichloroindophenol in acidic solution. The spectrophotometry method involves a direct treatment with phenylhydrazine to form a derivative detectable by spectrophotometer (Omaye et al., 1979; Arya et al., 2000). In this assay, L-AA is selectively and completely oxidized to DHAA by AAO. The product is then coupled with *o*-phenylenediamine (OPDA) to yield a chromophore, the absorbance of which is measured at 340 nm in a centrifugal analyzer. Polarographic assays involve electrochemical oxidation of L-AA on a dropping mercury electrode. A sensor has been developed that selectively catalyses the oxidation of L-AA at low potentials (+100 mV), and can be used in the detection of AA (L-AA, Arya, et al., 2000). The above methods have their shortcomings as they tend to over-estimate the AA quantities in samples due to the presence of oxidizable species other than L-AA and their inability to measure DHAA. In titrimetry, substances naturally present in fruits such as tannins, betannins, sulfhydril compounds, Cu(II), Fe(II), Mn(II) and Co(II) are oxidised by the dye and hence there is a likelihood to overestimate the AA content especially in ALVs. Moreover, the method is applicable only when the concentration of DHAA is low (Arya et al., 2000; Hernandez et al., 2006).

The separation techniques include capillary electrophoresis (Versari, et al., 2004), gas chromatography (Silva, 2005) and liquid chromatography (LC). LC avoids the problems of non-specific interference and ion-pair (Ke et al., 1994; Nováková, et al., 2008). In these methods, DHAA is determined as the difference between the total AA (after DHAA) reduction and L-AA content of the original sample. Also, DHAA can be determined after LC separation and detection by fluorimetry after a post-column derivatisation with *O*-phenyldiamine (Kall and Andersen, 1999; Nováková, et al., 2008).

Inactivation of degradative enzymes which can destroy L-AA during the extraction is important in the sample preparation stage (Davey et al., 2000). This can be achieved by performing extraction under acidic conditions since L-AA is readily oxidised under alkaline conditions. Acidic conditions are also important to precipitate proteins (including enzymes) and, therefore, remove them during subsequent steps of separation. Metaphosphoric acid is the most efficient extraction acid (Cano et al., 1997; Franke et al., 2004) however, it may cause serious analytical interactions with silica-based column materials, (RP-C_{18} or NH_2 bonded-phases) which can result in drifts in the baseline and retention time and eventually leading to the destruction of the column (Kall and Andersen, 1999; Nováková, et al., 2008). An alternative to metaphosphoric acid is oxalic acid (Ke et al., 1994; Kabasakalis et al., 2000) but it has a drawback of sometimes not being able to recover the total L-AA present in the sample and moreover the extracts are less stable than in metaphosphoric acid. In the sample preparation, a metal chelator such as EDTA is used so as to minimize metal-catalyzed oxidation (Nováková, et al., 2008).

2.2.3 Stability, degradation and cooking losses

The mechanism of AA degradation (**Figure 2.2)** depends on several factors (Tannenbaum, 1976, Esteve et al., 1999; Vieira et al., 2000), following either aerobic or anaerobic pathway. The aerobic pathway (oxidation pathway) can be divided into (a) a catalyzed pathway (b) an uncatalyzed pathway. The catalyzed pathway can be enzymatic or non enzymatic (presence of metals). In general, in the aerobic pathway, L-AA is easily degraded by oxidation, especially in aqueous solutions, and the presence of oxygen, heavy metal ions, and by alkaline pH and high temperature, and after several intermediary steps, the end product being DHAA. However, DHAA can be reduced to L-AA by reducing agents and also can be irreversibly oxidized to form diketogulonic acid (Parviainen and Nyyssonen, 1992; 2004; Serpen et al., 2007).

In the anaerobic pathway, L-AA undergoes ketonization to form the intermediate keto-tautomer (keto-ascorbic acid) which is in equilibrium with its anion (keto-monoanion ascorbic acid) which by further delactonization forms diketogulonic acid, DKGA, (Tannenbaum, 1976; Esteve et al., 1999; Vieira et al., 2000; Karhan et al., 2004; Serpen et al., 2007, (**Figure 2.2)**). In the presence of amino acids, L-AA, DHAA and their degradation products might be changed further by entering into Maillard-type browning reactions.

In summary, both pathways have common intermediate products and both lead to DHAA (reversible equilibrium occurs between L-AA and DHAA) which by further degradation forms 2,3-diketogulonic acid, DKGA, (under certain conditions such as

oxidizing and reducing properties, heat, light etc. over time (Fennema, 1977; Favell, 1998; 2004; Serpen et al., 2007).

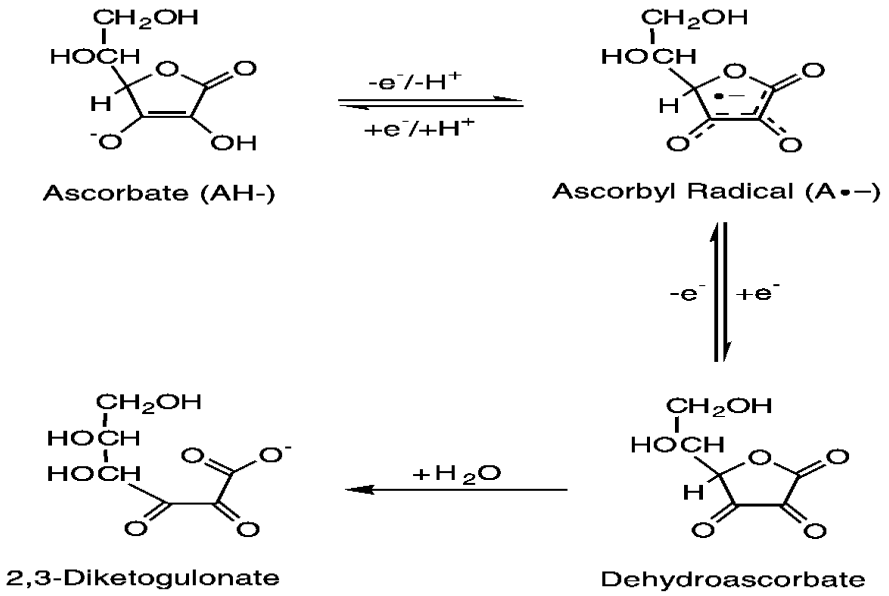


Figure 2.2 Ascorbic acid degradation

AA is often used as a general indicator of changes occurring in food. AA is most sensitive to destruction when the commodity is subjected to adverse handling and storage conditions (higher temperatures, low relative humidity, physical damage and chilling injury). Lee and Kader (2000) have extensively reviewed factors that influence the AA content of horticultural crops and they included the preharvest factors, harvest conditions and postharvest factors.

One of the most prominent harvest factors that affect AA content is vegetable maturity (Cebula and Kalisz, 1996; Sørensen et al., 1995; Lee and Kader, 2000, Biesiada et al., 2007). The needs of AA in the plants change at different stages of

plant growth, maturity and the response of the plants to different types and levels of stress (Smirnoff, 2000). Plants produce large amounts of AA to facilitate resistance to the oxidative stresses associated with myriad biotic and abiotic challenges inherent to photosynthesis (Giovannoni, 2007) and in turn the photosynthesis and photorespiration needs are dictated by among other things, the stage of plant (leaf) development (Kennedy and Johnson, 1981; Pallanca and Smirnoff, 2000). In general, photosynthesis increases during leaf development, often reaching a maximum at about full leaf expansion, then decreases rapidly during late developmental stages or senescence (Kennedy and Johnson, 1981; Pallanca and Smirnoff, 2000). These photosynthetic changes, therefore, are likely to affect not only the quantity but also the quality of AA in the plant and, thus, the vegetables.

One of the most common postharvest operations on vegetables is cooking. On the other hand cooking contributes to some to the largest losses of AA in the diet. Cooking losses of AA depend on the degree of heating, leaching into the cooking medium, surface area exposed to water and oxygen, pH, presence of transition metals, and any other factors that facilitate oxidation (Eitenmiller and Laden, 1999; Stešková et al., 2006). Mathooko and Imungi (1994) showed the effect of cooking (by varying the cooking time and amount of cooking water) of three indigenous Kenyan leafy vegetables (*A. hybridus*, *G. gynandra* and *S. nigrum* L.) on the retention of AA. Cooking for 20 minutes in boiling water led to losses of between 75 and 89% and these losses were minimized when the leafy vegetables were cooked in two and four volumes of water. The loss in AA as a function of cooking time was highest in *S. nigrum* while its loss as a function of volume of cooking water was

highest in *A. hybridus.* Losses in AA associated with cooking vegetables (cooking method and type of vegetable) have been reviewed by Lěsková et al., (2006).

While AA is very susceptible to chemical and enzymatic oxidation during processing, cooking and storage, thermal treatments (blanching and pasteurization) are important in preventing the action of AAO, which in turn is responsible for AA enzymatic losses. Other plant enzymes, including phenolase, cytochrome oxidase and POD, are also responsible for AA losses (Lěsková et al., 2006). Thus, processing conditions that inactivate these enzymes with little loss of AA are important.

2.2.4 Ascorbic acid oxidase (AAO)

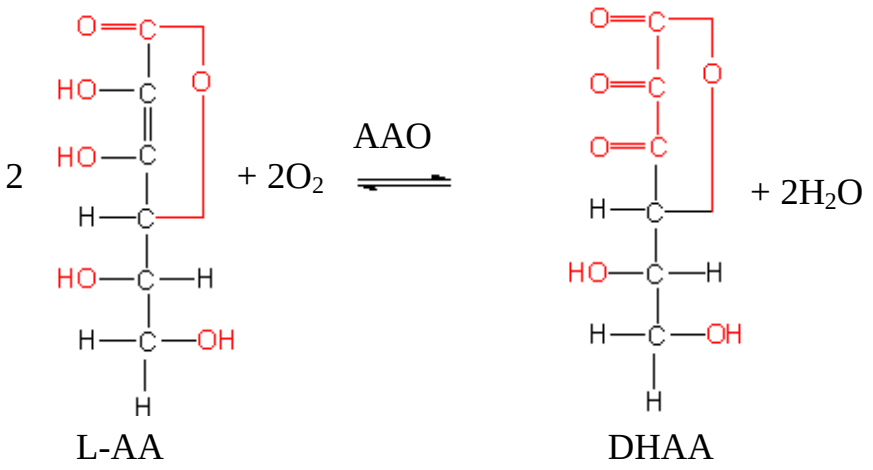
Ascorbic acid oxidase (AAO) is a glycoprotein widely distributed in higher plants and microorganisms, the main sources being members of the *Cucurbitaceae* (cucumber, zucchini, pumpkin, squash and melon) (Diallinas et al., 1997; De Tullio et al., 2007).

2.2.4.1 Occurrence and localization

In plants, AAO is associated with rapidly growing regions and its expression increases during fruit ripening (Al-Madhoun et al., 2003). It is mainly found in the cell wall as tightly bound to the cell wall or as loosely bound (soluble). This loosely bound AAO forms part of the AAO in the apoplast and cytosol, responsible for the oxidation of apoplast-L-AA to MDHAA (Hallaway et al., 1970; Loewus and Loewus, 1987; De Tullio et al., 2007).

2.2.4.2 Reaction catalyzed

Ascorbic acid oxidase (AAO, EC 1.10.3.3) is an oxidoreductase acting on diphenols and related substances (as donor) with oxygen as acceptor and it is involved in metabolism of L-AA, using copper as a cofactor. It catalyzes the oxidation of L-AA in the presence of oxygen to DHAA Equation (Eq.) 2.1. It is only released when the plant wilts or is cut (Diallinas et al., 1997; Horemans et al., 2000). It helps to maintain the cell redox system and helps to create a defensive mechanism by plants against the reactive oxygen species. L-AA (around 5%) is exported to the apoplast from the cytoplasm and in the apoplast; L-AA is oxidized to monodehydroascorbate (MDHAA) by the AAO. MDHAA is an unstable radical and rapidly disproportionates to yield DHAA. DHAA is then transported into the cytosol through the plasma membrane by a specific carrier that preferentially translocates the oxidized form in exchange for the reduced form, ensuring a continuous flux of reducing power to the cell wall while at the same time maintaining a defensive mechanism against potentially damaging external oxidants such as ozone, SO_2, and NO_2 (Horemans et al., 2000; Plöchl et al., 2000; Barnes et al., 2002).



2.2.4.3 Food technological implications of the enzymatic reaction

The enzymatic degradation of L-AA has a big impact on the quality of vegetables (Munyaka et al., 2010). Therefore, inactivation of AAO in vegetables is crucial in the processing of the vegetables. Indeed, vegetables where the AAO has been thermally inactivated not only show higher L-AA/DHAA ratio but the total AA is more thermal stable (Munyaka et al., 2010).

Several strategies can be employed in reducing the effect of AAO on the quality and quantity of AA in the postharvest system and they include

(a) Mechanical injury; Activities that bruise and cut the vegetables can lead to cell membrane disruption and also processing conditions like cutting, chopping, dicing. This cell membrane disruption brings the enzyme into contact with the substrate and hence leading to the enzymatic degradation of AA. There are two types of mechanical injury that can be incurred during harvesting and handling of vegetables; (i) cuts or punctures, and (ii) impacts leading to bruises (Toivonen

and Hodges, 2011). Thus, avoiding mechanical damage to the cell membrane structure is crucial in preserving of AA.

(b) Temperature treatments in the postharvest handling process of the vegetables. These temperature treatments include freezing, refrigeration and blanching. Freezing of vegetables is one of the most common processes for quality preservation, as it reduces deteriorative reaction rates to a minimum (Gonçalves et al., 2011). However, the quality of frozen vegetable is highly dependent on the characteristics of the raw vegetables (Simandjuntak et al., 1996), handling of the vegetables (e.g. cutting, bruising) before the freezing process (Alvarez and Canet, 2000), and pre treatments of the vegetables (e.g. blanching) before freezing (Jaworska et al., 2008; Olivera et al., 2008), and the freezing process itself (e.g. fast freezing, slow freezing and freezer storage temperature). Refrigeration also slows down enzyme activity by lowering respiration rate, and reducing water loss. Reducing the rate of water loss slows the rate of shrivelling and wilting, which can lead to disruption of the cell membrane leading to interaction between enzyme and substrate (Gonçalves et al., 2011). Also, wilting leads to an increased production of AAO by the plants (Diallinas et al., 1997; De Tullio et al., 2007). On the other hand, high temperature treatments, like blanching, inactivate enzymes (thermal denaturation of enzymes) (Ahern and Klibanov 1987; Dill et al., 1989).

(c) Reducing AAO yield during disruption of the cell matrix system. This is because AAO is found both as free and bound (tightly bound to the cell wall) enzyme (Pignocchi et al., 2003). Therefore, a high yield of AAO, comprising of both the

free and bound enzyme, is likely to lead to a faster degradation of AA, since increase in either substrate or enzyme concentration leads to increase in reaction rate.

(d) Denaturing of AAO. Like many enzymes, AAO, being a protein based molecule, gets denatured under extreme pH conditions or very high salt concentration (Makki and Durance, 1996).

2.3 Folates

2.3.1 Biological role, requirements, and occurrence

Folates are part of the water-soluble B-vitamins, comprising of eight vitamins including (a) vitamin B_1 (thiamine) (b) vitamin B_2 (riboflavin) (c) vitamin B_3 (niacin) (d) vitamin B_5 (pantothenic acid) (e) vitamin B_6 (pyridoxamine) (f) vitamin B_7 (biotin) (g) vitamin B_9 (folic acid) (h) vitamin B_{12} (various cobalamins).

Folate is a generic term referring to the mono- to poly-glutamate derivatives of pteroic acid that occur naturally in many foods (Gregory, 1989; Bagley and Selhub, 2000). The general chemical structure of folates (**Figure 2.3**) consists of a pteridine ring linked via carbon number 9 to a ρ-aminobenzoyl residue which is, in turn, linked to poly-γ-glutamate, and the number of glutamate residues varies up to eight with folic acid having only one glutamate residue (**Figure 2.3**).

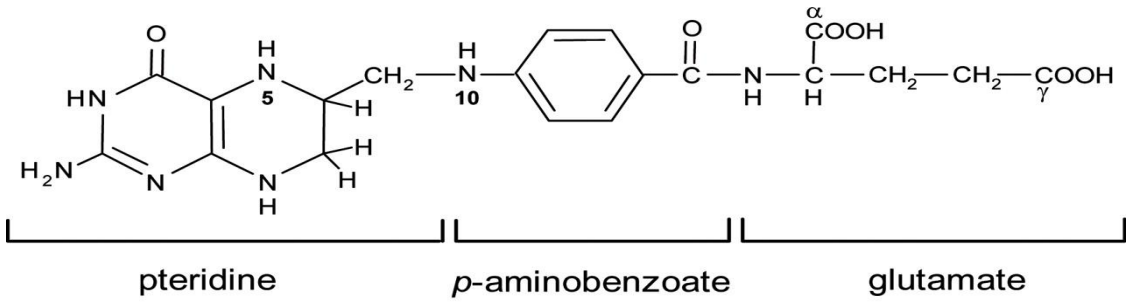
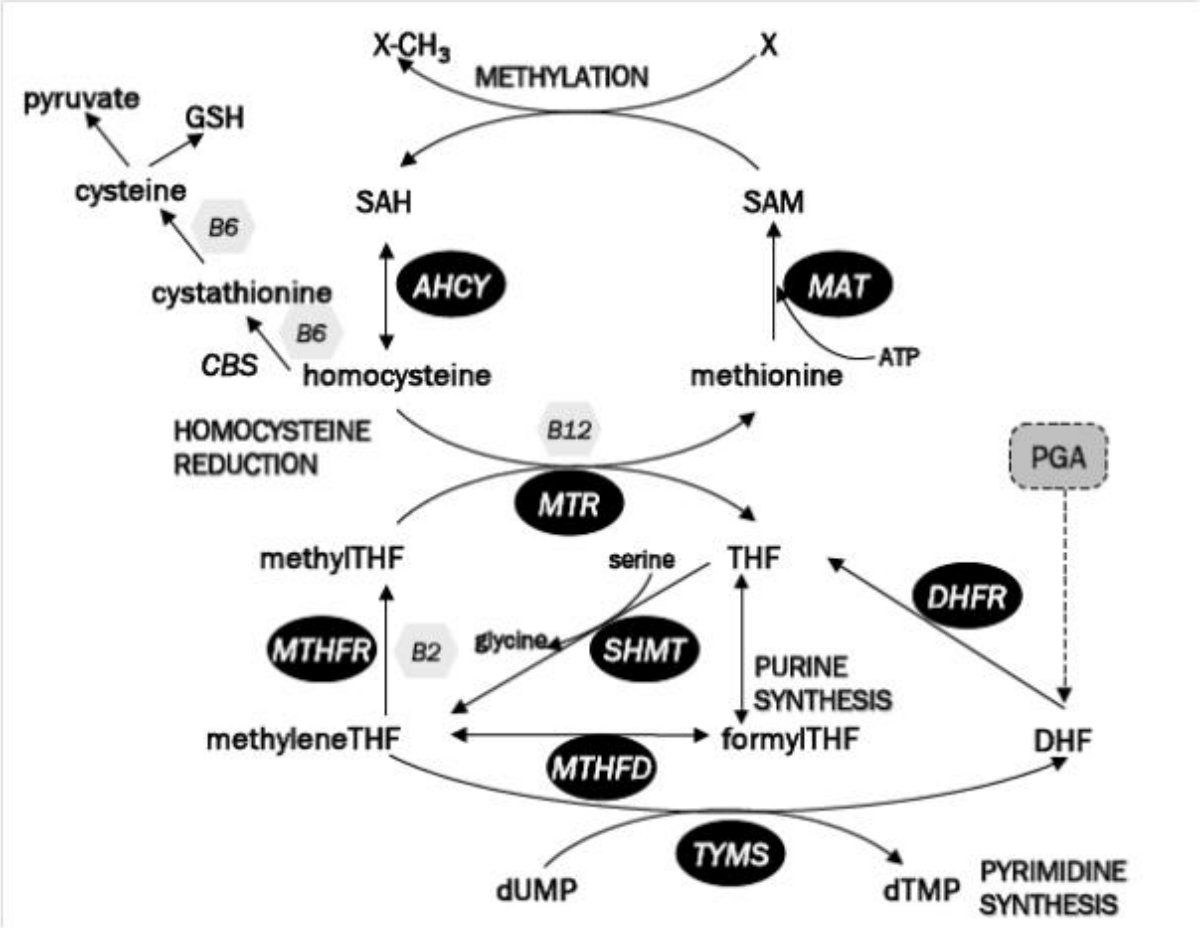


Figure 2.3 Generalized chemical structure of folate (folic acid).

The pteridine ring of folates can exist in tetrahydro-, dihydro-, and fully oxidized forms hence folates are a mixture of un-substituted polyglutamyl tetrahydrofolates and various substituted one-carbon forms of tetrahydrofolate (e.g., 10-formyl, 5,10-methylene, and 5-methyl). Folates undergo several reduction and methylation steps to yield 5-methyltetrahydrofolic acid (5-MTHF, 5-CH_3–H_4PteGlu_γ), which is the most biologically active form of the folates and the end product that is needed by the body (Ndaw et al., 2001). However, vitamin B_{12} is the only acceptor of MTHF and hence a deficiency of vitamin B_{12} can lead to a methyl-trap of THF and a subsequent folate deficiency (Hoffbrand and Weir, 2001). Folates are needed by the human body as a coenzyme substrate in many reactions (single carbon metabolism reactions) of amino acids and nucleotides to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in biological reactions (during rapid cell division and growth, such as in infancy and pregnancy) involving folate (FAO/WHO, 2005).



Abbreviations: **AHCY**: S-adenosylhomocysteine hydrolase; **ATP**: adenosine triphosphate; **B2**: vitamin B2, riboflavine; **B6**: vitamin B6, pyridoxine; **B12**: vitamin B12, cobalamine; **CBS**: cystathionine beta-synthase; **DHF**: dihydrofolate; **DHFR**: dihydrofolate reductase; **GSH**: gluthatione; **THF**: tetrahydrofolate; **MAT**: methionine adenosyltransferase; **MTHFR**: 5,10-methylenetetrahydrofolate reductase; **MTHFD**: methylenetetrahydrofolate dehydrogenase; **MTR**: 5-methyltetrahydrofolate-homocysteine-methyltransferase/methionine synthase; **PGA**: folic acid, pteryol glutamic acid; **SAH**: S-adenosyl homocysteine; **SAM**: S-adenosyl methionine; **SHMT**: serine hydroxymethyltransferase; **TYMS**: thymidylate synthase; **X**: DNA, RNA, lipids, proteins, neurotransmitters

Figure 2.4 Schematic representation of folate metabolism (adapted from Scott, 1999)

Chemically, folates accept one-carbon units from donor molecules and pass them on via various biosynthetic reactions (Scott and Weir, 1994; Bagley and Selhub, 2000). In the human body, the polyglutamyl chain is removed in the mucosal cells by the γ-glutamyl hydrolase (GGH, EC 3.4.19.9), and folate monoglutamate (5-methyltetrahydrofolate monoglutamate) (Scott and Weir, 1994; Bagley and Selhub, 2000) is absorbed (**Figure 2.4**).

Table 2.2 Estimated average requirement (EAR) and recommended nutrient intake (RNI) for folic acid expressed as dietary folate equivalents, by age group. (National Academy of Sciences, 1998; FAO/WHO, 2005)

Group	EAR (µg/day)	RNI (µg/day)
Infants and children		
0-6 months [a]	65	80
7-12 months	65	80
1-3 years	120	160
4-6 years	160	200
7-9 years	250	300
Adolescents, 10-18 years	300	400
Adults		
19-65 years	320	400
65+ years	320	400
Pregnancy	520	600
Lactation	450	500

[a] Based on a human milk intake of 0.75 l/day.

The daily requirements of folates in the diet vary between the age groups, sexes, and during pregnancy and lactation (National Academy of Sciences, 1998; FAO/WHO, 2005, **Table 2.2**).

The three main sources of folate and/or folic acid intake in the diet are (a) naturally occurring food folate (b) synthetic folic acid added to food (fortified foods), and (c) supplements containing folic acid (Berry et al., 2010). Leafy vegetables (including ALVs) have been identified as the major source of folate in human diet (Malin 1977; Leichter et al., 1979; Mullin et al., 1982; Suitor et al., 2000; USDA, 2009); however, the liver remains one of the best sources (in terms of density) of folates in the diet (Chanarin, 1979; FAO/WHO, 2005).

Other diets that contain adequate amounts of folates include other fresh vegetables and fruits, such as legumes, spinach, citrus fruits (Thomas et al., 2003) bananas, cabbage (raw/boiled), broccoli (raw/boiled), apples, tomatoes (fresh) and avocados, and other foods, such as hardboiled egg yolk, canned tuna, cheddar cheese and whole wheat bread (Machlin, 1991; FAO/WHO, 2005).

In many developed countries, due to the importance of folates in the diet especially in the early stages of pregnancy, folates (folic acid) fortification in cereals especially wheat and maize products has become the most common source of folates in the diet. This is because adequate consumption of folic acid before pregnancy and during the early weeks of gestation protects foetuses from developing neural tube defects (NTDs), (MRC, 1991; Czeizel and Dudas, 1992; Berry et al., 1999; Berry et al., 2010). Thus, many countries have permitted manufacturers to voluntarily fortify certain foodstuffs, such as breakfast cereals, and/or introduced mandatory fortification of a staple food with folic acid for the prevention of NTDs (Berry et al., 2010). While folic acid supplementation is popular among women with planned

pregnancies, however, it is recommended for all women of childbearing age since most pregnancies are not pre planned as such and yet folic acid intake is crucial in the first 28 days of conception (Ladipo, 2000). However, taking large amounts of folic acid can mask the damaging effects of low vitamin B_{12} deficiency, indeed high folic or folate levels, when combined with low B_{12} levels, has been associated with significant cognitive impairment among the elderly (Morris et al., 2007). In addition, a deficiency in B_{12} can generate a large pool of 5-MTHF that is unable to undergo reactions and will mimic folate deficiency (Hoffbrand and Weir, 2001).

2.3.2 Quantification of folates

The analysis of folates in food follows a three step process, that is, (a) pre-treatment (b) enzyme treatment/extraction and (c) detection. The pre-treatment procedure can involve a hot or cold extraction, using methanol (Puwastien et al., 2005), acid or alkaline treatment and with varying pH ranges depending on the food matrix system and the subsequent enzyme treatment procedure. The extraction involves homogenization of the sample under subdued light and in presence of high quantities of anti oxidants so as to avoid folate degradation (Gregory et al., 1990; Tamura et al., 1997; Lim et al., 1998; Rader et al., 1998; Munyaka et al., 2009).

Enzyme treatment is an analytical protocol in folate analysis and it can be divided into (a) mono-enzyme treatment (folate conjugase, Tamura and Stokstad, 1973; Kirsch and Chen, 1984; Puwastien et al., 2005) (b) di-enzyme treatment (such as the combinations of pancreas preparation and human plasma folate conjugase or α-amylase and pancreas preparation), or (c) tri enzyme treatment (conjugase, protease

and α-amylase). The order of enzyme treatments most suitable for food folate extraction varies from one food matrix to another (Tamura, 1998; Rader et al., 1998; Ndaw et al., 2001). If crude enzyme extracts are used, endogenous folate possibly present in the crude enzyme preparations should also be considered in the final calculation of food folate values.

Folate quantification can be performed by (a) microbiological assay (b) HPLC with UV detection, and (c) liquid chromatography−mass spectrometry, LC/MS (d) radiobinding assay. The microbiological assay involves the use of *Lactobacillus casei* or *Enterococcus hirae* (formally *Streptococcus faecalis*, ATCC 8043) as the test organism. However, *E. hirae* does not respond to 5-CH_3-H_4 folate, which is known to exist in foods (Tamura, 1990). In this assay the growth of assay organisms is measured by turbidity reading at wavelengths ranging from 530 to 655 nm in a 96-well microplate reader. In the HPLC-UV method the pre-column conversion of all folates present into 5-CH_3-H_4 folate and subsequent purification is crucial. This is because 5-CH_3-H_4 folate can easily be isolated by liquid chromatography (purification) and analyzed by fluorescence. It is also very stable in acidic conditions and it is the folate possessing the highest fluorescence quantum yield (Ndaw et al., 2001). Moreover, it can be produced from folic acid (PteGlu), 5-formyltetrahydrofolate (5-CHO-H_4PteGlu) and 10-formylfolate (10-CHO-PteGlu), thus, giving a more accurate representation of the total folate content (Ndaw et al., 2001). Just like the HPLC-UV method, the LC/MS method also requires a pre-column purification.

Radiobinding assay follows a heat extraction and folate conjugase treatment. However, monoglutamyl folates with different one-carbon substitutes and oxidation states respond differently in the radiobinding procedures (Shane et al., 1980; Puwastien et al., 2005), hence this assay method is not suitable for some samples, such as foods, which contain folates other than 5-CH_3-H_4folate or folic acid (Puwastien et al., 2005).

2.3.3 Stability, degradation and cooking losses

Folate losses during harvesting, storage, distribution, and cooking can be substantial as a result of a combination of thermal degradation and leaching of the vitamin into the cooking water. The degree of loss can be influenced by environmental factors, including temperature, pressure, pH, oxygen, light, metal ions, antioxidants and duration of heating (Keagy, 1985; Gregory, 1989; Hawkes and Villota, 1989; Eitenmiller and Laden, 1999; McKillop et al., 2002; Indrawati et al., 2004; Lěsková et al., 2006; Verlinde et al., 2008). The method of cooking also has effect on the degree of folate losses. For example, boiling of spinach or broccoli led to 51% and 56% reduction in the folate content (respectively) over the raw vegetables while steaming (for the same time) did not result in a significant loss of folate content (McKillop et al., 2002). In general, cooking methods that minimize the direct contact of food with the cooking water, such as pressure-cooking (Dang et al., 2000) and microwave cooking (Chen et al., 1983a, b; Klein 1989) have better folate retention than boiling. Lěsková et al., 2006 has reviewed the different cooking methods and their effect on folate retention in the food. Other processing operations like homogenizing, freezing, and thawing of fresh fruits and vegetables disrupts cell

membranes and releases endogenous enzymes that may oxidize and or hydrolyse the folates (Vahteristo, et al., 1997; Munyaka et al., 2009).

The presence of reducing agents (AA) in the food can increase folate retention during thermal processing by protecting folates from oxidation processes (Gregory, 1985; Ford, 1967; Chen and Cooper, 1979, Verlinde et al., 2008; Munyaka et al., 2009) and the presence of metals (Fe^{2+}) can increase folate loss (Gregory, 1985; Day and Gregory, 1983; Kall, et al., 2000). Common food additives including sodium nitrite used in some cured products can cause folate degradation. Indeed tertrahydrofolates are rapidly oxidized in the presence of sodium nitrite to produce ρ-aminobenzoylglutamate and several pterin products (Lynn and Archer, 1979; Kall, et al., 2000). Folate is quite stable in dry products in the absence of light and oxygen.

Folates present a special challenge in food processing because of the need to present them to the consumer in a reduced (non-oxidized) form, deconjugated, stable and highly extractable in the food matrix system (Indrawati et al., 2004; Verlinde et al., 2008; Munyaka et al., 2009). Besides, their bioavailability in the body is further complicated by the activity of intestinal γ-glutamyl hydrolase (GGH, EC 3.4.19.9) which is susceptible to inhibition by constituents found in some foods (Bhandari and Gregory, 1990). Thermal treatments, if properly done, can help to meet the above conditions for improving folate bioavailability in the vegetables (Indrawati et al., 2004; Verlinde, et al., 2008; Munyaka et al., 2009). Furthermore, modification of the thermal processing conditions, e.g. low temperature long time (LTLT) or high temperature short time (HTST) in combination with acidification, pressure and anti

oxidants (like ascorbic acid) can help to improve folate bioavailablity (Indrawati et al., 2004; Verlinde, et al., 2008; Munyaka et al., 2009).

2.4 Colour (Visual green colour)

The colour in the vegetables plays a crucial role in the consumer perception of vegetable quality. It is the major factor in vegetable appearance, (together with gloss, lesions and other attributes) detected by visual evaluation of the product and it contributes significantly to one's enjoyment of meals. Moreover, it can also serve as an index of physiological maturity or senescence and it can be used to grade vegetables and gives an idea of the preharvest, harvest and postharvest conditions and handling of the vegetables (indicator of physiological, mechanical or pathological injury). In the purchase and consumption of vegetables, colour is one of the major visual factors that forms a key means of evaluating vegetable quality i.e., a vegetable that has lost a lot of its original colour is unlikely to be purchased and even less likely to be consumed (Shewfelt, 2003). Plant pigments provide the chemical basis for vegetable visual colour in many fruits and vegetables (Vaclavik and Christian, 2008). These pigments are classified into two major groups (a) fat soluble pigments (chlorophyll and carotenoids) and (b) water soluble pigments, these include the flavonoids (anthocyanins and anthoxanthins) (Kidmose et al., 2002).

2.4.1 Chlorophyll: occurrence, localization and biological role

The visual green colour of leafy vegetables is mainly due to the green pigments in plants called chlorophyll (Kidmose et al., 2002).

Chlorophyll is a chlorin pigment, with a magnesium ion centrally placed in the chlorine ring and is present in almost all plants with green colour and it is contained mainly within the chloroplasts. The pigment is primarily made up of a chlorin ring that can have several different side chains, which give rise to the different types of chlorophyll (chlorophyll a, b, c_1, d and f) as illustrated in **Figure 2.5** below (Kidmose et al., 2002).

Chlorophyll plays two major roles in plants (a) it provides the visual green colour of vegetables and (b) plays a photosynthetic (photosynthetic oxidation-reduction reaction between carbon dioxide and water) role.

The visual green colour of vegetables is due to the magnesium element in the structure and magnesium does not absorb the middle spectrum of visible light (green) which is in turn leads the green colour being reflected to the human eye. The intensity or loss of this green colour gives an idea about the condition of the plant e.g. biotic or abiotic stress, senescence.

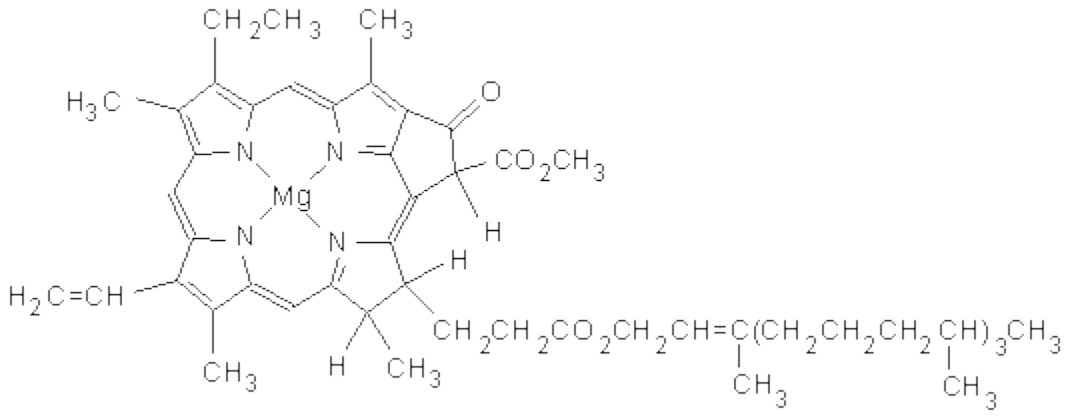


Figure 2.5 Structure of chlorophyll a (adapted from Schoefs, 2002).

Chlorophyll is rapidly degraded during senescence, exposing the lighter yellow pigments, a process called unmasking of chlorophyll (Gross, 1991; Kidmose et al., 2002). On the other hand, the photosynthetic role of chlorophyll is due to its ability to absorb light energy, causing excitation of an electron in chlorophyll and this electron in an excited state is more readily transferred to another molecule. This is subsequently followed by a chain of electron-transfer steps, ending up with an electron transferred to carbon dioxide. On the other hand, chlorophyll which gave up an electron draws an electron from another molecule, in this reaction, that is water (Kidmose et al., 2002).

2.4.2 Analysis and quantification of green colouration

There are three main methods for analyzing and quantifying the visual green colour in vegetables and these are (a) sensory evaluation (b) pigment analysis and (c) colourimetry (Shewfelt, 2003).

Sensory evaluation of vegetable colour can be performed by expert graders, descriptive panels or consumer panels. Expert grading consists of a small group of highly trained or experienced individuals using selected scales labelled with descriptors for at least half of the points on the scale. The difference with descriptive panels is the descriptive panels has a larger number of trained panellists and the colour evaluation is done in conjunction with flavour and textural attributes (Shewfelt, 1993; Hutchings, 1994; McFeeters, 2002) while on the other hand consumer panels are used when the primary objective is to determine preference (like/dislike; acceptable/not acceptable etc).

Pigment analysis is conducted when there is an established relationship between the quantitative measurements of pigment composition and vegetable colour, since all colour changes in vegetables are attributed to chemical changes in the constituent pigments (Gross, 1991; McFeeters, 2002). Chlorophylls are typically extracted with organic solvents such as acetone or methanol, followed by transfer into a more non-polar solvent like diethyl ether (Gross, 1991; Shewfelt, 2003) and measured spectrophotmetrically (Dere et al., 1998; McFeeters, 2002).

The colourimetry method describes colour mathematically in terms of human perception (Hunter and Harold, 1987; Hutchings, 1994; Shewfelt, 1993). It uses the CIE colour solid scale of (L*a*b*) whereby colour of objects can be expressed in terms of red-green character and blue-yellow character (Hunter and Harold, 1987; Hutchings, 1994; Shewfelt, 1993).

2.4.3 Chlorophyll stability

Handling, processing and storage procedures (thermal processing, cooking), changes in pH and a presence of minerals) of vegetables cause some deterioration of the chlorophyll pigments and subsequent loss of green colour. The loss of green colour in vegetables is primarily due to a process called pheophytinisation, that is, the displacement of the magnesium in the cholorophyll (from the porphyrin ring), leading to the formation of a dull olive brown called pheophytin (Kidmose et al., 2002). In the initial stages of cooking green vegetables, intra and intercellular air is removed leading to a bright green colour. Then the internal organic acids are released and hydrogen displaces magnesium, producing pheophytins, that is, magnesium-free

pheophytin a (grey-green pigment) or pheophytin b (olive-green pigment) (Kidmose et al., 2002). Apart from the direct effect of heat and pH, chlorophyll stability also depends a lot on enzyme chlorophyllase, an intrinsic membrane glycoprotein. Chlorophyllase does not usually interact with the chlorophyll substrate in intact tissue however, a substantial disruption of thylakoid membrane (where it is localized) leads to its interaction with chlorophyll. This disruption can be due to heat, mechanical damage, attack from pests and diseases (Lambers and Terpestra, 1985; McFeeters, 2002). Chlorophyllase works by hydrolyzing chlorophyll to chlorophyllides, which are water soluble. Deterioration of the chlorophyll pigments can also be due to an oxidative (photo oxidation) process which is accelerated by light (Jen and Mackinney, 1970; McFeeters, 2002).

2.4.4 Food technological implications of the enzymatic reaction

Since the degradation of chlorophyll and hence visual green colour in the vegetables is catalysed by acid and accelerated by heat, cooking vegetables is one of the most common ways of degradation of green colour in the vegetables. Thus, keeping the cooking pot uncovered during cooking helps to stabilize the visual green colour as the volatile plant acids that would otherwise remain in the cooking water and react to displace the magnesium, are allowed to escape. Also increasing the pH of the cooking medium helps to stabilize the visual green colour in vegetables during cooking. However, this is accompanied by loss of texture and change in flavour due to softening of hemicelluloses and destabilization of AA (Kidmose et al., 2002). The alkaline environment can be created by cooking the vegetables in sodium bicarbonate as the soda reacts with cholorophyll, displacing the phytyl and methyl groups on the

molecule and the green pigment forms a bright green water soluble chlorophyllin (Kidmose et al., 2002). Thermal processing can also accelerate cholorophyll degradation by activating chlorophyllase (60-80°C), however, blanching temperature of 100°C lead to stabilization of the green colour by inactivating chlorophyllase (Ogura et al., 1987). As earlier indicated, chlorophyllase leads to formation of chlorophyllides which are water soluble and hence lost in the cooking water. Apart from heat and pH, light can lead to degradation of green colour due to photo oxidation (photobleaching), and these can be mitigated by a modified atmosphere packaging and using an opaque material to pack the vegetables.

2.5 Minerals

Mineral substances are present as salts of organic or inorganic acids or as complex organic combinations (chlorophyll, lecithin, etc.). They play a variety of functions in plant metabolism and are essential nutrients required for plant growth and development. Their functions are of structural in nature especially in macromolecules such as nucleic acids and of energy transfer in metabolic pathways of biosynthesis and degradation, synthesis of chlorophyll maintaining turgor pressure and aiding in the opening and closing of stomata (Marschner, 1993). In the human body, these trace elements play a crucial role ranging from being cofactors in enzyme activities to being part of the structural make up of many body tissues (FAO/WHO, 2005). Green leafy vegetables are important sources of minerals (Ca, Mg, Na, K, P, Fe, Zn, Mn and Cu) in the diet of many in Africa (Aletor and Adeogun, 1995; Agte et al., 2000, van der Walt et al., 2009).

2.5.1 Biological role, requirements, and occurrence

Calcium is a divalent cation with an atomic weight of 40 (making up 1.9% of the body by weight) and it plays a crucial role in the human body structure (Nordin, 1976; FAO/WHO, 2005). Calcium salts provide rigidity to the skeleton by a form of calcium phosphate embedded in collagen fibrils. Apart from being an integral part of the human body structure, calcium ions take part in many metabolic processes such as phosphoinositide and cyclic adenosine monophosphate system (Brown et al., 1997; FAO/WHO, 2005). Magnesium plays a crucial role in the human body, mainly as a co-factor of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis, maintenance of the electrical potential of nervous tissues, cell membrane regulation, and metabolism of calcium (Classen, 1984; Waterlow, 1992; FAO/WHO, 2005).

Sodium is an essential nutrient that regulates blood pressure, blood volume, blood pressure (osmotic equilibrium and pH) and it is also critical for the functioning of muscles and nerves In the diet, sodium is available as sodium chloride (Lichtenstein et al., 2006).

Potassium is an important mineral in human diet and it is involved in electrical (brain and nerve) and cellular body functions. It influences osmotic balance between cells and the interstitial fluid (Campbell, 1987; Food and Nutrition Board, 2005; Hellgren et al., 2006).

Phosphorus is a mineral that makes up 1% of a person's total body weight. It plays a major role in biological functions by forming part of the structural framework of molecules (DNA and RNA), cellular membranes (phospholipids) and the process of phosphorylation. One of its main functions is in the formation of bones and teeth. Living cells also use phosphate to transport cellular energy in the form of adenosine triphosphate, ATP, a molecule the body uses to store energy (Food and Nutrition Board, 1997; Rakel, 2007; Yu, 2007).

Iron has several vital functions in the body; synthesis of hemoglobin and myoglobin for transporting oxygen, as a transport medium for electrons within cells, and as an integral part of important enzyme systems (that are important for energy production, immune defence and thyroid function) in various tissues (Hallberg, 1982; Bothwell, 1979; FAO/WHO, 2005).

Zinc is present in all body tissues and fluids. Zinc is an essential component of a large number (>300) of zinc-dependent enzymes participating in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids as well as in the metabolism of other micronutrients. Zinc stabilizes the molecular structure of cellular components and membranes and contributes in the maintenance of cell and organ integrity. Furthermore, zinc plays an important role in the immune system of the human body system (Shankar and Prasad, 1998; FAO/WHO, 2005).

Manganese is considered as one of the essential minerals in human diet, as it acts as a cofactor in several types of enzymes including oxidoreductases, transferases,

hydrolases, lyases, isomerases, ligases, lectins, and integrins, e.g arginase and superoxide dismutase, (Law et al., 1998; Powers et al., 2003). Copper is an essential trace mineral present in most body tissues. It forms part of enzymes including ascorbic acid oxidase, superoxide dismutases, the laccases and tyrosinases. Thus, together with iron, it helps in the formation of red blood cells and plays a role in the oxygen carrying system of the human body (Decker and Terwilliger, 2000; Trumbo et al., 2001).

The daily requirements for these trace elements vary based on sex, age and other environmental factors that can affect the bioavailability of these elements and the most vulnerable groups being pregnant women, lactating mothers and teenagers. (Ladipo, 2000). Deficiency of these minerals can lead to several diseases including iron deficiency anemia, hypocalcaemia, hypozincemia, etc (FAO/WHO, 2005).

For many Africans, dietary minerals are supplied to a large extend by the ALVs, including cowpea leaves (Agte et al., 2000). The gross content of minerals in fresh leafy vegetables varies widely (Gopalan et al., 1989) and the mineral content for many ALVs is not available in food composition tables. Furthermore, minerals, like many micronutrient contents get degraded or lost by handling, processing and storing of vegetables, thus limiting the use of food composition tables in estimating their intakes (Rahaman et al., 1990, Yadav and Sehegal, 1997; Bethke and Jansky, 2008).

2.5.2 Stability, degradation and cooking losses

Since minerals are water soluble, leaching presents the most common way in which they are lost in vegetables. This can happen at the preparation stage or during

cooking, if the cooking water is not retained with the vegetables (Bethke et al., 2008). There is also loss associated with the bioavailability of the minerals. Not all the minerals present in the food can be absorbed, and or be used by the body for the different intended roles. Low bioavailabilty of minerals can be due to mal-absorption conditions, presence of anti nutrients and presence or absence of other trace elements or micronutrients in the diet. For example, anti nutrients like phytates chelate minerals making them unavailable for the body (Wise, 1995; Mosha et al., 1995; Lestienne, et al., 2005). Uncorrected magnesium deficiency impairs repletion of cellular potassium, while increased sodium intake raises calcium excretion (FAO/WHO, 2005).

Table 2.3 Recommended dietary allowances and intakes for adolescent girls; nonpregnant, nonlactating women; and pregnant women

	Adolescent girls			Nonpregnant, nonlactating women			Pregnant women (3rd trimester)			% increase over nonpregnant, nonlactating women: pregnant women
	NRC	IOM	FAO/WHO	NRC	IOM	FAO/WHO				
Mineral	15–18 y	14–18 y	15–19 y	25–49 y	19–50 y	18–60 y	NRC	IOM	FAO/WHO	
					%					
Calcium (mg)	1200	1300	500–600	800	1000	400–500	1200	1000	1000–1200	140–150
Phosphorus (mg)	1200	1250	—	800	700	—	1200	700	—	—
Magnesium (mg)	300	360	—	280	310–320	—	320	350–360	—	—
Iron (mg)	15	—	—	15	—	—	30	—	—	—
Very low bioavailability	—	—	60	—	—	59	—	—	179–299	203–407
Low bioavailability	—	—	32	—	—	32	—	—	92–152	187–375
Medium bioavailability	—	—	16	—	—	16	—	—	46–76	187–375
High bioavailability	—	—	10	—	—	11	—	—	31–61	182–454
Zinc (mg)	12	—	1.5	12	—	1.4	15	—	2	43
Iodine (μg)	150	—	150	150	—	150	175	—	200	33
Selenium (μg)	50	—	21.5	55	—	21.5	65	—	27	26
Copper (mg)	—	—	1.15	1.5–3.0	—	1.15	1.5–3.0	—	1.15	0

NRC, National Research Council ; IOM, Institute of Medicine; y, years old

2.6 Optimizing nutrient and quality properties of the vegetables

Many preharvest, harvest and postharvest factors influence the nutrient and quality properties of horticultural crops. The preharvest conditions like climatic conditions (including light and average temperature) and cultural practices (e.g. variety of vegetable, application of fertilizer, irrigation, pruning, thinning, etc) have profound effect not only on the quality and quantity of the nutrients but also on the quality properties of vegetables. For example, the lower the light intensity during growth, the lower the AA content of plant tissues (Harris and Karmas, 1975; Lee and Kader, 2000) and nitrogen fertilizers, especially at high rates, seem to decrease the concentration of AA in many fruits and vegetables (Lee and Kader, 2000). Harvesting factors include maturity of the crop at the time of harvest and harvesting method. For example, total vitamin C of red pepper was about 30% higher than that of green pepper (Howard et al., 1994) while mechanical injuries such as bruising, surface abrasions, and cuts (associated with the method of harvest and handling operations) can result in accelerated loss of AA (Lee and Kader, 2000). Postharvest factors include (a) temperature and relative humidity management (Zepplin and Elvehjein, 1944; Lešková, 2006) (b) bruising, trimming, cutting and other size reduction operations (Mozafar, 1994; Klieber and Franklin, 2000) (c) chemical treatments e.g. calcium dips, cysteine hydrochloride (Bangerth, 1976; Mohamed et al., 1993) (d) storage (including controlled and atmosphere storage (Albrecht et al., 1990; Lešková, 2006) and (e) processing methods. Moreover, common postharvest operations such as blanching (Boushell and Potter, 1980; Artz et al., 1983; Kincal and Giray, 1987; Lešková, 2006), freezing (Katsaboxakis and Papanicolaou, 1996;

Lešková, 2006) dehydration (Jadhav et al., 1975; Lešková, 2006), or standing at various environmental temperatures (Lee et al., 1977; Lešková, 2006) can lead to AA losses in vegetables.

During processing, cooking and storage of vegetables, the nutrient and quality properties of vegetables can be degraded (Fennema, 1977; Lešková, 2006). Most of these vegetable properties are highly unstable and very vulnerable to chemical and or enzymatic degradation. However, proper thermal processing methods like blanching and pasteurization can improve the nutrient and quality properties of the vegetables by inactivating adverse enzymes.

Blanching inactivates enzymes and also helps to soften the vegetable tissues leading to an improvement in the palatability and digestion of the vegetables and product quality retention during subsequent storage procedures including freezing (Canet, 1989). The benefits of blanching have been widely documented (Kleinschmidt, 1971; Shams and Thompson, 1987; Canet, 1989; Préstamo et al., 1998; Reyes De Corcuera, et al., 2004); however, this thermal treatment has some adverse effects, especially on the quality attributes of the vegetables. On the other hand, an improper blanching process can lead to incomplete enzyme inactivation which may result in quality changes, such as texture, colour, flavour and nutritional losses, during storage.

Thus, it is important that a blanching process results in a complete inactivation of undesirable enzymes while having little effect on the quality properties of the vegetables. In order to achieve this, a blanching indicator can be used. In this context, one of the most widely used indicators is the enzyme peroxidase (POD,

E.C.1.11.1.7). For example, blanching and pasteurization prevent the action of AAO and other plant enzymes, including phenolase, cytochrome oxidase and POD, which are directly or indirectly responsible for enzymatic loss of AA. Thus, the biggest challenge in the processing of these vegetables is optimizing the processing conditions that inactivate the enzymes while at the same time retaining the nutrient and quality properties of the vegetables.

2.6.1 Peroxidase (POD)

The main function of PODs is catalyzing oxido-reduction between hydrogen peroxide and reductants and it is responsible for changes in flavour, colour and texture in fruits and vegetables.

2.6.1.1 Occurrence, classification and localization

PODs (E.C 1.11.1.) are found in animals, plants and microorganisms and divided into three main groups based on their structural and catalytic properties (Welinder, 1991; Hiraga et al., 2001). These groups include (a) PODs in animals (e.g glutathione peroxidase, myeloperoxidase, lactoperoxidase and eosinophill peroxidase) (b) catalases and include PODs in animals, plants bacteria, fungi and yeast (c) plant PODs. In turn the plant PODs can be divided into three classes. Class I plant PODs include the intracellular enzymes in plants, bacteria and yeast (e.g cytochrome *c* peroxidase, bacterial catalase-POD and ascorbate peroxidase. Class II plant POD are extracellular PODs from fungi (lignin POD and Mn^{2+}-dependent POD). Class III plant PODs are secreted outside the cells or transported into vacuoles, they are the most common of the plant PODs and responsible for much of flavour, colour and

texture changes in fruits and vegetables associated with PODs (Welinder, 1991; Hiraga et al., 2001).

2.6.1.2 Reaction catalyzed

The enzyme converts hydrogen peroxide to water, obtaining the two hydrogen atoms it needs for this from a "donor" molecule, e.g. guaiacol (commonly used in POD spectrophotometric assay). As hydrogen peroxide is being reduced, guaiacol is oxidized to tetraguaiacol (Bergmeyer, 1974; Eq. 2.2).

$$H_2O_2 + 4\ \text{Guaiacol} \xrightarrow{\text{POD}} 8\ H_2O + \text{Tetraguaiacol} \qquad \text{...(2.2)}$$

Plant PODs generally react to compounds containing a hydroxyl group(s) attached to an aromatic ring. Guaiacol is commonly used as a substrate for the measurement of PODs while ascorbic acid is the substrate in case of ascorbate peroxidase. In plants, it catalyzes the polymerization of hydroxycinnamyl alcohol species to lignin and catalyzes the incorporation of hydroxycinnamic acids containing aliphatic moieties to suberin (Fry, 1986; Quiroga et al., 2000). These macromolecules, when deposited on the extracellular surfaces, they strengthen the cell wall (giving structural strength to the plants) and prevent pathogen invasion. Suberin deposition is important for wound healing as it is deposited in wounded tissues and since suberin is a highly hydrophobic macromolecule (composed of hydroxycinnamic acid) and its derivatives contain conjugated in aliphatic moieties, its deposition around wounded tissues helps wound healing (Kolattukudy, 1980; Quiroga et al., 2000). Apart from catalyzing the lignifications and suberization of cell wall, POD catalyzes the oxidation of indole-3-

acetic acid (IAA) leading to suppression of root growth and eventual wilting of plants (Lagrimini et al., 1990; Gazaryan and Lagrimini, 1996; 1997; Quiroga et al., 2000). Plant PODs have also been found to play a crucial role in development of pathogen resistance by plants (Lovrekovich et al., 1968; Quiroga et al., 2000).

2.6.1.3 Food technological implications of the enzymatic reaction

As earlier indicated, PODs have been linked to flavour, colour and texture changes in fruits and vegetables in the postharvest system. Their effect is highly dependent not only on the number of isoenzymes but also on the quantities. Thus, POD inactivation is crucial in the maintenance of vegetable quality after harvest. Products with high POD activity must be blanched or treated with anti oxidant to decrease isoenzyme activity and those that exhibit low POD activity and mainly contain low-molecular-weight isoenzymes need not be blanched, however, they can be treated with anti oxidants if needed (Espinosa et al., 1984; Hiraga et al., 2001). PODs have a high thermal stability and therefore, their inactivation can lead to degradation of other nutrient and quality aspects of the vegetables (like AA). However, they can also be used in food processing as indicators of an effective thermal processing, since an inactivation of POD means all other enzymes that affect vegetable nutrients and quality are also inactivated. Due to the high number of isoenzymes, PODs can exhibit more than one form of thermal stability; those that are heat-labile and those that are heat-stable (Barret and Theerakulkait, 1995; Bahçeci et al, 2005; Gonçlaves et al., 2009).

2.6.2 Kinetics of thermal inactivation of enzymes/colour degradation

Two parameters are needed to characterize the thermal stability of the enzymes or degradation of the visual green colour

(a) The rate of inactivation at a specified temperature, expressed either as a rate constant, k, or as a D value (for a first order reaction)
(b) A measure of temperature sensitivity of the reaction rates as indicated by by either an activation energy (E_a) or a z value.

With these two parameters the rate of enzyme inactivation/ degradation of the visual green colour at any temperature, and accordingly the expected level of residual activity remaining after a given heat treatment, can be calculated. Furthermore, the time-temperature combination needed for complete inactivation of the enzyme can be estimated (Anthon and Barrett, 2002). Therefore, if thermal processing is combined with other enzyme inactivation strategies (e.g. pH, salt concentration, and high pressure treatment), it is possible to have a very effective process of enzyme inactivation and with suppressed quality/nutrient losses (Ludikhuyze et al., 2003; Munyaka et al., 2010).

The flow of the study is as illustrated in **Figure 2.6.**

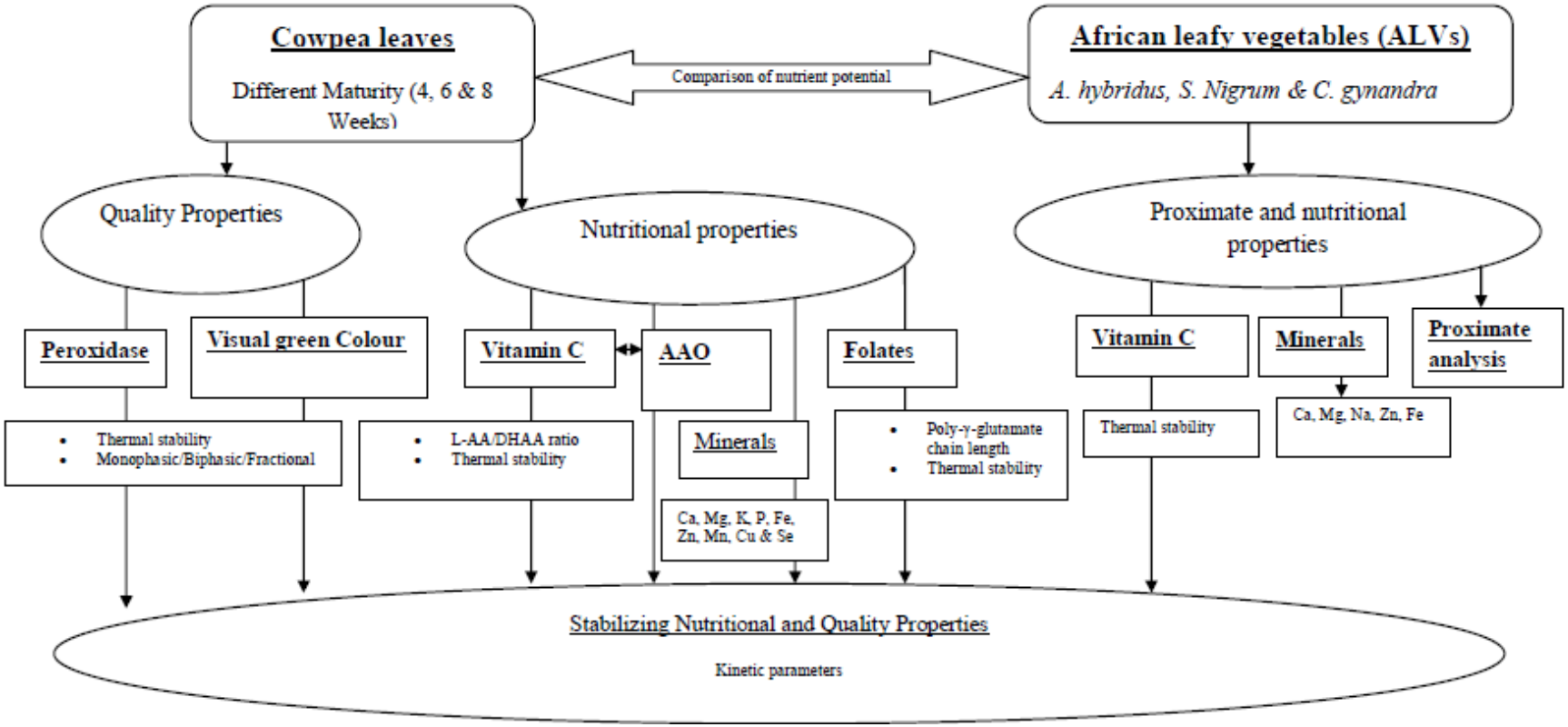


Figure 2.6 Summary of the research chronology and experiment set up

CHAPTER THREE

3.0. Thermal stability of ascorbic acid and ascorbic acid oxidase in African cowpea leaves (*Vigna unguiculata*) of different maturity

Abstract

Cowpea, an African leafy vegetable (*Vigna unguiculata*), contains a high level of vitamin C. The leaves harvested at 4 to 9 weeks old are highly prone to vitamin C losses during handling and processing. Therefore, the purpose of this research was to study the effect of thermal treatment on the stability of ascorbic acid oxidase (AAO), total vitamin C content (L-ascorbic acid, L-AA) and dehydroascorbic acid (DHAA) and L-AA/DHAA ratio in cowpea leaves harvested at different maturity (4, 6 and 8-week-old). The results showed that AAO activity, total vitamin C content and L-AA/DHAA ratio in cowpea leaves increased with increasing maturity (up to 8 weeks). Eight-week-old leaves were the best source of total vitamin C and showed a high ratio of L-AA/DHAA (4:1). Thermal inactivation of AAO followed first order reaction kinetics. Heating at temperature above 90^{o}C for short times resulted in a complete AAO inactivation, resulting in a protective effect of L-AA towards enzyme catalyzed oxidation. Total vitamin C in young leaves (harvested at 4 and 6 weeks old) was predominantly in the form of DHAA and therefore temperature treatment at 30-90^{o}C for 10 min decreased the total vitamin C content, whereas total vitamin C in 8 weeks old cowpea leaves were more than 80% in the form of L-AA so that a high retention of the total vitamin C can be obtained even after heating and/or reheating (30-90^{o}C for 10 min.) before consumption. The results

indicated that the stability of total vitamin C *in situ* was strongly dependent on the plant maturity stage and the processing conditions applied.

3.1 Introduction

Cowpeas (*Vigna unguiculata*) are tropical leafy vegetables from the family *Fabaceae* (known as well as *Leguminosae*). They are widely grown and consumed as a basic diet in the African region (Oomen and Grubben, 1978). The leaves are harvested by plucking them from the stalk in a period from 4 weeks after emergence of seedlings to the onset of flowering (between 6 to 9 weeks depending on the cultivar) (Grubben et al., 2004). After harvest, the leaves are usually washed and cut into pieces for subsequent meal preparation. The preparation of leaves into a meal entails heating them for about 8-15 minutes either by frying, steaming followed by frying, or boiling them together with tomato and/or onion in a small amount of water. The leaves are consumed as a side dish for ugali (a paste prepared from maize meal), and to a lesser extent as part of a main meal when they are cooked with maize kernels, legumes, green bananas or potatoes. Due to their widespread consumption in Africa, cowpea leaves may contribute to the daily diet as sources of vitamins such as vitamin C (ascorbic acid) and folates; fibre and minerals (Abe et al., 1982; Gomez, 1982; FAO, 2006; Aletor and Adeogun, 1995; Fasuyi, 2006).

Ascorbic Acid in vegetables comprises of L-ascorbic acid (L-AA) and dehydroascorbic acid (DHAA). L-ascorbic acid is the principal biologically active form of AA while DHAA, an oxidation product, also exhibits biological activity but to a lesser extent. Since DHAA can be easily converted into L-AA in the human

body it is important to measure both L-AA and DHAA in vegetables as total AA activity (Lee and Kader, 2000). During processing of vegetables, it is important to maintain AA in the form of L-AA because L-AA acts as a scavenger of reactive oxygen species, where it is effective against superoxide radical anions, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Rose, 1989; Weber et al., 1996) while DHAA has no radical-scavenging activity (Takamura et al., 2002). Also, the degradation of DHAA to 2, 3-diketogulonic acid (DKGA) is not reversible (Fennema, 1977; Favell, 1998; Lee and Kader, 2000) as opposed to the degradation of L-AA to DHAA. During cooking, AA losses can be due to enzymatic and chemical degradation, heating or leaching (Mathooko and Imungi, 1994). In vegetables, when cell disruption occurs, L-AA oxidation to mono-dehydroascorbic acid (MDHAA) is accelerated by ascorbic acid oxidase (AAO). MDHAA is an unstable radical and rapidly disproportionates to yield DHAA. AAO (EC 1.10.3.3) is a multi-copper oxidase (Malmstrom et al., 1975) glycoprotein widely distributed in higher plants and microorganisms, the main sources being members of the *Cucurbitaceae* family (Diallinas et al., 1997). It catalyzes one-electron oxidation of L-AA with concomitant reduction of molecular oxygen to water (Loewus, 1980). Under aerobic conditions, thermal degradation of L-AA can be described by apparent first order kinetics. L-AA is oxidized to form DHAA followed by hydrolysis and oxidation to form DKGA and oxalic acid. Under anaerobic conditions, L-AA undergoes ketonization to form the intermediate keto-tautomer (keto-ascorbic acid) which is in equilibrium with its anion (keto-monoanion ascorbic acid) which by further delactonization forms DKGA (Tannenbaum, 1976; Lin and Agalloco, 1979).

Both DHAA and AAO play important roles in plant growth. Total AA content and L-AA/DHAA ratio in leaves change at different stages of maturity. Young leaves likely contain a lower L-AA/DHAA ratio than older leaves. Several mechanisms whereby AAO controls cell growth have been proposed (Esaka, 1998; Smirnoff, 2000). In plants, AAO occurs as free or cell wall bound enzyme (Ohkawa et al., 1989; Esaka et al., 1990). The bound enzyme occurs tightly adsorbed to cell walls while the free enzyme is found as a soluble protein in the cytosol (Kivilaan et al., 1961; Loewus and Loewus, 1987). Furthermore, AAO exists as different isoenzymes (Al-Madhoun et al., 2003). Under stress condition during plant growth such as pathogen attack, chemical exposure or adverse weather conditions, the AAO content in leaves increases (Loewus and Loewus, 1987).

Hereto, the main objective of this study was to analyze the effect of thermal treatment on the stability of AAO and AA in cowpea leaves at different growth stages (maturity). Detailed kinetics on thermal inactivation of AAO *in situ* were also investigated. This integrated study from farm to processing provided different strategies to achieve high AA content with a high L-AA/DHAA ratio after thermal processing.

3.2 Materials and Methods

3.2.1 Growth condition of cowpea vegetables

Cowpea (*Vigna unguiculata*) seeds were purchased from Kenya Seed Company in Nairobi, Kenya. They were planted indoor (*Katholieke Universiteit Leuven* green house, Heverlee, Belgium); in the period from April 2008 to May 2009 (Appendix

1). The parameters for growth conditions such as temperature, relative humidity and light inside the green house were automatically controlled and monitored. From 07.00 to 23.00 hours, the temperature averaged 25.5°C and the relative humidity averaged 80%. From 23.00 to 07.00 hours, the temperature averaged 20°C while the relative humidity averaged 60%. Artificial light automatically switched on at any time between 06.00 and 19.00 hours when the light was <200 W/m^2. The plants were grown in perforated pots rested in a shallow trough filled with commercial potting compost (containing fertilizer of calcium and magnesium, 0.8 kg/m^3 composed fertilizer NPK 12-14-24, 35% dry matter, 22% organic matter, pH (water)-zone between 5 and 6.5, and electric conductivity of 350 ECµS/cm). Watering was done once a day by flooding the trough, thus, enabling uptake by the plants through the pot perforations. In this study, no commercial fertilizer was used and there was no sign of nutrient deficiency. The plants begun to pod from the 9^{th} week after planting, therefore, the leaves were harvested at the ages of 4, 6 and 8 weeks (as usually used for consumption in Kenya).

3.2.2 Sample preparation after harvest

Cowpea leaves were packed and cooled immediately after harvest to slow down respiration and transpiration during transport from the field to the research laboratory. On arrival, the leaves were rinsed with water, chopped into pieces (simulated as normally done in African cuisines) and packed (approximately 10g) in plastic pouches (140 x 200 x 40 mm, DaklaPack United Kingdom, Chiswick, London). The packages were vacuumized at 34 millibars (Multivac C200 Vacuum Chamber, Sepp Haggenmueller GmbH & Co, Wolfertschwenden, Germany). Fresh

leaves were then subjected to different treatments to study AA stability and determination of AAO content while the rest of the fresh leaves were frozen in liquid nitrogen and stored at -80°C for moisture content determination and AAO thermal stability studies (**Figure 3.1**).

3.2.3 *In situ* thermal stability of AAO in cowpea leaves

3.2.3.1 Thermal treatment

The effect of freezing (in liquid nitrogen and storage at -80°C) and vegetable maturity on AAO activity for material harvested at all stages of maturity was initially investigated using fresh (**Figure 3.1(a), sample a**) and thawed cowpea leaves (**Figure 3.1(a), sample e**). After study on the effect of freezing on AAO activity, in situ thermal stability of AAO in fresh and thawed frozen leaves was studied at different predefined temperatures for all stages of maturity.

In case of fresh leaves, approximately 10g (unfrozen) of fresh leaves at age of 6 weeks were vacuum packed in plastic pouches and afterwards treated at temperatures between 30-90°C for 10 minutes (**Figure 3.1(a), sample b**) in a thermo-stated water bath (Memmert water bath WBU 45, Memmert GmbH + Co. KG, Schwabach, Germany, Appendix 3). To stop thermal inactivation, the samples were immediately immersed in an ice-water bath for 20 minutes (Appendix 3) and the residual AAO activity was determined. The control samples (non treated samples) were used to identify the initial enzyme activity (A_0).

In case of frozen (fresh samples were initially frozen in liquid nitrogen and immediately followed by storage at -80°C) samples, plastic pouches each filled with

10g of frozen cowpea leaves that had been harvested at all stages of maturity were thawed at 25°C (30 min.) in a thermo-stated water bath (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany). After thawing, the plastic pouches were heated for 10 minutes at a predefined temperature from 30 up to 90°C (Figure 1a, sample C) in a thermo-stated water bath (Memmert water bath WBU 45, Memmert GmbH + Co. KG, Schwabach, Germany). Pouches containing leaves that were not subjected to any thermal treatment after thawing were used to assess the initial enzyme activity (A_0). After the heat treatment, the leaves were cooled in an ice water bath for 20 minutes and the residual AAO activity was determined.

3.2.3.2 Kinetics of thermal inactivation of AAO

Plastic pouches each filled with 10g of frozen (in liquid nitrogen and storage at -80^{o}C) cowpea leaves that had been harvested at 6 weeks were thawed at 25°C in a thermostatic water bath (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany). The samples were heated at predefined temperatures between 55-65°C for different time intervals (**Figure 3.1(a), sample (d**) in a thermo-stated water bath. Leaves that were not subjected to any thermal treatments after thawing were used as blank (initial enzyme activity, A_0). After heat treatment, the leaves were cooled in an ice water bath for 20 minutes and then subjected to AAO extraction.

3.2.3.3 Extraction of AAO from cowpea leaves

The procedure of AAO extraction was initially optimized. AAO in thawed cowpea leaves harvested at 6 weeks was extracted at different concentrations of sodium chloride (0-1.4 M) and pH levels (pH 4 to 7). The extraction was done using sodium

phosphate buffer (Na_2HPO_4, 4 mM) with different molar concentrations of sodium chloride and at different pH's.

The extraction of AAO was done on cowpea leaves samples, fresh or thawed, depending on the experiment as illustrated in **Figure 3.1(a)**. The extraction of AAO was done in a mixer (magic bullet blender, J-26, Ningbo Vanguard Import & Export Co, Ningbo, China) by mixing 10g of vegetable sample with 20ml of sodium phosphate buffer (Na_2HPO_4, 4 mM, pH 5.6 containing 1M NaCl). After mixing, the extract was filtered through sieves with 1 mm^2 pores, with aid of a vacuum pump. The filtrate was then centrifuged (Beckman J2-HS, Pali Alto, CA, USA) at 13300 g and 4°C for 20 minutes. AAO crude extract was obtained by 80% ammonium sulfate precipitation at 4°C for 30 minutes. The mixture was then centrifuged at 17700 g and 4°C for 20 minutes (Beckman J2-HS, Palo Alto, CA, USA). The supernatant was discarded and the residue dissolved in sodium phosphate buffer (Na2HPO4, 4 mM, pH 5.6). This crude extract solution was afterwards frozen in liquid nitrogen and stored at -80°C until AAO assay.

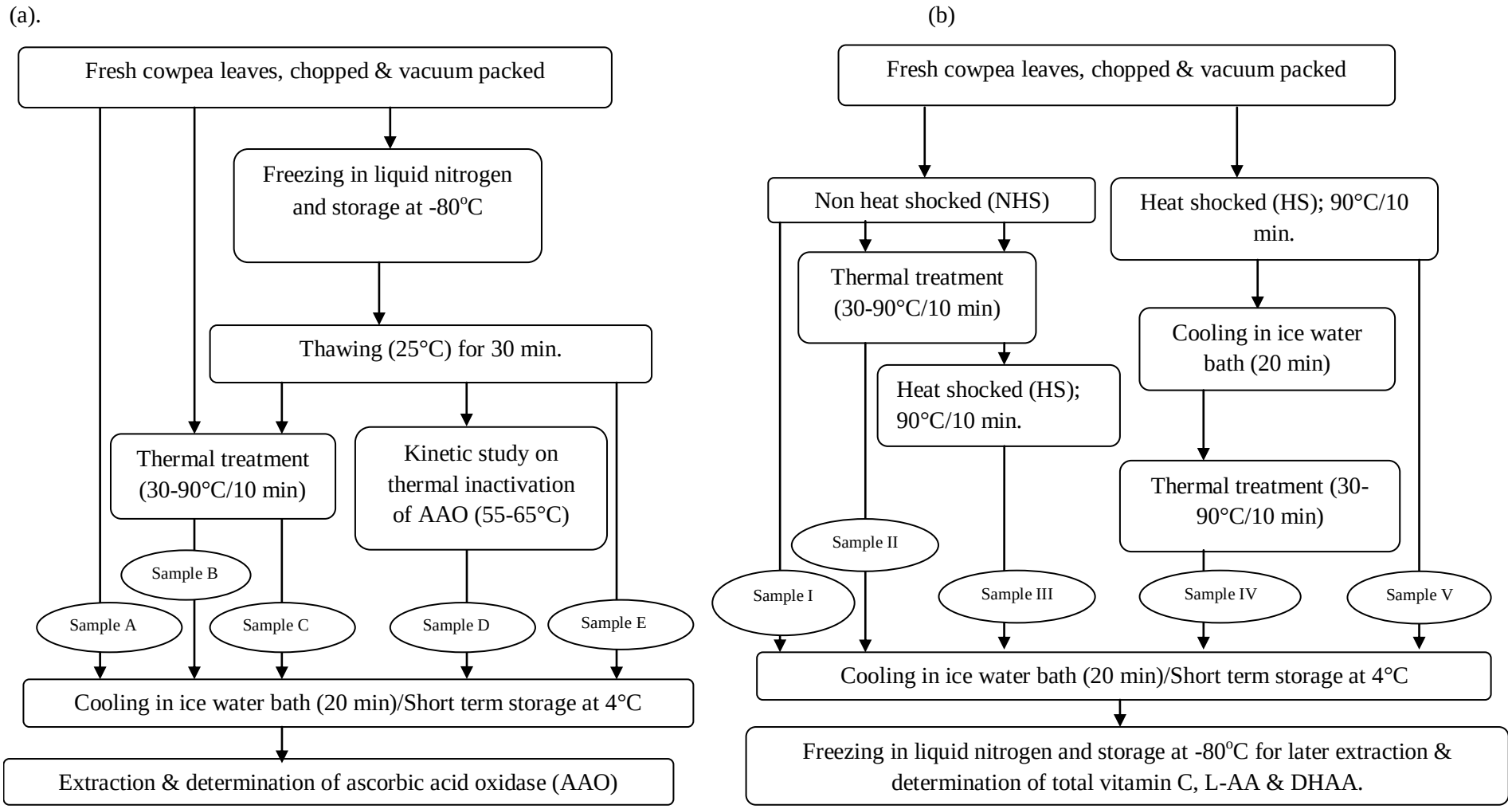
3.2.3.4 AAO activity

A polarographic assay (Strathkelvin model 78 1 B oxymeter, Glasgow, Scotland, Appendix 4) based on oxygen consumption due to AAO reaction was used to determine AAO activity. The assay was performed at 25°C in a reaction cell (4 ml) by homogenizing a mixture of 2.9 ml of air saturated phosphate buffer (4 mM and pH 5.6 containing 0.5 mM EDTA); 100 µL of substrate solution (0.5 mM L-ascorbic acid dissolved in sodium phosphate buffer (4 mM, pH 5.6) and 750 µL of cowpea leaves AAO extract/concentrate. All solutions were thermo-stated at 25°C before the

enzyme assay. The decrease in oxygen concentration was followed and the AAO activity (expressed in units) was calculated based on Eq. 3.1.

$$\text{AAO activity (units)} = \frac{\Delta[O_2]}{t} * \frac{1}{32} * V \quad \text{.....(3.1)}$$

Where $\Delta[O_2]$ is the change in oxygen consumption due to oxidation, in parts per million (ppm) over reaction time (t in seconds); the value of 32 the molecular weight of oxygen and V is the total reaction volume (liters). One unit of AAO activity is the amount of enzyme needed to catalyze the oxidation of 1 µmol of L-AA per second at 25°C and pH 5.6.



Sample A, C & E were cowpea leaves at maturity stages of 4, 6 & 8 weeks, while B & D were cowpea leaves at maturity stages of 6 weeks. Sample I to V were cowpea leaves at maturity stages of 4, 6 & 8 weeks.

Figure 3.1 Flow chart of sample preparation for determination of AAO stability and AA content in cowpea leaves.

3.2.4 Ascorbic acid stability

3.2.4.1 Sample preparation

Freshly harvested (4, 6 and 8-weeks-old) leaves, rinsed with water, chopped into pieces and vacuum packed (approximately 10g) in plastic pouches were divided into 2 types of samples: Non heat shock samples (NHS samples) and heat shock samples (HS samples) as illustrated in **Figure 3.1(b)**. NHS samples were not subjected to any thermal treatments after harvest while in plastic pouches (approximately 10 grams per package), (**Figure 3.1(b), sample I**). HS samples were prepared by boiling fresh cowpea leaves at 95°C for 10 minutes and immediately cooling down for 20 minutes in an ice water bath to inactivate AAO prior to further investigations (**Figure 3.1(b), sample V**). These samples were stored at 4^{o}C before further treatments for less than 24 hours after harvest.

3.2.4.2 Thermal treatment of cowpea leaves

In order to investigate the thermal stability of AA and the effects of AAO activity on AA content and L-AA/DHAA ratio, different strategies of thermal treatment were adopted. The L-AA/DHAA ratio were monitored using NHS (in presence of AAO activity) and HS (in absence of AAO activity) samples. In this setup, plastic pouches containing approximately 10g of the leaves (NHS) were subjected to thermal treatment at 30-90°C for 10 minutes (**Figure 3.1(b), sample II**) and then cooled down in an ice-water bath for 20 minutes, frozen in liquid nitrogen and stored at -80^{o}C before analysis. In addition, by using the NHS and HS samples, effects of different strategies on total AA content and L-AA/DHAA ratio were studied. The study was conducted by heating NHS samples at 30-90°C for 10 min. and

immediately followed by heat shock in boiling water bath for 10 min. (**Figure 3.1(b), sample III**). To stop thermal treatment, the samples were cooled down in an ice-water bath for 20 minutes, frozen in liquid nitrogen and stored at -80^{o}C until analysis. HS samples were used to validate the thermal stability of AA, L-AA and DHAA. This was done by heat shocking the samples in a boiling water bath for 10 min., followed by thermal treatment at 30-90°C for 10 min. (**Figure 3.1(b), sample IV**). To stop the reaction, the samples were cooled down in an ice-water bath for 20 minutes, frozen in liquid nitrogen and stored at -80^{o}C until analysis. Thermal treatments were carried out in a thermo-stated water bath (Memmert water bath WBU 45, Memmert GmbH + Co. KG, Schwabach, Germany). During the thermal treatments, the time to achieve the desired temperature (non isothermal condition) was followed and monitored for each experiment.

3.2.4.3 AA extraction

Frozen (in liquid nitrogen and storage at -80^{o}C) cowpea leaves samples were thawed in a water bath at 25°C (30 min.) (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany). The extraction of AA was done under subdued light by mixing 10g of sample with 50 ml of cold extraction buffer (NaH_2PO_4 solution (20 mM, pH 2.1 acidified with 1N HCl) containing 1 mM EDTA) in a mixer (magic bullet blender, J-26, Ningbo Vanguard Import and Export Co, Ningbo, China) for 30 seconds. Subsequently, the extract was centrifuged (Beckman J2-HS, Pali Alto, CA, USA) at 4°C and 17700 *g* for 30 minutes. The supernatant was filtered using a 1.2 µm membrane filter paper (Whatman, Maidstone, England) with an aid of a vacuum pump. The pH of the supernatant was brought to 4 by adding NaOH (1N) or HCl

(1M). The volume of NaOH and HCl solution was determined and incorporated in the further quantification. The supernatant was frozen in liquid nitrogen and stored at -80°C until the HPLC analysis of L-AA, DHAA and total AA content.

3.2.5 Ascorbic acid (AA) content

The supernatant obtained from the extraction procedure above was thawed in a water bath at 25°C (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany) and divided into two portions for determination of L-AA and total AA content. To determine the content of L-AA, the supernatant was filtered using a 0.45 µm cellulose acetate syringe filter (Macherey–Nagel, Düren, Germany) and brought to the auto sampler (4°C) for HPLC analysis. Total AA content was determined by performing a pre-column reduction of DHAA to L-AA using a reducing agent, Tris (2-carboxy-ethyl) phosphine-HCl/TCEP solution (2.5 mM dissolved in NaH_2PO_4 solution (20 mM, pH 3.5) containing 1 mM EDTA). Two parts of TCEP solution were added to one part of the supernatant and incubated at 25°C for 30 min. This solution was filtered using a syringe filter, 0.45 µm cellulose acetate syringe filters (Macherey–Nagel, Düren, Germany) and brought to the auto sampler (4°C) for HPLC analysis.

HPLC analysis (Agilent 1200 Series, Agilent technologies, Diegem, Belgium) was conducted to identify and quantify L-AA using a reverse phase C18 column (Prevail C18, 250mm x 4.6mm, 5µm particle size, Grace, Deerfield, IL, United States) at 25°C equipped with a DAD detector (G1315B, Agilent Technologies, Diegem, Belgium) at 245 nm. The separation was done isocratically using an ammonium

acetate solution (10 mM, pH 3.0, 1 mM EDTA) with an elution rate of 0.8mL/min for a total elution time of 30 min. The injection volume was 40 µL. Quantification was done by comparing the peak area/height of L-AA in the sample with known concentrations based on an external calibration curve of standard solutions, prepared on the day of use (500 µg/ml L-AA dissolved in NaH_2PO_4 (20 mM, pH 4.0, 1 mM EDTA)). The content of DHAA was calculated by subtracting the content of L-AA after and before TCEP reduction. The AA content was expressed as microgram or milligram L-AA per gram of dry matter (µg or mg L-AA/g DM). Example of HPLC chromatogram for AA is shown in Appendix 5.

3.2.6 Moisture content

Moisture content of cowpea leaves was determined gravimetrically based on the A.O.A.C method (2000).

3.2.7 Data analysis to estimate kinetic parameters for AAO inactivation

AAO inactivation was described by a first-order kinetic model (Eq. 3.2) and the temperature dependence of the inactivation rate constant was estimated by Arrhenius equation (Eq. 3.3), Morales-Blancas, et al., 2002).

$$A = A_0 \exp(-k_t t) \quad ..(3.2)$$

$$k_t = k_{ref} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad ..(3.3)$$

Where A is AAO activity (in units) after heat treatment for time (t) in minutes at a given temperature while A_0 is the initial AAO activity (in units), k_t is the inactivation rate constant (min^{-1}), k_{ref} is the inactivation rate constant (in min^{-1}) at reference

temperature, E_a is activation energy (in kJ/mol), R is the ideal gas constant (0.008314 kJ/mol.K), T is the actual treatment temperature (in K), and T_{ref} is the reference temperature (in K, in this study T_{ref} = 60°C).The k values were estimated based on nonlinear regression analysis. The E_a value was estimated by integrating the kinetic models considered (Eq. 3.2) with Arrhenius equation (Eq. 3.3) i.e. substituting k_t in Eq. 3.2with Eq. 3.3.

D values, the time required in reducing the AAO content by 90%, and z values, the temperature required for a 90% decrease in D value (Ariahu and Ogunsua, 2000), were estimated as follows

$$A=A_0*10^{(-t/D)} \quad ..(3.4)$$

$$A= A_0*10^{\left(\frac{-t}{D_{ref}*10^{((T_{ref}-T)/Z)}}\right)} \quad ..(3.5)$$

Whereby D_{ref} is the D value at temperature T_{ref}.

The standard errors at 95% confidence intervals were used to assess the precision of the estimates while the fitting of the model was evaluated by randomness and normality of residuals (Hill and Grieger-Block, 1980). SAS 9.1 software (SAS Institute Inc., Cary, N.C., U.S.A.) was used for nonlinear regression analysis and ANOVA analysis procedures (using least squares estimation and Levenverg–Marquart method, for minimizing the sum of squares of the deviations between experimental values and the ones predicted by the mathematical model).

3.3 Results and Discussion

3.3.1 Preliminary study on enzyme extraction and storage

The enzyme extraction yield was affected by several factors including the maturity stage of the vegetables, the environment under which the extraction was done (with or without NaCl) and the storage conditions (frozen, refrigerated or ambient temperature conditions). Firstly, the extraction procedure of AAO from cowpea leaves harvested at different maturity stages was optimized. It was observed that NaCl addition and the pH of the extraction buffer affected the AAO extraction yield. Increasing pH up to 5.4-5.8 and increasing NaCl concentration up to 1 M led to an increase in enzyme extraction yield. Extraction in absence of NaCl led to partial extraction while NaCl addition, up to 1 M, led to an increase in the AAO activity by up to 45% due to extraction of both free and membrane bound enzyme. This could indicate that the proportion of free and membrane bound AAO was almost evenly distributed, as also observed in cabbage by Hallaway et al., (1970). Optimum cowpea leaves AAO activity was found at pH around 5.4-5.8 similar to the optimum pH of AAO in other plants such as cabbage (5.5-6.6), as observed by Tauber et al., (1935) and pH of 5-6 in star fruit as observed by Saari et al., (1999).

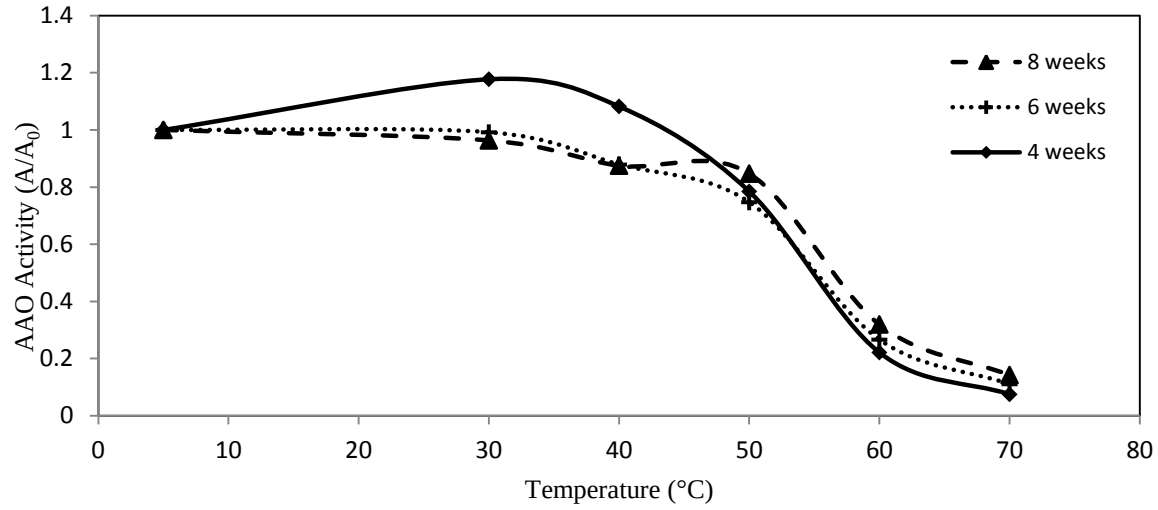
Secondly, the optimal storage conditions for AAO were studied. In this experiment, the storage conditions (quick freezing under liquid nitrogen followed by storage at -80°C) did not lead to loss of enzyme activity. This can be attributed to fast freezing in liquid nitrogen since fast freezing is associated with formation of small crystals that do not damage cell compartmental structure by the ice crystals leading to less leaching of enzyme on thawing (Zaritzky and La Plata, 2000). Apart from fast

freezing, losses due to leaching were minimized by carrying out the treatments of the leaves in plastic bags and the extraction was done by emptying all the contents in the mixer.

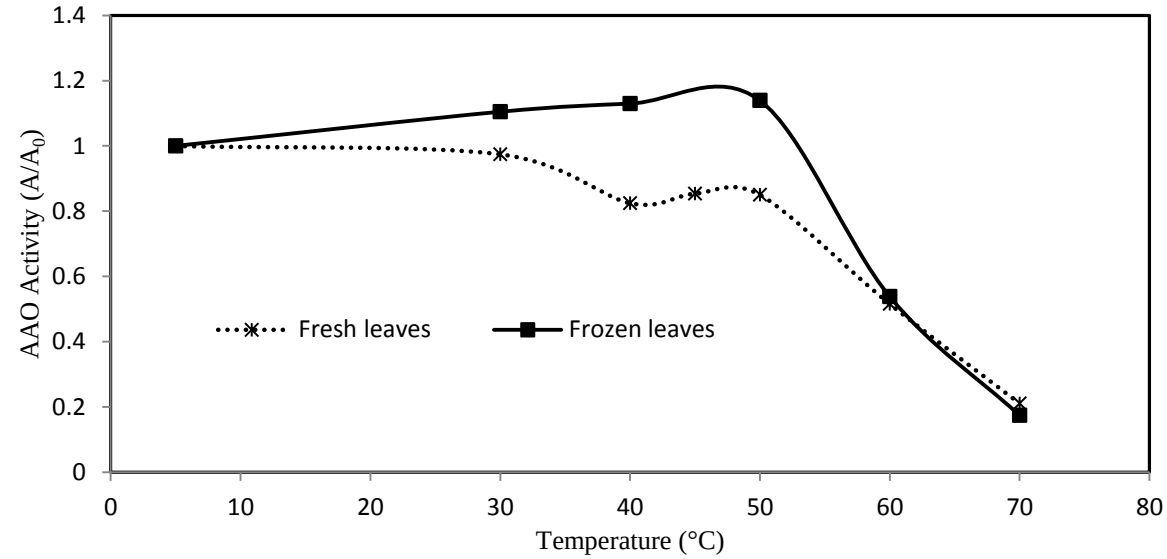
3.3.2 Effect of maturity of the leaves on thermal stability of AAO

Enzyme AAO plays a role in plant growth by controlling cell growth (Esaka, 1998; Smirnoff, 2000). In this study, AAO activity increased during maturation. Parallel to this investigation, moisture content of cowpea leaves at different stages of maturity was followed. The moisture content slightly decreased during maturation, i.e. 84.75±1.27 (4 weeks old); 82.76 ± 1.11 (6 weeks old) and 81.81 ± 0.80 (8 weeks old).

Figure 3.2(a) illustrates the thermal stability of AAO in cowpea leaves at different stages of maturity (4, 6 and 8 weeks). The results indicate that AAO thermal stability was not affected by harvesting age (4, 6 and 8 weeks old). AAO remained stable up to temperatures around 50°C. This is similar to AAO in star fruit, which is stable up to 45°C as indicated by Saari et al. (Saari et al., 1999). AAO was largely inactivated by elevating temperature above 50^{o}C. Due to the detection limit of the polarographic assay used in this study, enzyme activity after heating at temperature above 70^{o}C was not detected. The residual enzyme activity after heating >70^{o}C was 5-7% of the initial activity at 50^{o}C. High amounts of interfering substances (minerals, chlorophyll and other enzymes like polyphenol oxidase and lipoxygenase) that involve depletion of oxygen in the enzyme reactor cell resulted in a high baseline for the detection of AAO. Vegetables harvested at 6 weeks old were used to investigate the effect of freezing on the thermal stability of AAO **(Figure 3.2(b))**.



a.



b.

Figure 3.2 Temperature stability of AAO in cowpea leaves freshly harvested at 4, 6 and 8 weeks old (a) and harvested at 6 weeks old followed by freezing (b). Thermal treatment for 10 min. was conducted.

The results indicated that in 6-week-old fresh leaves the thermal stability of AAO was lower ($p<0.05$) than in thawed cowpea leaves (fast freezing done in liquid nitrogen prior to storage at -80^{o}C) (**Figure 3.2(b)**). AAO activity in fresh leaves gradually decreased when the heating temperature was increased up to 50^{o}C and afterwards significantly depleted at temperature above 50^{o}C, as also observed in thawed cowpea leaves. Therefore, further kinetic studies on thermal inactivation of AAO were carried out in the temperature range between 50 and 70^{o}C using frozen leaves.

In situ thermal inactivation of AAO in cowpea leaves followed first order kinetics with an Arrhenius dependence. The model fitting of a one-step nonlinear regression analysis based on integrating Eq. 3.3 into Eq. 3.2, the relation between predicted and measured enzyme retention and a linear Arrhenius plot between inactivation rate constants (ln (k)) and temperature (1/T) are depicted in **Figure 3.3**

The estimated kinetic parameters of AAO inactivation (k, D, z and E_a values) are summarized in **Table 3.1**. AAO inactivation was enhanced by increasing heating temperature. From the kinetics studies, a complete inactivation of AAO occurred at temperatures >80°C (based on the detection limit of the assay used in this study). AAO inactivation in cowpea leaves (E_a=238.4 ± 11.2 kJ/mol) is comparable to other green vegetables such as broccoli as experientiated by Munyaka et al., (2010) at 260-266 kJ/mol.

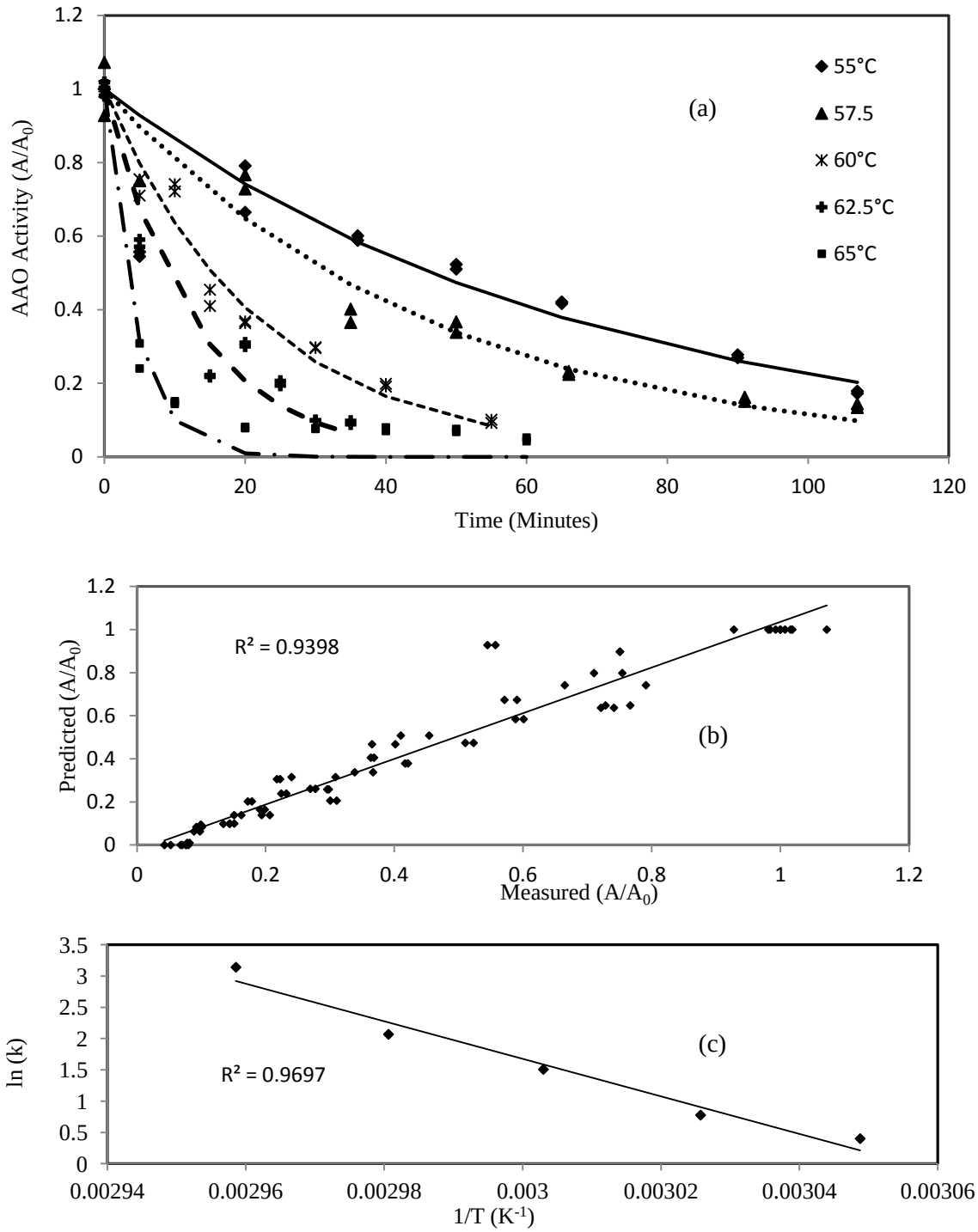


Figure 3.3 (a) Residual activity of AAO in 8-week cowpea leaves as a function of time at different temperatures. Symbols represent experimental values, lines correspond to individual fittings of the first order kinetic model to each temperature with an Arrhenius temperature dependence using non linear regression analysis; **(b)** Scatter plot of predicted versus measured residual activity of AAO activity in 8-week cowpea leaves; **(c)** Arrhenius plot between natural logarithm of reaction rate constant versus 1/T for thermal inactivation of AAO in cowpea leaves

Table 3.1 Estimated kinetic parameters for thermal inactivation of ascorbic acid oxidase (AAO) in cowpea leaves harvested at 6 weeks old.

Temperature (°C)	Parameter Estimate k($*10^{-2}$ min^{-1})	Parameter Estimate; D value (min)
55	1.5 ±0.2	154.1 ±19.3
57.5	2.2 ± 0.2	106.1 ± 7.7
60	4.5 ± 0.2	51.0 ± 2.6
62.5	7.9 ± 0.6	29.2 ± 2.1
65	23.1 ± 2.4	10.0 ± 1.0
T_{ref} = 60°C	**k_{ref} ($*10^{-2}$ min^{-1})** 4.9 ± 0.2	**D_{ref} (min)** 47.1 ± 2.1
	E_a (kJ/mol) 238.4 ± 11.2	**Z value (°C)** 8.9 ± 0.4

*The standard errors (for duplicate samples) are defined at 95% confidence level

3.3.3 Effect of AAO on thermal stability of AA

Based on the study above, it was clear that AAO was able to be inactivated by heating. Since AA is very heat sensitive, the stability of AA in vegetables during thermal inactivation of AAO is questioned. Therefore, an integrated experiment following AAO activity, L-AA content, DHAA content and total AA content at different heating temperature was conducted. For this purpose, 8 week old non heat shock (NHS) cowpea leaves were used, as illustrated in **Figure 3.1(b), sample II,** because they contained a higher AAO activity compared to 4 and 6-week-old cowpea leaves. Total AA content remained stable during thermal inactivation of AAO, even up to 90^{o}C for 10 minutes (**Figure 3.4**). Most of AA (>97%) was present in the form

of DHAA, especially when AAO was still active even at very low residual enzyme activity.

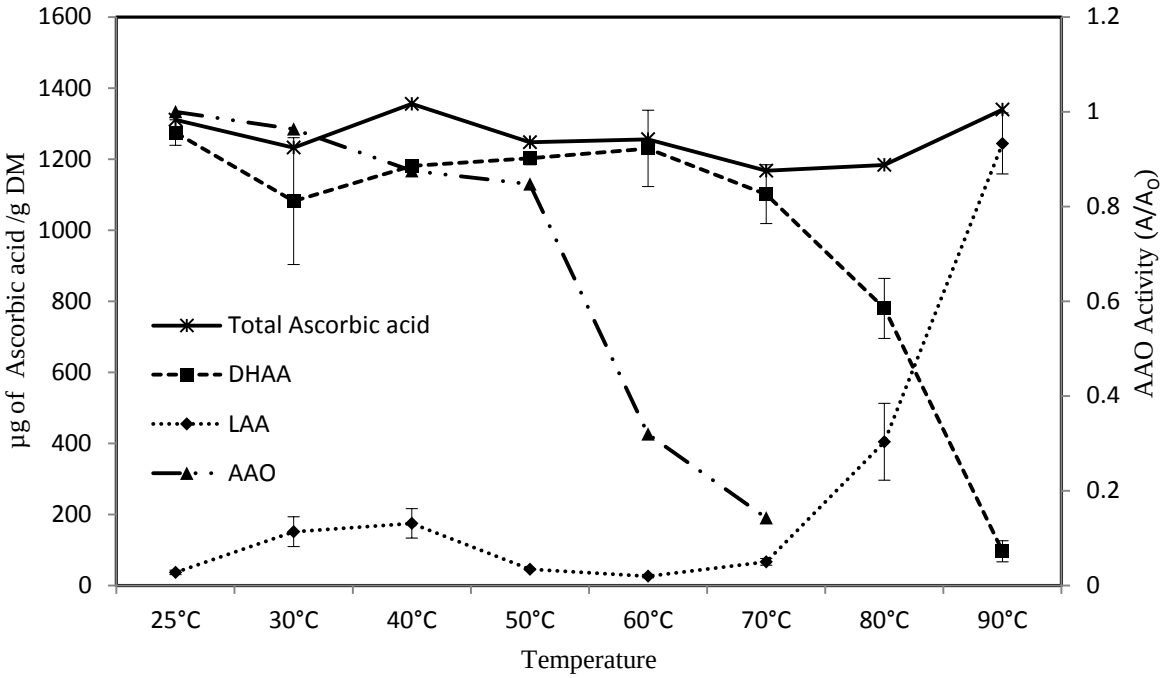


Figure 3.4 Evolution of the content of total AA, DHAA, L-AA and AAO residual activity in 8-week cowpea leaves not subjected to heat shock as a function of temperature during heating (10 min.).

As mentioned previously, AAO activity after heating at temperature of 80 and 90^{o}C for 10 minutes was not measureable due to the detection limit of the assay, however, a high proportion of DHAA to L-AA was still found even after complete inactivation of AAO (> 80^{o}C for 10 minutes, **Figure 3.4**). This is due to the fact that during heating, the temperature of the leaves in the package rose to equilibrium approaching the surrounding temperature (i.e. the temperature of the water bath). In this period, the samples passed through the temperature regime where AAO still has high

stability and activity (≤ 50°C). For example, the leaves that were thermally treated at 60°C for 10 minutes experienced non isothermal condition for 2.10 minutes before reaching 60°C (≤ 60°C) in which AAO was still active leading to the enhancement of enzymatic oxidation of L-AA to DHAA.

At high temperatures (e.g. 80 and 90°C), the temperature gradient between the sample and the surrounding environment was greater so that the samples went through the temperature regime favourable for AAO activity (≤ 50°C) faster and immediately followed by AAO inactivation. As a result, most of AA was present in the form of L-AA due to limited enzymatic oxidation of L-AA to DHAA (high L-AA/DHAA ratio). Despite the fact that the vegetables were vacuum packed before thermal treatments, vacuum packing only removed 'free' oxygen inside the package and not dissolved oxygen trapped in the plant cells which also plays an important role in the oxidation reduction process. Similar phenomenon was also observed in broccoli by Munyaka et al., (2010) and in carrots by Leong and Oey, (2012).

3.3.4 Ascorbic Acid (AA) content

Total AA, L-AA and DHAA content in cowpea leaves was followed at different stages of maturity (**Figure 3.5**). On average, the amount of total AA (1-1.3 mg/g dry weight) was comparable to total AA in cowpea leaves (1.64 mg/g dry weight) purchased from a local market in Uganda (Africa) (Ndawula et al., 2004).

At all stages of maturity, the total AA content in cowpea leaves (1.7-2.2 $x10^4$ µg/100 g fresh weight) was higher than in lettuce (1.6 $x10^3$ µg /100 g fresh weight) as determined by Yamaguchi et al., (2003). However, the total AA content in the leaves

(1-1.3 x10^3 µg /g dry weight) was less than in broccoli florets (7 x10^3 µg/g dry weight) or broccoli stalks (1.2 x10^4 µg/g dry weight) as reported by Munyaka et al., (2010). It is worth noting that the total AA content at the maturity stages of 4 and 6 weeks, was mostly in the DHAA form, even though AAO had been inactivated by cooking in boiling water (heat shocked/HS samples).

In fresh cowpea leaves (NHS samples, **Figure 3.5 (a)**), 87-98% of AA was in the DHAA form as also noticed by Munyaka et al., (2010) in broccoli. The high amount of DHAA can be attributed to the activity of AAO which was released during the extraction process and therefore, lead to the oxidation of L-AA to DHAA.

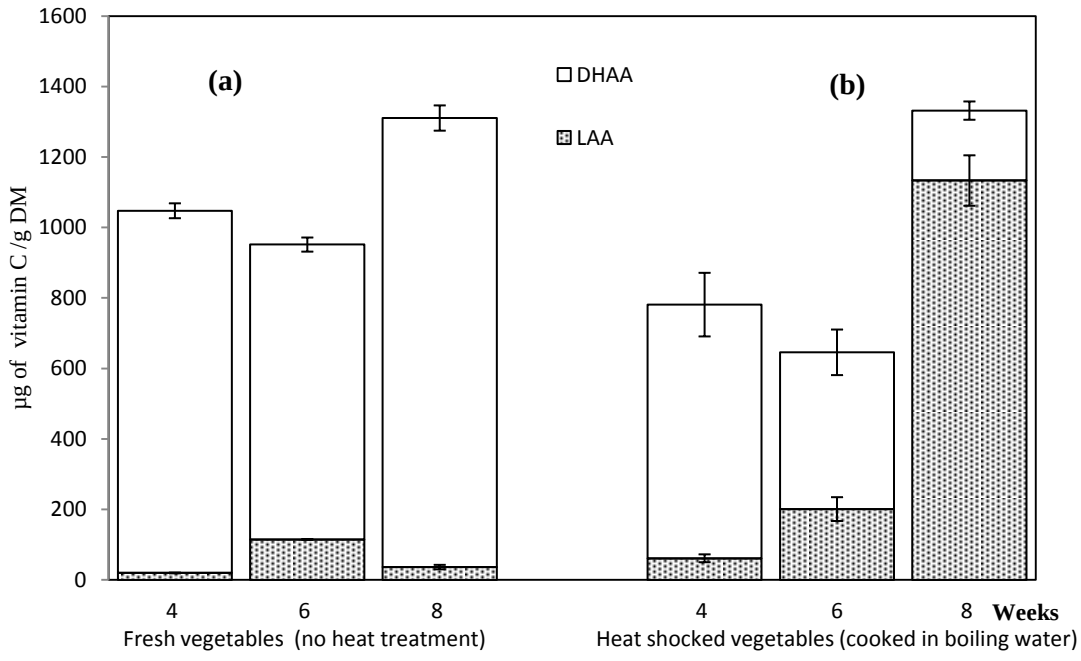


Figure 3.5 Total AA, L-AA and DHAA content in fresh cowpea leaves (a) and heat shocked (boiled for 10 minutes) cowpea leaves (b) at different levels of maturity.

Cooking the vegetables in boiling water for 10 minutes (heat shock) did not result in any significant loss of total AA for leaves harvested at 8 weeks (**Figure 3.5(b)**) and most of AA was in the form of L-AA instead of DHAA. It was also observed that heat shock treatment resulted in decrease in total AA content, i.e. by approximately 26% in 4-week-old leaves (from 1047 to 781 µg/g DM) and by approximately 32% in 6-week-old leaves (from 951 to 645 µg/g DM) when compared to the fresh uncooked leaves.

However, the heat shock pretreatment prior to extraction led to not only AAO inactivation resulting in protection of L-AA but also DHAA degradation.

Higher L-AA content after a heat shock treatment was also noticed in broccoli florets (from 0-83%) and broccoli stalks (from 17%-93%) by Munyaka et al., (2010). The results were in agreement with other findings made by Maeda et al., (1992) and Yamaguchi et al., (2001) in which cooked vegetables (burdock, lettuce and broccoli) exhibited higher radical-scavenging activity than in fresh ones due to inactivation of AAO (by cooking) leading to higher content of L-AA, which L-AA has higher radical scavenging activity than DHAA (Takamura, et al., 2002). Even though there is no significant difference in total AA content between the fresh sample (NHS) as shown in **Figure 3.5 (a)** and the HS samples (**Figure 3.5 (b)**), failure to inactivate AAO in fresh leaves led to a reduced redox state of AA.

When compared with evolution of AAO with maturity of the vegetables (previously discussed), there was no relationship found between the level of AAO activity in the vegetables and the content of total AA or the L-AA/DHAA ratio. This is because

AAO is majorly linked to cell expansion and plant growth while AA is linked to other oxidative compounds influencing the antioxidant capacity of the vegetable. High L-AA/DHAA ratio for the older leaves (8 weeks old) showed that the conversion of L-AA to DHAA by AAO occurs during cell disruption and this can be mitigated by heat shocking. For the younger leaves L-AA could be converted to DHAA by AAO in the apoplast during the plant growth and hence heat shocking only partially protects the L-AA or high level of AAO inhibitors could be present in older leaves.

The fact that even with total thermal inactivation of AAO there was still some conversion of L-AA to DHAA **Figure 3.4** showed the possible activity of other enzymes that use L-AA as indirect substrate in presence of oxygen, such as ascorbate peroxidase. Ascorbate peroxidase reduces hydrogen peroxide in the chloroplasts to water, utilizing L-AA as electron donor and in turn leading to the conversion of L-AA to DHAA (Foyer and Halliwell, 1977; De Tullio et al., 2007). This is the only plausible explanation since non enzymatic oxidation had been minimized by the precautions taken during the handling of the samples, for example working keeping samples in an ice wter bath, covering the conical flasks and test tubes with aluminium foil etc).

The results in **Figure 3.5** indicated that heat shock treatment could be an appropriate pretreatment step prior to extraction in order to obtain a complete inactivation of AAO with an expectation of protecting L-AA against enzyme oxidation, however, the practice of this pretreatment step should be taken into consideration especially for young leaf vegetables in which AA is predominantly in the form of DHAA. The heat

shock treatment could lead to underestimation of the L-AA, DHAA and total AA content because of DHAA degradation.

3.3.5 Strategies to optimize AA content and L-AA/DHAA ratio in cowpea leaves

Blanching is used to inactivate undesirable enzymes and to prolong the shelf life of cowpea leaves after harvest. In this study, it is clear that AAO should be inactivated to achieve a high level of L-AA at the moment of consumption. Different strategies of cowpea leaves preservation and effect of different cooking steps on the total AA content and L-AA/DHAA ratio in cowpea leaves at different stages of maturity were examined following the flow chart as illustrated in **Figure 3.1(b)**. Strategy 1 consisted of cooking the fresh vegetables at different temperatures as a single heating step (**Figure 3.1(b), sample II**), strategy 2 consisted of heating fresh vegetables at different temperatures (30-90°C, using NHS samples) immediately followed by blanching (95°C, 10 minutes) to inactivate the residual AAO activity (**Figure 3.1(b), sample III**) and strategy 3 consisted of blanching the fresh vegetables (95°C, 10 minutes) followed by re-heating at different temperatures, 30-90°C (**Figure 3.1(b), sample IV**).

In strategy 1, there was a definite increase in the amount of L-AA (and subsequent reduction in the amount of DHAA) with increasing temperature treatments at all the stages of maturity of the leaves. A profound increase was noted in cowpea leaves harvested at 8 weeks **(Table 3.2)**. Increase in L-AA content was noticed at temperatures >70°C. The 8-week leaves had 34% of AA being in the L-AA form at 80°C and 92% of AA being in the L-AA form at 90°C, while at the temperatures < 70°C maximally only about 12% of AA is present in the form of L-AA. These results

indicated that at temperatures < 70°C the enzyme AAO was still active and hence high amount of DHAA was produced while at temperatures >70°C, the gradual increase in L-AA content shows a decrease in AAO activity, as observed previously (**Figure 3.4**).

When strategy 2 was applied to preserve cowpea leaves after harvest, the L-AA content was very low for 4 and 6 weeks old leaves (sometimes below the detection limit) while for the 8 weeks old leaves the percentage of L-AA was high and remained stable with increasing temperatures (**Table 3.2**). There was also a general decline in the total AA content with increased heating temperature. This was due to the fact that by the time the heat shock treatment was done, AAO had already catalyzed the oxidation of L-AA to DHAA, which is more heat-labile than L-AA.

Strategy 3 decreased the total AA content respectively by 50% and 79% but increased the proportion of L-AA (**Table 3.2**). There was a marginal loss of total AA content for leaves harvested at 8 weeks and a marginal increase in the percentage of L-AA of the total AA during these thermal treatments. The decline in the total AA in the younger leaves compared to the 8 weeks samples is due to the higher amount of DHAA in the younger leaves compared to the older leaves. DHAA was less heat-stable than L-AA, therefore, the younger leaves showed a general decline in the total AA content during reheating. Since a high amount of DHAA was present in the younger leaves (4-6 weeks old), boiling pretreatment for the younger leaves (for AAO inactivation) did not give an advantage to the preservation of L-AA as witnessed in the 8 weeks leaves. The results of this investigation indicated that the age of cowpea leaves at the time of harvest and the processing/preservation methods

determine the nutritional value of cowpea leaves in terms of the AA. This integrated study implies that high retention of AA in vegetables before consumption can be obtained by optimizing conditions starting from farm to processing/meal preparation. Harvesting cowpea leaves at age of 8 weeks (before the development of the pods) is the best harvest time to have high content of AA in the form of L-AA. Since the over-expression of AAO concurrently takes place during maturity, high AAO activity can jeopardize the stability of L-AA leading to losses of nutritional value during maturation. With process optimization during blanching or thermal processing, AA can be kept in the L-AA form. Therefore, conditions of blanching and/or other thermal treatments should be appropriately designed.

Table 3.2 Effect of different strategies (Strategy 1; single heating step, Strategy 2; Single heating phase followed by blanching, Strategy 3; blanching followed by single heating phase) conducting thermal treatments on total AA content (mg/g DM) and the percentage of L-AA and DHAA.

		Strategy 1			Strategy 2			Strategy 3		
Temperature (^{o}C)	Maturity (weeks)	Total Vitamin C	L-AA (%)	DHAA (%)	Total Vitamin C	L-AA (%)	DHAA (%)	Total Vitamin C	L-AA (%)	DHAA (%)
30	4	0.96 ± 0.05	0.0	100.0	0.74 ± 0.05	0.0	100.0	0.78 ± 0.07	32.2	67.8
	6	0.94 ± 0.05	1.7	98.3	0.85 ± 0.06	2.4	97.6	0.86 ± 0.01	41.5	58.5
	8	1.23 ± 0.14	12.3	87.7	1.37 ± 0.07	84.3	15.7	1.41 ± 0.00*	82.8	17.2
40	4	1.00 ± 0.05	0.0	100.0	0.74 ± 0.05	0.0	100.0	0.61 ± 0.10	38.9	61.1
	6	1.07 ± 0.05	1.9	98.1	0.84 ± 0.05	2.2	97.8	0.67 ± 0.13	54.6	45.4
	8	1.36 ± 0.03	12.9	87.1	1.44 ± 0.00*	88.5	11.5	1.43 ± 0.06	72.3	27.7
50	4	1.02 ± 0.00*	0.0	100.0	0.79 ± 0.00*	0.0	100.0	0.68 ± 0.05	32.8	67.2
	6	1.06 ± 0.00*	0.9	99.1	0.78 ± 0.02	2.2	97.8	0.82 ± 0.04	42.6	57.4
	8	1.25 ± 0.02	3.6	96.4	1.38 ± 0.08	87.4	12.6	1.44 ± 0.05	81.6	18.4
60	4	0.77 ± 0.20	0.0	100.0	0.74 ± 0.00*	0.0	100.0	0.65 ± 0.04	25.9	74.1
	6	0.97 ± 0.02	0.0	100.0	0.75 ± 0.20	0.0	100.0	0.79 ± 0.14	38.2	61.8
	8	1.26 ± 0.10	2.1	97.9	1.26 ± 0.05	89.6	10.4	1.32 ± 0.09	85.5	14.5
70	4	0.85 ± 0.12	0.0	100.0	0.62 ± 0.04	0.0	100.0	0.65 ± 0.04	34.7	65.3
	6	0.90 ± 0.12	0.0	100.0	0.68 ± 0.12	0.0	100.0	0.78 ± 0.01	46.0	54.0
	8	1.17 ± 0.09	5.7	94.3	1.27 ± 0.01	81.6	18.4	1.18 ± 0.06	91.7	8.3
80	4	0.75 ± 0.00*	0.0	100.0	0.62 ± 0.04	2.5	97.5	0.51 ± 0.02	32.0	68.0
	6	0.84 ± 0.00*	3.3	96.7	0.63 ± 0.00*	0.0	100.0	0.76 ± 0.03	40.7	59.3
	8	1.18 ± 0.02	34.1	65.9	1.19 ± 0.09	85.1	14.9	1.24 ± 0.03	93.3	6.7
90	4	0.66 ± 0.00*	0.0	100.0	0.57 ± 0.05	3.0	97.0	0.51 ± 0.01	26.7	73.3
	6	0.77 ± 0.00	2.6	97.4	0.54 ± 0.00	0.0	100.0	0.76 ± 0.04	40.2	59.8
	8	1.34 ± 0.06	92.9	7.1	1.21 ± 0.02	82.7	17.3	1.30 ± 0.07	92.3	7.7

Values are means ± standard deviation of duplicate samples, at 95% confidence interval. *Standard deviation (< 5µg/g DM)

3.4 Conclusions and Recommendations

Several strategies can be used to inactivate or slow down AAO activity during handling, processing, and storage and in return have the potential to improve total AA stability. These include adjusting the pH, reducing the amount of salt in the medium of the leaves and thermal treatment. Avoiding the optimum conditions for AAO activity (pH of 5.6, increased salt concentration (1M), high temperature (50-70°C)) can go a long way in preserving AA in the vegetables. In terms of AA, the preharvest/harvest conditions (maturity of cowpea leaves at the time of harvest) and postharvest conditions (the processing/preservation methods) determine the nutritional value of cowpea leaves in terms of AA. This integrated study implies that high retention of AA in vegetables before consumption can be obtained by optimizing conditions starting from farm to processing/meal preparation.

Harvesting cowpea leaves at age of 8 weeks (before the development of the pods) is the best harvest time to have high content of AA in the form of L-AA. Since the over-expression of AAO concurrently takes place during maturity, high AAO activity can jeopardize the stability of L-AA leading to losses of nutritional value during maturation. With process optimization (during blanching or thermal processing), the AA is kept in the L-AA form. Therefore, conditions of blanching and/or other thermal treatments should be correctly designed. There is a need to investigate the thermodynamics (activation enthalpy, entropy and activation free energy) for AAO inactivation so as to be able to characterize fully AAO in cowpea leaves. There is a need to explore processing conditions such as pressure/temperature that can lead to enzyme inactivation and AA retention.

CHAPTER FOUR

4.0. Effect of harvest age and thermal processing on poly-γ-glutamate folates and minerals in African cowpea (*Vigna unguiculata*) leaves

Abstract

Cowpea vegetable, (*Vigna unguiculata*) is greatly consumed in Africa because it is easy to cultivate and highly disease/drought tolerant. The vegetables are harvested between 4-9 weeks of maturity. The objectives of this study were to (i) investigate the effect of vegetable maturity on the total folate content; the poly-γ-glutamate profile and the mineral content (ii) evaluate the effect of thermal treatments on folate stability and its poly-γ-glutamate profile changes. The total folate content and the folate poly-γ-glutamate chain length increased with increasing maturity of the vegetables. Folate occured predominantly as tri-γ-glutamates (55%); chain lengths of up to tetra-γ-glutamates (14%) for vegetables harvested at 4weeks old. For 6 and 8 weeks old vegetables, they occurred mainly as penta-γ-glutamates (78 and 60% respectively); chain lengths of up to hexa and hepta- γ-glutamates respectively. On crushing raw vegetables, at all stages of maturity, there was enzymatic conversion to short chain folate poly-γ-glutamates indicating the presence of endogenous γ-glutamyl hydrolase. Heating (30-70°C/10 minutes) affected the stability and profile of folates. There was a general decline (>60%) in the levels of minerals during maturation of the vegetables. In conclusion, the maturity stage is an important factor in optimizing the type and quantity of these micronutrients.

4.1 Introduction

Micronutrients are key components in securing the nutritional status of individuals; of particular importance are vitamin A, folates and other B vitamins, AA and minerals such as iodine, iron, zinc and calcium. Micronutrient deficiencies could be due to poor dietary quality/low nutrient density, poor bioavailability due to presence of inhibitors, mode of food preparation and interactions with other ingredients. The majority of the Kenyan population does not consume fortified cereals or dietary supplements in order to offset the inadequacy of the micronutrients in the diet. Previous surveys have shown that less than 10% of the population consumes fortified wheat flour and maize meal, and even if fortification were mandatory, this number would only rise to 20% (ECSA-HC, 2009). This means that the majority of the people mainly rely on traditional foods as their nutrient source. Cowpea is a common traditional horticultural product and one of the most important food legume crops in Kenya and Africa. It is a warm-weather drought-tolerant crop, well-adapted to the drier regions of the tropics, where other food legumes do not grow well. It also grows well in poor soils and is consumed both as leaves and seeds (Singh et al., 2003). Cowpea leaves, like many other leafy green vegetables, are a good source of micronutrients like folates and minerals.

Natural folates mostly exist as polyglutamate folates containing different numbers of glutamic acids depending on the raw materials. Due to the chemical instability of folates, there is a significant loss of the folate content during handling processing and storage; more than half of the folate content may be lost during processing (Blakley,

1969). Folate stability during processing is influenced by many factors including light, pH, oxygen/antioxidants, pressure, temperature and treatment time (Indrawati et al., 2004; Verlinde et al., 2008; Munyaka et al., 2009). The quantity and the type of folates present in the vegetables depend on various factors during maturation of the leaves (Gambonnet et al., 2001) while the bioavailability of folates depends on many factors including mal-absorption conditions and the chain length of folate polyglutamates. The chain length of folate polyglutamates could affect folate bioavailability because this chain should be hydrolyzed in the mucosal cells by folate conjugase prior to absorption (Scott and Weir, 1994; FAO/WHO, 2004) hence deconjugation of long polyglutamate chains before absorption is crucial.

Cowpea leaves, like many green leafy vegetables, are an excellent source of minerals in the human diet. They provide an inexpensive and abundant supply of minerals such as calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), phosphorus (P), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and selenium (Se). Minerals play an important role in the different body functions (FAO/WHO, 2004). The mineral content of many African traditional vegetables changes at different stages of maturity (Ibrikci et al., 2003; Makobo et al., 2010). During vegetable processing, the greatest loss in minerals occurs due to leaching. Surveys show that folate and mineral deficiencies are common in Africa (Fleming, 1989; WHO/FAO, 2006) and the consequences of folate and mineral deficiencies have been widely documented (FAO/WHO, 2004).

A substantial amount of work has been done on cowpea seeds as a source of nutrients for the population (Singh et al., 2003) especially on the protein content of cowpea seeds as summarized in **Table 4.1**.

Table 4.1 Proximate composition (g kg^{-1} dry matter) of cowpea (*Vigna unguiculata* L. Walp var. IT86D-719) whole meal (WM), processed meal (PM) and protein concentrate (PC) (Olivera-Castillo et al., 2007).

Item	WM	PM	PC
Moisture	91.90 ± 1.50a	77.27 ± 0.50b	77.48 ± 1.00b
Ash	32.39 ± 0.61a	25.84 ± 0.98b	32.27 ± 0.28a
Crude fat	18.07 ± 2.72b	11.45 ± 0.29c	58.47 ± 0.22a
Crude protein	247.53 ± 7.50b	257.00 ± 1.91b	786.00 ± 3.40a
Crude fibre	35.40 ± 2.90a	24.23 ± 1.03b	3.97 ± 0.49c
NFE[b]	666.61 ± 6.79a	681.48 ± 2.71a	119.29 ± 7.00b

[a] Values are mean ± standard deviation of three determinations. Different letters in the same row indicate significantly different ($P < 0.05$) means.
[b] Nitrogen-free extract, estimated by difference.

However, there is very little information on cowpea leaves. Being one of the main sources of these micronutrients (folates and minerals) in the Kenyan diet, there is a need to increase the nutrient intake from the cowpea leaves. This can be achieved by consuming the vegetables when these nutrients are at optimal levels and improving their retention during processing. One of the strategies to improve the nutrient content of vegetables is harvesting at the optimal age. There is also need for proper handling and storage and the optimization of the processing (thermal processing) of

the leaves to maximize nutrient retention and hence availability at the time of consumption.

In the context of nutrient security, the main objective of this study was to evaluate the potential of cowpea leaves to improve the supply of micronutrients, in particular folates and minerals, in the diet. The effects of maturity at harvesting time on the folate content and the poly-γ-glutamate profile as well as the mineral content was studied. In addition, the effect of thermal treatments on the folate stability and poly-γ-glutamate profile was investigated.

4.2 Materials and Methods

4.2.1 Cultivation of African cowpea leaves

A batch of cowpea (*Vigna unguiculata*) seeds, imported from Kenya (purchased from Kenya Seed Compant), was planted indoor (*Katholieke Universiteit Leuven* green house), Heverlee, Belgium in 2008 and 2009 in randomized complete blocks. The green house conditions were as follows; temperature (25.5°C from 7am to 11pm and 20°C from 11pm to 7 am), relative humidity (80% from 7am to 11pm and 60% from 11pm to 7 am) and light (switched on at any time between 6am to 7pm when the light was <200 W/m^2 inside the green house). The plants were grown in perforated pots rested in a shallow trough filled with commercial potting compost (containing fertilizer of calcium and magnesium, 0.8 kg/m^3 composed fertilizer NPK 12-14-24, 35% dry matter, 22% organic matter, pH (water)-zone between 5 and 6.5, and electric conductivity of 350 ECμS/cm). In this study, no additional commercial fertilizer was used and there was no sign of nutrient deficiency. Watering occurred

once a day by flooding the trough, thus, enabling uptake by the plants through the pot perforations. The leaves were harvested at the ages of 4, 6 and 8 weeks after planting (WAP) as commonly done for consumption in Kenya before the development of pods starting from the 9^{th} WAP.

The green house conditions were set so as to ensure a standard sample and thus reduce the errors that could arise from fluctuating daily or seasonal weather conditions outdoors.

4.2.2 Sample preparation after harvest

Cowpea leaves of vegetables harvested at 4, 6 and 8 WAP were packed and cooled immediately to slow down the respiration and transpiration during transport from the field to the research centre. On arrival, the leaves were cleaned (by rinsing them under flowing tap water), chopped into pieces which was simulated as normally conducted in African cuisines) and afterwards packed (approximately 10g/pouch) in plastic pouches (140 x 200 x 40 mm, DaklaPack United Kingdom, Chiswick, London). The packages were vacuum-sealed at 34 millibars (Multivac C200 Vacuum Chamber, Sepp Haggenmueller GmbH and Co, Wolfertschwenden, Germany) to eliminate folate oxidation. The packed fresh leaves were divided into two batches of samples, i.e. non heat shock samples (NHS samples) and heat shock samples (HS samples). HS samples were used to study the effect of maturity on the folate content and its poly-γ-glutamate profile, moisture content and mineral content. NHS samples were used to study the effect of thermal treatment on folate stability and poly-γ-glutamate folate deconjugation. To inactivate endogenous plant γ-glutamyl hydrolase (GGH), HS samples were prepared by boiling fresh cowpea leaves (approximately

10 grams per package) at 95°C for 10 minutes and immediately cooled down for 20 minutes in ice bath (0-4^{o}C). All samples were frozen in liquid nitrogen and stored at -80^{o}C for further extraction and folate and mineral analyses. Prior to folate extraction, the frozen samples were ground (Grindomix, GM 200, Germany) for 20 s to frozen powder in order to obtain homogenous samples.

4.2.3 Folate content and stability

4.2.3.1 Reagents and folate standards

All chemicals used were of analytical or HPLC grade. Unless otherwise stated, all reagents were prepared using double demineralized water (18 MΩcm at 25°C) produced with a water purification system (Simplicity 185, Millipore Massachusetts, USA). The following folate poly-γ-glutamate standards were obtained from Schircks Laboratories (Switzerland) with 85-95% and 98-99.5% purity according to HPLC analysis by the manufacturer: (6R,S)-5-methyl-5,6,7,8 tetrahydro poly-γ–glutamic acid (5-CH_3–H_4PteGluγ_{2-5}) trihydrochloride salts; pteroylhexa-γ-L-glutamic acid (PteGluγ_6) and pteroylhepta-γ-L-glutamic acid (PteGluγ_7). 5-CH_3–H_4PteGluγ_6 and 5-CH_3–H_4PteGluγ_7 were obtained by chemical conversion of PteGluγ_6 and PteGluγ_7 as outlined by Ndaw et al. (2001). (6S)-5-Methyl-5,6,7,8-tetrahydrofolic acid calcium salt (5-CH_3–H_4PteGlu$_1$) was obtained as a gift from Merck Eprova AG (Schaffhausen, Switzerland).

4.2.3.2 Thermal treatment of the leaves

For determination of the native folate poly-γ-glutamate profile of the leaves, HS samples (γ-glutamyl hydrolase/GGH inactivated prior to crushing) were used. To

study the effects of GGH activity on the folate poly-γ-glutamate profile and content of the leaves, NHS samples (in presence of GGH activity) were used. In this set up, approximately 10g (unfrozen fresh leaves at all stages of maturity vacuum-packed in plastic pouches) were treated at temperatures between 30 and 90°C for 10 minutes in a thermo-stated water bath (Memmert water bath WBU 45, Memmert GmbH + Co. KG, Schwabach, Germany). To slow down the reactions (activity or inactivation depending on the temperature level), the samples were immediately immersed in an ice-water bath (0-4°C) for 20 minutes, frozen in liquid nitrogen and stored at -80^{o}C before analysis.

4.2.3.3 Folate extraction, chemical conversion and analysis

Folate analysis was carried out using three vegetable samples through extraction, chemical conversion of poly-γ-glutamate folates to 5-CH_3–H_4PteGluγ_n, folate purification, identification and quantification. The extraction procedure was performed according to the modified method used for broccoli by Verlinde et al. (2008) and Munyaka et al. (2009) and optimized for cowpea leaves. Firstly, the folate extraction was done by mixing 20 g HS or NHS samples with 50 ml cold or 50 ml boiling extraction buffer (phosphate buffer (0.1 M, pH 7, containing 0.5 g ascorbic acid)) respectively. Then the mixtures were homogenized (Ultra Turrax T25, IKA Labortechnik, Staufen, Germany) for 30 s. The NHS samples were extracted by immersing the tubes containing the sample extract in boiling water for 10 min and subsequently cooled in ice water. The HS samples did not receive an additional hot extraction so that both categories of samples received approximately the same total heating intensity. The pH of the sample was adjusted to 7.0 using HCl

(37%) and NaOH (5M) prior to bi-enzyme digestion carried out at 37^{o}C for 4 hours using 50µl of *Bacillus subtilisin* protease (1mg/mL, Sigma-Aldrich, Steinheim, Germany) and 50µl of amylase (Termamyl 120L, Novo Nordisk, Bagvaærd, Denmark). To inactivate the enzymes, the extract was heated in boiling water for 5 min. and afterwards cooled down in an ice water bath to stop heating effect. The extraction mixture was centrifuged at 20000*g* and 4^{o}C for 30 min. and the obtained supernatant was used for further analysis.

Secondly, poly-γ-glutamates of different folate derivatives were chemically converted to 5-CH_3–H_4PteGluγ_n. In this procedure, poly-γ-glutamate folates of different folate derivatives were converted to 5 methyltetrahydrofolylpolyglutamates (5-CH_3–H_4PteGlu$_{\gamma n}$) according to the procedure described by Ndaw et al. (2001) and Verlinde et al. (2008). The obtained supernatant (5 ml containing a potential maximum of 20 nmol folates after method optimization) was added to 15 mL Tris buffer (66 mM, pH 7.8) with 2.0 g sodium ascorbate and 1 mL 2-octanol in a conical flask. The sodium ascorbate acted as an antioxidant in order to protect folates from oxidation while the 2-octanol was added to prevent foaming during subsequent procedures. This solution was mixed with a magnetic stirrer until all the sodium ascorbate was dissolved. Afterwards, 10 mL of $NaBH_4$ (3.2 M) was added using a burette and the mixture was incubated for 10 min. In excess of NaBH4 compared to the quantity of the folates to be converted, reduction of PteGlu to H_4PteGlu and also a partial conversion of 5-CHO–H_4PteGlu to H_4PteGlu were achieved. The pH of the mixture was adjusted to pH 7.4 using CH_3COOH (5 M) and 80 µL of formaldehyde, HCHO (37%) was added to convert H_4PteGlu to 5,10-CH_2–H_4PteGlu. This mixture

was stirred vigorously for 30s and subsequently followed by addition of $NaBH_4$ (3.2 M, 10 mL) within 30s.

The conversion solution was adjusted to an approximate pH 0.85 using HCl (37%) and the mixture was incubated for 10 min. After incubation, the pH was increased to pH 5 with NaOH (5 M) and another 10 mL of $NaBH_4$ (3.2 M) was added. Further addition of $NaBH_4$ led to a high degree of conversion of folates, in the order of 95%, to 5-CH_3–H_4PteGlu. This converted solution was incubated for 20 min and the volume was adjusted to 100 mL. 2-Octanol was skimmed from the converted extract and aliquots were stored at -80°C until purification. The entire conversion process was conducted under subdued light by wrapping the samples with aluminium foil to prevent folate degradation.

Thirdly, folate purification, done using folate binding protein (FBP) affinity chromatography, were performed as described by Indrawati et al. (2004) and Verlinde et al. (2008). In this procedure, the purification was done on thawed and filtered conversion mixtures obtained from the above procedure. Purified bovine FBP (Scripps Laboratories, San Diego, CA, USA) immobilized on an *N*-hydroxysuccinimide ester resin (Affi-gel 10, BioRad Laboratories, Hercules, CA, USA) was used for affinity chromatography columns. Sample (5 mL) was applied on previously equilibrated FBP columns using 5 mL phosphate buffer (0.1 M, pH 7.0). Afterwards, the columns were washed using 5 mL phosphate buffer (25 mM, pH 7.0) containing NaCl (1 M) followed by rinsing the columns with 5 mL phosphate buffer (25 mM, pH 7.0). Folates were eluted using 4.6 mL of trifluoroacetic acid

(CF_3COOH, 20 mM) with 1,4-dithioerythritol (20 mM) solution (pH 6.35). The eluate was collected in a 5 mL volumetric flask containing 5 µL β-mercaptoethanol, 200 µL of L-ascorbic acid solution (25%, w:v), and 40 µL of a KOH solution (60%, w:v). The volume of the eluate was adjusted to 5 mL with the eluent solution. The entire purification procedure was performed in a dark room at 4°C in order to prevent degradation of folates.

5-CH_3–H_4PteGluγ_{1-7} derivatives were identified and quantified using reverse - phase HPLC (Agilent 1200 Series HPLC system, Agilent Technologies, Diegem, Belgium) equipped with diode array (G1315B, Agilent Technologies, Diegem, Belgium) and fluorescence (RF-10AxL, Shimadzu, Kyoto, Japan) detectors. Separation of converted poly-γ-glutamate folates was performed using step-wise linear gradient elution at 25°C using a Prevail C_{18} column (250 x 4.6 mm, 5 µm particle size; Alltech, Deerfield, IL, USA), protected with C_{18} guard cartridge (7.5 x 4.6 mm, 5 µm particle size; Alltech, Deerfield, IL, USA). Fluorescence detection was applied at excitation and emission wavelengths of, respectively, 295 and 356 nm at 20 Hz at the highest sensitivity magnification factor. Two solutions were used as eluents, i.e. solvent A - NaH_2PO_4 solution (50 mM, pH 4.5) and (ii) solvent B: 20% (v:v) acetonitrile in NaH_2PO_4 (50 mM) solution (pH 4.5) at a flow rate of 0.8 mL/min. Sample injection was ranging from 5 to100 µL. First linear gradient was performed by raising the concentration of solvent B to 50% within 23.40 min and followed by the second gradient using solvent B from 50% to 100% within 13.00 min. Afterwards, the column was washed for 10.40 min with 100% B, followed by equilibration for 10.40 min with 100% solvent A. Folate identification and

quantification were done using external folate standards (Schircks Laboratories, Jona, Switzerland) of 5-CH_3–H_4PteGlu$\gamma_{1\text{-}7}$. Peak height was used to estimate the content of folate poly-γ-glutamates in the form of 5-CH_3–H_4PteGlu$_{\gamma n}$. The detection limit for various poly-γ-glutamates and monoglutamates ranged from 0.015 to 0.100 pmol on the column. Example of HPLC chromatogram for poly-γ-glutamates in the form of 5-CH_3–H_4PteGlu$_{\gamma n}$ is shown in Appendix 6.

4.2.4 Mineral and moisture content

The contents of Ca, Mg, Na, K, P, Fe, Zn, Mn, Cu and Se were determined at the laboratory of Bodemkundige Dienst van België (B.D.B) in Heverlee, Belgium. For Ca, Mg, Na, K, P, Fe, Zn, Mn and Cu, 10g of freshly harvested leaves of vegetables (at 4, 6 and 8 WAP) were dried in the oven and then pulverized in a grinding mill. Approximately 2.5g of this homogenous sample was weighed exactly in a crucible and heated to ash in a muffle oven at a temperature of 550°C. To make the extract, the crucible was filled with 1N HNO_3 and placed in a warm water-bath of 70°C for 1 hour. The digested sample was filtered into a 100 ml flask and made up to the mark with the 1N HNO_3. The mineral values of Ca, Mg, Na, K and P were measured with an inductive coupled plasma optical emission spectrometry, ICP-OES, (PerkinElmer Optima™ 4300 DV; PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) and on the other hand the mineral values of Fe, Zn, Mn and Cu were determined by a PerkinElmer Optima™ 5300 DV ICP-OES; PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). For Se, a reflux-digestion system was used. That is, the dried sample was weighted in destruction tubes and $HClO_4$ was added. The tubes were heated in a destruction block until there was no more production of orange-

brown fumes (at this point the sample in the destruction tube was dry). Then the digestion tubes were removed from the destruction block and put on a table to cool at room temperature. After cooling the samples were filtered into a 100 ml flask and made up to the mark with the 1N HNO_3, and the quantification was done by ICP emission spectrometer, (PerkinElmer Optima™ 3300 DV; PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). The following spectral emission lines (nm) were used in the quantification of the minerals using the ICP-OES system: Ca, 315.887; Mg, 279.077; Na, 589.592; K, 766.490; P, 214.914; Fe, 259.939; Zn, 206.200; Mn, 257.610; Cu, 327.393 and Se, 196.026. The moisture content of the leaves was determined based on the A.O.A.C method (2002).

4.3 Results and Discussion

4.3.1 Folate content and stability

4.3.1.1 Profile of native folate poly-γ-glutamates

The total folate content and the native folate poly-γ-glutamate profile in cowpea leaves were compared for heat shocked (HS) samples (in which endogenous enzymes including folate conjugase had been inactivated) and non-heat-shocked (NHS) samples (in which all endogenous enzymes are still active). Regarding the profile of native folate poly-γ-glutamates (obtained from HS samples), folates in cowpea leaves predominantly existed as poly-γ-glutamate forms consisting of 4 up to 7 glutamic acids. Folate with a longer poly-γ-glutamic acid chain was found in older leaves (the chain length of folate poly-γ-glutamates increased with maturity, (**Table 4.2**).

In HS samples, folates existed mainly as tri-γ-glutamates (55%) in leaves of vegetables harvested at 4 weeks WAP and as penta-γ-glutamates in leaves of vegetables harvested at 6 and 8 WAP (78 and 60% respectively). The longest folate poly-γ-glutamate chains were tetra, hexa and hepta- γ-glutamates, respectively for vegetables harvested at 4, 6 and 8 WAP. Young vegetables (4 WAP) contained a higher amount of mono and di-γ-glutamates (30% of the total folate content) compared to the older vegetables (max. 20% of the total folate content). In this study, it was also observed that the content of short chain folate poly-γ-glutamates in NHS samples was higher than in HS samples indicating that the leaves contained active GGH. Deconjugation of the long chain folate poly-γ-glutamates in NHS samples occurred due to cell rupture during folate extraction (involving mixing and homogenization with extraction buffer) facilitating contact between GGH and folate poly-γ-glutamates. Therefore, the folate poly-γ-glutamate profile differed between cowpea leaves with and without heat shock treatment before extraction.

As found in NHS samples, endogenous GGH catalysed conversions could result in a high amount of folate monoglutamates at all stages of maturity. Hence, GGH was active in the raw samples at all stages of maturity and could result in deconjugation of long chain to short chain poly-γ-glutamate folates including monoglutamates. Changes in folate profiles of vegetables ware also observed in broccoli by Munyaka et al., (2009), whereby folates in heat shocked broccoli (inactivating endogenous plant GGH) predominantly occurred as hexa- and hepta-γ-glutamates while in raw crushed broccoli low concentrations of hexa and hepta-γ-glutamates and higher concentrations of tri, di-γ-glutamate and monoglutamate folates were observed.

Other vegetables like spinach and peas show a wide range of poly-γ-glutamates (1–6-γ-glutamyl residues) as reported by Ndaw et al., (2001) while cauliflower, lettuce, cabbage and carrot contained 2–8-γ -glutamyl residues (Zheng et al., 1992).

The total folate content in cowpea leaves increased during maturation (in this study observed between 4 and 8 weeks of maturity). The folate content of leaves of vegetables harvested at 8 WAP was twice as high as that of 4 WAP. In general, heat shocked samples contained a higher amount of folates compared to raw (non heat shocked) samples. These differences in total folate content could be due to enzyme inactivation including oxidative enzymes such as ascorbate oxidase during a heat shock thermal treatment resulting in limited degradation of most antioxidants especially ascorbic acid (protecting folates against oxidation reactions), as further discussed in the text. Older leaves (8 WAP) are deemed to be the best source of folate since they show significantly higher ($p<0.05$) quantities of folates compared to those harvested at 4 and 6 WAP. If the cowpea leaves are consumed as raw vegetables (e.g. salad), 8 weeks old vegetables will provide not only the highest total folate content but also the highest amount of di- and monoglutamates (>70%).

The highest total folate content recorded for cowpea leaves (78 µg/g fresh weight) was less than that reported for spinach (191.8 µg/100g fresh weight) (McKillop et al., 2002; using the microbiological assay), approximately 4 times lower than in broccoli florets and approximately 2 times lower than in broccoli stalks as reported by Munyaka et al., (2009) (used the HPLC method), but it was higher than values (57 µg/g fresh weight) reported for Amaranth leaves (another traditional vegetable

consumed in Kenya) and English cabbage (33 µg/g fresh weight) (Devi et al., 2008; these studies used microbiological assay); white cabbage (Vahteristo et al., 1997; Souci et al., 2000; Jägerstad et al., 2004) where the HPLC method was used. However it was higher than 1.41 µg/g fresh weight as found in cowpea seeds.

4.3.1.2 Effect of thermal treatment on total folate content and native folate poly-γ-glutamate profile in the leaves

Traditionally cowpea leaves are consumed cooked. In order to improve the health benefits to the consumer there is need to increase the retention of folates in cowpea leaves by obtaining and or maintaining high concentrations of tri, di-γ-glutamate and monoglutamate folates which implies a higher folate bioavailability. At all stages of maturity, thermal treatments at temperatures lower than 70^{o}C resulted in a high content of short chain poly-γ-glutamates (di- and monoglutamate foms) because in this range, a temperature increase enhances the enzymatic deconjugation. In this study, increasing temperature above 70^{o}C retarded the deconjugation of long chain poly-γ-glutamates which was indicated by lower amounts of short chain folate poly-γ-glutamates, in particular di- and monoglutamate forms. This mechanism can be easily seen in leaves of vegetables harvested at 4 WAP because the profile of native folate poly-γ-glutamates is less complex (up to 4 glutamyl acid residues) than those at 6 and 8 WAP. In these older leaves, this phenomenon was less obvious because of the complex starting profile and the different parallel folate interconversions (deconjugations) occurring. Hence, the results obtained in this research indirectly indicate that GGH was highly active in the temperature range of 30 to 50^{o}C as also observed in broccoli (Verlinde et al., 2008; Munyaka et al., 2009). Temperatures

above 50^{o}C retarded the deconjugation of folate poly-γ-glutamates in cowpea leaves resulting in long chain folate poly-γ-glutamates.

With regard to the total folate content determined using the experimental design of this study, increasing the heating temperature resulted in a high total folate content (**Table 4.3**). Compared to folates in leaves treated at 90^{o}C and above, heating at lower temperature decreased the total folate content ranging from 60% to 30% depending on vegetable maturity. Folate degradation in vegetables harvested at 8 WAP due to heating was lower than in those at 4 and 6 WAP. It is likely that the stability of folates in cowpea leaves is depending on the presence of other antioxidants such as ascorbic acid. In **Chapter 3,** (Wawire et al., (2011)) have shown that the AA content, the ratio between L-ascorbic acid (L-AA) and dehydro-ascorbic acid (DHAA) and the activity of ascorbic acid oxidase (which plays an important role in L-AA degradation resulting in lower antioxidant capacity of AA) are changing with leave maturity at the time of harvest. Indeed another study, Indrawati et al., (2004) has shown that ascorbic acid enhanced both the thermo- and pressure-stabilities of 5-CH_3-H_4 folate. Therefore, the higher folate content in HS samples (of vegetables harvested at all stages of maturity) and the vegetables harvested at 8 WAP for NHS maybe be due to the stabilizing effect of L-AA just as much as these samples inherently having more folate.

In this study, we observed that simultaneous complex enzymatic and chemical reactions during thermal treatment affect the retention of micronutrients. Furthermore, this should be taken into account when applying these findings because

the loss of micronutrients during heat treatments is not only related to the temperature stability of the compounds of interest but also related to how the vegetables are cooked (i.e. linked to cooking preparation and technique). From a practical point of view, avoidance of leaching (as exemplified in the experiments where the samples were thermally treated in plastic pouches and all the contents used in the experiments), avoiding the direct exposing of the vegetables directly to boiling water and elimination of free and dissolved oxygen are advisable in order to eliminate the loss of folates in cowpea leaves.

Table 4.2 The folate poly-γ-glutamate profile and total folate content (5-methyltetrahydrofolates polyglutamate) of heat shocked (95°C/10 min) and non-heat shocked (raw) cowpea leaves at different stages of maturity (4, 6 and 8 weeks old).

		Folate derivative (nmol/g DM)							
Treatment	Maturity (Weeks)	Hepta-γ-glutamates	Hexa-γ-glutamates	Penta-γ-glutamates	Tetra-γ-glutamates	Tri-γ-glutamates	Di-γ-glutamates	Mono-γ-glutamate	Total folates
		nmol/g DM							
Raw (NHS) samples	4	n.d.	n.d.	n.d.	n.d.	n.d.	1.44±0.24	2.27±0.92	3.71±1.16
	6	n.d.	0.28±0.01*	n.d.	n.d.	n.d.	0.26±0.03	2.43±0.19	2.97±0.23
	8	n.d.	0.31±0.03	0.55±0.03	n.d.	1.13±0.30	1.76±0.53	3.28±0.78	7.02±1.69
Heat shocked samples (HS)	4	n.d.	n.d.	n.d.	0.77±0.34	2.93±0.43	0.41±0.82	1.22±0.34	5.33±2.20
	6	n.d.	0.44±0.02	7.40±0.23	0.44±0.02	0.24±0.05	0.16±0.10	0.77±0.30	9.45±0.70
	8	0.25±0.01	0.39±0.11	6.32±1.36	0.38±0.00**	0.99±0.47	0.36±0.23	1.70±0.60	10.41±2.78

n.d. : not detected (under detection limit of the measurements; Limit of detection= 0.015 nmol/g DM)

*: means ± standard deviation of triplicate samples (95% confidence interval, n=3 samples from each maturity)

**: lower than 5 pmol/g DM therefore rounded off. DM: Dry matter

Table 4.3 Folate poly-γ-glutamate and total folate content (5-methyltetrahydrofolate polyglutamate), of leaves of cowpea vegetables harvested at different stages of maturity (4, 6 and 8 weeks old) after different thermal treatments (10 min.).

Treatment	Maturity (Weeks)	Folate derivative (nmol/g DM)							
		Hepta-γ-glutamates	Hexa-γ-glutamates	Penta-γ-glutamates	Tetra-γ-glutamates	Tri-γ-glutamates	Di-γ-glutamates	Mono-γ-glutamate	Total nMol
		nmol/g DM							
25°C	4	n.d.	n.d.	n.d.	n.d.	n.d.	1.44±0.24	2.27±0.92	3.71±1.16
	6	n.d.	0.28±0.01*	n.d.	n.d.	n.d.	0.26±0.03	2.43±0.19	2.97±0.23
	8	n.d.	0.31±0.03	0.55±0.03	n.d.	1.13±0.30	1.76±0.53	3.28±0.78	7.02±1.69
30°C	4	n.d.	n.d.	n.d.	n.d.	n.d.	1.01±0.081	2.41±0.55	3.42±0.63
	6	n.d.	0.43±0.22	n.d.	n.d.	n.d.	0.40±0.10	2.31±0.19	3.15±0.42
	8	n.d.	0.36±0.11	0.41±0.00**	n.d.	1.36±0.74	1.40±0.12	3.44±0.45	6.97±0.68
50°C	4	n.d.	n.d.	n.d.	n.d.	n.d.	0.74±0.05	2.15±0.29	2.88±0.34
	6	n.d.	0.25±0.01	0.76±0.25	n.d.	0.43±0.15	0.53±0.05	2.60±0.75	4.57±0.30
	8	n.d.	0.39±0.00**	0.92±0.40	0.39±0.06	0.45±0.16	1.07±1.33	3.72±1.32	6.95±3.30
70°C	4	n.d.	n.d.	n.d.	n.d.	1.48±0.39	1.04±0.31	1.44±0.17	3.96±0.86
	6	n.d.	0.34±0.08	0.84±0.45	n.d.	0.45±0.06	0.38±0.12	1.92±0.16	3.93±0.83
	8	n.d.	0.26±0.02	2.09±0.54	0.45±0.07	0.35±0.08	0.34±0.11	3.44±0.60	6.94±1.42
90°C	4	n.d.	n.d.	n.d.	n.d.	3.35±0.77	1.10±0.2	0.64±0.02	5.09±1.22
	6	n.d.	0.52±0.15	1.59±0.68	0.36±0.03	0.48±0.05	0.52±0.17	2.49±0.56	5.96±1.65
	8	0.28±0.01	0.30±0.09	5.26±1.65	0.50±0.15	0.64±0.35	0.46±0.18	2.07±0.56	9.51±3.00
95°C	4	n.d.	n.d.	n.d.	0.766±0.34	2.93±0.43	0.41±0.82	1.22±0.34	5.33±2.20
	6	n.d.	0.44±0.02	7.40±0.23	0.44±0.02	0.24±0.05	0.16±0.10	0.77±0.30	9.45±0.70
	8	0.25±0.01	0.39±0.11	6.32±1.36	0.38±0.00**	0.99±0.47	0.36±0.23	1.70±0.60	10.41±2.78

n.d. : not detected (under detection limit of the measurements; Limit of detection= 0.015 nmol/g DM) *means ± standard deviation of triplicate samples (95% confidence interval, n=3 samples from each maturity and treatment) **: lower than 5 pmol/g dry matter DM therefore rounded off

4.3.2 Mineral content

Except for Na, a general decrease (>60%) in the amounts of Ca, Mg, K, P, Fe, Zn, Mn Cu and Se was observed during maturation of the leaves of vegetables harvested between 4 and 8 WAP (**Table 4.4**). The moisture content (%) slightly decreased during maturation, i.e. 84.75 ± 1.27 (4 weeks old); 82.76 ± 1.11 (6 weeks old) and 81.81 ± 0.80 (8 weeks old). Among the minerals determined in this research, Zn, Fe and Se showed the highest decrease (up to 97%) during maturation. For most minerals, the greatest decrease took place between 6 and 8 WAP, i.e. Zn, Fe and Mn recording the highest decrease (88, 85, 81% respectively) followed by Ca, Mg, K and P (70, 66, 69, 70% respectively) while Na showed a moderate decrease of 36% and no changes were observed in Se content. Regarding Ca, the decrease during maturation could be explained by the relocation of Ca from the terminal organs (Marshner, 1986; 1995; Makobo et al., 2011) like the leaves, to the pods that start to develop from the 9th week of maturity. Between 4 and 6 WAP, there was an increase in the amounts of Na and Mn respectively;

The rest of the minerals decreased and even though the decrease was significant ($p<0.05$) it was relatively modest compared to that between 6 and 8 WAP. The results indicate that harvesting the vegetables at 4 WAP provides high mineral levels (Ca, Mg, P, Fe, Zn, Cu and Se) and harvesting at older age leads to lower mineral levels.

Table 4.4 Mineral content (mg/g D.M) in cowpea leaves at different maturity stages (4, 6 and 8 weeks old).

Mineral	Maturity		
	4 Weeks	6 Weeks	8 Weeks
	Content (mg/g DM)		
Ca	32.00±0.52[b]	27.23±0.07	8.05±0.27
Mg	8.08±0.17	7.64±0.15	2.59±0.04
Na	0.29±0.09	0.49±0.02	0.31±0.02
K	36.17±0.64	37.77±1.89	11.56±0.44
P	7.16±0.04	6.39±0.19	1.90±0.10
	*10^{-3} mg/g DM[a]		
Zn	128.10±16.00	30.40±14.50	3.44±0.17
Fe	250.00±3.00	115.50±23.50	16.20±2.60
Mn	70.20±14.00	190.50±35.00	36.10±5.80
Cu	5.40±0.80	n.d	n.d
Se	0.29±0.00[c]	0.03±0.00[c]	0.03±0.01

*D.M: Dry Matter *n.d. : not detected (under detection limit of the measurements; Limit of detection=0.01 µg/g DM)

[a]: the content of Zn, Fe, Mn, Cu Na and Se was very low. [b]: means ± standard deviation of triplicate samples (95% confidence interval, n=3 samples from each maturity) [c]: lower than 5 ng/g DM

In literature, it has also been reported that the maturity stage of the vegetables has an impact on the net accumulation of certain minerals in snap bean plants (Pomper and Grusak, 2004) and other higher plants (Marshner, 1986; 1995; Makobo et al., (2011). The trend in the changes of the mineral content of the leaves during maturation was also observed by Makobo et al., (2011) in Amaranth (*Amaranths cruentus* L.) and in chickpeas by Ibrikci et al., (2003).

On average, the cowpea leaves contained more Ca than other leafy vegetables (spinach 1062 mg/100g DM and cabbage 536 mg/100g DM), more P (cabbage 301 mg/100g DM), but less Mg (spinach 850mg/100g DM and cabbage134 mg/100g DM), K (spinach 5951 mg/100g DM and cabbage 1993 mg/100g DM) and P (spinach 531 mg/100g DM) (USDA/ARS, 2011).

4.4 Conclusions and Recommendations

This study indicates that cowpea leaves are a good source of micronutrients. This finding provides scientific evidences that harvest time plays an important role in optimizing the intake of micronutrients. Harvesting at the 6^{th} week after planting is the optimal maturity to obtain high levels of both total folates and minerals however for Zn and Fe, the highest quantities are obtained at 4 WAP. Eating raw vegetables for leaves at 4 WAP gives folates of shorter chains and thus higher bioavailabilty while those of 8 WAP gives a higher total folate content but lower bioavailabilty due to the longer chain. Since cowpea leaves are mostly consumed as cooked/blanched vegetables, combining the optimal leave maturity at harvest time and processing

conditions (95°C/10 mins) would be the best and the most effective “natural” way to optimize the health benefits of cowpea leaves.

There is need to explore processing conditions of pressure/time/temperature combinations on folate stability in the cowpea leaves and also the effect of traditional processing methods like sun drying and or fermentation on the stability of the folates and minerals in the cowpea leaves.

CHAPTER FIVE

5.0. Kinetics of peroxidase inactivation and visual green colour degradation of cowpea (*Vigna unguiculata*) leaves

Abstract

The thermal inactivation of peroxidase (POD) was used to evaluate the adequacy of blanching cowpea leaves, while the thermal stability of the visual green colour (as 'a' value) was evaluated as an indicator for consumer perception of quality degradation of the vegetables. The effect of thermal treatment on POD inactivation in the leaves was studied in the range of 75-100°/120 mins while that of the visual green colour was in the range of 55-80°C/90 mins. The kinetics of the enzyme *in situ* thermal inactivation that of the visual green colour shows a first-order, biphasic and fractional conversion model respectively, however they both follow an Arrhenius model of the temperature sensitivity of the reaction rates. The kinetic parameters k_{ref}, (k at reference temperature, T_{ref}=80°C), and E_a, (at T_{ref}) were estimated at was k_{ref} =11.52 ± 0.95*10^{-2} $min^{-1}$and E_a of 109.67 ± 6.20 kJ/mol for the heat-labile phase while heat-stable isoenzyme fractions had a k_{ref}=0.29± 0.07*10^{-2} min^{-1} and E_a of 256.93 ± 15.27 kJ/mol. The kinetic parameters for the thermal degradation of the visual green colour (k_{ref} and E_a), at a reference temperature (T_{ref}=70°C) were estimated and k_{ref} =13.53 ± 0.01*10^{-2}.min^{-1} and E_a,=88.78 ± 3.21 kJ/mol.

5.1 Introduction

Processing methods like canning of cowpea leaves can go a long way in preserving and value-addition of the leaves. These thermal processes are normally preceded by other important unit operations like blanching. Blanching of the vegetables is one of the most common methods of preparing cowpea (*Vigna unguiculata*) leaves before consumption. Blanching inactivates enzymes and also helps to soften the vegetable tissues leading to an improvement in the palatability and digestion of the vegetables and product quality retention during subsequent storage procedures including freezing (Canet, 1989). The benefits of blanching have been widely documented (Kleinschmidt, 1971; Shams and Thompson, 1987; Canet, 1989; Préstamo et al., 1998; Reyes De Corcuera, et al., 2004); however, this thermal treatment has some adverse effects, especially on the quality attributes of the vegetables. On the other hand, an improper blanching process can lead to incomplete enzyme inactivation which may result in quality changes, such as texture, colour, flavour and nutritional losses, during storage.

Thus, it is important that a blanching process results in a complete inactivation of undesirable enzymes while having little effect on the quality properties of the vegetables. In order to achieve this, a blanching indicator can be used. In this context, one of the most widely used indicators is the enzyme peroxidase (POD, E.C.1.11.1.7). It is one of the most widely distributed enzymes in vegetables and due to its presence in most plant tissues, its high thermal stability and its ease of assay it is used to monitor and evaluate the effectiveness of blanching (Gonçalves et al., 2007). POD inactivation is mostly used as a blanching index, that is, if POD is

inactivated, it is a reasonable assumption that other quality-related enzymes have also been inactivated.

The specific role of POD on quality impact during food storage is not yet clear, and this is mainly due to its (POD) high number of isoenzymes and also the fact that the quantity and type of these isoenzymes change at different stages of plant growth (Garrote et al., 1987; Halpin and Lee, 1987; Aparicio-Cuesta et al., 1992). Furthermore, plant PODs show specific affinity for different substrates (Siegel and Siegel, 1970; Mustapha and Selselet-Attou, 2007) and their activity changes in response to a wide range of environmental conditions (Gaspar et al., 1982; Mustapha and Selselet-Attou, 2007). However, there is an empirical relationship between the prevention of off flavour development in frozen vegetables and inactivation of POD (Guyer and Holmquist, 1954; Burnette, 1977; Williams et al., 1986; Lopez and Burges, 1995; Mustapha and Selselet-Attou, 2007). Vegetable POD, like many other vegetable enzymes, is located in the soluble form in the cell cytoplasm, and in the insoluble form being ionically or covalently bound to the cell wall (Gkinis and Fennema 1978; McLellan and Robinson 1981; Vamós-Vigyázó 1981, Morales-Blancas et al., 2002).

Because POD is more than the effective food spoilage related enzymes, its complete inactivation by thermal processing may lead to an excessive heat treatment of the product and cause other quality problems, such as pigment modifications, tissues softening and nutritional losses (Howard et al., 1999; Murcia et al., 1999; Oboh, 2005; Gonçalves et al., 2007). Thus, an optimized thermal process, in terms of a balance between thermal inactivation of enzymes and degradation of quality

attributes of the vegetables is of importance. This can be achieved by monitoring changes of some quality parameters, such as colour in the case of cowpea leaves, of the vegetables during thermal processing.

Colour is a primary consumer-perceived product characteristic and plays an important role in food. Furthermore, the degree of greenness, attributed to chlorophyll pigments, is important in determining the final quality of thermally processed green vegetables and is often expected to be close to the raw ones (MacDougall, 2002). Thermally processed green vegetables exhibit poor green-colour quality as compared to the fresh ones, due to chlorophyll degradation. Chlorophyll degradation can be due to the action of chlorophyllase or tissue damage (Heaton and Marangoni, 1996; McFeeters, 2002). The chlorophyll degradation pathway entails the replacement of magnesium in the centre of the chlorophyllic group by hydrogen ions to form pheophytin and further formation of the degradation products such as pheophorbides and chlorins (White et al., 1963; Schwartz and Von Elbe, 1983; Canjura et al., 1991; Schwartz and Lorenzo, 1991; Heaton and Marangoni, 1996; Van Boekel, 1999, 2000). Pigment degradation can be related to physical colour measurements (Bao and Chang, 1994; Martins and Silva, 2002; Muftugil, 1986; Sims et al., 1993; McFeeters, 2002).

The study of the kinetics of the thermal inactivation of POD (to represent the effectiveness of the blanching procedure) and the kinetics of the thermal degradation of the green colour (to represent the consumer perception of the degree of adverseness of the blanching procedure) forms a framework for optimizing the blanching process of cowpea leaves. The commonly used kinetic models and

parameter estimates, for POD inactivation and colour degradation for a number of fruits and vegetables have been reviewed by Gonçalves et al., in 2007. In summary they are described by zero or first-order kinetics. However, when there are different heat-resistant fractions available (heat labile and heat stable fractions), a biphasic first-order model is used to describe their thermal inactivation kinetics (Ling and Lund, 1978).

The objective of this study was to evaluate the kinetics of cowpea leaves POD inactivation and geen colour changes during blanching. To the best of our knowledge, this information is not available in open literature.

5.2 Materials and Methods

5.2.1 Growing of the cowpea leaves and sample preparation after harvest

Cowpea (*V. unguiculata*) vegetables were grown under conditions described in **Section 4.2.1**. After harvesting (6 WAP) the leaves were immediately packed in a cooler box so as to aid in slowing down the respiration and transpiration during transport from the field to the research centre. On arrival at the laboratory, the leaves were rinsed under flowing tap water, and the excess water on the leaves was removed by drabbing them in pieces of dry cotton cloth. Then they were chopped into pieces and afterwards packed (approximately 10g/pouch) in plastic pouches (140 x 200 x 40 mm, DaklaPack United Kingdom, Chiswick, London) followed by vacuum-sealing at 34 millibars (Multivac C200 Vacuum Chamber, Sepp Haggenmueller GmbH and Co, Wolfertschwenden, Germany) to minimize oxidative reactions.

The packed fresh leaves were divided into two batches of samples, whereby one batch was subjected to the study on colour degradation after thermal treatment while another batch was frozen in liquid nitrogen and stored at -80^{o}C for later thermal treatment, extraction and measurement of POD.

5.2.2 *In situ* thermal stability of POD in cowpea leaves

5.2.2.1 Thermal treatment

The frozen samples were thawed at 25°C in a thermo-static water bath (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany). After thawing, the plastic pouches were heated for 10 minutes at a predefined temperature from 30 up to 100°C in a thermo-static water bath (Memmert Water Bath WBU 45, Memmert GmbH + Co. KG, Schwabach, Germany). Pouches containing leaves that were not subjected to any thermal treatment after thawing were used to assess the initial enzyme activity (A_0). After the heat treatment, the leaves were cooled in an ice water bath for 20 minutes to a temperature of just about 0°C, and the residual POD activity was determined.

5.2.2.2 Kinetics of thermal inactivation of POD

Plastic pouches each filled with 10g of frozen leaves that had been harvested at 6 weeks were thawed at 25°C in a thermostatic water bath (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany). The samples were heated at predefined temperatures between 75-85°C for different time intervals in a thermo-static water bath and from 90°C-100°C for different time intervals in a thermo-static oil bath (Memmert oilr bath model 1, Memmert GmbH + Co. KG, Schwabach, Germany). It took approximately 2.10 minutes for the samples to heat up (non

isothermal condition). Leaves that were not subjected to any thermal treatments after thawing were used as blank (initial enzyme activity, A_0). After heat treatment, the plastic pouches containing the samples were cooled in an ice water bath (it took approximately 10 minutes to reach the ice water bath temperature of just above 0°C) for 20 minutes and then subjected to POD extraction.

5.2.2.3 Extraction of POD

The procedure of POD extraction was optimized, Bergmeyer, 1974, (in terms of the dilution ratio of enzyme extract to buffer and the mode of extraction i.e. magic bullet blender, J-26, (Ningbo Vanguard Import and Export Co, Ningbo, China), and a grinder (Grindomix, GM 200, Germany).

Plastic pouches containing (approximately 10g) samples after thermal treatment (**Section 5.2.2.2** above) and blank samples were opened and the contents emptied in a blender (magic bullet blender, J-26, Ningbo Vanguard Import and Export Co, Ningbo, China) and then mixed with sodium phosphate buffer (0.1M, pH 6) containing 1M NaCl and 1% polyvinylpyrrolidone (PVPP). This mixture was mixed for 20 seconds and then the extract was filtered through a 1mm² sieve with aid of a vacuum pump. The filtrate was then centrifuged at 17 700g at 4°C for 30 minutes. The filtrate was discarded and the supernatant diluted five times in sodium phosphate (0.1M, pH 6.5). This crude extract solution was afterwards frozen in liquid nitrogen and stored at -80°C until POD assay.

5.2.2.4 POD activity

The supernatant obtained from the extraction procedure above was thawed in a water bath at 25°C (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany) and subjected to a POD assay. The measurement of POD activity was done spectrophotometrically as described by Bergmeyer (1974), with slight modifications, with 1% (v/v) guaiacol as a substrate and 0.1% (v/v) H_2O_2 as a co-substrate, and monitored the rate of change in absorbance, A_{470} nm in a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden) kitted with portable PC and SWIFT II software (Amersham Biosciences, Uppsala, Sweden).

The reaction mixture contained 20 µL of enzyme extract, 125 µL H_2O_2 (0.1%) and 1075 µL of 1% guaiacol. The increase in absorbance was followed for 5 minutes. The blank sample included 100µL sodium phosphate buffer (0.1M, pH 6.5), 125 µL H_2O_2 (0.1%) and 1075 µL guaiacol (1%). The assay was done at 25°C in a 3 liter re-circulating bath, Haake C10-B, (Karlsruhe, Germany).

POD activity is defined as the amount of enzyme which oxidizes 1µmol guaiacol per minute at pH 7 and 25°C. The specific activity was expressed as units (U) of activity per gram of dry matter of the cowpea leaves.

$$\mathrm{POD}\left(\frac{\mathrm{U}}{\mathrm{ml}}\right)=\frac{\mathrm{A}\times\mathrm{c.f}\ \times\mathrm{d.f}\times\mathrm{V_e}\times\mathrm{V_t}}{\mathrm{l}\times\varepsilon\times\mathrm{t}\times\mathrm{V_s}} \qquad ..(5.1)$$

And

$$\text{POD}\left(\frac{\text{U}}{\text{g dry matter}}\right)=\text{POD}\left(\frac{\text{U}}{\text{ml}}\right)\times 1.689 \quad ..(5.2)$$

Whereby

$\frac{A}{t}$ is the absorbance per minute.

Whereby, c.f is the conversion factor from guaiacol to tetraguaiacol (4), d.f is the dilution factor of the extract prior to measuring in the spectrophotometer (5), V_e is the volume of the extraction buffer (20ml), ε is the micro-molar extinction co-efficient of tetraguaiacol, (26.6/(mM.cm)), ℓ is the light path (1cm), V_t is the total reaction volume and V_s is the sample volume (Bergmeyer, 1974).

5.2.3 Data analysis to calculate kinetic parameters for POD thermal inactivation

Due to the presence of POD isoenzymes with different thermo-stability (most often two fractions, i.e. heat-labile and heat-stable fraction) in which each isoenzyme is inactivated in parallel according to first order reaction, the inactivation can be described using Eq. 5.3

$$A = A_L + A_S \quad ..(5.3)$$

In case of a first order kinetic, at constant temperature (rate constants being independent of time) Eq. 5.3 becomes Eq. 5.4 (Ludikhuyze, 2003; Chen and Wu, 1998).

$$A_L + A_S = A_{L0}\exp(-k_L t) + A_{S0}\exp(-k_S t) \quad ..(5.4)$$

The temperature dependence of the rate constant is normally described by an Arrhenius behaviour

$$k = k_{ref} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad ..(5.5)$$

D values, the time required in reducing the POD content by 90%, and z values, the temperature required for a 90% decrease in D value (Ariahu and Ogunsua, 2000), were estimated as follows

$$A_L + A_S = A_L * 10^{(-t/D_L)} + A_S * 10^{(-t/D_S)} \quad ..(5.6)$$

$$A_L + A_S = A_L * 10^{\left(\frac{-t}{D_{refL} * 10^{((T_{ref}-T)/Z_L)}}\right)} + A_L * 10^{\left(\frac{-t}{D_{refS} * 10^{((T_{ref}-T)/Z_S)}}\right)} \quad ....(5.7)$$

Where A is the total residual, experimentally measured, POD activity (in units), A_L is the residual enzyme activity (in units) of the thermolabile fraction while A_S is the residual enzyme activity (in units) of the thermostable fraction after heat treatment for time (t) in minutes at a given temperature, A_{L0} and A_{S0} is the initial enzyme activity at t=0 for the thermolabile and thermostable fractions of the enzyme respectively, k is the inactivation rate constant (min^{-1}) whereby for thermolabile phase k= k_L and for the thermostable phase k=k_S, k_{ref} the inactivation rate constant (in min^{-1}) at reference temperature, E_a is activation energy (in kJ/mol), R is the ideal gas constant (8.314 J/mol.K), T is the actual treatment temperature (in K), and T_{ref} is the reference temperature (in K, in this study T_{ref} = 80°C=353K).

The k values (k_L and k_S) were estimated based on non-linear regression analysis (Eq.5.4). The E_a value was estimated by integrating the kinetic models considered (Eq.5.4) with Arrhenius equation (Eq.5.5) i.e. substituting k_L and k_S in Eq.5.4 with

Eq. 5.5 (Arabshahi and Lund, 1985; Lund, 1983). The reference temperature used was the average value of the range considered (i.e. T_{ref} = 80°C), aiming at improving parameter estimation.

D_{refL} and D_{refS} is the D value at temperature T_{ref} (80°C) for the thermolabile and the thermostable enzyme fractions respectively. The D values (D_L and D_S for the thermolabile and the thermostable enzyme fractions respectively) were estimated based on non-linear regression analysis (Eq.5.6) while the Z values (Z_L and Z_S for the thermolabile and the thermostable enzyme fractions respectively) were estimated based on non-linear regression analysis (Eq. 5.7).

Parameters' precision was evaluated based on confidence intervals at 95%, and the quality of the regression was assessed by the coefficient of determination (r^2), and randomness and normality of residuals (Hill and Grieger-Block, 1980), thus allowing best model selection. SAS 9.1 software (SAS Institute Inc., Cary, N.C., U.S.A.) was used for all regression analysis procedures (using least squares estimation and Levenverg–Marquart method, for minimizing the sum of squares of the deviations between experimental values and the ones predicted by the mathematical model).

5.2.4 Moisture content

The moisture content of the leaves was determined by the A.O.A.C method (2002).

5.2.5 Thermal stability of the visual green colour

5.2.5.1 Thermal treatment

Approximately, 10g of fresh leaves, vacuum packed in plastic pouches (from **Section 5.2.1** above) were used for the determination of the thermal stability of green colour in cowpea leaves. These leaves, in their plastic pouches, were heated for 10 minutes at a predefined temperature from 30 up to 85°C in a thermo-static water bath (Memmert water bath WBU 45, Memmert GmbH + Co. KG, Schwabach, Germany). Samples that were not subjected to any thermal treatment were used to assess the initial green colour (C_0). After the heat treatment, the leaves were cooled in an ice water bath for 20 minutes and the residual green colour was measured.

5.2.5.2 Kinetics of thermal degradation of green colour

Plastic pouches each filled with 10g of fresh cowpea leaves that had been harvested at 6 weeks were thawed at 25°C in a thermostatic water bath (Memmert water bath WB 22, Memmert GmbH + Co. KG, Schwabach, Germany). The samples were heated at predefined temperatures between 55-65°C for different time intervals in a thermo-stated water bath. Leaves that were not subjected to any thermal treatment were used to assess the initial green colour (C_0). After heat treatment, the leaves were cooled in an ice water bath for 20 minutes to a temperature of just about 0°C (to the equilibrium temperature with ice water), and then subjected to colour measurement.

5.2.6 Colour properties

The colour of the leaves samples was measured using a handheld tristimulus colourimeter (Minolta Chroma Meter CR-300, Osaka, Japan) and a CIE standard illuminant C to determine CIE colour space co-ordinates, L*a*b* values (Francis and

Clydesdale, 1975). The L* value (lightness value), indicates how dark/light the sample is (varying from 0 for black to 100 for white), a* value is a measure of greenness/redness (varying from -60 to +60), and b* value is a measure of blueness/yellowness (also varying from -60 to +60). The colourimeter was calibrated against a standard white reference tile. The colour measurements were performed on the samples in the plastic pouches in which they had been previously packed.

5.2.7 Data analysis to calculate kinetic parameters for colour thermal degradation

A fractional conversion model was used to describe the thermal degradation of visual green colour in the leaves. This model represents a first order process reaching a final stable color value after prolonged heating (Arabshahi and Lund, 1985; Lund, 1983).

$$C = C_\infty + (C_0 - C_\infty)\exp(-k_t * t) \quad ..(5.8)$$

Where C_0 and C_∞ refer to the initial colour measurements and to the residual colour measurements respectively (after reaching the degradation plateau).

The temperature dependence of the rate constant is normally described by an Arrhenius behaviour (Arabshahi and Lund, 1985; Lund, 1983).

$$k_t = k_{ref} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad ..(5.9)$$

Where C is the measured colour (in units), after heat treatment of time (t) in minutes at a given temperature. C_0 refers to the initial measured colour (before heat treatment, at t=0) and C_∞ is the measured colour after reaching a colour loss (degradation)

plateau while k_t is the inactivation rate constant (min^{-1}). k_{ref} is the inactivation rate constant (in min^{-1}) at reference temperature, E_a is activation energy (in kJ/mol), R is the ideal gas constant (8.314 J/mol.K), T is the actual treatment temperature (in K), and T_{ref} is the reference temperature (in K, in this study T_{ref} = 70°C).

The k values were estimated based on non-linear regression analysis (Eq.5.8). The E_a value was estimated by integrating the kinetic models considered (Eq.5.8) with Arrhenius equation (Eq.5.9) i.e. substituting k_t in Eq.5.8 with Eq. 5.9 (Arabshahi and Lund, 1985; Lund, 1983). The reference temperature used was the average value of the range considered (i.e. T_{ref} = 70°C), aiming at improving parameter estimation.

Parameters' precision was evaluated based on confidence intervals at 95%, and the quality of the regression was assessed by the coefficient of determination (r^2), and randomness and normality of residuals (Hill and Grieger-Block, 1980), thus allowing best model selection. SAS 9.1 software (SAS Institute Inc., Cary, N.C., U.S.A.) was used for all regression analysis procedures (using least squares estimation and Levenverg–Marquart method, for minimizing the sum of squares of the deviations between experimental values and the ones predicted by the mathematical model).

5.3 Results and Discussion

5.3.1. Extraction of POD

The total POD enzyme extraction yield (units/g dry weight) was recorded at 2793.71±332.59 and the moisture content (% wet basis) was 81.8±0.4. This total POD activity represents the sum of the free and bound enzyme since sodium chloride was used in the extraction medium to release the POD fraction ionically bound to the cell wall (Mieslel et al., 1991). The high quantities of POD in the vegetable will facilitate thermal inactivation studies since the large quantities of POD means that there will be measurable POD levels over more than one log-unit of inactivation allowing reliable parameter estimation. In addition, POD extraction, as for many other enzymes, forms an important part in subsequent enzyme inactivation studies. An optimal enzyme extraction procedure is crucial in determination of the thermal stability of POD (as expressed in its thermal inactivation kinetic parameters).

5.3.2 *In situ* thermal stability of POD

5.3.2.1 Thermal stability of POD

As indicated earlier, POD is known to exist of a large number of isoenzymes, often leading to a heat-labile and heat-stable fraction of the enzyme. A thermal stability screening study is crucial in determining the temperature range of inactivation and revealing whether the POD in the leaves exhibits a monophasic or biphasic behaviour. This, in turn, is important in determining the design of kinetic experiment for studying the thermal inactivation of POD in the leaves.

The results on the thermal stability screening of POD after ten minutes of thermal treatments are indicated in **Figure 5.1**. Here, the residual enzyme activities, presented as ratio of measured enzyme activity to initial enzyme activity, as a function of heating temperature were plotted, obtaining a nonlinear curve for the POD extract. In general, the plot showed that POD is thermally stable up to around 65°C. However, POD was inactivated at temperatures >65°C where a fraction of it was inactivated between 65 and 80°C and a second fraction was inactivated between 85 and 100°C. The first fraction is characterized by a steeper slope indicating a higher rate of inactivation when compared with the second fraction. These results were important in designing the kinetic experiment to study the thermal inactivation of POD in cowpea leaves, that is, setting of experiments that allow the simultaneous estimation of kinetic parameters of the two separate POD fractions.

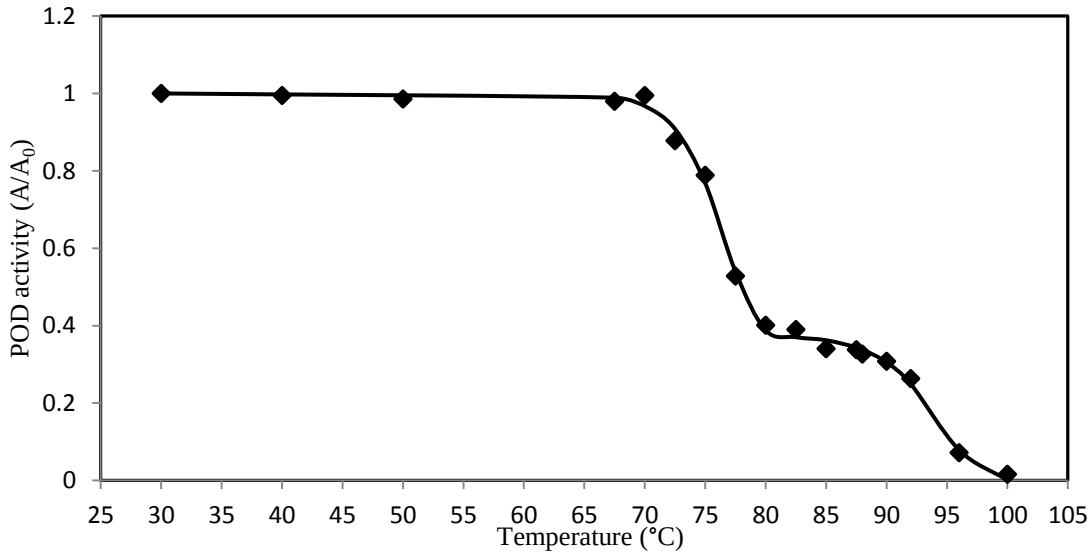


Figure 5.1 Residual activity of peroxidase (POD) as a function of temperature for 6-week old cowpea leaves after a thermal treatment of 10 minutes and subsequent extraction of POD using a blender (magic bullet).

5.3.2.2 Thermal inactivation kinetics of POD

Starting from the POD thermal stability screening results, the determination of the thermal inactivation kinetics of POD in the leaves was carried out in a temperature range of 75-100°C for a time of up to 120 minutes depending on the experimental temperature. Experimental data are presented in **(Figure 5.2)**.

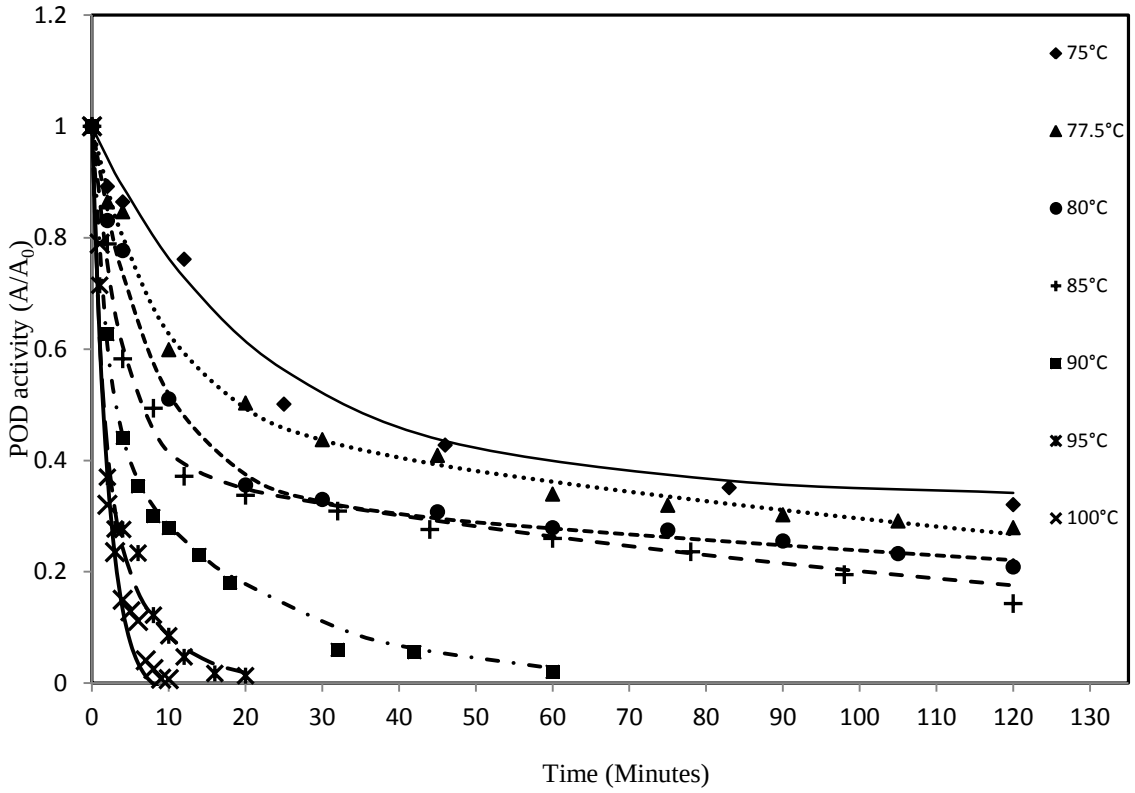


Figure 5.2 Residual activity of peroxidase (POD) in 6-week cowpea leaves as a function of time at different temperatures. Symbols represent experimental values, lines correspond to individual fittings of the first order kinetic model to each temperature with an Arrhenius temperature dependence using nonlinear regression analysis.

The figure shows two sets of distinct curves; those above 85°C and those below 85°C. The curves also showed an initial steep straight line, an intermediate curved portion and a final straight line with a shallow slope. These results corroborate those obtained for the thermal stability of POD, above. At a given temperature, the inactivation results can be described with the biphasic (2-fraction model; Weng et al., 1991; Saraiva et al., 1996; Rodrigo et al., 1997).) first-order model proposed by Ling and Lund (1978) based on the presence of two isoenzyme groups with distinct thermal stabilities, a heat-labile fraction (initial steep straight line, **(Figure 5.2)**, that inactivates rapidly and a heat-stable fraction which cannot be inactivated completely (a final straight line with a shallow slope, **(Figure 5.2)**.

5.3.2.3 Kinetic parameters

Thermal inactivation kinetic parameters were obtained as described in the materials and methods whereby the rate constants were obtained from Eq.5.4 while the kref, E_a and the amounts of the contributing fractions (heat labile and heat stable fractions) were obtained from Eq. 5.5. The results are given in **Table 5.1**. In general, k values increase with increasing temperature (**Table 5.1**) and those for heat-labile fractions (k_L) are several times (from 5 to 51) higher than those for heat-stable (k_S) fraction (**Table 5.2**). This explains the higher decrease in enzyme activity during the steep slope section of the graphic presentation of the data (**Figure 5.2**) of the thermal treatments.

The temperature-dependence of the inactivation rate constant (k) is explained with the concept of activation energy (E_a). Calculated E_a values for cowpea leaves POD

are shown in **Table 5.1**. The activation energy of the heat-stable fractions was almost double than that of the heat-labile fractions. This can be attributed to the fact the two different isoenzymes (proteins) posses' different thermal stability with the most common mechanism of inactivation being denaturing of proteins and to some extend loss of heme POD (Anthon and Barrett, 2002; Tamura and Morita, 1975; Lu and Whitaker, 1974). The higher activation energy of the heat-stable fractions than in the heat-labile fractions phenomena was also observed by Günes and Bayindirh (1993) whereby POD in peas (4.1 x 104 J/mol against 7.5 x 104 J/mol), green beans (5.7 x 104 J/mol against 7.7 x 104 J/mol), and carrots (5.2 x 104 J/mol against 5.7 x 104 J/mol) was lower in the heat-labile fraction that the heat-stable fraction.

The calculated E_a values for POD in the leaves were higher than those reported for broccoli (florets), green asparagus (tip), green asparagus (stem), carrot (cortex), and carrot (core) (Morales-Blancas et al., 2002) where the ratios ranged from 61-97 kJ/mol for the heat-labile fraction and 43-83 kJ/mol for the heat-stable fractions respectively, however, the cowpea leaves POD compared well with those recorded by Gonçalves et al., 2009 (159 kJ/mol) for experiments that were performed in situ on broccoli samples. These experimental results obtained for POD in cowpea leaves are, satisfactorily described by a first-order kinetic model (biphasic), for all temperatures tested **(Figure 5.3(a))** where the quality of the model fit was assessed by analyses of a scatter plot between measured and predicted values of A/A_0 and their corresponding values of R^2. On the other hand the Arrhenius model described the temperature dependence **(Figure 3(b))** where the quality of the model fit was indicated by the R^2 generated by the Arrhenius plot (1/T (K^{-1}) vs ln(k) both for the

heat labile and heat stable fraction. Having established that cowpea leaves POD has both the heat-labile and heat-stable isoenzymes and keeping in mind the fact that the amount of residual activity plays a big role in the vegetable shelf life, it is important to determine the amount of heat-labile versus heat-stable fraction of the enzyme in the vegetable. The results are shown in **Table 5.1**, where approximately 64% of the enzyme is in the heat-labile fraction while 36% is heat-stable. These results are similar to those obtained by Morales-Blancas et al., (2002) for the carrot core section (35.1 ± 6.1%). A higher ratio of heat-labile/heat-stable enzyme gives an indication of a better shelf life of the product after thermal treatment since the enzyme will behave like a monophasic enzyme while a smaller ratio of heat-labile/heat-stable enzyme means that one needs a high time/temperature thermal treatment of the vegetables in order to achieve a 90% reduction in the enzyme activity, and in turn this means that other quality and nutritional properties (like visual green colour) of the vegetables could be compromised.

Residual enzyme activity is also dependent on the initial enzyme load since a low percentage of residual enzyme activity could still represent a significant enzyme activity if the initial one was high enough.

Table 5.1 Estimated kinetic parameters and corresponding confidence intervals at 95% for thermal inactivation of peroxidase (POD) in cowpea leaves harvested at 6 weeks old.

Temperature (°C)	Parameter Estimate			
	Heat-labile fraction		Heat-stable fraction	
	k(*10^{-2} min^{-1})	D(min)	k(*10^{-2} min^{-1})	D(min)
75	4.67±1.24	49.33±13.13	0.09 ±0.003	2610.90±89.97
77.5	11.41 ± 1.27	20.19±2.24	0.50 ± 0.07	459.20±61.96
80	12.64 ± 0.92	18.21±1.33	0.38± 0.07	605.70±11.20
85	25.09 ± 2.47	9.18±0.90	0.68 ± 0.09	340.80±45.18
90	50.53 ± 6.60	4.56±0.59	4.72 ± 0.52	48.74±5.40
95	75.38 ± 23.9	3.06±0.97	15.30 ± 0.05	15.05±4.92
100	N.D	N.D	45.84 ± 10.45	5.69±0.81
T_{ref} = 80°C	**k_{ref} (*10^{-2} min^{-1})** 11.52 ± 0.95 **E_a (kJ/mol)** 109.67 ± 6.20	**D_{ref} (min)** 19.63±1.71 **Z value (°C)** 22.75±1.34	**k_{ref} (*10^{-2} min^{-1})** 0.29± 0.07 **E_a (kJ/mol)** 256.93 ± 15.27	**D_{ref} (min)** 687.4±148.2 **Z value (°C)** 10.03±0.59
	Fraction of enzyme in each phase			
	0.63±0.099		0.38±0.099	

*N.D= not detected. Values are means ± standard deviation at 95% confidence interval

Table 5.2 Ratio of the estimated kinetic parameters (k_L/k_S) for thermal inactivation of POD in cowpea leaves harvested at 6 weeks old.

Temperature (°C)	Ratio of k_L/k_S
75	51.9
77.5	22.8
80	33.3
85	36.9
90	10.7
95	4.9
100	N.D

*N.D= not detected.

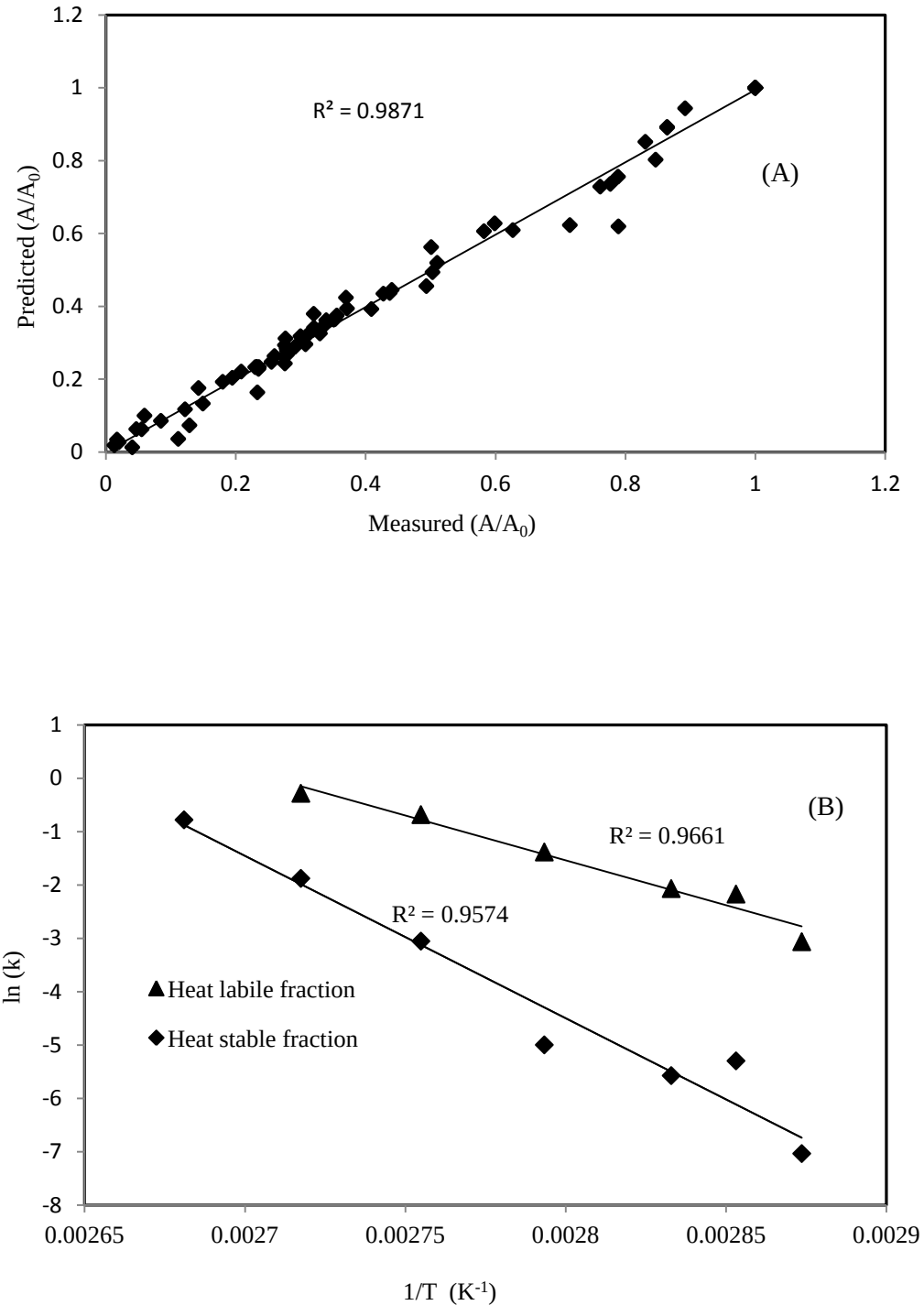


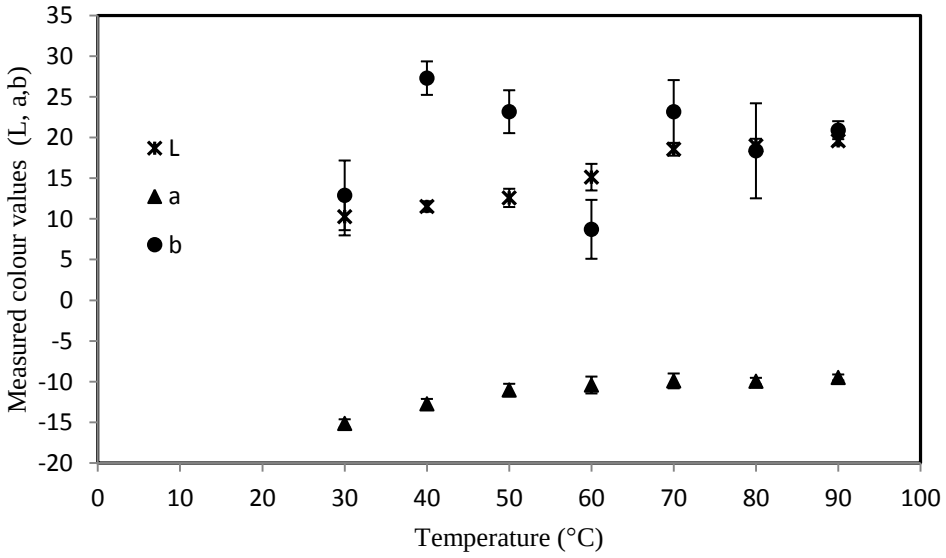
Figure 5.3 Scatter plot of predicted versus measured residual activity of peroxidase (POD) activity in 6-week cowpea leaves (a), and Arrhenius plot for thermal inactivation of the heat-labile and heat-stable fraction of POD in cow-pea leaves (b).

5.3.3 Visual green colour

5.3.3.1 Effect of temperature on visual green colour of cowpea leaves

Figure 5.4 shows the effect of temperature on the visual green colour expressed as Hunter L*a*b* values of the leaves. There is a consistent decrease in L* and '-a*' values with an increase in the treatment time and temperature. However, there was no consistent change in b* values.

This phenomenon was also observed by Nisha et al., (2004) in thermal treatments on spinach. The change in L* and b* values may be due to pheophytin–pyripheophytin conversion or due to degradation/ reaction of other components present in the vegetables (Weemas et al., 1999).



*Initial **L*a*b*** values are10.55 ± 1.5, -16.21 ± 0.54 and 12.72 ± 4.49 respectively. Symbols represent experimental values (Values are means ± standard deviation of triplicate samples, 95% confidence interval).

Figure 5.4 Changes in the colour of cowpea leaves (harvested at 6 weeks old), measured as colour values L*a*b* as a function of temperature after a thermal treatment of 10 minutes. Symbols represent experimental values. The error bars are standard errors at 95% confidence interval. *Initial L*a*b* values are10.55 ± 1.5, -16.21 ± 0.54 and 12.72 ± 4.49 respectively.

Thus, since the greenness is indicated by '-a*', and due to the fact that the green is the dominant colour for visual quality attributes of vegetables, the kinetics studies were carried out only with respect to '- a*' values.

Figure 5.4 also indicates a rapid loss of green colour between 30°C and 70°C and from 70°C the '-a*' values reach a plateau phase and flatten out. This indicates a biphasic model of the fractional conversion type as opposed to POD which indicated a further POD inactivation after the plateau (or interphase or flattening out), a characteristic of the biphasic model of the consecutive step type.

5.3.3.2 Degradation kinetics of visual green colour

Using nonlinear regression, the visual green colour degradation data were analyzed using Eq. 7.7 and 7.8 to determine the overall order and rate constant for the degradation reaction. The degradation rate constants are shown in **Table 5.3**, indicating a general increasing trend of the k values with increasing temperature, as corroborated by **Figure 5.5**.

Table 5.3 also shows that there is an almost even distribution between the thermally degradable visual green colour (C_0- C_∞) and the thermally non-degradable green colour (C_∞). This means that the green colour of the vegetables will rapidly deteriorate in the first few minutes of cooking and afterwards the colour will remain stable for much of the entire cooking time. This loss of green colour in the leaves could be explained by the pheophytinisation pathway, that is, the displacement of the magnesium in the cholorophyll (from the porphyrin ring), leading to the formation of a dull olive brown called pheophytin (Kidmose et al., 2002). The action of cholorophylaase can be disounted in these experiments since the leaves were not

crushed prior to the thermal treatments and subsequent measurements. Cholorophyllase, like many enzymes is compartmentalized in the cell structure and it only comes in contact with the substrate (cholorphyll) if there is cell disruption.

Table 5.3 Estimated kinetic parameters and corresponding confidence intervals at 95% for thermal degradation of green colour (measured as '-a*' values) in cowpea leaves harvested at 6 weeks old.

Temperature (°C)	Parameter Estimate k($*10^{-2}$ min^{-1})
55	1.76±0.01
60	6.00±0.01
65	7.83±0.02
70	11.4±0.02
75	19.4±0.03
80	29.67±0.05
T_{ref} = 70°C	**k_{ref} ($*10^{-2}$ min^{-1})** 13.53 ± 0.01
	E_a (kJ/mol) 88.78 ± 3.21
	C_0- C_∞ = 0.536; **C_∞** = 0.464

***C_0- C_∞** = Proportion of 'degradable' green colour; ***C_∞** = Proportion of 'non-degradable' green colour. Values are means ± standard deviation of triplicate samples (95% confidence interval)

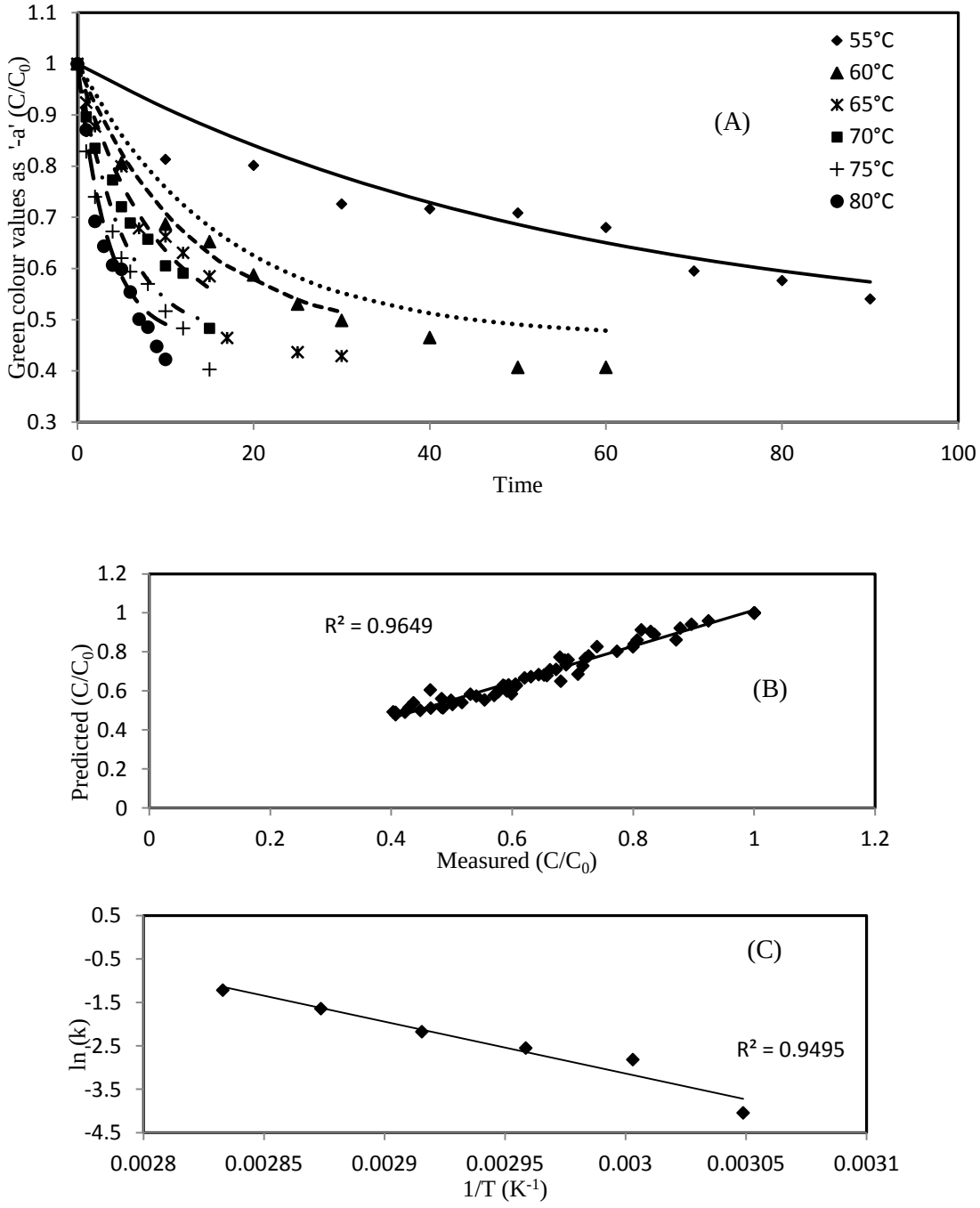


Figure 5.5 (a) Residual green colour, (measured as '-a*' values) in 6-week cowpea leaves, as a function of time at different temperatures. Symbols represent experimental values, lines correspond to individual fittings of the first order kinetic model to each temperature with an Arrhenius temperature dependence using nonlinear regression analysis **(b)** Linearized scatter plot of predicted versus residual green colour (measured as '-a*' values) in 6-week cowpea leaves, **(c)** Arrhenius plot between natural logarithm of reaction rate constant versus 1/T for thermal degradation of green colour (measured as '-a*' values) in cowpea leaves.

The results in **Table 5.3** and **Figure 5.5** show that the thermal degradation of the visual green colour in vegetables is the first order kind with an Arrhenius temperature dependence.

The activation energy, E_a, for visual green colour in the leaves (88.78 kJ/mol) was higher than that in spinach puree, approximately 37.57 kJ/mol (Nisha et al., (2004), and E_a of 28.55, 41.15 and 34.01 kJ/mol for spinach puree, mustard leaves and a mixed puree, respectively (Ahmed et al., 2002).

Ahmed et al., (2000) reported an E_a of 11.34–15.98 kJ/mol for colour degradation in green chilli puree while Weemas et al., (1999) reported an E_a of 72.01 kJ/mol for heated broccoli juice. These variations may be attributed to the differences in the raw material due to variations in levels of pH, salt, minerals and the general matrix structure that can affect the green colour stability and the temperature ranges used in these studies.

5.3.4 Thermal inactivation of POD versus retention of visual green colour

The impact of a thermal process on quality attributes of the vegetables can be gauged by monitoring a parameter that represents the 'cooked quality' of the processed food product, and in this case the cooked value can be represented by the visual green colour (Tucker and Featherstone, 2010). In order to optimize product quality, it is desirable to keep this cooked value as low as possible, while assuring the required time needed to inactivate the undesirable micro organisms and enzymes as indicated by the inactivation of POD.

If we assume an average cooking temperature of around 90°C for most cooking, then it takes approximately 4.6 and 48.8 minutes to inactivate 90% of the heat-labile and heat-stable POD, respectively (D-value) and on the other hand it takes 3.06 minutes to degrade 90% of the visual green colour of the vegetables (Eq. 5.6 and 5.7). For a good cooking process, a 50% green colour retention is normally targeted, and in the experiments this would take around 1 minute (D value). These results show that colour degradation in the leaves is far much faster than the POD inactivation and, therefore, other methods of stabilizing the green colour (pH, use of salts, Tijskens et al., 2001; Nisha et al., 2004) need to be used while inactivating POD. It also shows that colour degradation during thermal processing can be used to adequately describe the cook value of the leaves.

5.4 Conclusions and Recommendations

Experimental results indicate that the biphasic first order (consecutive step) model provides an adequate description for the nonlinear thermal inactivation curves of total POD at 75 to 100°C while the biphasic first order (fractional conversion) model provides an adequate description for the nonlinear thermal degradation of the visual green colour in cowpea leaves. For successful predictions of the residual enzyme activities and retention of the green colour after cooking the vegetables, it is necessary to know the kinetic parameters (k and E_a), the total initial enzyme activities and proportion of the heat-labile and heat-stable isoenzyme fractions and the enzyme distribution in plant tissue. The Arrhenius model describes the temperature dependence of the reaction rate constant of all the thermal inactivation of POD and the thermal degradation of the green colour. These results together with a

heat penetration model could be used to optimize the blanching process in vegetables, reducing the process time and, thus, minimizing the loss of nutritional and sensory properties especially in a canning process. This canning process can be an important step in value addition to cowpea leaves hence and also in improving the shelf life of the leaves.

Given the widespread use of POD as an indicator enzyme and visual green colour as a quality indicator, there is a need to carry out POD thermodynamic investigation as well as further characterization of POD and visual green colour inactivation kinetics under a variety of conditions e.g. pH, varying salt concentration.

CHAPTER SIX

6.0. Nutrient stability during cooking of selected African leafy vegetables (*Amaranthus hybridus, Solanum nigrum* and *Cleome gynandra*) consumed in Kenya

Abstract

The African leafy vegetables (ALVs) *Amaranthus hybridus*, *Solanum* and *Cleome gynandra* were purchased from Thika municipal market, in Thika district of central Kenya and after multi stage sampling, samples were used to quantify their nutrient content using standard A.O.A.C methods. The content of moisture, AA, total carotenoids, crude protein, total fibre, ash, and crude fat in the raw leaves per g dry matter (D.M) ranges from 81.0-89.9 mg/g, 5.6-7.3 mg/ g, 15.8-23.5mg/g, 9.2-12.4g/g, 6.8-11.2 mg/g, 14.6-17.7 mg/g and 2.3-8.3, respectively. The vegetables also exhibit high levels of phytates ranging between 0.42-0.89 mg/g D.M. The molar ratio of phytates to the different minerals was also calculated so as to gauge the bio-availability of these minerals on consumption. There is a degradation of the total AA during thermal processing. A two step linear method can be used to evaluate the time dependence parameters (k values) and the temperature dependant of k values (E_a value) for the thermal degradation of AA in these vegetables. The thermal degradation of AA follows a first order reaction and the temperature dependence of k values can be described by Arrhenius relation.

6.1 Introduction

Cowpea leaves are part of a larger group of vegetables, the African leafy vegetables (ALVs) together with the slim amaranth or pigweed (*Amaranthus hybridus*) from the family Amaranthaceae, black night shade (*Solanum nigrum*) from the family solanaceae, and cat's whiskers (*Cleome gynandra*) from the family capparidaceae, among others (Aletor and Adeogun, 1995; Agte, 2000; Borah et al., 2009; Makobo et al., 2010). These vegetables form part of basic daily diet, making them the main source of nutrients in a large section of the population in Kenya (Oomen and Grubben, 1978). The nutrient potential of these vegetables has been greatly discussed (Sreeramulu et al., 1983; Imungi and Potter, 1983; Mathooko and Imungi, 1994; Aletor and Adeogun, 1995; Chweya and Mnzava; 1997, Agte, 2000; Fasuyi, 2006; Borah et al., 2009; Makobo et al., 2010). Schonfeldt et al. (2011) studied the nutrient content (proximate, vitamin B_2, ß-carotene, iron, zinc, magnesium, calcium and phosphorus) of five (*Amaranthus tricolor, Curcubita maxima, Cleome, Vigna unguiculata* and *Corchorus olitorius*) traditional dark green leafy vegetables, traditionally consumed by rural inhabitants of South Africa (SA), whereby the moisture, protein, ash and fat content in the raw leaves per 100g ranged from 8-1.089.9 g/100g, 3.49–5.68 g/100g, 1.42–3.23g/100g and 0.12–0.36 g/100g respectively. The zinc content ranged from 0.5 towards 1.0 mg/100g while the magnesium ranged from 54.7 mg to 146 mg/100g.

There is a need to maximize the intake of nutrients from these vegetables, by studying stability of the nutrient and quality properties of these vegetables. The stability of nutrient and quality properties of cowpea leaves has been studied in

earlier sections (**Chapter 3, 4** and **5**); however, it is necessary to establish a comparison between the stability of nutrient and quality properties of cowpea leaves with the other ALVs and amongst the other ALVs mentioned above. This is due to the fact that in many dishes, these vegetables are mixed during cooking or alternated over time depending on many factors including consumer preferences, the harvesting season among others. In this research, thermal stability of AA in these vegetables was studied because these vegetables are usually prepared by cooking (boiling them in water) before consumption and it could result in loss of nutrients including ascorbic acid and organoleptic properties (Esteve et al., 1999; Osundahunsi, 2008). Therefore, it is also vital to study the degradation kinetics of AA in these vegetables to ascertain optimal process condition. Besides determining the total AA content, total carotene content and a proximate analysis of crude protein, crude fat, ash, total dietary fibre, and moisture content were determined. It is also crucial to determine the phytate content in these vegetables since phytates are anti nutrients, and lower the bioavailability of minerals. Phytates are the main storage form of phosphorus in many plant tissues, but they are not digestible by humans, hence, they are not considered to be a source of either inositol or phosphate if eaten directly. Moreover, phytic acid is a strong chelator of divalent minerals such as iron, zinc, calcium, copper, and magnesium (Jackman and Black, 1951; Lonnerdal, 1989; Sandström and Sandberg, 1992; Wise, 1995) and its presence can be used to lower the bioavailabity of the above minerals. Beyond nutrients, stability of overall green colour of the vegetables was also studied.

The objectives of this study were to determine the stability of total AA content and colour of the leaves of vegetables *A. hybridus, S. nigrum, C. gynandra* during thermal treatment. Furthermore, total carotene content, a proximate analysis, of the leaves and the phytate content were also determined and compared with cowpea leaves.

6.2 Materials and methods

6.2.1 Sample preparation after harvest

Slim amaranth (*A. hybridus*), black night shade (*S. nigrum*), and cat's whiskers (*C. gynandra*) fresh leaves were purchased from Thika municipal market, in Thika district of central Kenya. The samples were purchased in bulk (approximately 4kg) from different vendors and the final samples of analysis arrived at through multi stage sampling. The bulk sampling followed by multistage sampling was crucial so as to minimize errors arising from sample variability caused by the differences in vegetable maturity, environmental conditions under which they were cultivated, cultural practices, and days after harvest. Unlike cowpea leaves that were grown indoors, these vegetables were purchased from the market due to their difficulty in growing uniformly even under green house conditions and therefore making it difficult to discern the maturity levels and furthermore their delayed germination had an impact on the time frame of the thesis.

The leaves were transported to the research laboratory at the department of food science and technology at the Jomo-Kenyatta University of Agriculture and Technology in Juja, Kenya. The samples were transported under cool conditions in a

cooler box. At the laboratory, the vegetables were prepared by rinsing them with water, chopping them into pieces. They were then taken through a multistage sampling procedure and vacuum packed in plastic pouches (approximately 10g per pouch). From this, samples were taken and analyzed for total AA content and thermal stability, proximate analysis of crude protein (or Kjeldahl protein), crude fat, ash, total dietary fibre, and moisture content), mineral composition, green colour and phytate content.

6.2.2 Total AA stability

6.2.2.1 Kinetics of thermal inactivation of AA in the vegetables

Plastic pouches each filled with 10g of fresh leaves of the different vegetables were heated at predefined temperature between 70-95°C for different time intervals in a thermo-static water bath. Fresh leaves that were not subjected to any thermal treatments were used as blank. After the heat treatment, the leaves were cooled in an ice water bath for 20 minutes and then subjected to total AA extraction procedure.

6.2.2.2 Total AA content

Contents of a pouch of plastic package (from **Section 6.2.1**. above) containing approximately 10g of the sample together with 20ml of the extract solution (3% metaphosphoric acid and 10% trichloroacetic acid, TCA, (w/v)) were emptied in a mixer (magic bullet blender, Homeland housewares, Ningbo, China.) and homogenized for 30 seconds. This homogenized sample was transferred quantitatively into a 100ml volumetric flask and made to the mark with the TCA solution. This was mixed well and centrifuged (Kokusan H-950, Tokyo, Japan) at 4°C and 17700 g for 30 minutes to clarify the solution. In order to stabilize AA in the

extract solutions 1 mM tert-butylhydroquinone (TBHQ) was added to the supernatant. For determination of AA, A.O.A.C's visual titrimetric method, using the 2, 6 dichlorophenolindophenol (DCIP) solution (A.O.A.C, 1990) was used. In brief, 10ml of this supernatant was titrated with indophenol solution (25% DCIP and 21% $NaHCO_3$ in water) until a light but distinct rose pink colour appeared and persisted for more than 5 seconds. The indophenol solution was standardized daily with ascorbic acid solution. All determinations were repeated ten times. For blank, 10ml of the extract solutions was pipetted and distilled water equivalent to the volume of indophenols solution used in above was added. This was titrated with indophenol solution until a pink colour appeared.

6.2.3 Data analysis to estimate kinetic parameters for total AA degradation

AA thermal degradation was described by a first-order kinetic model (Eq. 6.1) and the temperature dependence of the inactivation rate constant was estimated by Arrhenius equation. (Eq. 6.2), Morales-Blancas et al., 2002).

$$C = C_0 \exp(-k_t t) \quad ..(6.1)$$

$$k_t = k_{ref} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad ..(6.2)$$

Where C is the total AA content (mg/100 g) after heat treatment for time (t) in minutes at a given temperature while C_0 is the initial total AA content (mg/100 g), k_t is the inactivation rate constant (min^{-1}), k_{ref} is the inactivation rate constant (in min^{-1}) at reference temperature, E_a is activation energy (in kJ/mol), R is the ideal gas constant (8.314 J/mol.K), T is the actual treatment temperature (in K), and T_{ref} is the

reference temperature (in K, in this study T_{ref} = 80°C). Kinetic parameters for total AA degradation were estimated by a common two-step linear regression method (Esteve et al., 1999), where (i) the logarithm of the values of AA was plotted against time of thermal treatments to obtain k_t values at each temperature and (ii) the natural logarithm of the reaction rate constant (ln (k)) was regressed against $\left[\left({}^{1}/_{T_{ref}}\right) - \left({}^{1}/_{T}\right)\right]$ as described by Karhan et al. (2004). The slope of this curve, the Arrhenius plot, is equal to ${}^{E_a}/_{R}$ and allowed for the calculation of the activation energy. D values, the time required in reducing the total AA content by 90%, and z values, the temperature required for a 90% decrease in D value, were estimated according to Ariahu and Ogunsua (2000).

$$C=C_0*10^{(-t/D)} \quad ..(6.3)$$

$$C= C_0*10^{\left(\frac{-t}{D_{ref}*10^{((T_{ref}-T)/Z)}}\right)} \quad ..(6.4)$$

Whereby D_{ref} is the D value at temperature T_{ref}.

The **s**tandard errors at 95% confidence intervals were used to assess the precision of the estimates.

6.2.4 Analyses

6.2.4.1 Proximate and nutrient composition

The crude protein, crude fat, ash, total dietary fibre, and moisture content were determined by following the methods described in the Official Methods of Analysis

(A.O.A.C, 1995; 2000). The mineral content was determined by atomic absorption spectrophotometer (AA-6200, Shimadzu Corporation, Kyoto, Japan) method as described in the Official Methods of Analysis (A.O.A.C, 1995). Quantitative determination of phytates was carried out using the method of A.O.A.C (1990). The total carotene was determined by the standard spectrophotometric method (Booth, 1957). The colour of the green vegetables was measured by the Minolta colour difference meter CR-300 (Nippon Denshoku Industry. Co. Ltd, NF 333, Japan). The data were taken by measuring the colour from six different places on a spread of leaves. This was done on both sides of the leaves spread. The determinations were done in triplicate. The results from the colour difference meter were expressed in L*a*b* colour space. The L value stands for the lightness variable (the more positive the value, the lighter is the material) while the a* value is a measure of redness (positive), greyness (zero) or greenness (minus).The b* value is a measure of yellowness (positive), greyness (zero) or blueness (minus).

6.2.4.2 Data analyses

Reported leaf mineral concentrations are the mean ±standard deviation of three independent samples of each accession. Statistical comparisons of group means were performed with an unpaired t-test (Excel Vista software; Microsoft Corp, Seattle, WA, USA).

6.3 Results and Discussion

6.3.1. Nutritional composition of ALVs

In their raw form, the vegetables showed high contents of AA (5.6 mg/g dry matter for *A. hybridus*, 6.2 mg/g dry matter for *S. nigrum,* and 7.3 mg/g dry matter for *C. gynandra* as indicated in **Table 6.1.**

Table 6.1 Proximate and nutritional composition of raw vegetables.

Nutritional/proximate component	Vegetable Source		
	A. hybridus	*S. nigrum*	*C. gynandra*
		mg/g D.W	
Total AA	5.60±0.19	6.20±0.63	7.30±0.33
Total carotene	15.76±2.64	16.98±3.02	23.49±2.91
Crude protein	9.19±0.64	12.40±0.45	12.28±0.69
Crude fat	2.30±0.35	8.34±1.52	2.42±0.38
Total ash	17.70±0.81	16.10±1.89	14.61±1.48
Total dietary fiber	6.80±1.66	11.21±2.56	7.79±1.98
Moisture content	81.20±2.6	89.35±1.0	86.42±1.51
Phytates	0.42±0.03	0.89±0.10	0.86±0.11

*D.W: Dry Weight

Values are means ± standard deviation of triplicate samples (95% confidence interval)

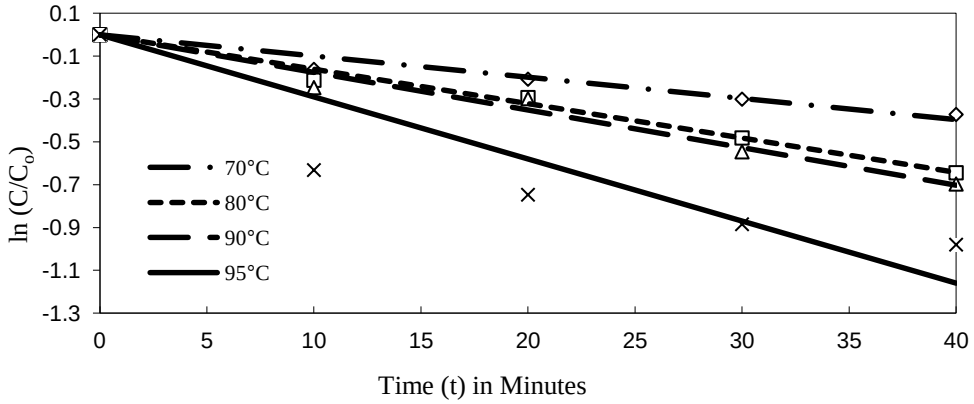
These AA contents were higher than those found in cowpea leaves (**Table 3.2**). This big difference can be attributed to the fact that the above three vegetables were harvested at a later maturity stage when compared to the cowpea leaves and vegetable maturity plays a big role in the quality and quantity of AA. Indeed, the overall nutrient content of many vegetables is affected by, among other things, the degree of maturity at time of harvest (Lee and Kader, 2000; Wawire et al., 2011), since nutrient content, like minerals and AA, change with maturity of the vegetables, furthermore, over mature vegetables are too fibrous and can be rejected by the consumer. As earlier indicated, AA is a temperature-sensitive molecule hence its conservation during cooking is of uttermost importance; furthermore it provides an indication of the loss of other nutrients and organoleptic properties (Esteve et al., 1999). Thus, it is crucial to define the degradation kinetics of AA in these vegetables as this can be used to ascertain optimal cooking conditions.

6.3.2 Kinetic parameters for AA degradation during thermal processing

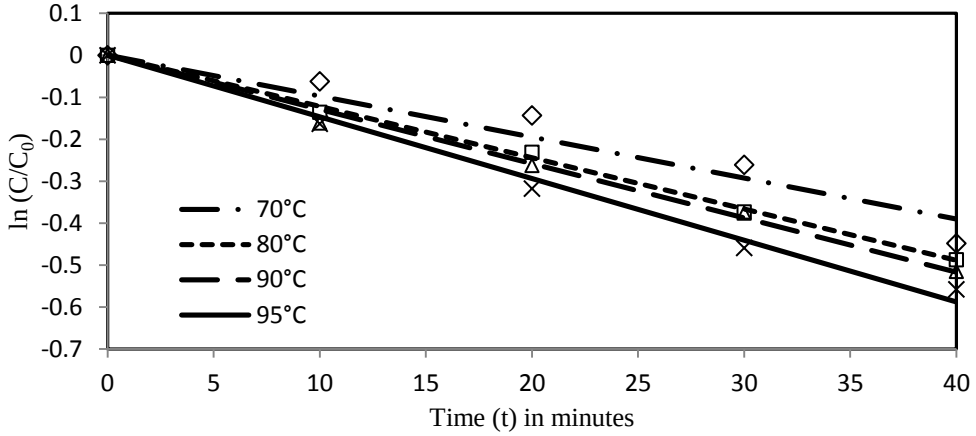
To establish the kinetic model, a two-step method was used as outlined above. The first step involved the use of a regression analysis to calculate the reaction rate constant for each experiment. **Figure 6.1** shows the decrease in ascorbic acid content in the leaves of the vegetables during thermal treatment with time by fitting to a first-order equation. The kinetics showed a good fit for a first-order reaction. This first-order reaction was also previously observed (Johnson et al., 1995; Esteve et al., 1998, 1999; Manso et al., 2001; Karhan et al., 2004). The rate constants determined from the slopes of the regression lines are presented in **Table 6.2.** The second step

involved the use of the Arrhenius equation to define the temperature dependence of the reaction rate constants as illustrated in **Figure 6.2**, for each of the vegetables.

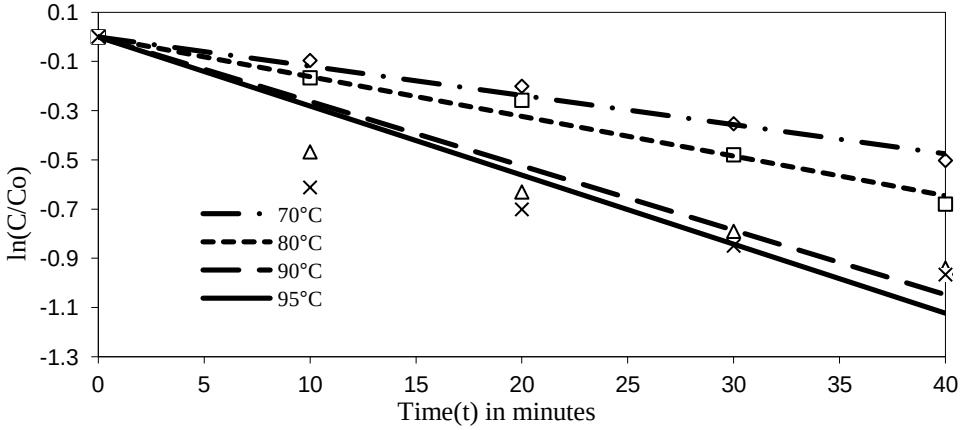
The model shows a good fit (R^2 values of 0.87, 0.95, 0.99 for *A. hybridus, S. nigrum* and *C. gynandra,* respectively; (**Table 6.2**) for the Arrhenius plot furthermore, the plot of ln(k) vs $(1/T)$ as indicated in **Figure 6.2 (a)**, **(b)** and **(c)** shows no breaks; an indication that the thermal degradation of AA in the three vegetables was monophasic, meaning that only one form of the AA was present since L-AA and DHAA exhibit different thermal stability. The Arrhenius equation allowed for the evaluation of the E_a values and the calculation of the D and Z values as indicated in **Table 6.2**. The E_a values estimated were 39.2, 35.6 and 38.7 kJ/mole for *A. hybridus, S. nigrum* and *C. gynandra,* respectively. High E_a values (low Z values) are an indicator of the AA vulnerability during cooking in a given vegetable. This means that of the three vegetables, *A. hybridus* showed least stable AA during cooking, therefore, more care needs to be taken when cooking it. The differences in the E_a values of the vegetables can be attributed to the difference in the matrix systems of the vegetables as the food matrix plays a big role in heat transfer rates through a system and hence influences the thermal effect on the food. Indeed previous studies by Stešková et al. (2006) have shown that the food matrix system and the form of AA (L-AA or DHAA) are the main factors that affect the stability of AA in foods and beverages. The food matrix system includes fibre content, pH, salts, metals, heat conductivity and the state (i.e. liquid or solid). In fact one of the major components of the food matrix is the fibre content.



(a) *A. hybridus*



(b) *S. nigrum*



(c) *C. gynandra*

Figure 6.1 Plot of loss of total AA content ($\ln(C/C_0)$ in leaves as function of thermal treatment time. Symbols represent experimental values, lines correspond to individual fittings of the first order kinetic model to each temperature using linear regression analysis (Eq.6.1)

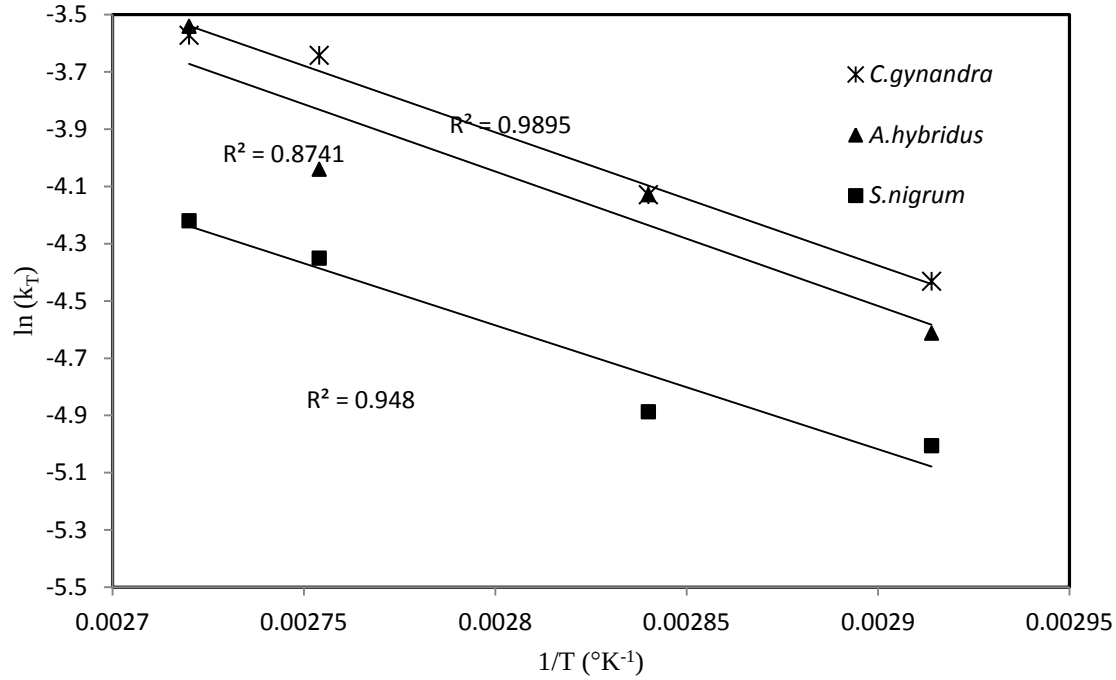


Figure 6.2 Arrhenius plot between natural log of reaction rate constant, $\ln(k_T)$ against 1/T (°K) for AA degradation during thermal treatment in the vegetable leaves of *C. gynandra, A. hybridus and S. nigrum.*

The impact of the food matrix system on the E_a values is illustrated when the E_a of the vegetables are compared to fruit juices and the E_a values of these vegetables were lower than those of orange juice at 115.5 kJ/mole (Johnson et al., 1995) and 75 kJ/mole for cupuacu (Vieira et al., 2000) since juices have a faster heat transfer rate and, thus, a faster thermal degradation of AA. The E_a values of the vegetables are in good agreement with the values of 14.4–47.4 kJ/mole for guava fruits stored at 30 and 50°C (Uddin et al., 2002) and 47.5 kJ/mole in rose hip pulp (Karhan et al., 2004).

Table 6.2 (a) Estimated kinetic parameters for thermal degradation of total AA in *A. hybridus*, **(b)** *S. nigrum* **(c)** *C. gynandra*, at various temperatures.

(a) *A. hybridus,*

T(^{0}C)	k_T (*10^{-2} min^-	r^2	D value (min)	Ea (kJ/mole).	Z value (^{0}C)
70	0.99±0.01	0.943	232.63±10.51		
80	1.61±0.02	0.986	143.04±23.61	39.17	61.34
90	1.76±0.01	0.972	130.85±4.88		
95	2.9±0.03	0.702	79.41±15.22		

(b) *S. nigrum*

T(^{0}C)	k_T (*10^{-2} min^-	r^2	Dvalue (min)	Ea (kJ/mole).	Z value
70	0.67±0.01	0.968	343.73±3.33		
80	0.75±0.01	0.959	305.30±2.22	35.62	68.03±12
90	1.29±0.03	0.924	178.53±4.77		
95	1.47±0.01	0.989	156.67±8.22		

(c) *C. gynandra*

T(^{0}C)	k_T (*10^{-2} min^-	R^2	D value (min)	Ea (kJ/mole).	Z value
70	1.19±0.02	0.984	193.53±4.85		
80	1.61±0.01	0.981	143.04±9.34	38.68	62.89
90	2.62±0.04	0.876	87.90±15.49		
95	2.81±0.01	0.727	81.96±18.22		

*r^2 is the regression for the inactivation rate constant for thermal degradation of total AA in the leaves of *A. hybridus* (a) *S. nigrum* (b) and *C. gynandra* (c) as exhibited in **Figure 6.1**. k_T is the reaction rate constant (slopes of linear graphs in **Figure 6.1**). Z_{value} is calculated from the inverse of the slope of the graph in **Figure 6.2.**

6.3.3 Nutritional composition and nutrient potential of the vegetables

The data in **Table 6.1** showed that the vegetables (*A. hybridus, S. nigrum* and *C. gynandra)* are good sources of several essential nutrients including AA (as earlier discussed), carotenes and minerals (as indicated by the ash content). However, the amounts of proteins, and fat in the vegetables is too small for them to be considered as important sources. The vegetables also contain high quantities of dietary fibre and moisture content.

The amount of total carotene, found to range between 15.76 mg/g in *A. hybridus,* 16.98mg/g in *S. nigrum* and 23.49mg/g in *C. gynandra* in dry weight, indicated that the vegetables were a good source of carotene among the leafy vegetables especially when compared to cowpea leaves (0.57mg/g on dry weight basis) as reported by Imungi and Potter (1983), or African spinach (0.69 mg/g on dry weight basis) as reported by Maeda and Salunkhe (1981). However, it is comparable to what is reported for *Talinum triangulare* at 210mg/100g and spinach at 290 mg/100g of fresh weight (Renqvist et al., 1978).

Ash content in plants varies due to the differences in mineral status of the soil, the species, and part of the plant as well as maturity stage at harvest (Mziray et al., 2001). In the three vegetables the ash content ranged between 14-18% of the weight on dry weight basis. This compares well with values of approximately 13% in *V. unguiculata* leaves (Imungi and Potter, 1983) and 8% in *M. oleifera* leaves (Gidamis et al., 2003). These results are also in good range with those reported for tropical vegetable leaf meals from Nigeria at between 10% and 20% (Fasuyi, 2006). The high

ash content is a good indicator of the mineral potential of the vegetables. This was corroborated by data in **Table 6.3 (a)**.

The vegetables were good sources of Fe, Zn, Mg, Mn and Ca. In comparison to cowpea leaves (**Table 4.3**), these vegetables had in general lower mineral content compared to the leaves of the younger cowpea vegetables (four and six week old) but their mineral content was comparable to the leaves of the older vegetables. However, the Ca and Na levels in *A. hybridus* were ten times less and 500 times less, (respectively) than those quoted in *Amaranthus cruentus* (Makobo et al., 2010).

Table 6.3 (a) Mineral concentrations (mg/g D.M) in leaves of *A. hybridus*, *S. nigrum* and *C. gynandra* vegetables.

Mineral	Vegetable Source		
	A. hybridus	*S. nigrum*	*C. gynandra*
	mg/g D.M		
Ca	2.48±0.38	1.06±0.12	2.99±0.14
Mg	3.92±0.093	2.07±0.38	2.59±0.17
Na	0.17±0.93	0.26±0.084	0.84±0.16
	*10^{-3} mg/g D.M		
Zn	50.0±0.4	60.0±2.0	50.0±1.3
Fe	370.0±5.5	500.0±9.7	340.0±9.3

*D.M: Dry Matter. Values are means ± standard deviation of triplicate samples (95% confidence interval)

The total dietary fibre is usually a sum of lignin and cellulose and most of it is contained in the plant cell wall. Fibre is considered important in diet to improve digestion by increasing the water retention in the stomach and intestines (Agte et al., 2001). The fibre content in these leaves was comparable with approximately 12% that is found in *Moringa oleifera* leaves (Gidamis et al., 2003), 14% that was found in *V. unguiculata* (Imungi and Potter, 1983), and approximately 8% to 13% that was found in tropical vegetable leaf meals from Nigeria (Fasuyi, 2006).

These vegetables also exhibited high moisture content, ranging between 81% in *A. hybridus* and 89% in *S. nigrum*. High moisture content in vegetables means that they can inherently contain water-soluble nutrients and make them available to the consumer. However, high moisture content has its drawbacks in that it increases the perishability of the vegetables. These results are comparable with another traditional African leafy vegetable, of about 82% moisture content for *V. unguiculata* leaves as earlier indicated (**Section 3.3.2**).

The high amounts of phytates in the vegetables (0.42 mg/g in *A. hybridus,* 0.89 mg/g *S. nigrum* and 0.86 mg/g in *C. gynandra* in dry weight) are of concern since the presence of phytates in food has been reported to lower the bio-availability of minerals (di- and trivalent minerals ions), and inhibit proteases and amylases (Mosha et al., 1995). The effect of phytate on the bioavailability of minerals depends on the amount of phytate and minerals in the diets (Nävert et al., 1985; Brune et al., 1992) and the ratio of phytate/minerals. Therefore, the phytate/minerals molar ratios can be used to predict the inhibitory effect on the bioavailability of minerals in the vegetables (Turnlund et al., 1984; Fordyce et al., 1987; Morris and Ellis, 1985;

Morris and Ellis, 1989; Sandberg et al., 1987; Davies et al., 1985; Bindra et al., 1986). The phytate/calcium molar ratio >0.24 will impair calcium bioavailability (Morris and Ellis, 1985). The phytate/iron molar ratio >1 is indicative of poor iron bioavailability (Hallberg et al., 1989a, b). Zinc absorption is greatly reduced and results in negative zinc balance when phytate/zinc molar ratio is >15 (Turnlund et al., 1984; Bindra et al., 1986; Oberleas and Harland, 1981; Ferguson et al., 1993; Huddle et al., 1998). Most plant-based diets have low calcium contents which does not inhibit zinc absorption. When diets are high in both phytate and calcium, phytate × calcium/zinc is better used to assess the zinc bioavailability than phytate/zinc molar ratio (Fordyce et al., 1987).

Table 6.3 (b) Molar ratio of phytates to Ca, Mg, Zn and Fe in leaves of *A. hybridus*, *S. nigrum* and *C. gynandra* vegetables.

Mineral	Vegetable Source		
	A. hybridus	*S. nigrum*	*C. gynandra*
Phytate/Ca	0.17	0.84	0.29
Phytate/Mg	0.11	0.43	0.33
Phytate/Fe	1.14	1.78	2.53
Phytate/Zn	8.40	14.83	17.20
Phytate × Calcium/Zinc	0.0085	0.050	0.014

Values are means ± standard deviation of triplicate samples (95% confidence interval)

From **Table 6.3 (b)**, the phytate/calcium molar ratio was >0.24 in two of the vegetables (*S. nigrum and C. gynandra*) at 0.84 and 0.29, respectively indicating good calcium bioavailability in *A. hybridus* while all the vegetables exhibited a phytate/iron molar ratio >1, a strong indication of poor bioavailability of iron in these vegetables. However, only one vegetable, *C. gynandra* had a negative zinc balance with a phytate/zinc ratio at >15. Overall, these vegetables had high phytate content when compared to other leafy vegetables like *M. oleifera* leaves (Gidamis et al., 2003). Previuos studies have shown that quantities of phytates in food especially cereals, can be reduced by sprouting and subsequent fermentation, a process that stimulates enzyme phytase which in turn breaks down phytic acid (Eklund-Jonsson et al., 2006).

6.4 Conclusions and Recommendations

High amount of AA was found in the ALVs, and the three vegetables are also good sources of AA, total carotene, fibre and minerals. The Na and Ca amounts in the vegetables were found too low for these vegetables to be considered and depended on as good sources in the diet. Phytates need to be reduced before consumption and there is need to explore vegetable fermentation or soaking of vegetables in fermented milk as a way of reducing phytates. The ALVs are known to harbour a bitter taste, therefore, there is need to explore ways to reduce the bitter taste so as to increase their appeal, especially among the young population in the country. In the case of calcium, there is a need to carry out further tests to quantify how much calcium in the vegetables is available to the consumer and in which form, since calcium in the form of calcium oxalate is not available for the body to use.

CHAPTER SEVEN

7.0. Recommendations, applications and conclusions

7.1 Recommendations

There is a need:

1) To investigate anti nutritional factors (eg phytates) in cowpea leaves especially in relation to the mineral content
2) To explore the addition of salt or lowering of the pH during thermal processing so as to stabilize the green colour; however, the addition of salt or lowering of pH can also lead to enhance inactivation of AAO and POD and the stability of AA.
3) To investigate the presence of toxic alkaloids in some ALVs such as *S. nigrum*.
4) To investigate the properties of the bitter taste in *C. gynandra* and find ways to reduce it in the cooked vegetables.
5) To investigate presence of phytochemicals and impact on heat processing
6) To investigate the degree of lignification and fiber content in the vegetables.

7.2 Applications

1) Since thermal processing was ineffective in stabilising AA in young vegetables (4 weeks) it is advised to consume young leaves without thermal processing for example vegetable salad or vegetable juice from a blender. The older leaves, 8 weeks old were ideal for processes that require thermal processing since blanching had a profound effect on stabilizing the AA in the vegetables.

2) For domestic cooking of the vegetables, the results indicate that in order to optimize AA in the vegetables, 8 weeks old vegetables are most suitable, and the vegetables should be added in an already boiling pot of water rather than the usual practice of bringing both the vegetables and the water to a boil. Adding the vegetables in an already boiling pot of water means that the vegetables will spend very little time in a temperature zone that is optimal for AAO activity and hence AA degradation. This situation is exacerbated if the water that is being brought to a boil also has some salt since salt might lead to increased extractability of AAO and hence increased AA degradation. Thus, the ideal cooking practice would be to add salt on the table or when the vegetables are sufficiently blanched, i.e. after 10 minutes of boiling. Other traditional cooking practices like adding fermented milk in the vegetables during cooking could help improve AA intake from the vegetables as this lowers the pH of the cooking medium, leading to a reduced AAO activity and a more stable AA.
3) In order to optimize the intake of folates from cowpea leaves, there is need to consume the vegetables at all stages of maturity and there is also a need to vary the consumption of vegetables from raw vegetables (in vegetable salad) to cooked vegetables. The consumption of vegetables from different maturity stages means that there is, on overall, an uptake in terms of quality (short chain poly-γ-glutamate) and quantity (total folate).
4) Complete thermal inactivation of POD is accompanied by degradation of organoleptic properties (as indicated by loss of green colour in the leaves) and, thus, steps need to be taken to reduce the thermal degradation of green colour and other organoleptic properties during thermal processing.

5) Other ALVs are also good sources of micronutrients; therefore, they can be prepared in combination with cowpea leaves to have a complementary effect on nutrient composition.

7.3 Conclusions

Cowpea leaves form an important part of the diet of many Kenyans and, thus, optimization of the nutritional and quality properties of cowpea leaves is of uttermost importance so as to have an improved nutrient intake from these vegetables. In this research, cowpea leaves were good sources of AA, folates and minerals in the diet, however, the quantity and quality of these nutrients heavily depends on the maturity stage of the leaves and the treatments they are subjected to during handling, processing and storage. Indeed the quantity (total AA) and quality of AA (LAA/DHAA ratio) in cowpea leaves was increased by a combination of harvesting the cowpea leaves at a maturity of 8 weeks and thermal treatments that lead to reduction of AAO activity. The research shows that as far as thermal stability of AA is concerned, the LAA/DHAA ratio plays an important role and the higher the LAA/DHAA ratio, the more thermal stable is the total AA. Kinetics of thermal inactivation of AAO revealed that the enzyme is thermally stable up to 50°C and completely inactivated at 80°C within a short time. Thus, blanching (>80°C) of vegetables before any further thermal treatments was the most appropriate strategy in optimizing the quantity and quality of AA in the vegetables as it protected the LAA from the enzymatic degradation by AAO.

Blanching of cowpea leaves led to longer chain folate poly-γ-glutamate, due to the inactivation of enzyme GGH and it also led to a general increase in total folate

content. The increased total AA content and LAA/DHAA ratio from blanching could have also improved the protection of folate from oxidative degradation leading to their improved stability and content. Thus, for an increased bioavailability of folates in the cowpea leaves diet, blanching is not desirable, however, to increase total folate content in the cowpea leaves diet, blanching is an important processing step. The stage of maturity of the vegetables and thermal treatments also affects the total folate content and the folate poly-γ-glutamate chain length. Thus, in view of balancing between shorter folate poly-γ-glutamate and high total folate content, cowpea leaves harvested at 6 weeks offered the best option in optimization of folates.

In order to optimize the nutrient and quality properties of cowpea leaves, an optimized thermal processing is desirable; that can inactivate harmful enzymes while retaining the desirable nutrient and quality properties of the vegetables. In this research, POD in the leaves was adequate in gauging the effectiveness of the thermal processing. The kinetics of POD inactivation showed that it exhibited two phases; a heat-labile phase and a heat-stable phase. Thus, thermal inactivation of POD was appropriate in ensuring that all other harmful enzymes were also inactivated. However, in order to ensure that the thermal processing does not adversely affect the quality properties of the vegetables, the visual green colour properties of the vegetables were tracked through the thermal processing. The green colour of the vegetables was thermally unstable in the initial thermal processing but stabilized with further processing. Therefore, the conditions for complete thermal inactivation of Enzyme POD in the leaves were also accompanied by high thermal degradation of the visual green colour, a clear indicator of potential degradation of other quality

properties like texture and taste. In this research, thermal processing of the vegetables must be optimized to ensure a total inactivation of undesirable enzymes without degrading the quality properties of the vegetables.

In summary, the optimization of nutrients from cowpea leaves at different levels of maturity strongly depends on the type of nutrient, whereby at 4 weeks, the mineral content is at optimum, at 6 weeks the folate content is at optimum (in terms of the balance between folate poly-γ-glutamate chain length and quantity) while at 8 weeks the AA content (both in terms of total content and LAA/DHAA ratio) is at optimum. Thermal processing was an important process in optimizing the nutrient content of the cowpea leaves, depending on the type of nutrient and stage of maturity. For AA, thermal processing was effective for stabilizing the quality and quantity in 8 weeks old leaves while it was ineffective for 4 week old leaves, as for folates, it was effective in achieving higher folate content with long chain in 8 weeks old leaves but ineffective as far as short chain folate poly-γ-glutamate.

The ALVs *A. hybridus* (pig weed), *S. nigrum* (black night shade) and *C. gynandra* (cat's whiskers) showed high nutrient content and the total AA was relatively thermo-stable. Therefore, the preharvest, harvest and postharvest factors play a big role in the nutrient and quality stability of cowpea leaves. The maturity of the vegetable at the time of harvesting is crucial in determining the quality and quantity of nutrients in the vegetables. Thermal processing can also affect the stability of the nutrients. The ALVs are good sources of essential micronutrients.

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications

1) **Wawire, M.,** Oey, I., Mathooko, F.M., Njoroge, C.K., Shitanda, D., and Hendrickx, M. (2011). Thermal stability of ascorbic acid and ascorbic acid oxidase in African cowpea leaves (*Vigna unguiculata*) of different maturity. Journal of Agricultural and Food Chemistry, 59: 1774-1783.

2) **Wawire, M.,** Oey, I., Mathooko, F.M., Njoroge, C.K., Shitanda, D., and Hendrickx, M. (2011). Stability of ascorbic acid oxidase in African cowpea leaves (*Vigna unguiculatta*) at different maturity stages. Poster presentation at the "US-NZ Science and Technology Workshop on Postharvest Innovation for safe, more attractive and nutritious horticultural produce", Dunedin, New Zealand, August 23-25 2011, page 70.

3) **Wawire, M., Oey,** I., Mathooko, F.M., Njoroge, C.K., Shitanda, D., Sila, D., Hendrickx, M. (2012). Effect of harvest age and thermal processing on poly-gamma-glutamate folates and minerals in African cowpea leaves (*Vigna unguiculata*). Journal of Food Composition and Analysis, **25**, 160–165. Doi: 10.1016/j.jfca.2011.11.003.

Accepted for publication

1) **Wawire, M.,** Oey, I., Njoroge, C.K., Shitanda, D., Hendrickx, M., and Mathooko, F.M. (2011). Evolution and stability of ascorbic acid oxidase in African cowpea (*Vigna unguiculatta*) leaves of different maturities and grown under different conditions; proceedings of the conference at the Institute of

Tropical Medicine and Infectious Diseases (ITROMID), at the Jomo-Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

Seminar participations

1) **Wawire, M.,** Oey, I., Mathooko, F.M., Njoroge, C.K., Shitanda, D., and Hendrickx, M. (2011). Stability of ascorbic acid oxidase in African cowpea leaves (*Vigna unguiculatta*) at different maturity stages. Poster presentation at the "US-NZ Science and Technology Workshop on Postharvest Innovation for safe, more attractive and nutritious horticultural produce", Dunedin, New Zealand, August 23-25 2011.
2) **Wawire, M.,** Oey, I., Mathooko, F.M., Njoroge, C.K., Shitanda, D., and Hendrickx, M. (2011). Evolution and stability of ascorbic acid oxidase in African cowpea (*Vigna unguiculatta*) leaves of different maturities and grown under different conditions. **ITROMID** Scientific Conference on "Health research for sustainable development", Nairobi, Kenya, 19^{th} – 20^{th} May 2011.

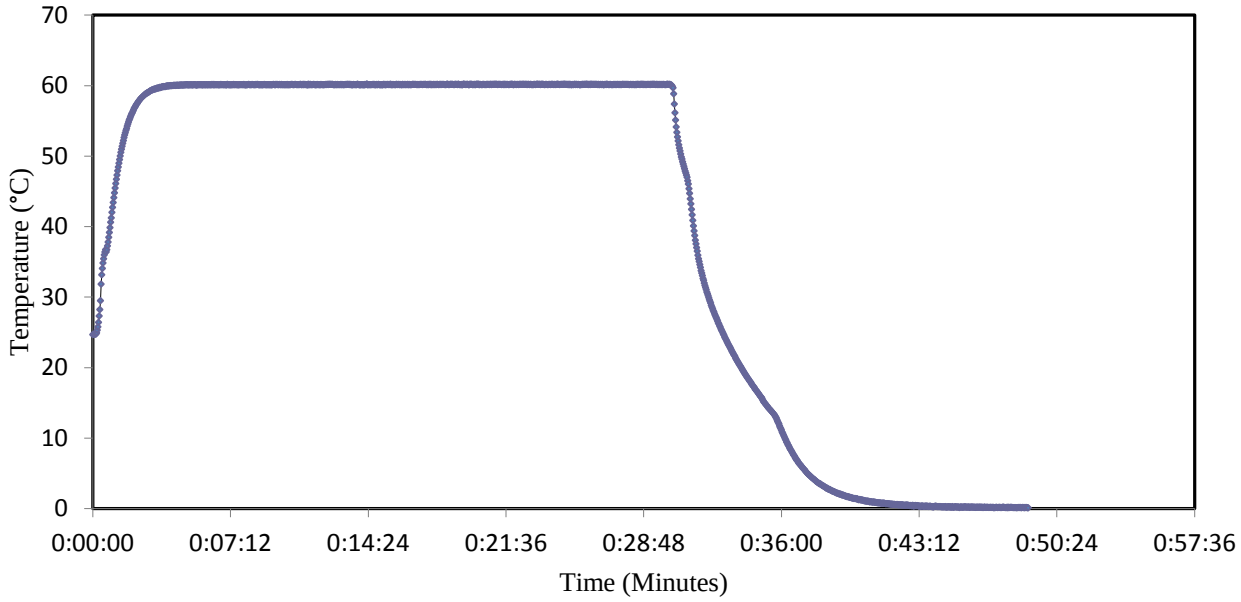
APPENDICES

Appendix 1 Cowpea vegetable in the green house

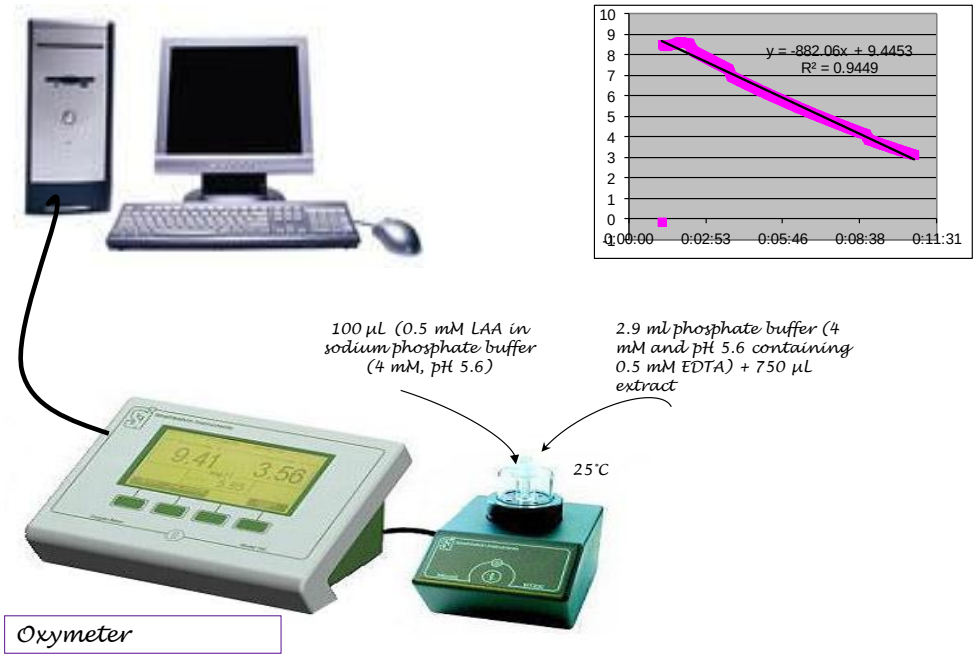
Appendix 2 (a) Black night shade (*Solanum nigrum*) (b) Pigweed (*Amaranthus hybridus*) (c) Cat's whiskers (*Cleome gynandra*)

Appendix 3 (a) The Memmert waterbath

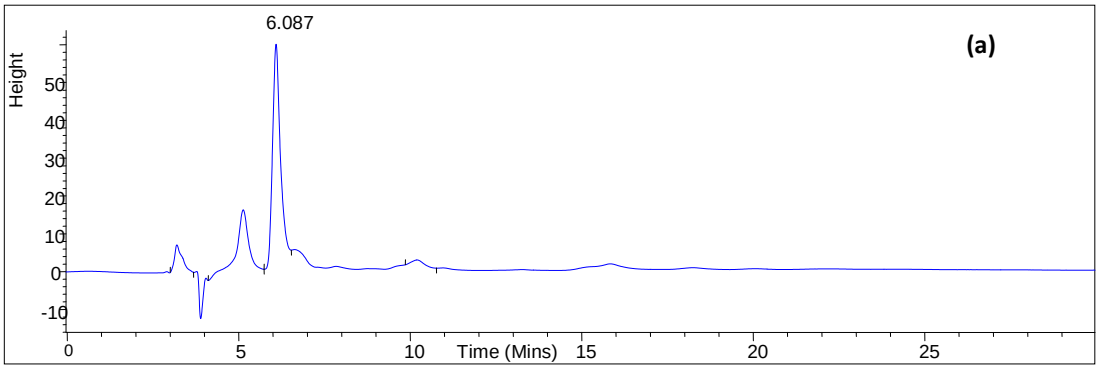
Appendix 3 (b) The temperature profile of cowpea leave[s] (approximately 10g) vacuum-packed (at 34 millibars) in plastic pouches (140 x 200 x 40 mm, DaklaPack) during thermal processing in a water bath (Memmert Water Bath, WBU 45) at 60°C and subsequent cooling in an ice water bath.

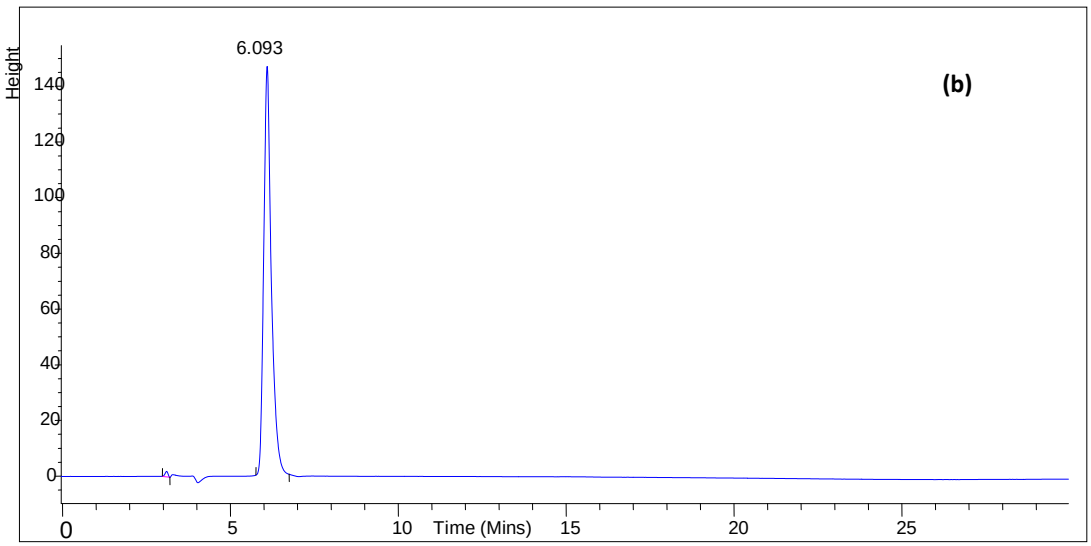


Appendix 4 A polarographic assay for ascorbic acid oxidase determination



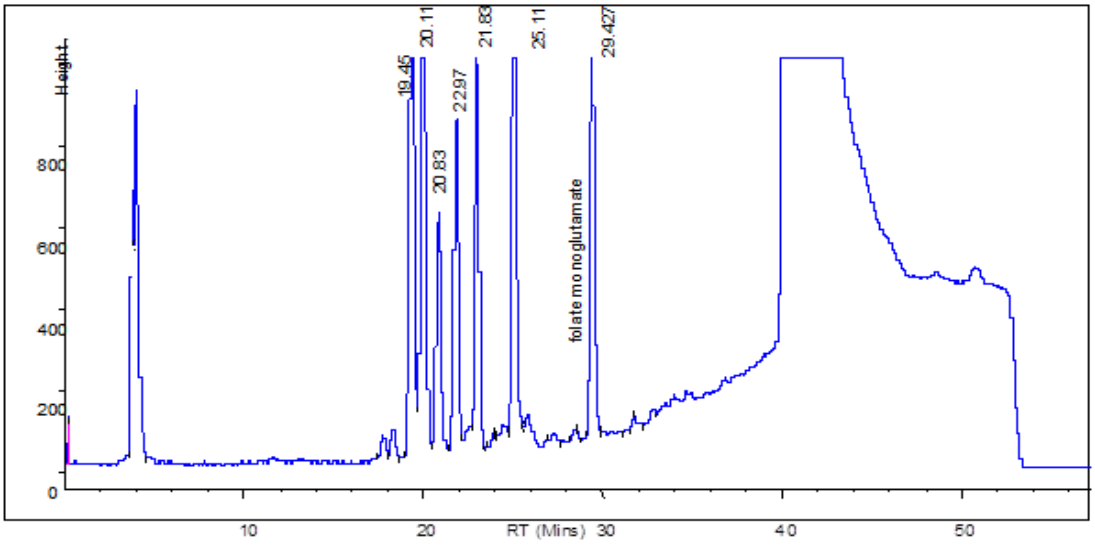
Appendix 5 Chromatogram showing the peak area/height of L-AA in the cowpea leaves sample (a) and known concentrations based on external calibration curve of standard solution (b), prepared on the day of use (500 µg/ml L-AA dissolved in NaH_2PO_4 (20 mM, pH 4.0, 1 mM EDTA)).





*The time of 6.087 minutes is the retention time of L-AA in the sample and the time of 6.093 minutes is the retention time of L-AA in the standard solution

Appendix 6 (a) Chromatogram showing the peak area/height of folate poly-γ-glutamate derivatives in in 4 weeks spiked sample (with known concentrations of folate standards of 5-CH_3–H_4PteGluγ_{1-7}, at ratio of 2:1) of different retention times (RT, minutes) of 19.61 (Hepta), 20.27 (Hexa), 21.042 (Penta), 22.125 (Tetra), 23.236 (Tri), 25.27 (Di) and 29.834 (Mono) γ-glutamates.



Appendix 6 (b) Chromatogram showing the peak area/height of folate poly-γ-glutamate derivatives in known concentrations based on external calibration curve, using external folate standards of 5-CH_3–H_4PteGluγ_{1-7} of different retention times (RT, minutes) of 19.45 (Hepta), 20.11 (Hexa), 20.83 (Penta), 21.83 (Tetra), 22.972 (Tri), 25.11 (Di) and 29.427 (Mono) γ-glutamates.