Optimisation of Baobab *(Adansonia digitata)* fruit processing and handling techniques for increased human nutrition and commercialisation in Malawi

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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I dedicate this piece of work to my parents Tryson and Tryness Tembo for their endless love and continuous spiritual and moral support. I also dedicate this thesis to my wife Nthandosi and children Joel and Bridget Tembo for their patience. During the past four years of this research work I gave them little attention.

I thank God for the good health and keeping me stronger during the entire period of this work.

Abstract

The influence of thermal processing and preservation techniques on selected bioactive compounds of baobab fruit pulp and juice from Malawi were analysed using RP-HPLC whereas total phenol content (TPC) and antioxidant activity were measured by spectrophotometry. ^{1}H NMR-based metabolomics was used to compare the metabolic profile of untreated (UT), high temperature short time (HTST) pasteurised and high temperature short time pasteurised with added citric acid (CAHTST) baobab juice samples and to analyse the temporal changes upon different storage conditions. Malawi baobab pulp contains high levels of procyanidin B2 (533.3 ± 22.6 mg/100 g FW), vitamin C (465.8 ± 2.5 mg/100 g FW), gallic acid (68.5 ± 12.4 mg/100 g FW) and (-)-epicatechin (43.0 ± 3.0 mg/100 g FW) and showed high TPC and antioxidant activity. Vitamin C content of dry baobab pulp and 10% juice (w/v) was retained during heat treatment by microwaving and pasteurisation (72 °C, 15 s) respectively. However vitamin C level in pasteurised juice significantly decreased during storage compared to untreated control, suggesting the demand for advanced processing methods including non-thermal pasteurisation. Seed liquor enhanced phenolic compounds in the juice but its influence on vitamin C needs further investigation. Principal Component Analysis (PCA) of ^{1}H NMR data produced distinct clusters separated according to treatment and storage conditions. For HTST and CAHTST juice samples, the most important accumulating metabolites were fructose, glucose and galacturonic acid, while sucrose decreased over time. The more rapid production of ethanol in UT juice is a clear indication that pasteurisation of

juice alone or in combination with the addition of citric acid can minimise fermentation in baobab juice. Results from this study will enhance understanding of composition of baobab fruit products and promote development of quality novel functional foods to tackle malnutrition as well as expand market opportunities for Malawi.

Publications

1. Tembo, D.T., Holmes, M.J., Marshall, L.J, 2016. Effect of thermal treatment and storage on bioactive compounds, organic acids and antioxidant activity of baobab fruit (*Adansonia digitata*) pulp from Malawi. *Journal of Food composition and Analysis*.

2. Tembo, D.T., Marshall, L.J., and Holmes, M.J, 2014. Optimisation of baobab (*Adansonia digitata*) fruit processing and handling techniques for increased human nutrition and commercialisation in Malawi. The 28th EFFosT International Conference, 25th - 28th November 2014, Uppsala, Sweden.

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List of Abbreviations

(-)-Epicatechin	EC
(-)-Epigallocatechin	EGC
(-)-Epigallocatechin-3-O-gallate	EGCG
2, 4, 6-Tris-2-pyridyl-s-triazine	TPTZ
2, 6-Dichlorophenol-indophenol	DCIP
2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)	ABTS
2,2–Diphenyl-1-picrylhydrazyl	DPPH
2,3-Diketogulonic acid	DKGA
2-Carboxy ethyl phosphine hydrochloride	TCEP
5-Hydroxymethlyfurfural	HMF
Activation energy	E_a
African Union	AU
Agroforestry tree products	AFTPs
Ascorbate oxidase	AO
Ascorbic acid	AA
Butylated hydroxyanisole	BHA

Butylated hydroxytoluene	BHT
Dehydroascorbic acid	DHA
Deoxyribonucleic acid	DNA
Electron transfer	ET
Ferric reducing antioxidant power	FRAP
Folin-Ciocalteu Reagent	FCR
Food and Agricultural Organisation	FAO
Gallic acid	GA
Gallic acid equivalent	GAE
Gallocatechin	GC
High temperature short time	HTST
High temperature short time with added citric acid	CAHTST
Human Immunodeficiency Virus and Acquired Immune Deficiency Syndrome	HIV/AIDS
Hydrogen atom transfer	HAT
International Centre for Research in Agroforestry	ICRAF
L- Ascorbic acid	L-AA
L-Dehydroascorbic acid	L-DHA

Limit of detection	LOD
Limit of quantification	LOQ
Low temperature long time	LTLT
Maillard reaction products	MRPs
Metaphosphoric acid	MPA
Multivariate Data Analysis	MVA
Nuclear magnetic resonance	NMR
Orthogonal Partial Least Squares Discriminant Analysis	OPLS-DA
Photodiode array absorbance detector	PDA
Pectin methylesterase	PME
Polyphenol oxidase	PPO
Principal Component Analysis	PCA
Proanthocyanidins	PAs
Procyanidin B2	PRO
Reactive oxygen species	ROS
Reversed Phase High Performance Liquid Chromatography	RP-HPLC
Southern Africa Development Community	SADC

tert-Butylhydroquinone	TBHQ
Total phenol content	TPC
Trolox equivalent antioxidant capacity	TEAC
World Health Organisation	WHO

Chapter 1 **Introduction and Literature Review**

1.1 Background: Food security and malnutrition

Millions of rural people in Eastern and Southern Africa, including Malawi are food insecure and chronically malnourished yet there are more than 200 edible species of indigenous fruits as well as tropical fruits available and distributed throughout the year (Malembo et al., 1998). Food insecurity and malnutrition has been a long standing challenge for people in this region, which is exacerbated by the emergence of HIV/AIDS (African Union, 2006). Since HIV/AIDS is a disease of the immune system, new strategies, including specific dietary nutrients, can be helpful to improve immune functions, quality of life and thus improve survival in infected individuals. The deficiency of vital micronutrients and vitamins in the dietary system are a form of 'hidden hunger' that could be addressed through availability of fruits and fruit products (Akinnifesi et al., 2004). The main victims of hunger and malnutrition are largely women and children. In the Sub-Saharan Africa region, children benefit from wild fruits since they are an important source of food as tropical fruits are often out of reach for most rural communities or are unaffordable due to widespread poverty (Akinnifesi and Kwesiga, 2006; Saka et al., 2007).

In Malawi, markets continue experiencing escalating prices of maize, the main staple food. Commercial imports and food aid deliveries have been meagre in spite of the significant amounts pledged by international donors (AU, 2006). In Sub-Saharan Africa forest food

including indigenous fruits could offer vital insurance against famine and malnutrition during times of seasonal food shortages or emergencies such as drought and floods (FAO, 1989; Saka et al., 2004). Moreover most rural households also rely on indigenous fruit trees as a source of cash and subsistence in the Southern Africa Development Community (SADC) (Akinnifesi and Kwesiga, 2006). Fruits and other products from indigenous fruit trees constitute the cheapest yet rich source of food on which the poor can survive. Interest has therefore grown of these fruits in the region (Tiisekwa et al., 2004).

There is adequate evidence to show that indigenous fruits contribute to the socio-economic, health and nutritional welfare of the rural people in Sub-Saharan Africa. They are sold fresh or processed in local markets (Saka and Msonthi, 1994; Akinnifesi and Kwesiga, 2006). However their contribution is limited to areas where the trees grow. The indigenous fruit supply chain is now well developed in Malawi and thus new processing, products and postharvest handling techniques will promote this initiative and provide market opportunities.

In Malawi farmers prefer simultaneous exploitation of indigenous and exotic fruits for product development in order to expand product range and meet market needs. Thus full exploitation of primary indigenous fruits may provide opportunities for product diversification (Saka et al., 2002). The presence and diversity of essential micronutrients including antioxidants in native fruits can be used to improve the quality of existing or local food products through fortification or development of novel functional foods (Netzel et al., 2006).

Farmers generally learn about fruit processing from their parents and this compromises quality of the final products (Tembo, 2008). Local processing and commercialisation of indigenous fruit-based products in Southern Africa is limited because of the lack of a sustainable supply of indigenous fruits, inadequate information on technologies and lack of market potential and benefits to farmers (Dietz, 1999). Empowering the community with appropriate processing techniques is thus essential and would contribute to the World Agroforestry program of indigenous fruit tree domestication (Tembo, 2008). Significant domestication headway could be made if it is accompanied by rigorous product development and commercialisation (Simons and Leakey, 2004; Akinnifesi and Kwesiga, 2006).

1.2 Distribution and domestication of indigenous fruits

The distribution of the miombo woodlands of Southern Africa corresponds roughly to the Zambezian Regional Centre of Endemism recognised for their floristic richness and the widespread occurrence of the tree genera *Brachystegia*, *Julbernardia* and *Isoberlinia*. The woodlands cover large areas of Angola, Malawi, Mozambique, Tanzania, Zaire, Zambia and Zimbabwe and for a long time have been a useful source of various forests products and services the subsistence needs of rural communities. The miombo woodlands are rich in the variety and quantity of indigenous fruit trees (Saka et al., 2002). In Malawi, a survey conducted in Zomba, Mwanza, Mangochi, Dedza, Lilongwe, Mzimba, Rumphi and Nkhata Bay revealed over 200 indigenous tree species including baobab (*Adansonia digitata*), mobola plum (*Parinari curatellifolia*) and monkey oranges (*Strychnos cocculoides*) as edible (Malembo et al., 1998). Rural communities have for many years

depended on natural forests for their household requirements including fruits and medicine (Akinnifesi and Kwesiga, 2006; Saka et al., 2007).

Until recently there has been little effort to cultivate, improve or add value to indigenous fruits. However, since 1989, the International Centre for Research in Agroforestry (ICRAF: now World Agroforestry Centre) has implemented a research and development initiative to domesticate and commercialise indigenous fruit trees in SADC countries. This is now seen as an important strategy to reduce poverty and hunger and to create employment opportunities in rural areas. The domestication of trees producing agroforestry tree products (AFTPs) should ensure that this resource is added to future national food production statistics, and that indigenous fruits supplement staple foods, so promoting food and nutritional security, moving towards the achievement of the United Nation Millennium Development Goals (Akinnifesi and Kwesiga, 2006). Therefore the present study "Optimisation of baobab fruit processing and handling techniques for increased human nutrition and commercialisation in Malawi" is adding value to the World Agroforestry Centre initiative. Research outputs may encourage conservation, domestication, processing and commercialisation of huge natural resources which currently are underutilised due to the lack of sufficient scientific knowledge in terms of chemical composition and quality changes during processing and storage.

1.3 General composition of fresh fruits and health benefits

The chemical compositions of fresh fruits are strongly influenced by genetic variation, climacteric type, fruit maturity and region of growth. The major constituents are sugars,

polysaccharides and organic acids. Nitrogen compounds and lipids are present in lesser extents. Minor constituents include pigments and aroma substances of importance to organoleptic quality and health, and vitamins and minerals of nutritional importance (Belitz et al., 2009). Some phytochemicals in fruits act as antioxidants in the human body by scavenging harmful free radicals, which are implicated in most degenerative diseases including cancer (Genovese, 2008; Caluwé et al., 2010; Vermaak et al., 2011; Benhura et al., 2012). These include fibre, polyphenols, flavonoids, conjugated isomers of linoleic acid, vitamins and tocopherols (Netzel et al., 2007; Kubola et al., 2011). Fruit phenolic compounds have attracted increasing demand and attention as potential agents for preventing and treating many oxidative stress-related diseases (Haminiuk et al., 2012). Interest has therefore grown on fruits that impart health benefits as well as basic nutrition.

1.4 Chemical composition of selected indigenous fruits

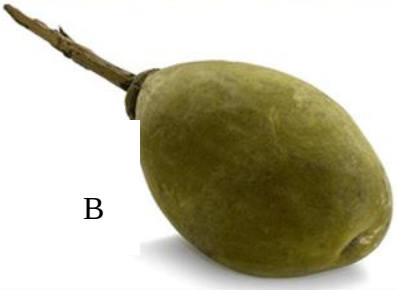
Research studies have shown that indigenous fruits are a rich source of essential micronutrients (Saka and Msonthi, 1994; Sanmartin et al., 2000; Adubiaro et al., 2011; Benhura et al., 2012; Kim et al., 2013) and can contribute significantly to meeting the recommended daily allowance of nutrients and help to reduce diseases associated with oxidative stress (Prior, 2004). For example, Tembo (2008) reported that mobola plum contained 134 mg/100 g of vitamin C and far surpassed oranges famous vitamin C content of 56.1 ± 1.0 mg/100 g (Scherer et al., 2012), and one variety of Chinese apples (*Ziziphus mauritiana*) a common indigenous fruit tree of Malawi reached levels as high as 1000 mg/100 g. African chewing gum (*Azanza garkeana*), a valuable edible indigenous fruit of Botswana, is also rich in vitamin C, as well as crude protein, total carbohydrate, and

important minerals necessary for human nutrition (Mojeremane and Tshwenyane, 2004). Adekunle and Oyerinde (2004) reported on food potential of some indigenous fruits of South West Nigeria. *Piper guinensis*, an indigenous fruit of South West Nigeria showed highest crude protein content (352.95 ± 0.16 g/kg, DW) while roasted and boiled *Artocarpus altilis* had considerable crude fibre content at 555.30 ± 0.19 and 544.25 ± 0.30 g/kg respectively. Fruits with appreciable protein content can replace animal protein usually absent in the diet of the poor in developing countries of the world.

1.5 Baobab (*Adansonia digitata L.)*

Baobab (*Adansonia digitata L.*) is a multipurpose tree species belonging to the *Malvacea* family (Bremer et al., 2009) and is a deciduous tree (Yazzie et al., 1994). Baobab fruit tree occurs naturally in dry areas of Africa, mainly in the Sahelian, Soudano-Sahelian and Soudanian zones; the distribution extends through the woodlands, savannas, and grasslands of Sub-Saharan Africa to about 25 °S. It is characterised by its massive size, reaching to a height of 18 – 25 m. The bark is smooth, reddish-brown, greyish-brown or purplish-grey, soft and fibrous (Chadare et al., 2008). In Malawi baobab fruit trees are found along the shores of Lake Malawi and Shire River. The fruits are in season from April to September (Saka et al., 2002; Tembo, 2008). Ripe fruits are large, egg shaped; 15 to 20 cm long with a hard woody outer shell covered with yellowish brown hairs and is filled with a dry white powdery pulp that covers brownish seeds having a bean-like structure (Figure 1.1). The pulp falls off upon cracking the shell and is eaten fresh like sweets and having a slightly lemon sherbet texture and taste.

B



C

D

Figure 1.1-Baobab fruit trees (A); baobab fruit (B); white powdery pulp (C) and brown seeds (D)

1.5.1 **Traditional use of baobab fruit**

Throughout Sub-Saharan Africa every part of baobab fruit is used where trees are found. Baobab is used as a source of food, traditional medicine, as well as sold (fresh fruits or processed) for household income. Seeds, leaves and bark are used for the treatment of malaria, tuberculosis, fever, diarrhoea, anaemia, dysentery and toothache while fruits are used for treatment of microbial infections (Caluwé et al., 2010; Kaboré et al., 2011; Vermaak et al., 2011).

Because of the multipurpose and growing efforts and interests by rural communities to process into different products, baobab fruit was selected for this study amongst several priority indigenous fruits of Malawi. Further postharvest handling and transportation of baobab fruit samples to the United Kingdom was easier as the fruits are not perishable and well protected with a hard outer shell (Figure 1.1B). Unlike other indigenous fruits, transportation of baobab was cheaper and possible at ambient conditions without any loss of quality.

Baobab fruits are processed into different products including juice, yoghurt, gruel, sour dough, oil, a coffee-like drink and dried as food reserves (Saka et al., 2002). However these products are achieved through local processing knowledge and often of low quality. Baobab fruit products are already gaining popularity on the international market including the UK because of global increasing demand for dietary sources of bioactive compounds. Most western organic food and drug companies are realising or becoming aware of

potential health benefits of baobab from traditional knowledge of its use by local African people.

Currently baobab pulp (a dry white powder) is purchased from Malawi and used by a few companies in European countries including the UK for manufacturing of health organic food products, medicines and cosmetics. In the UK several baobab products including Minvita Superfruit Powder, Minvita Baobab Body Oil, Baobab Vitamin C Capsules and Baobars were identified in supermarkets (Holland & Barrett, Leeds) (Figure 1.2). These products are sold at much higher prices compared to cost of raw materials. For instance, pulp powder (5000 g) costs less than £5 including transportation to the city centre within Malawi. However the cost of 250 g baobab pulp powder called Minvita Baobab Superfruit Powder (Figure 1.2D) is £14.99 (Hollard and Barrett, Leeds, UK).

A B C

D E F G

Figure 1.2-Baobab fruit products; Baobars (A), Baobab oil (B), Baobab Superfruit juice (C), Minvita Baobab Superfruit Powder (D), Minivita Baobab Body Oil (E) Baobab Capsules (F) and Aduna Baobab (G).

Therefore, this study targets a potential indigenous fruit that can not only improve nutritional, health and social-economic status of local people but also promote international trade for Malawi by adding value to existing products. Rural communities especially women are trying out different products using simple affordable methods and techniques but the lack of scientific knowledge retards development of their initiatives. It is hoped that some of the research output will encourage local processing and investment either by government or the business community thereby adding value to huge natural resources which are currently being underutilised.

1.6 Bioactive compounds and nutritional attributes from baobab fruit

Bioactive compounds are components of foods that influence physiological or cellular activities resulting in a beneficial health effect (Kris-Etherton et al., 2004). Unlike nutrients which prevent deficiency diseases, bioactive compounds may reduce the risk of chronic diseases such as cardiovascular disease and cancer. Examples of bioactive compounds include carotenoids, proanthocyanidins and phenolic acids which are widely available in fruits and vegetables, cereals, tea and coffee.

Baobab pulp contains very high levels of minerals (calcium, iron, magnesium, and sodium), vitamin C, organic acids, essential amino acids, sugars, sterols, saponins, triterpenes, flavonoids (high levels of procyanidin B2 and (-)-epicatechin), cellulose, fibres and tannins which may be responsible for its antioxidant, anti-inflammatory, antipyretic, analgesic, hepatoprotective, antimicrobial, antiviral, anti-trypanosoma, antidiarhoea properties and healing effects (Kaboré et al., 2011); seeds contain essential oils with provitamin A carotenoids and fatty acids (high concentration of linoleic and oleic acids and less amounts of palmitic, linolenic, stearic and arachidonic acids); leaves contain good quality proteins (Shahat, 2006; Chadare et al., 2008; Tembo, 2008; Caluwé et al., 2010; Adubiaro et al., 2011; Kaboré et al., 2011; Kamatou et al., 2011; Coe et al., 2013). Phytosterols including ß-sitosterol, campesterol, cholesterol and stigmasterol had been identified in seed oil (Kamatou et al., 2011).

The presence of flavonoids and vitamins in most indigenous fruits provide evidence of antioxidant properties. For instance because of high natural vitamin C content, baobab fruit pulp has a well-documented antioxidant capability and may have a role in the extension of shelf-life for derived foods and beverages as well as cosmetics (Vermaak et al., 2011). The food/beverage industry could introduce baobab fruit pulp into food in order to act as a preserving ingredient by preventing oxidation of lipids in the food (Afolabi and Popoola, 2005). Baobab aqueous extract (800 mg/kg) gave comparable anti-inflammatory effects to phenylbutazone (15 mg/kg), a classical drug used as painkiller (Kaboré et al., 2011). Baobab extract showed high polyphenol content and total antioxidant activity *in vitro* and reduced starch digestion and glycemic response (GR) in humans (Coe et al., 2013).

1.7 Vitamin C

1.7.1 Structure and chemical properties

Ascorbic acid (AA) and its oxidised product, dehydroascorbic acid (DHA) are referred to as vitamin C. Ascorbic acid is found naturally in citrus fruits and many vegetables and is an essential nutrient in human diets. Several enzymes are suggested for its biosynthesis (L-galactono-1,4-lactone dehydrogenase), oxidation (ascorbate oxidase, ascorbate peroxidase) and recycling (monodehydroascorbate reductase and dehydroascorbate reductase) especially during development and ripening of fruits (Luiza et al., 2008). Ascorbic acid ($C_6H_8O_6$) is a white, odourless, crystalline solid, high melting point (190-192 °C) and with sharp acidic taste. It has a density of 1.65 g/ml, pH 3 (5 mg/ml), 2 (50 mg/ml) and pK_1 and pK_2 of 4.17 and 11.57 respectively. It is polar and highly water

soluble (0.33 g/ml) but insoluble in fats and oils (Sanmartin et al., 2000). L-Ascorbic acid (L-AA) is very stable when dry but readily oxidises in solution especially in presence of metal ions.

Ionisation of ascorbic acid is enhanced by delocalisation of the π- electrons over the C_2-C_3 conjugated enediol system which stabilises the molecule (Sanmartin et al., 2000). The second ionisation, dissociation of the C_2 hydroxyl, is less favourable and results to the formation of L-dehydroascorbic acid (DHA). L-DHA exhibits approximately the same vitamin C activity as L-AA in humans and is almost completely reduced to L-AA in the human body (Fennema, 1996). Ascorbic acid is readily and reversibly oxidised to DHA which is present in aqueous media as a hydrated hemiketal (Figure 1.3). The stereoisomers of L-AA and L-DHA including L-isoascorbic acid, L-isodehydroascorbic acid, D-ascorbic acid and D-dehydroascorbic acid exist but have no vitamin C activity (Fennema, 1996). Oxidation of ascorbic acid takes places as either one-electron transfer processes or as a single two-electron reaction without detection of the semidehydroascorbate intermediate (Figure 1.3B). In one electron, the first step involves transfer of an electron to form the free radical semidehydroascorbic acid in equilibrium with its hemiketal form (Figure 1.3C). Loss of an additional electron yields DHA (Figure 1.3D) in equilibrium with hemiketal form (Figure 1.3E). DHA is highly unstable because of the susceptibility to irreversible hydrolysis of the lactone bridge resulting into the formation of 2, 3-diketogulonic acid (Figure 1.3F) which is responsible for the loss of vitamin C activity. The oxidation of ascorbic acid is catalysed by metal ions including copper (Cu^{2+}) and iron (Fe^{3+}). Heat and light accelerate the process, while pH, oxygen

concentration, and water activity strongly influence the rate of degradation (Fennema, 1996; Igual et al., 2010; Remini et al., 2015).

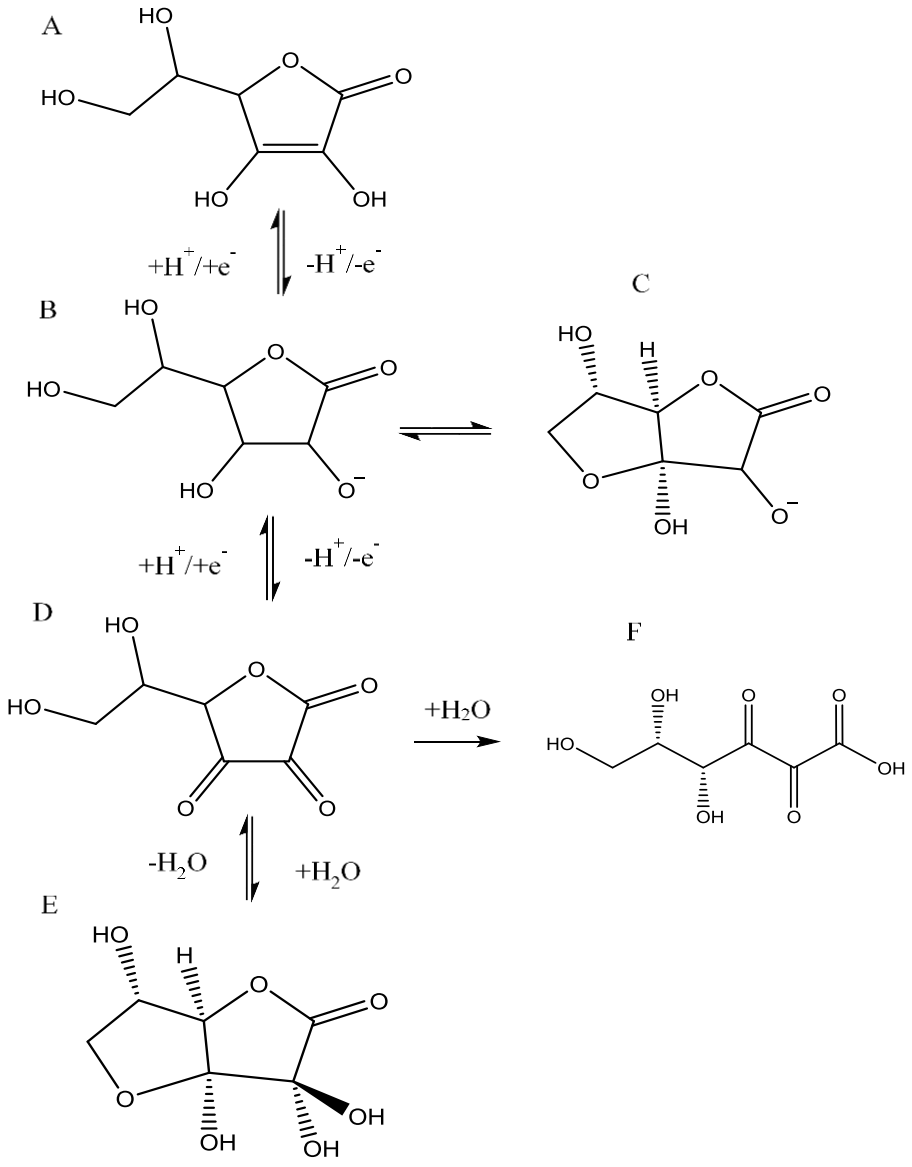


Figure 1.3- Ascorbic acid (A) and oxidation products including semidehydroascorbic acid (B) and its hemiketal form (C); dehydroascorbic acid (D) and its hydrated hemiketal form (E) and 2,3 diketogulonic acid (F) (Fennema, 1996; Belitz et al., 2009).

1.7.2 **Stability and mode of degradation in food products**

The high solubility of ascorbic acid (AA) in aqueous solutions is responsible for significant losses by leaching from fresh fruit and vegetable products. The degradation product of ascorbic acid (2, 3-diketogulonic acid) may further oxidise to form 2-furoic acid, 2-furaldehyde, 3-deoxypentosone and xylosone (Fennema, 1996). The oxidation and dehydration processes closely parallel dehydration reactions of sugars that lead to many unsaturated products and polymers. The primary factors affecting the rate, mechanism, and qualitative nature of AA degradation products include pH, oxygen concentration, and the presence of trace metal catalysts and degradative enzymes. Buettner (1988) reported that the rate of oxidative oxidation of AA is a nonlinear function of pH because the various ionic forms of the AA differ in their susceptibility to oxidation: fully protonated (AH_2) < ascorbate monoanion (AH^-) < ascorbate dianion (A^{2-}). Under conditions relevant to most foods, pH dependence of oxidation is governed mainly by the relative concentration of AH_2 and AH^- species, and is in turn, governed by pH (pK_{a1} = 4.04). The presence of significant concentration of the A^{2-} form, as controlled by pK_{a2} of 11.4 yields an increase in rate at pH ≥ 8 (Fennema, 1996).

The rate of oxidative degradation of AA is generally observed to fit first order reaction kinetics with respect to the concentration of the ascorbate monoanion (HA^-), molecular oxygen and the metal ions (Gamboa-Santos et al., 2014; Remini et al., 2015). Moreover in the absence of metal ions the rate of degradation of AA is believed to be very low at pH 7 (Buettner, 1988). Therefore trace metals in foods or experimental conditions are responsible for much of the oxidative degradation of ascorbic acid. It is also reported that

rate of metal catalysed oxidation of AA is proportional to the partial pressure of dissolved oxygen over the range of 1.0 to 0.4 atm and is independent of oxygen concentration at partial pressures below 0.2 atm (Khan and Martell, 1967). In contrast oxidation of AA catalysed by metal chelates is independent of oxygen concentration (Khan and Martell, 1967). The type of the metal ion affects the magnitude of metal-catalysed oxidation of AA. For instance Cu (II) has more influence than Fe (III) and the chelate of Fe (III) and ethylenediaminetetraacetic acid (EDTA) complex is 4 fold more than free Fe (III). The rate expression (1.1) of ascorbate oxidation can be presented as metal ion concentration and the k_{cat} for the metal ions can be used to estimate the rate of AA degradation in simple solutions, where [TA] is the concentration of total ascorbic acid.

$$-d\,[TA]/dt = k_{cat} \times [AH^{-}] \times [Cu\ (II) or\ Fe(III)] \quad \text{.....1.1}$$

The situation in real food samples may be different because trace metals may associate with other constituents including amino acids and may participate in other chemical reactions some of which may generate reactive free radicals that may hasten oxidation of ascorbic acid. The mechanism of AA degradation may differ depending on the nature of the food system or reaction medium. Metal catalysed degradation of AA has been proposed to occur through formation of a ternary complex of ascorbate monoanion, O_2, and metal ion. The ternary complex of ascorbate, oxygen, and metal catalyst appears to yield DHA as the product, without detectable formation of the product of one-electron oxidation, semidehydroascorbate radical (Khan and Martell, 1967).

The loss of vitamin C activity during oxidative degradation of AA through hydrolysis of DHA to yield DKGA is favoured by alkaline conditions otherwise DHA is most stable at pH 2.5- 5.5. The stability of DHA at pH > 5.5 is very poor, and becomes more so as pH increases. For instance the half-life of DHA hydrolysis at 23 °C is 100 and 230 min at pH 7.3 and 6.6 respectively. The rate of DHA hydrolysis markedly increases with increasing temperature but is unaffected by the presence or absence of oxygen. In view of the labile nature of DHA at neutral pH, analytical data showing significant quantities of DHA in foods should be viewed with caution because elevated DHA concentration may also reflect uncontrolled oxidation during analysis. On the other hand, Scarpa et al. (1983) reported that metal-catalysed oxidation of the ascorbate monoanion forms superoxide (O_2^-) in the rate determining step (1.2) and subsequent steps involving superoxide as a rate enhancer, effectively doubling the overall ascorbate oxidation (1.3 and 1.4).

$$AH^- + O_2 \xrightarrow{Catalyst} AH^{\cdot} + O_2^{-\cdot} \quad \text{(1.2)}$$

$$AH^- + O_2^{-\cdot} \xrightarrow{H^+} AH^{\cdot} + H_2O \quad \text{(1.3)}$$

$$AH^{\cdot} + O_2^{-\cdot} \xrightarrow{H^+} AH^{\cdot} + H_2O_2 \quad \text{(1.4)}$$

Termination also can occur through the reaction of two ascorbate radicals (1.5)

$$2AH^{\cdot} \xrightarrow{-H^+} DHA + AH^- \quad \text{(1.5)}$$

Anaerobic degradation of ascorbic acid is relatively insignificant as a means of loss of the vitamin in foods (Fennema, 1996). It may only become significant in canned products including vegetables and fruit juices after depletion of residual oxygen. However it is regarded as the most predominant mechanism for loss of AA during storage especially in dehydrated juices. The mechanism includes direct cleavage of the 1, 4-lactone bridge without prior oxidation to DHA and it is proposed that it may follow an enol-keto tautomerisation. In contrast to oxidative degradation of AA conditions anaerobic loss exhibits maximum rate at pH 3 - 4. In many food packages loss of ascorbic acid is attributed to both oxidative and anaerobic processes due to residual oxygen.

Products of ascorbic acid degradation participate in production of flavour compounds and as precursors of nonenzymatic browning reactions. The concentration of such compounds is largely influenced by temperature, pH, and water activity, concentration of oxygen, metal catalysts and presence of active oxygen species. In general three classes of degradative products include (i) polymerised intermediates, (ii) unsaturated carboxylic acids containing 5 and 6 carbon chains length and (iii) fragmentation products with 5 or fewer carbons. Some of these compounds contribute to change in flavour and odour that take place in citrus juices during storage or excessive processing (Fennema, 1996). Major products of AA breakdown products in neutral and acidic solution include L-xylosone, oxalic acid, L-threonic acid, tartaric acid, 2-furaldehyde (furfural), furoic acid and a wide range of carbonyl and other unsaturated compounds. DHA and dicarbonyls formed during degradation may participate in Strecker degradation with amino acids forming sorbanic

acid which can form dimers and polymers with reddish or yellowish colour (Fennema, 1996).

Apart from factors (oxygen, catalyst and pH) outlined affecting stability of AA, environmental factors including increased moisture content and water activity influences degradation of ascorbic acid. This may be attributed to increased availability of water to act as a solvent for reactants and catalysts. It has also been suggested that sugars (ketoses) including sucrose can increase the rate of anaerobic degradation of ascorbic acid while some sugars may protect against oxidative degradation of ascorbic acid by binding metal ions and reducing their catalytic potency (Fennema, 1996). It is therefore not surprising that high sugar concentration is widely used as a preservative.

1.7.3 **Functions of vitamin C**

Vitamin C or L-ascorbic acid is an electron donor (reducing agent or antioxidant), and probably all its biochemical and molecular roles can be accounted for by this function (WHO and FAO, 2004). In general three main types of biological activity in plants and humans can be defined for L-AA. It is an enzyme cofactor, radical scavenger and a donor/acceptor in electron transport either at the plasma membrane or in the chloroplasts (Luiza et al., 2008).

1.7.4 **Functions of vitamin C in foods**

Both L-isoascorbic acid and L-ascorbic acid are widely used as food ingredients because of their reducing and antioxidant activity especially inhibiting enzymatic browning in fruits and vegetables by reducing ortho-quinone products. Other functions include

protection of certain oxidisable compounds like folates by reductive effects, free radical scavenging, and oxygen scavenging and inhibiting nitrosamine formation in cured meats and reducing metal ions. The antioxidative role of ascorbic acid is multifunctional, with ascorbate inhibiting lipid autoxidation by several mechanisms including (i) scavenging singlet oxygen, (ii) reduction of oxygen and carbon-centred radicals, with formation of less reactive semidehydroascorbic acid radical or DHA, (iii) preferential oxidation of ascorbate, with concurrent depletion of oxygen and (iv) regeneration of other antioxidants, such as through reduction of the tocopherol radical (Fennema, 1996). In plants L-AA appears to be a substrate for oxalate and tartrate biosynthesis.

As enzymatic cofactor L-AA modulate a number of important enzymatic reactions in plants (Sanmartin et al., 2000). Enzymes that require L-AA as a cofactor or modulator of activities include 4-hydroxylphenylpyruvate dioxygenase, 1-aminocyclopropane-1-carboxylate oxidase and thioglucoside glucohydrolase for decarboxylation and hydroxylation of 4-hydroxylphenylpyruvic acid to homogenistic acid; oxidation of 1-aminocyclopropane to ethylene and cycloformic acid; and hydrolysis of S-glucosides respectively. The function of L-AA is to maintain the transition metal ion centres of these enzymes in reduced form, and L-AA is an important enhancer of activity (Sanmartin et al., 2000). During plant growth, ascorbate and ascorbate oxidase are linked to modulation of cell proliferation and elongation. Ideally ascorbate oxidase (AO) catalyses the oxidation of L-AA to semidehydroascorbic acid with the concomitant reduction of molecular oxygen to water. AO is linked to modulation of cell expansion and or cell division possibly via control of the oxidation status of L-AA/DHA redox pair (Sanmartin

et al., 2000). All these enzymatic activities are likely responsible for the loss of L-AA in many situations including during product development and storage. Controlling such activities may improve retention of L-AA in products.

As an antioxidant L-AA interacts both enzymatically and non-enzymatically with damaging radicals and their derivative reactive oxygen species (ROS). L-AA terminates free radical chain reactions by dispropotionation to non-toxic, non-radical products including DHA and 2, 3-diketogulonic acid. L-AA is only mildly electronegative and can donate electrons to wide range of substrate thereby preventing quality loss through oxidative degradation of nutritional attributes. Proper understanding of these functions is necessary for extensive comprehension of mechanisms leading to its loss in fresh fruits and derived products and may facilitate adaption of best practices for quality control during processing and storage.

1.7.5 **Functions of vitamin C in human metabolic processes**

L-Ascorbic acid is very important in the human body. Most of its biological properties are linked to its redox properties. L-AA status of humans influences many metabolic systems including lipid, steroid, and peptide metabolism, the immune system, endocrine function, control of blood pressure, iron and copper balance, haemostasis, endothelial function and fatty acid transport. L-AA scavenges reactive species within aqueous systems of the body, protecting protein and DNA from damage (Halliwell and Gutteridge, 1990; Wiseman and Halliwell, 1996; Iqbal et al., 2004). Vitamin C helps in the synthesis of collagen in connective tissues in the skin, cartilage, tendons, ligaments and vascular

system. Its antioxidant activity is essential in preventing damages due to free radical substances that destroy DNA; and has been reported to have a protective effect against cardiovascular diseases, common cold, cancer, osteoarthritis, age-related macular degeneration, pre-eclampsia, and asthma (Nakka et al., 2014). It is needed to maintain healthy teeth, bones, gums, cartilage, vertebrae discs, joint linings, skin and blood vessels. It also promotes the healing of cuts, abrasions and wounds (Harris, 1996), helps fight infection, and reduces the effects of oxidative stress. Both haemoglobin (Hb) and cell membranes appear to be the principal sites of damage induced to red blood cells upon exposure to oxidative stress. It is reported that L-AA may recycle vitamin E, the main lipid-soluble antioxidant that may prevent lipid peroxidation.

Ascorbic acid lessens the risk of developing high blood pressure and heart diseases, helps regulate cholesterol levels, prevent the development of scurvy, lower the risk of developing cataracts, aid iron absorption and reduce levels of load in the blood (Walingo, 2005). It thus has got a recommended daily intake, usually given in milligrams prescribed for all stages of life ranging from babies (25 mg) through children (30 mg) to pregnant (55 mg) and lactating women (70 mg) to smokers (FAO and WHO, 2004). The general recommended daily intake of vitamin C is 45 mg/day (FAO and WHO, 2004).

1.7.6 **Dietary sources of vitamin C and limitations to supply**

Ascorbic acid occurs naturally in fruits and vegetables and to some extent in animal tissues and animal derived products. Primates (including humans) have lost the ability to synthesize ascorbic acid and must obtain it in through diet. Citrus fruits and juices are

particularly rich sources of vitamin C but other fruits including cherries, kiwi fruits, mangoes and strawberries contain variable amounts of vitamin C (WHO and FAO, 2004). L-AA is a universal constituent of all green plants with the exception of dormant seeds and it is not difficult to obtain an adequate supply in the daily diet. It is reported that in developed countries deficiencies are usually associated with alcoholism and poverty (Sanmartin et al., 2000). In many developing countries, the supply is often determined by seasonal factors including availability of water and time and the short harvesting season for many fruits. The lack of technological advancement to process and preserve fresh fruits aggravates the situation. Vitamin C content of food is thus strongly influenced by season, variety, provenance, harvesting period, postharvest handling and processing practices (Tembo, 2008). Regularity of food intake is critical to maintain vitamin C requirement in the human body. In food, vitamin C occurs naturally almost exclusively in the reduced L-ascorbic acid form. The concentration of DHA found in foods is almost always substantially lower than AA and is a function of the rates of ascorbate oxidation and DHA hydrolysis (Fennema, 1996).

1.8 5-Hydroxymethyfurfural (HMF)

5-Hydroxymethylfurfural (HMF) is furanic compound and one of the decomposition products of ascorbic acid in fruit juices (Burdurlu et al., 2006). It is produced in orange juice from sugars by heating during processing and can cause browning reactions with amino compounds and sugars (Solomon et al., 1995). HMF is used as an index of heat treatment and deterioration in food such as fruit juices (Burdurlu et al., 2006).HMF formation is influenced by low pH and a high processing and storage temperature

(Ghaderi et al., 2015). Ascorbic acid is decomposed to furfural which is known to undergo polymerisation and, as an active aldehyde, may combine with amino acids and contribute to the browning and loss of juice quality (Davies and Wedzicha, 1994; Solomon et al., 1995). HMF could be metabolised to 5-sulfooxymethylfurfural making it potentially harmful (Arribas-Lorenzo and Morales, 2010). A level of 8.57mg HMF/day is considered for risk assessment. Thus fruit juices with high ascorbic acid may be associated with HMF or produced during processing and storage under desirable conditions of pH and temperature.

1.9 Flavan-3-ols

1.9.1 Structure and chemical properties

Flavan-3-ols are among the main sub classes of plant secondary metabolites (flavonoids) including flavonols, flavones, flavan-3-ols, anthocyanins, flavanones and isoflavones that have received growing interests because of their potential health beneficial activities (Harnly and Doherty, 2006; Aron and Kennedy, 2008; Khanal et al., 2009; Pugliese, 2013; Rio et al., 2013; Wojdyło et al., 2014). Flavan-3-ols are reported to be available in significant amount in baobab fruit products (Shahat, 2006).

Flavan-3-ols are the most complex subclass of flavonoids ranging from simple monomers to the oligomeric and polymeric proanthocyanidins (condensed tannins). Their monomeric skeletal structure comprises of 15 carbons, with two aromatic rings connected by a three carbon bridge, hence C6–C3–C6 (Figure 1.4). They are non-polar due to the saturated C3 element. The two chiral centres at C2 and C3 of the monomeric flavan-3-ol

(A) produce four isomers for each level of B-ring hydroxylation including (+)-catechin (B), (-)-epicatechin (C), (-)-catechin (D) and (+) – epicatechin (E) of which (+)-catechin and (-)-epicatechin are widely spread in nature (Figure 1.4) (Rio et al., 2013).

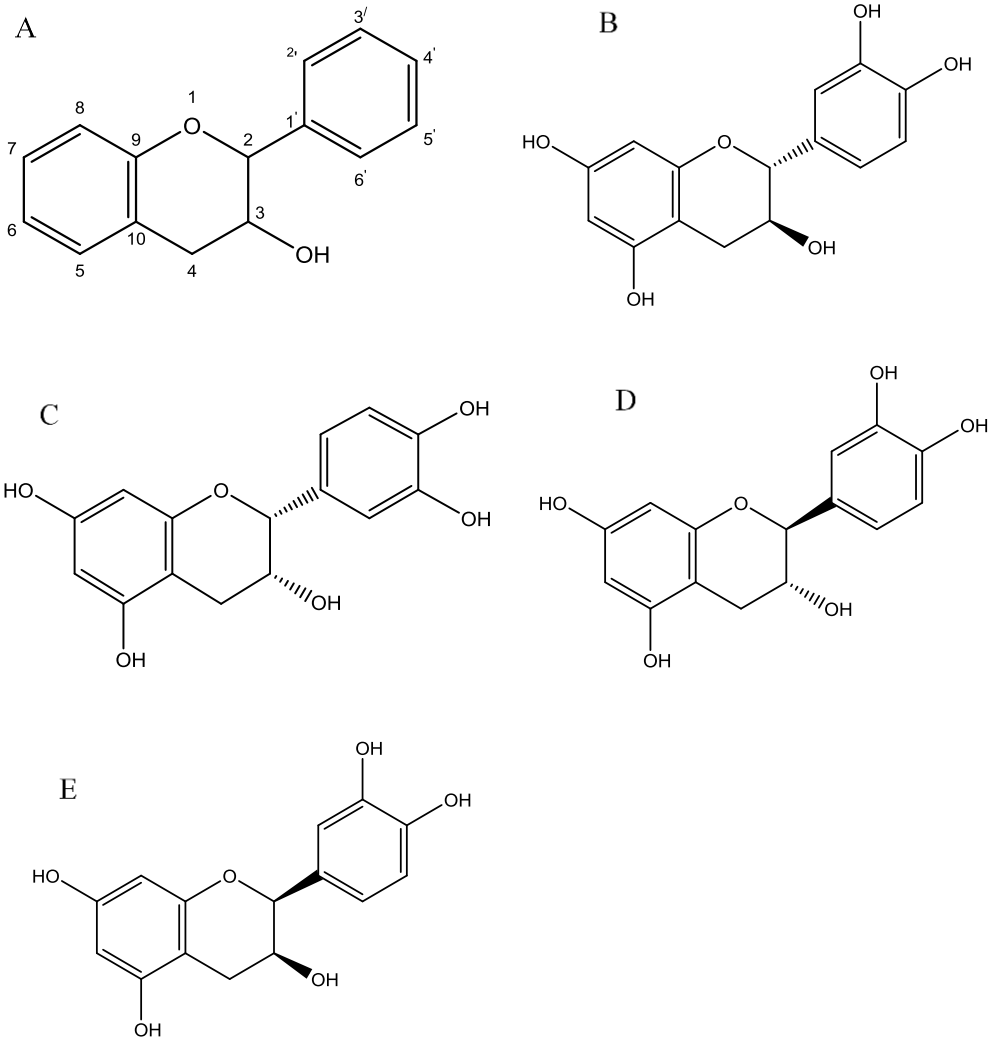


Figure 1.4-Flavan-3-ols skeleton (A) and monomeric structures of (+)-catechin (B) and isomers (-)-epicatechin (C), (-)-catechin (D) and (+)-epicatechin (E) (Crozier et al., 2009; Rio et al., 2013).

Figure 1.5 shows an example of products of hydroxylation and esterification of flavan-3-ols. (+)-Catechin and (-)-epicatechin can be hydroxylated on the B ring to (+)-gallocatechin (A) and (-)-epigallocatechin (B). Under suitable conditions, (-)-epicatechin and (-)-epigallocatechins may also undergo esterification with gallic acid to form (-)-epicatechin-3-O-gallate (C) and (-)-epigallocatechin-3-O-gallate (D) respectively (Figure 1.5)(Rio et al., 2013).

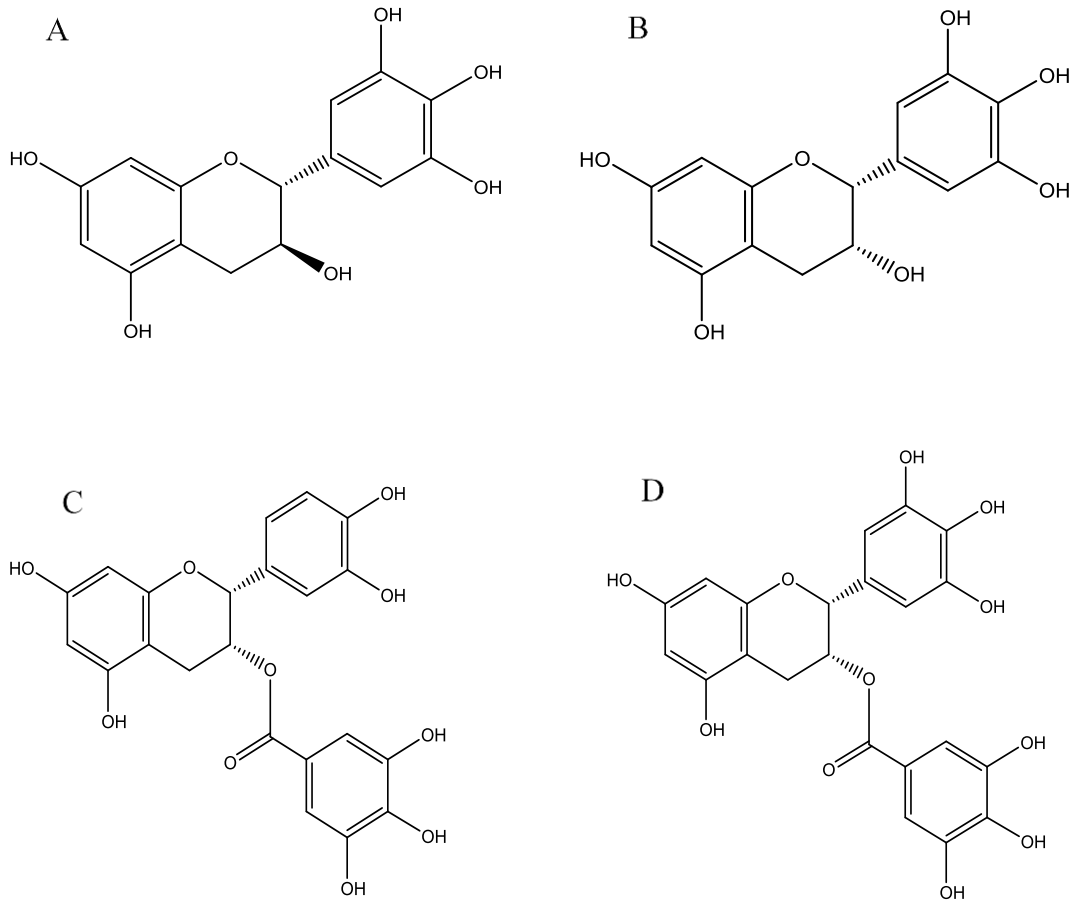


Figure 1.5 - (+)-Gallocatechin (A), (-)-epigallocatechin (B), (-)-epicatechin-3-O-gallate (C) and (-)-epigallocatechin-3-O-gallate (D) (Rio et al., 2013).

Polymerisation of monomeric flavan-3-ols is possible and may produce a series of structures including type A and B proanthocyanidins (Figure 1.6). Type B proanthocyanidins are formed from (+)-catechin and (-)-epicatechin with oxidative coupling occurring between the C4 of the heterocycle and the C6 or C8 positions of the adjacent unit to create oligomers or polymers. Dimers of this type include proanthocyanidin B1 (A), B2 (B) and B5 (C). Proanthocyanidins that consist exclusively of (-)-epicatechin units are called procyanidins and are the most common in plants (Crozier et al., 2009). Type A proanthocyanidins have an additional ether bond between C2 and C7 producing compounds including proanthocyanidin A2 dimer (D). Soluble proanthocyanidin polymers typically possess molecular weight (MW) averages of 1000-6000 Da, but sometimes MW of up to 20,000 Da do exist (Aron and Kennedy, 2008).

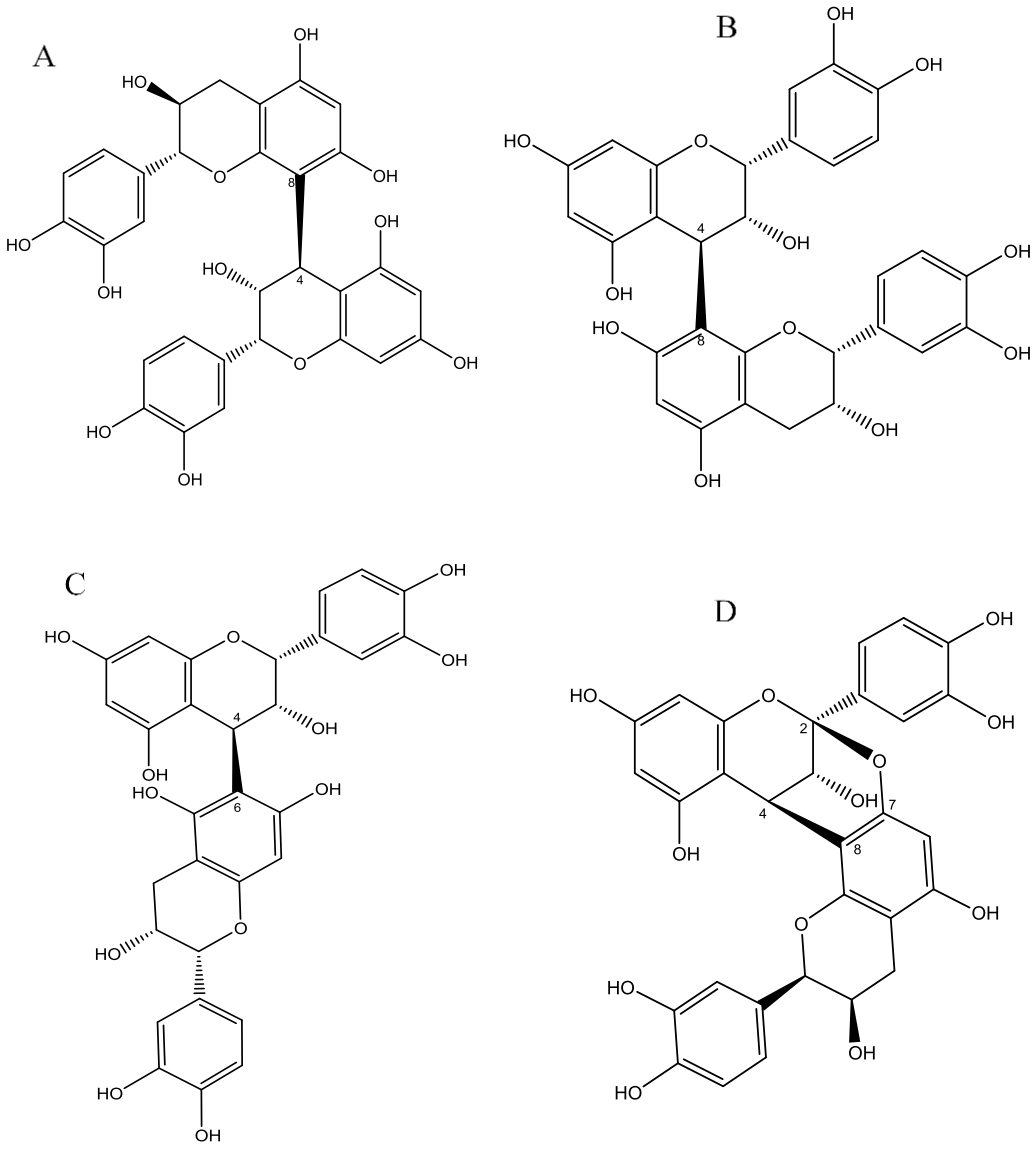


Figure 1.6-Polymerisation products of flavan-3-ols including proanthocyanidin B1 (A) procyanidin B2 (B), proanthocyanidin B5 (C) and proanthocyanidin A2 (D).

1.9.2 **Stability and mode of degradation of flavan-3-ols in foods**

The stability of flavan-3-ol content in food can be affected by many factors such as variety, environmental, food processing, and food storage conditions (Aron and Kennedy, 2008). Epimerisation, degradation and depolymerisation of oligomers and polymers are known to occur during food processing and storage producing a series of compounds described previously (Prior and Gu, 2005). Enzymes (polyphenol oxidase and peroxidase) and heat treatment may facilitate transformation of flavan-3-ols to a wide range of compounds including (-)-epigallocatechin-3-O-gallate, (-)-epicatechin-3-O-gallate, (+)-gallocatechin and (-) - epigallocatechin. For instance in tea the primary substrates for polyphenol oxidase are the flavan-3-ols which are converted to quinones which may be reduced back to phenols in the presence of gallic acid (Crozier et al., 2009). Therefore processing conditions that affect activity of enzymes will in essence influence the level of flavan-3-ols in the final product. Flavan-3-ols may also undergo esterification with hydroxycinnamates including p-coumaric acid or caffeic acid and with various levels of methylation. (-)-Epicatechin and (+)-catechin may epimerise during processing or storage to form (+)-epicatechin and (-)-catechin. Molecules such as (-)-epicatechin and (+)-epicatechin or (-)-catechin and (+)-catechin are nonidentical (enantiomers) because their mirror images cannot be superimposed and will rotate plane-polarised light differently (McMurry, 1992). Such organic molecules are said to be optically active and will usually have different chemical and biological properties. By convention rotation to the left is given a minus sign (-), and rotation to the right is given a plus sign (+) (McMurry, 1992). Transformation of type B to A procyanidin dimers has been reported in other fruits.

However the rate and extent of epimerisation may depend on a number of factors including composition, temperature, pH and matrix of plant foods (Chang et al., 2006). The degradation of the overall proanthocyanidins (PAs) and the depolymerisation of the higher oligomers and polymers appear to occur during food processing (Prior and Gu, 2005).

1.9.3 **Functions of flavan-3-ols in plants**

In plants flavan-3-ols have protective role against harmful intruders such as microbes, fungi, insects and herbivorous animals and harsh environment. Flavan-3-ols chelate metals such as iron and other essential minerals thereby limiting growth of invasive microorganisms by causing severe essential mineral-depletion. (–)-Epicatechin in plant tissue may also provide a similar resistance against fungal attack (Aron and Kennedy, 2008). In food products flavan-3-ols have shown a protective role against microbial and nutrition degradation (Donkor et al., 2014).

1.9.4 **Health benefits of flavan-3-ols**

Research studies have reported that dietary phenolic compounds including flavan-3-ols have several health benefits by acting as antioxidants through free radical scavenging, anti-viral and anti-HIV activities, transitional metal chelating properties as well as the mediation and inhibition of enzymes (Saito et al., 1998; Shahat et al., 1998; Prior and Cao, 2000; Kaur and Kapoor, 2001; Kris-Etherton et al., 2004; Aron and Kennedy, 2008; Fu et al., 2011; Luo et al., 2011; Haminiuk et al., 2012). Flavan-3-ols are good antioxidants because they easily donate electrons to free radical species and their derived

radical is generally more stable and less harmful than the initial radical species due to electron delocalisation.

Research studies indicate that proanthocyanidin monomers, dimers and hydroxylated metabolites including phenolic acids are absorbed into human plasma and may be associated with improved vascular health effects after short or long-term consumption of proanthocyanidins or foods and supplements containing them. These effects may include vasodilation, decreased platelet aggregation, reduced sensitivity of low-density lipoproteins (LDL) to oxidization, and modulation of several reactions associated with inflammation (Beecher, 2004; Wang et al., 2016). Further, results from a variety of experiments indicate proanthocyanidins may modulate several reactions involved in cancer processes (Beecher, 2004). *In vivo*, increased consumption of flavan-3-ol procyanidins has been implicated in improved antioxidant status and decreased DNA damage in humans (Simonetti et al., 2002; Rasmussen et al., 2005). Engler et al.(2004) reported that in a randomized, double-blind, placebo-controlled study conducted over a 2 week period with 21 healthy adult subjects, consumption of flavonoid-rich dark chocolate improved endothelial function and was associated with an increase in plasma epicatechin concentrations in healthy adults relative to intake of a low-flavonoid dark chocolate bar. Several research studies report that the biological effects of flavan-3-ols including procyanidins are generally attributed to their more readily absorbed colonic breakdown products, the phenolic acids (Williams et al., 2004; Crozier et al., 2009; Stoupi et al., 2010). Several *in vitro* and *in vivo* studies claim that proanthocyanidins may have protective effects against cancer in humans and other animals. For instance, Prior and Gu

(2005) reported that grape seed procyanidin extract (GSPE) exerted a chemopreventive effect in an animal model of breast cancer, therefore suggesting that diet may have great influence on anticarcinogenicity. Positive effects were also observed in mice where feeding with varying amounts of GSPE showed preventive effects against indicators of cancer including photocarcinogenesis.

1.9.5 **Dietary sources of flavan-3-ols**

Plants contain different amounts of monomeric, oligomeric and polymeric flavan-3-ols. In particular green tea and trees from genus *Cinnamomum* are widely reported to contain exceptionally high levels of flavan-3-ols (Gu et al., 2004). Common unprocessed foods containing high amounts of flavan-3-ols include fruits (choke berry > cranberry > blue berry > plum > black currant > strawberry > apple), cereals and beans (sorghum > cocoa > beans) and nuts (hazel nuts > pecan > pistachios > almond) (Beecher, 2004; Prior and Gu, 2005; Han et al., 2007; Aron and Kennedy, 2008; Crozier et al., 2009). Products including beverages (grape juice > red wine > beer), spices (ground cinnamon > curry), chocolate and dried grape seeds also contain significant amount of flavan-3-ols. These common fresh and processed foods and beverages contain different proportions of flavan-3-ols. For instance green tea contains highest levels of (-)-epicatechin and (+)-catechin monomers and transformation derivatives such as gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-O-gallate (ECG) and (-)-epigallocatechin-3-O-gallate (EGCG)(Crozier et al., 2009). Shahat (2006) reported that the pericarp of baobab fruit contains proanthocyanidins including monomeric (-)-epicatechin and procyanidin

B2. However the actual levels of these bioactive compounds in baobab fruit were not indicated.

1.10 Factors affecting quality attributes of fruit products

Nutritional and sensory properties of fruit products are affected by several factors including species, ripening stage at harvest, harvesting season, processing techniques and storage condition (Kaur and Kapoor, 2001; Tembo, 2008; Chen et al., 2013). Plant cell turgor pressure declines during ripening and changes during processing and this affects rigidity and firmness of final product. Similarly colour is imparted to plant tissues by a number of lipid-soluble and water-soluble pigments including phenolics and anthocyanins located either in the acidic plant cell vacuole or in the cytoplasm. Loss of compartmentalisation, due to normal senescence or processing-induced changes including homogenisation may result in interaction of enzymes and substrates (lycopene and β-carotene) that affect colour (Gonzalez and Barrett, 2010). A number of plant tissues have distinct flavour or aroma but are perceived only after loss of compartmentalisation during processing. Off-flavour production and an environment for microbial growth have also been associated with loss of compartmentalisation (Barry-Ryan and O'Beirne, 2000; Paull and Chen, 2000). Compartmentalisation increases exposure to oxygen and subsequent loss of vitamins and minerals as well as phytonutrients including phenolics, glycosinolates and carotenoids.

1.10.1 **Effect of processing**

Major emphasis of food processing is preservation or shelf life extension by preventing undesirable changes in the wholesomeness, nutritive value, and sensory qualities. This is completed by controlling chemical, biochemical, physiological and microbiological activities using various techniques including removal and addition of heat, removal of moisture, controlling water activity, addition of preservatives, sugar, salt and acids (Ramaswamy and Marcotte, 2006). Preservation techniques may be categorised into thermal, non-thermal, chemical and their combinations. Uncontrolled processing techniques may lead to loss of desirable quality characteristics.

In this study, thermal processing and the use of natural preservatives will be considered because they are affordable and common approaches used for the preservation of baobab fruit juice in Malawi. Non-thermal pasteurisation methods such as high hydrostatic pressure (HHP), high pressure homogenisation (HPH), pulsed electric field (PEF) and ultrasound (US) have greater potential of providing fresh-like and safe fruit juices, but are very expensive and not affordable in developing countries. The effects of processing on the stability of the bioactive compounds in foods vary across treatments thus the choice of processing method for a particular food products is very important for quality control (Nora et al., 2014).

1.10.1.1 **Thermal processing: Roasting, pasteurisation, sterilisation, blanching and microwaving**

Thermal processing may facilitate preservation and product diversification. Preservation aims at eliminating microbial pathogens that endanger public health and microorganisms and enzymes that deteriorate food during storage (Igual et al., 2010). In both cases safe products with specific quality attributes and extended shelf life are sought. Such conditions are unfortunately not uniform for all fruit species due to differences in composition, matrices or acidity. Heat processes include roasting, pasteurisation, sterilisation, microwaving and blanching. These processes have a wide range of effects on vitamins, phenolic compounds (Sommano et al., 2013; Gamboa-Santos et al., 2014) that may not be generalised.

Several authors reported that thermal processing increased antioxidant activity and total phenol content due to formation of new compounds such as Maillard reaction products (MRPs) (Kim et al., 2011). On the other hand Chandrasekara and Shahidi (2011) reported that extracts of roasted cashew nut showed considerable antioxidant efficiency in model system, however the effect was not significantly ($p \leq 0.05$) different from raw counterparts. Most reports indicate that the physical condition of plant material also matters (Parada and Aguilera, 2007; Aron and Kennedy, 2008).

Pasteurisation is a mild heat treatment given to foods, with the purpose of destroying selected vegetative microbial species especially pathogens or inactivating the enzymes. Thermal pasteurisation is widely used in fruit juice and is based on 5 log reduction of the most resistant micro-organisms of public health significance (Chen et al., 2013). Two

types of thermal pasteurisation exist including low temperature long time (LTLT) and high temperature short time (HTST) (Chen et al., 2013). Thermal pasteurisation may cause degradation of vitamins and some phenolic compounds in juices. The efficacy of HTST treatment can also be affected by complexity of the product and microorganisms. Therefore conditions effective for one fruit product may not apply to all (Irina and Mohamed, 2012). The severity of heat treatment and shelf life depends on nature of product, pH, types of microorganism or enzyme and type of heat application (Ramaswamy and Marcotte, 2006). For instance orange juice is normally pasteurised at 80 °C, for 30 s in industry (Polydera et al., 2003) in order to preserve bioactive compounds and antioxidant activity.

Skrede et al. (2000) reported that polyphenolic and anthocyanin losses in blueberries were relatively low when pasteurised single-strength juice was concentrated, except for the procyanidins, which showed marked reduction. Thermal treatment of aqueous model solution of flavonols quercetin and rutin showed different rates of degradation and were also influenced by pH (Buchner et al., 2006). Similarly, the level of bioactive compounds of fruit smoothies were affected differently with thermal and high hydrostatic processing (Keenan et al., 2011). While the level of vitamin C decreases during thermal processing of most fruit and vegetable products (Igual et al., 2010; Gamboa-Santos et al., 2014), an increase in antioxidant activity due to release of membrane bound phenolic compounds was reported (Leong and Oey, 2012). Moreover thermally treated products may be stable during storage due to inactivation of degradative enzymes such as ascorbic acid oxidase, polyphenol oxidase and peroxidase (Awuah et al., 2007; Igual et al., 2010).

Blanching is a mild heat treatment used to inactivate the oxidative enzymes in fruits and vegetables, and given prior to further processing which otherwise will result in undesirable changes in colour, flavor, and nutritive value of the product during handling and storage (Ramaswamy and Marcotte, 2006).

Sterilisation is the application of more severe heat treatment intended to destroy microorganisms of both spoilage and public health concern, after packaging the food in a hermetically sealed container. It involves exposing food to a high enough temperature for sufficiently long time to render them commercially sterile. It takes into account heat resistance of spore formers in addition to their growth sensitivity to oxygen, pH and temperature. Low acid foods that support their growth are processed at elevated temperature (115 °C to 125 °C) whereas acidic foods can be processed at 100 °C or lower.

Microwaving has a wide range of applications in food processing including reheating, cooking, roasting and crisping. Heat production is achieved by molecular friction of water molecules (Ramaswamy and Marcotte, 2006). The technique is efficient, fast and retains nutritional attributes in the final product. For instance, Igual et al. (2010) reported that microwave pasteurisation retained more ascorbic acid, vitamin C, total phenol content and antioxidant activity in grapefruit juice compared to conventional pasteurisation.

1.10.2 **Effect of preservatives on quality of fruit products**

Apart from thermal pasteurisation, fruit products are often treated with permitted chemical additives for the extension of shelf-life of fruit juices. These suppress activity of oxidative enzymes such as polyphenol oxidases by altering their substrate or limit

availability of oxygen for microbial growth. The commonly used preservatives in fruit juices are potassium sorbate and sodium benzoate (Aneja et al., 2014). However, there is greater demand for the use natural antimicrobials that are safe and environmentally friendly. Natural antimicrobials such as ascorbic acid, citric acid, malic acid and cinnamon bark oil are commonly used in foods as acidulant and preservative (Mosqueda-Melgar, 2012). Ascorbic acid and organic acids prevent deterioration of food caused by oxidation, enzymatic and microbiological activity. Organic acids may therefore be used to reduce quality loss in fruit products thereby extending shelf life. Moreover ascorbic acid has health benefits through its antioxidant property. Citric acid in combination with pasteurisation has been successfully used in apple, pear, mango, orange and strawberry juices and retained bioactive compounds and extended microbiological shelf life (Mosqueda-Melgar et al., 2008; Robles-Sánchez, 2009).

1.10.3 **Effect of storage condition**

Fruit juice undergoes a number of deteriorative reactions such as ascorbic acid degradation, microbial spoilage and change in organoleptic properties. The rate of degradation of quality attributes depends on storage time and temperature, packaging and the processing method employed (Polydera et al., 2003). Common storage conditions include refrigeration and freezing. Refrigerated foods are fresher because they are subjected to minimal processing and do not induce significant structural changes but have a short shelf life (Ramaswamy and Marcotte, 2006). Pasteurised products need to be stored under conditions of refrigeration or special environment with or without additives because the process does not eliminate all vegetative bacteria. However the change in

quality attributes may also depend on species, type of bioactive compound as well as treatment received prior to storage. Longer storage time and higher temperature influence loss of ascorbic acid. For instance, Remini et al. (2015) reported that 20% of the initial ascorbic acid was lost after 28 days of storage of pasteurised blood orange juice at 4 °C and a complete degradation was observed at higher storage temperatures. The loss of ascorbic acid in pasteurised juice during storage is likely attributed to non-enzymatic pathways since enzymatic degradation is eliminated during processing (Burdurlu and Karadeniz, 2003). Igual et al. (2010) reported that frozen non-treated and conventional pasteurised grapefruit juice served about 75% and 20% of the total phenols and antioxidant capacity, respectively whilst in frozen microwave pasteurised juices this preservation was of 82 and 33%. Similarly other authors (Tavarini et al., 2008) reported that the level of total phenol content in Hayward kiwifruit did not change significantly during storage at 0 °C for 2 months but observed a significant increase after a long storage (6 months). During 6 months storage of two brands of commercial orange juice at 18, 28 and 38 °C, the level of free caffeic acid decreased significantly, while the concentration of ρ-coumaric and ferulic acids were increasing under the same storage conditions (Klimczak et al., 2007).Total phenol content decreased by 7, 11 and 20 % respectively during the first 4 months but a significant increase was observed at the end of storage (6 months) (Klimczak et al., 2007).

Fluctuation in the level of organic compounds, total phenol content and antioxidant activity of commonly consumed fruit juices (black currant, cherry, cranberry, pomegranate and strawberry) during storage has been widely reported and attributed to

both enzymatic and non-enzymatic chemical reactions (Piljac-Žegarac et al., 2009; Igual et al., 2010). Consumption of juices within 48 hours of opening was encouraged for potential health benefit (Piljac-Žegarac et al., 2009). Degradation kinetics enables accurate prediction of quality loss and shelf-life determination of juice at a specific temperature. Successful degradation kinetics models are important for quality control and shelf-life estimation of foods. Data may help to improve processing and preservation techniques employed to food samples. Degradation of ascorbic acid or anthocyanins in orange and strawberry juice best fit first order reaction kinetic models (Derossi et al., 2010; Remini et al., 2015). This suggests that degradation only depends on concentration of ascorbic acid and anthocyanins in orange and strawberry juice respectively.

1.11 Standard analytical methods and techniques of chemical analyses

Characterisation of plant foods including changes during processing and storage is important for quality control. There are many standard methods and techniques of quantitative chemical analysis with varying degree of sensitivity, selectivity and accuracy. In this study RP-HPLC, ^{1}H NMR spectroscopy and spectrophotometry were used and described in chapter 2.

1.11.1 **Vitamin C and organic acid analysis**

The assessment of vitamin C (AA & DHA) and organic acids in plant materials involves extraction and determination using different methods and techniques. Ascorbic acid is readily oxidised under alkaline conditions and in the presence of degradative enzymes

and therefore extraction is done under acidic conditions. This is necessary to supress metabolic activities upon disruption of the cells and to precipitate proteins thereby fixing the AA/DHA equilibrium (Sanmartin et al., 2000). The amount of ascorbic acid in the extract may therefore depend on physicochemical properties of extracting solvent (concentration, pH and temperature) and extraction time which need to be optimised to ensure high yield and more accurate results. For less acidic plant materials solvent systems with a higher ionic strength are recommended (Tembo, 2008). Most researchers recommend the use of metaphosphoric acid (MPA) because it gives high stability and recoveries of ascorbic acid. Because both ascorbic acid (AA) and dehydroascorbic acid (DHA) possess vitamin C property it is easier to determine the sum of these through reduction of dehydroascorbic using appropriate reducing agent such as 2-carboxy ethyl phosphine hydrochloride (TCEP) (Chebrolu et al., 2012). However it may be necessary to optimise reduction of DHA into AA in the extract (Figure 1.7) before analysis in order to increase the yield (total vitamin C).

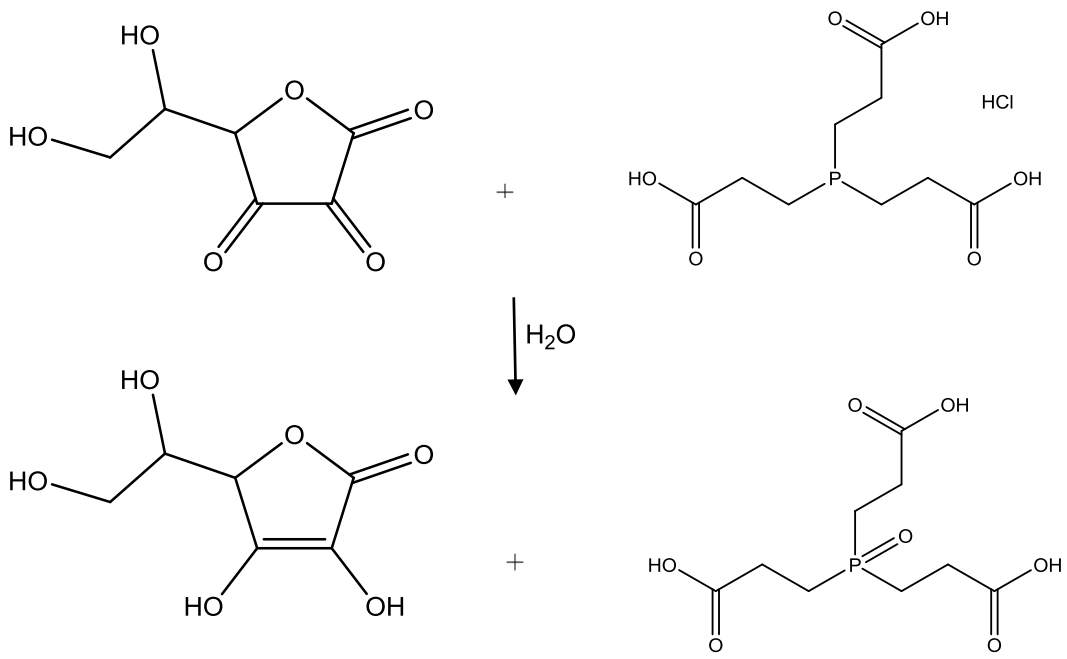


Figure 1.7-Reduction of dehydroascorbic acid to ascorbic acid using TCEP

The most common methods and techniques used for determination of ascorbic acid are 2, 6-dichlorophenol-indophenol (DCIP) dye titrimetric and high-performance liquid chromatography (HPLC). Although the DCIP titrimetric method is simple and cheap it is challenging to determine the titration end point and this may lead to overestimation or underestimation of ascorbic acid. Further, the extract is exposed to air and light which may lead to a loss of ascorbic acid before analysis. A more reliable reversed phase HPLC method with UV detection is commonly used in developed countries for simultaneous determination of vitamin C and organic acid under isocratic or gradient elution. HPLC has several advantages over DCIP-titrimetric methods: it avoids the problems of non-

specific interference, and the analysis is highly sensitive and selective. However the method is not affordable for general laboratory as it requires expensive equipment.

1.11.2 ^{1}H NMR food metabolomics

Metabolomics is a holistic study aimed at identifying and quantifying metabolites in biological system (Mannina et al., 2012; Piras et al., 2013; Marincola et al., 2015; Trimigno et al., 2015). Plant foods contain a complex mixture of metabolites in terms of structure, chemical properties and concentration specific to species. Many components are already present in the crude products whereas others can arise during storage and processing. Understanding the biochemical response to postharvest treatments is of great importance for the selection of better processing and storage conditions (Oms-Oliu et al., 2013). There are several analytical platforms for carrying out metabolic studies including nuclear magnetic resonance (NMR), gas chromatography mass spectroscopy (GC-MS) and liquid chromatography mass spectroscopy (LC-MS) (Sotelo and Slupsky, 2013). In metabolomics, NMR is recognised as one of the main analytical methodologies giving a complete view of the foodstuffs metabolites and, together with a suitable statistical analysis, providing relevant results in terms of foodstuffs quality, processing, raw material, safety and so on (Mannina et al., 2012; Oms-Oliu et al., 2013). For instance, De Oliveira et al. (2014) followed degradation of orange juice during storage using ^{1}H NMR technique and chemometric tools. Metabolites including formic, fumaric acid and acetic acids were identified; and an increase in the level of succinic and lactic acids and ethanol with storage time and temperature was reported (De Oliveira et al., 2014). Further, NMR

spectroscopy allowed metabolic fingerprinting of mature grape fruit berries, raw mullet roes and green tea (Le Gall et al., 2004; Pereira et al., 2005; Piras et al., 2014).

1.12 Problem statement and justification

Most of the rural communities in Malawi are chronically malnourished yet there is a widespread availability of indigenous fruits which can help to reduce the situation if fully exploited. There is lack of sufficient data on the nutritive values and functional attributes of most indigenous fruit products including *Adansonia digitata* available on the market of Malawi. Processing and preservation conditions affect the level of bioactive compounds including vitamin C, phenolic compounds, antioxidant activity and metabolic profile of fruit products. However, changes in quality attributes as a result of processing and preservation conditions are different due to other factors such as species and environmental conditions. There is sufficient knowledge and information on the influence of processing and storage on quality attributes of commonly consumed fruits including oranges, apples and grapes but such information is scarce for most indigenous fruits of Malawi.

1.13 Aims and objectives of the study

The aim of this research was to optimise thermal processing and investigate the influence of storage on selected bioactive compounds and metabolic profile of baobab fruit juice from Malawi. The specific objectives of the study were five-fold:

i. Optimisation of ascorbic acid and total phenolics extraction and determination,

ii. Quantification of ascorbic acid, vitamin C (AA + DHA), organic acids, phenolic compounds, caffeine, 5-hydroxymethylfurfural and antioxidant activity of fresh baobab pulp and juice,

iii. Influence of thermal treatment on vitamin C, phenolic compounds and antioxidant activity,

iv. Influence of storage temperature and time on ascorbic acid, vitamin C, organic acids phenolic compounds, antioxidant activity and metabolic profile and

v. Influence of seed liquor on vitamin C, phenolic compounds and antioxidant activity of baobab juice.

Table 1.1 provides a summary of all quality attributes analysed in baobab pulp and juice. Metabolic profile include amino acids (alanine, arginine, histidine, isoleucine, leucine, threonine and valine), organic acids (acetic, ascorbic, citric, formic, fumaric, malic, lactic and succinic acid), carbohydrates (glucose, ethanol, fructose, methanol and sucrose), phenolics ((-)-epicatechin, ferulic acid, gallic acid and N-phenylacetylphenylalanine) and caffeine.

Table 1.1 A summary of quality attributes analysed in baobab pulp and juice from Malawi

Organic acids	**Phenolic compounds**	**Metabolic profile**
Ascorbic acid	(-)-Epicatechin	Amino acids
Dehydroascorbic acid	Procyanidin B2	Organic acids
Vitamin C	(-)-Epigallocatechin-3-O-gallate	Carbohydrates
Citric acid	Gallic acid	Phenolics
Malic acid	**Others**	Caffeine
Tartaric acid	5-Hydroxymethylfurfural	
	Total phenol content	
	Antioxidant activity	

Chapter 2 **Materials and Methods**

In this chapter chemicals and reagents, fruit samples, preparation of required solutions and reagents, general equipment, general analytical methods and techniques are described. Specific methods and techniques are described in individual chapters.

2.1 Chemicals and Reagents

Only chemicals of analytical and HPLC grade were used. Standards (ascorbic acid, citric acid, dehydroascorbic acid, malic acid, tartaric acid, gallic acid, (+)-catechin, (-)-epicatechin, procyanidin B2, (-)-epigallocatechin-3-O-gallate, caffeine, 5-hydroxymethylfurfural), formic acid, sodium carbonate, iron (III) chloride anhydrous, hydrochloric acid and sodium acetate trihydrate were purchased from Fisher Scientific (UK); Folin-Ciocalteu phenol reagent, potassium persulphate, potassium dihydrogen phosphate (KH_2PO_4), ferric chloride hexahydrate, sodium acetate trihydrate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2–diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris-2-pyridyl-s-triazine (TPTZ) and metaphosphoric acid (HPO_3) were from Sigma (USA); deuterium oxide (D_2O, 99.9%), concentrated potassium deuteroxide (KOD) and sodium 3-trimethylsilyl-propionate-2, 2, 3, 3,-d4 (TSP, 98 atom% D) were acquired from Sigma-Aldrich (Milan, Italy); 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was from Calbiochem (Germany); acetonitrile, ethanol and methanol were from BDH chemicals (UK). All other chemicals and reagents were of analytical grade.

2.2 Baobab fruits and commercial baobab juice

Fresh fruits of baobab *(Adansonia digitata)* were collected at the peak of harvesting season (May and June 2013) from different sites in Malawi including Chikwawa, Machinga, Balaka, and Mangochi districts (Figure 2.1). Fruits from different sites were kept separately in sack cloth and transported by air at ambient conditions to the School of Food Science and Nutrition, University of Leeds, for pretreatment, processing and subsequent analysis. Only undamaged fruits with no signs of infection such as discolouration or rotting were selected for the study. Commercial baobab juice (CBJ) samples were purchased from Shoprite supermarket (Lilongwe, Malawi) in September, 2014 and deep frozen (-20 ºC) immediately after arrival (within 24 hours).

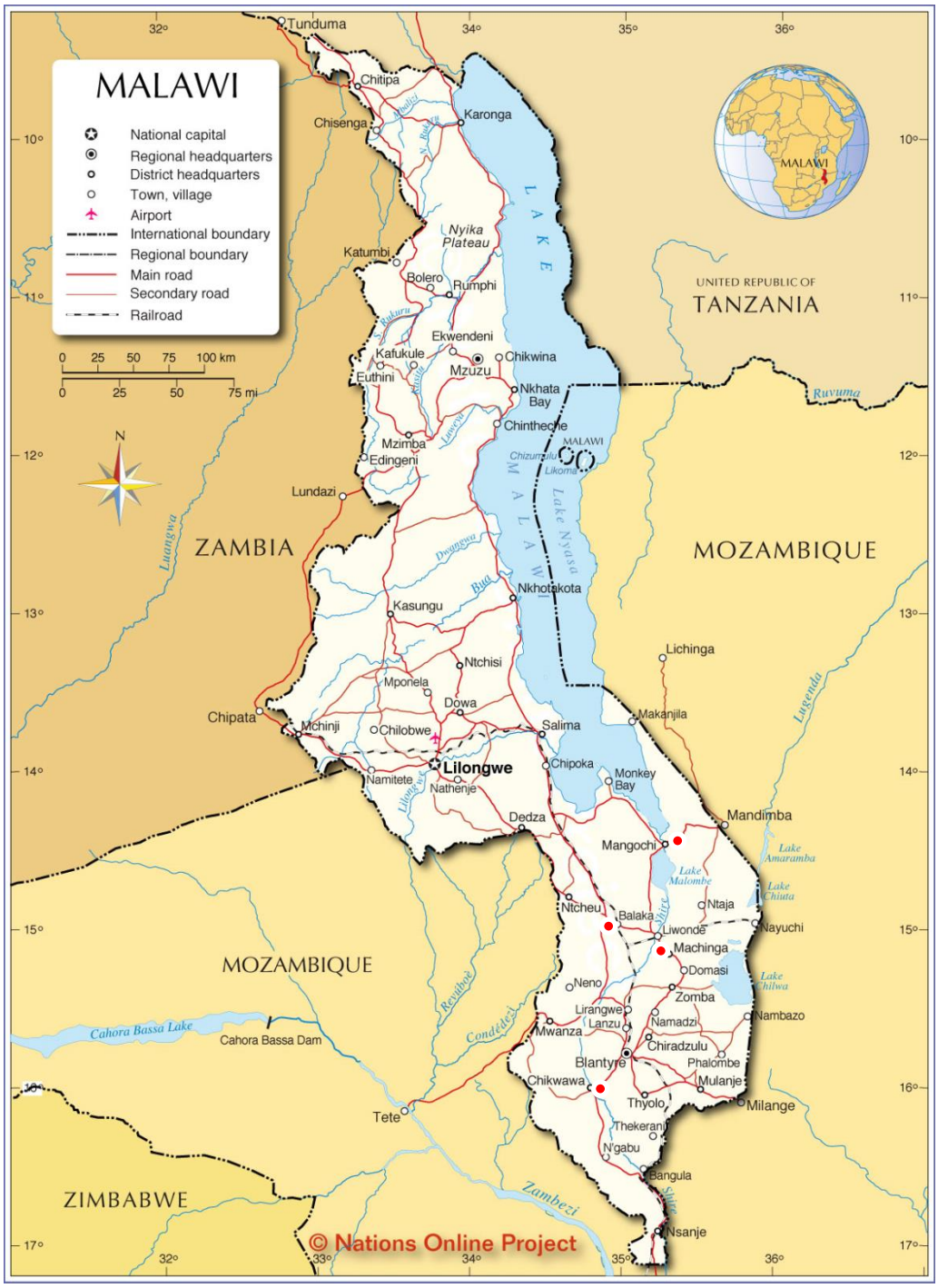


Figure 2.1 - Map of Malawi including sites (Balaka, Chikwawa, Machinga and Mangochi districts) where baobab fresh fruits were collected (●)(Nations Online, 2016).

2.3 Preparation of required solutions and reagents

General solutions used for experimentation are presented. Aqueous solutions were prepared using deionised water.

2.3.1 **Metaphosphoric acid (HPO_3) (3 g/100 mL)**

HPO_3 chips (60%, 15 g) were dissolved in deionised water (200 mL) and diluted to 500 mL in a volumetric flask.

2.3.2 **2-Carboxy ethyl phosphine hydrochloride (TCEP) (10 mM)**

TCEP (> 98%, 286.6 mg) was dissolved in deionised water and diluted to 100 mL in a volumetric flask.

2.3.3 **Potassium dihydrogen orthophosphate buffer (10 mM, pH 2.6)**

Concentrated orthophosphoric acid (H_3PO_4) (85%, 680 µL) and KH_2PO_4 (99.5%, 1367.74 mg) were dissolved in 200 mL deionised water and diluted to 2 L in a volumetric flask. The solution was degassed using a vacuum box for 1 h before use in the HPLC.

2.3.4 **Methanol (80%, v/v)**

Methanol (99.9%, 800 mL) was diluted to 1 L in a volumetric flask.

2.3.5 **Sodium carbonate solution (20%, w/v)**

Sodium carbonate anhydrous (99.9%, 200 g) was dissolved in deionised water and diluted to 1 L in a volumetric flask. The solution was immediately covered with aluminium foil and left at ambient temperature.

2.3.6 **Folin-Ciocalteu's reagent (FCR)**

Folin-Ciocalteu phenol reagent (2 N) was diluted 10 fold with deionised water in amber bottle prior to analysis of total phenol content. FCR (0.2 N, 1.5 L) was used in the assay.

2.3.7 **Hydrochloric acid (HCl) (40 mM)**

Concentrated HCl (36%, 343 µL) was diluted to 100 mL with deionised water in a volumetric flask.

2.3.8 **2, 4, 6-Tris-2-pyridyl-s-triazine (TPTZ) (10 mM)**

TPTZ (> 98%, 312.34 mg) was dissolved in 100 mL HCl (40 mM) in a volumetric flask.

2.3.9 **Sodium acetate buffer (300 mM, pH 3.6)**

Sodium acetate trihydrate ($C_2H_3Na.3H_2O$) (99.5%, 0.31 g) was dissolved in glacial acetic acid (16 mL) and diluted with deionised water to 1 L in a volumetric flask.

2.3.10 **Ferric chloride hexahydrate ($FeCl_3.6H_2O$) (20 mM)**

$FeCl_3.6H_2O$ (> 99%, 0.535 g) was dissolved in deionised water and diluted to 100 mL in a volumetric flask.

2.3.11 **FRAP reagent**

Sodium acetate buffer (300 mM, pH 3.6), TPTZ (10 mM) and ferric chloride hexahydrate (20 mM) were mixed in the ratio 10:1:1 (Xu et al., 2008).

2.3.12 **2, 2–Diphenyl-1-picrylhydrazyl (DPPH) (0.10 mM)**

DPPH (99%, 7.886 mg) was dissolved in 100 mL methanol (99.9%) in a volumetric flask.

2.3.13 **2, 2′-Azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (7.0 mM)**

ABTS, $C_{18}H_{24}N_6O_6S_4$ (99%, 384.03 mg) was dissolved in 100 mL of deionised water in a volumetric flask.

2.3.14 **Potassium persulfate ($K_2O_8S_2$) (140 mM)**

$K_2O_8S_2$ (99.99%, 946.127 mg) was dissolved in 25 mL of deionised water in a volumetric flask.

2.3.15 **$ABTS^{\cdot+}$ radical generation**

$K_2O_8S_2$ (140 mM, 1.76 mL) was added to ABTS solution (7.0 mM, 100 mL) and the mixture was kept in the dark at 25 °C for 16 h. The resultant solution was diluted 85 fold with deionised water to give an absorbance of 0.70 ± 0.02 at 734 nm in a Cecil CE3021 3000 Series Spectrophotometer.

2.3.16 **Phosphate buffer (0.5 M, pH 7.35)**

KH_2PO_4 (99.98%, 1.700 g) was dissolved in deuterated water (D_2O, 24.36 mL) and volume made up to 25 mL in a volumetric flask using concentrated KOD (640 μL).

2.3.17 **Sodium 3-trimethylsilyl-propionate-2, 2, 3, 3,-d_4 (TSP, 98 atom% D, 10 mM)**

TSP (99.98%, 0.01723 g) was dissolved in deuterated water (10 mL) in a volumetric flask.

2.4 Equipment

2.4.1 **General Equipment**

A list of general equipment used includes:

- Baumatic BC190.2TCSS 90 cm Gas Range Cooker – 365 Electrical (Leicester, UK)
- Plate heat pasteuriser P20-HB – Alfa Laval (Sweden) coupled with a level-controlled tank balance, Gryphon B.S 2048 centrifugal pump (Brook Motors Limited, Huddersfield, UK), Grant water bath (Grant Instruments Limited, Cambridge, UK) and an RCU6 Chiller unit (5.5 kcal/h) (F&R. Cooling Limited, Somerset, UK)
- Household electric kettle WMK2K2W012 – PLC (Bradford, UK)
- Kenwood microwave K25MMS12 (power input 1400 – 1450 W; power output 850-900 W and frequency 2450 MHz) – Kenwood (China)
- Pawkit water activity meter – Decagon devices (Aqualab, Pullman, WA 99163, USA)
- Top Mix Vortex mixer, FB 1503 (230 V, 50 Hz, ZX) – Wizard Fisherbrand, Fisher Scientific (Loughborough, UK)
- Sanyo Incubators MIR-153 – Sanyo Electric Co. Ltd. (Japan)
- Cold-room (-18 °C and 6 °C) – COLSEC GROUP (Droitwich, UK)
- Rodwell Autoclave MP25 – Rodwell Scientific Instruments (Essex, UK)

- Ultrasonic Water Bath XUB18 – Grant Instruments (Cambridge, UK)
- Select Purewater 300, 14.7 MΩ – SUEZ Environment Water Purification System – Purite Limited (Thame, UK)
- Allegra X-22 Centrifuge – Beckman Coulter (California, USA)
- Genevac EZ-2 – Genevac Limited (Ipswich, UK)
- Cecil CE3021 3000 Series Spectrophotometer – Talbot Scientific Limited (Cheshire, UK)
- Plastic cuvettes (12.5 x 12.5 x 45 mm- 2.5 mL), 100 pcs – Plastibrand – Fisher Scientific (Loughborough, UK)
- Hobart grinder – Hobart manufacturing company (London, UK)
- Endecotts test sieve shaker – Endecotts Limited (London, UK)
- Chromacol PVDF filters (17 mm, 0.2 µm pore size) – Lot No. 00180577 – Thermo Scientific (Germany)
- pH/ORP Meter HI2211 – Hanna Instruments (Bedfordshire, UK)
- Mettler Toledo Balance XS 104 – Precision Instruments (Leicester, UK)
- Waterbath VFP – Grant Instruments Limited (Cambridge, UK)
- Miropipette (25 – 100 µL) – Micromaster (Barcelona, Spain)
- Finnpipettes (0.5 – 5mL) – Thermo Scientific (Finland)
- Amber HPLC vials (2 mL) – Thermo Scientific (UK)
- Caps for HPLC vials – VWR International (UK)
- Gemini C18 column (250 x 4.6 mm, pore size, 5 µm) – Phenomenex Ltd (Cheshire, UK)

- Organic acid reversed-phase column (4.0 mm x 150 mm, pore size, 5 µm) – Thermo Scientific (Leicestershire, UK)
- High performance liquid chromatography (HPLC) (UFLCXR, Shimadzu) equipped with a degasser (DGU-20 A5), a pump system (LC-20 AD XR), an auto sampler (SIL-20 AC XR), a column oven (CTO-20 AC), a diode array detector (DAD; SPD-M20A) and a communications bus module (CMB-20A) coupled with LC chromatographic software (Version 1.25).
- Varian Unity Inova 500 NMR spectrometer (Agilent Technologies, CA, USA) coupled with Carr-Purcell-Meiboom-Gill (CPMG) for spectral acquisition.

2.5 Experimental design

An overview of key activities undertaken during baobab fruit processing and analyses are provided in Figure 2.2.

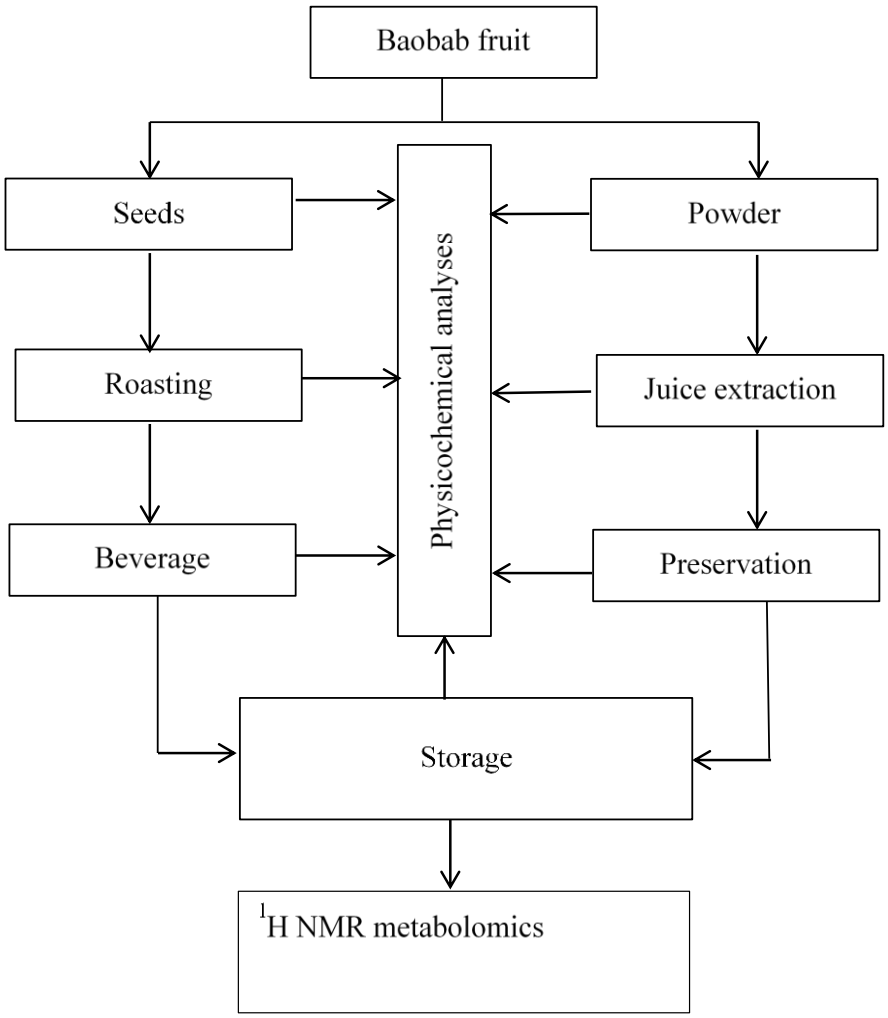


Figure 2.2 - Overview of key steps undertaken during processing and analysis of baobab fruit products.

2.5.1 **Baobab pulp powder**

Baobab fruits were cracked, and the pulp (which is in the form of a powder) was separated from the hard shell and seeds using a mortar and pestle. Pulp powder was sieved to one size (1 mm) using Endecotts test sieve shaker. A composite baobab powder sample was obtained by homogenising baobab pulp powder with a sieve shaker from different sites in equal proportions. Untreated pulp powder was kept in air tight plastic containers at dry ambient conditions until juice preparation. A small portion of pulp powder was microwaved (Kenwood, K25MMS12) for 60 s to investigate the effect of this heat treatment on vitamin C.

2.5.2 **Baobab seeds**

Baobab seeds were polished with tap water to remove pulp and air dried (25 °C, 8 h) in aluminum trays. Dried seeds were kept in plastic containers at room temperature until moisture content determination and roasting.

2.5.3 **Baobab juice preparation**

Baobab juice was prepared following the protocol commonly used by small holder farmers in Malawi with modifications. Tap water (Leeds, UK) was heated and held at boiling point (100 °C, 10 min) to inactive potential spoilage microorganisms. Fresh untreated baobab pulp powder (100 g) was added to the cooled, boiled water (1 L, 15 °C) and the mixture homogenised using a domestic blender (Kenwood Chef Classic, model KM 330) operated at medium speed for 10 min. The resultant mixture was transferred into falcon tubes (50 mL) and centrifuged at 4000 rpm for 20 min at 4 °C in a Beckman

Coulter Centrifuge (Allegra, model X-22) to separate the pulp from the remaining liquid juice. Afterwards the juice was filtered through a muslin cloth into containers, degassed using a vacuum box (Mill wall) for 10 min and immediately refrigerated (6 °C). Samples representing time zero were immediately deep frozen and the rest distributed into three storage temperatures (6, 15 and 30 °C).

2.5.4 **Low temperature long time (LTLT) pasteurisation of baobab juice**

To obtain LTLT pasteurisation, fresh juice (10%, 10 mL) (2.5.3) was heated (60 °C) in a falcon tube (15 mL) using a thermostatic water bath (Grant, model VFP). The juice took 5 min to reach 60 °C and was held at this temperature for 30 and 60 min. Temperatures were constantly monitored by incorporating thermocouples in representative falcon tubes filled with the same volume of juice. Treated juice was immediately cooled in ice-water to a final temperature of 20 °C.

2.5.5 **High temperature short time (HTST) pasteurisation of baobab juice**

To obtain HTST pasteurisation, fresh baobab juice (10%, 5L) (2.5.3) was heated in a plate heat pasteuriser (Alfa Laval, model P20-HB) at 72 °C and 85 °C for 15 s after disinfecting the equipment as described by Lewis and Heppell (2009). The regeneration section of the pasteuriser cooled samples to 4 °C. Part of pasteurised juice sample was immediately frozen (-18 °C) to represent time zero and the rest distributed into three storage temperatures (6, 15 and 30 °C). Separate storage containers were used to avoid oxygen and microbial contamination during time sampling.

2.5.6 **Preservatives**

Citric acid (CA, 0.5%) was added to HTST pasteurised juice (72 °C, 15 s) (2.5.5) to evaluate the effect on ascorbic acid, total phenol content, selected phenolic compounds, antioxidant activity and metabolic profile of baobab juice during storage.

2.5.7 **Roasted baobab seed powder**

Baobab seeds (200 ± 0.5 g) were roasted in a Baumatic gas oven at 250 °C for 60 min. Thermocouples was used to monitor the temperature in the oven. After roasting, seeds were immediately cooled in a desiccator at room temperature. Total weight loss was determined and seeds were finally transferred in sealed plastic containers and stored at room temperature until further processing. Roasted seeds were ground in a Hobart grinder (2.4.1) at a speed of 8 rpm. To obtain uniform particle sizes, samples were passed through mesh (500 µm sieve) using Endecotts test sieve shaker (2.4.1). Sieved samples were kept in tightly closed bottles and stored at -18 °C until preparation of baobab juice with seed liquor.

2.5.8 **HTST pasteurised juice with seed liquor (SL)**

Baobab seed liquor is generally made by dissolving roasted and ground baobab seeds in boiling water (100 °C) and filtering the resulting solution. The seed liquor is taken like coffee with or without addition of sugar or milk.

Pasteurised juice (10%, 72 °C, 15 s) was used to obtain juice samples with different percentage of seed liquor (SL). Different amounts (6, 12 and 18 g) of roasted (250 °C, 1

h) and ground seed powder (2.5.7) were dissolved in boiling water (240 mL), homogenised for 10 min using a domestic blender and cooled to room temperature (20 °C). Each seed liquor was diluted to 600 mL with pasteurised juice (10%, 72 °C, 15 s) and homogenised for a further 10 min. The resultant mixture was transferred into falcon tubes (50 mL) and centrifuged at 4000 rpm for 20 min at 4 °C in a Beckman Coulter Centrifuge to obtain a clear liquid juice. Afterwards samples were degassed using a vacuum box (Mill wall) for 10 min and immediately deep frozen until further treatment.

Subsequently HTST juice (6% pulp) samples with different concentrations of seed liquor (0, 1.0, 2.0 and 3%) were obtained and used to evaluate the protective effect of seed liquor against vitamin C degradation as well as changes in total phenol content, antioxidant activity (FRAP, DPPH and ABTS), procyanidin B2 and (-)-epicatechin of the juice during storage.

2.5.9 **Storage**

To determine the effect of storage temperature and time on the chemical and metabolic profile of baobab juice including commercial baobab juice samples were kept in screw capped falcon tubes (15 mL) in racks at isothermal temperatures (6, 15 and 30 ºC) in a cold room and Sanyo incubators and left to stand for a maximum of 60 days. Each storage sample was stored in a separate container to avoid oxygen and microbial contamination during time sampling.

2.6 Physicochemical and metabolic profile analysis

Quality attributes in all baobab samples were analysed using standard methods and techniques.

2.6.1 Extraction of soluble phenolic compounds

Extraction of phenolics from baobab juice was undertaken as described by Sun et al. (2013) with some modification. Baobab juice (1 mL) was dissolved in methanol/water (80:20, v/v) to a final volume of 10 mL in a falcon tube (15 mL). The resultant mixture was vortexed (20 s), centrifuged (4000 rpm, 4 °C, 10 min) and filtered using Whatman No.1 paper. Sample extract was deep frozen (-18 ºC) until determination of antioxidant activity or further extraction. For HPLC analysis, methanolic extract (5 mL) was evaporated to dryness using the EZ-2 Genevac (Genevac Limited, Ipswich, UK) applying the low boiling point method (32 ºC for 5 hrs) and reconstituted to 2.5 mL with methanol/water (80:20, v/v) representing a final five-fold dilution of the original sample.

2.6.2 Total phenolic content

Total phenol content is an important indicator of the total antioxidant capacity. Moreover several researchers have reported a significantly high positive correlation between total phenol content and total antioxidant activity (Yang et al., 2010; Maizura et al., 2011). It is used as a screening tool to study phenolic antioxidants in several biological systems. The assay is based on sample's reducing capacity (by phenolic compounds) of the commercially available Folin-Ciocalteu reagent (FCR) and may be therefore equivalent to other antioxidant assays such as FRAP and TEAC (Singleton et al., 1999; Huang et al.,

2005; Shahidi and Zhong, 2015). Phenolic compounds react with FCR only under basic conditions adjusted by sodium carbonate solution (pH 10). The dissociation of phenolic protons form phenolate anion which eventually reduce FCR forming a blue coloured compounds (maximum absorbance at 765 nm) which are independent of structure of phenols involved (Huang et al., 2005).

The total phenol content (TPC) in baobab extracts was measured through spectrophotometric determination according to the Folin–Ciocalteu procedure (Singleton et al., 1999) with some modification. Tenfold diluted sample extract was used. Within 3-8 min, to sample extract / standards (1 mL), Folin reagent (5 mL) and sodium carbonate solution (1.0 M, 4 mL) were added, vortexed (10 s) and left to stand for 2 hours at 26 °C in a waterbath. Absorbance of samples and blank [80% v/v methanol (1 mL) + Folin reagent (5 mL) + sodium carbonate (4 mL)] was measured at 765 nm using a spectrophotometer. Solutions of gallic acid standards with concentrations between 0.025 and 0.15 mg/mL were used to construct a standard calibration curve ($R^2 = 0.998$). Total phenolic content was expressed as mg gallic acid equivalent (GAE) per 100 g of fresh weight (FW).

2.6.3 **Ferric reducing antioxidant power (FRAP)**

The FRAP assay is generally considered to offer an index of antioxidant or reducing power of biological fluids applying electron transfer (ET) reaction mechanism. Under suitable conditions of pH the transition metal iron forms a complex with 2, 4, 6-tris-2-pyridyl-s-triazine (TPTZ) which gives a distinctive blue colour in different oxidation state.

FRAP measures directly the ability of antioxidants (AO) in the sample to reduce a ferric tripyridyltriazine [Fe (III) $(TPTZ)_2]^{3+}$ to the ferrous tripyridyltriazine complex [Fe (II) $(TPTZ)_2]^{2+}$ at low pH (Benzie and Strain, 1996;Frankel and Meyer, 2000; Huang et al., 2005).

The ferric reducing antioxidant power (FRAP) of the juice was measured according to Benzie (Benzie and Strain, 1996). Juice extract (200 µL) and freshly prepared FRAP reagent (6 mL) were transferred into a falcon tube, vortexed (10 s) and left in the dark at room temperature for 10 min. The absorbance of the samples was measured at 593 nm using Spectrophotometer against reagent blank consisting of 80% (v/v) methanol (200 µL) instead of juice extract. Solutions of Trolox standards with concentrations between 0.01 and 0.25 mg/mL were used to construct a standard calibration curve ($R^2 = 0.999$) and results expressed as Trolox equivalent antioxidant capacity per gram of fresh weight (TEAC, µM/g FW).

2.6.4 **DPPH radical scavenging activity**

The DPPH assay is based upon the reduction of a commercially available organic nitrogen radical (DPPH) in methanol solution with a maximum absorption at 515 nm (Brand-Williams et al., 1995; Frankel and Meyer, 2000; Huang et al., 2005; Thaipong et al., 2006). Then reduction of DPPH radical by antioxidants (AH) or free radical species ($R^{\cdot}$) can be followed by monitoring the decrease in absorbance at 515 nm. The interaction of a potential antioxidant with DPPH radical depends on its structural conformation (Brand-Williams et al., 1995). Antioxidants with greater number of hydrogens on hydroxyl groups available for donation tend to react more rapidly with DPPH radical.

The DPPH radical scavenging activity of juice was measured according to the Brand-Williams method (Brand-Williams et al., 1995). Sample extracts (0.1 mL) were added to methanol DPPH solution (0.1 mM, 3.9 mL), vortexed for 20 s and allowed to stand in the dark for 30 minutes at room temperature. Then, absorbance of the sample and control, which consisted of 80% (v/v) methanol (0.1 mL) in place of sample extract, was measured at 517 nm using a Spectrophotometer. Results were expressed as % DPPH calculated according to the equation:

$$\% \text{ DPPH} = (1 - A_s/A_c) \text{ x } 100$$

Where A_s and A_c are absorbance of sample and control, respectively.

2.6.5 **ABTS radical scavenging ability**

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay is widely used for screening both lipophilic and hydrophilic antioxidants such as flavonoids, hydroxycinnamates, carotenoids and plasma antioxidants (Re et al., 1999). In principle the assay involves decolourisation of blue/green free radical ($ABTS^{\cdot+}$) upon reduction. Antioxidants in sample extracts can neutralise the radical cation by either direct reduction via electron or hydrogen donation, and a balance of these mechanisms is influenced by structure of antioxidants and pH of the system (Re et al., 1999; Huang et al., 2005; Zulueta et al., 2009; Shahidi and Zhong, 2015). Thus although TEAC (ABTS) assay is classified as an electron transfer (ET)-based method, the hydrogen atom transfer (HAT) mechanism also applies. The antioxidant activity of sample is quantified using standards.

The ABTS antioxidant activity was measured according to Re method (Re et al., 1999) with some modification. Sample extracts (diluted 20 fold with 80% (v/v) methanol, 0.1 mL) or standards (0.1 mL) were added to $ABTS^{\cdot+}$ aqueous solution (3.9 mL) that had achieved an absorbance of 0.7 ± 0.02 at 734 nm after dilution with deionised water. The mixture was kept at 25 ^{o}C for 106 min to ensure complete reaction. The absorbance of sample extracts or standards was measured at 734 nm using a spectrophotometer against blank, which consisted of 80% (v/v) methanol (0.1 mL) instead of sample extract. Solutions of Trolox standards with concentrations between 0.01 and 0.12 mg/mL were used to construct a standard calibration curve and results expressed as Trolox equivalent antioxidant capacity per 100 gram of fresh weight (mg TEAC/100 g FW) and (mg TEAC/100 mL) for commercial juice.

2.6.6 **Optimisation of extraction and stability of ascorbic acid**

Ascorbic acid was extracted according to the method of Pimpão et al. (2013) with some modification. Baobab juice was diluted 2 fold with metaphosphoric acid (3 g/100 mL), vortexed for 20 s at medium speed and centrifuged (4000 rpm, 4 °C) for different time interval (10 – 30 min) to investigate the effect of centrifugation time on the amount of ascorbic acid extracted from the sample. The supernatant was filtered using Whatman No.1 paper to ensure clear filtrate. The solution was further filtered through a PTFE (0.45 µm) filter into amber HPLC vials and refrigerated until analysis. Sample extracts were also kept at ambient temperature (21 ºC) for a maximum of 206 hours to assess the stability of ascorbic acid in MPA (3 g/100 mL).

2.6.6.1 **Reduction of dehydroascorbic acid (DHA)**

Total vitamin C was determined after reduction of dehydroascorbic acid to ascorbic acid using 2-carboxy ethyl phosphine hydrochloride (TCEP) as described by Chebrolu et al. (2012). Three concentrations of TCEP (2.5, 5.0 and 10 ppm) were used for optimisation. Optimised reducing agent (TCEP, 5 ppm) was used to reduce DHA in the rest of the fruit samples. Briefly, baobab fruit samples that had been extracted with metaphosphoric acid as described in 2.6.6 were diluted two fold with TCEP (5.0 ppm), vortexed (20 s) and left in a waterbath (25 ºC) for 25 min to allow for complete reduction.

2.6.7 **Extraction of organic acids**

Organic acids (citric, malic and tartaric acids) were extracted according to the method described by other researchers (Veberic et al., 2009; Chebrolu et al., 2012) with some modification. Baobab juice was diluted 2 fold with metaphosphoric acid (3 g/100 mL), vortexed for 20 s at medium speed and centrifuged (4000 rpm, 10 min, 4 °C). The supernatant was filtered through Whatman No.1 paper to obtain clear filtrate. Finally the solution was passed through PTFE filters (0.45 µm) and into amber HPLC vials.

2.6.8 **HPLC analysis of vitamin C and organic acids**

Quantification of ascorbic acid, vitamin C (ascorbic acid plus dehydroascorbic acid) and organic acids (citric acid, malic acid and tartaric acid) was conducted using HPLC (2.4.1). A sample volume of 20 µL was injected and separations were achieved on a Gemini C18 column (250 x 4.6 mm, 5 µm) operated at 25 °C. Separation was carried out under isocratic conditions (0.5 mL/min; 15 min) using potassium dihydrogen orthophosphate

buffer (10 mM pH 2.6) as the mobile phase (2.3.3). Chromatograms of organic acids and ascorbic acid were detected at 210 and 254 nm respectively. Quantification was achieved using the external standard method (Klimczak et al., 2007). A mixture of organic acid standards with concentration ranging from 5 to 100 µg/mL was used for calibration and the amount expressed in mg/100 g FW. Spiked samples were included for identification, determination of sensitivity and reproducibility of the method, to account for the percent loss through recoveries and to monitor any shift in retention time of individual organic acids. Reaction kinetics were performed in order to obtain comprehensive data on evolution of vitamin C in baobab juice. The loss of vitamin C in baobab juice during storage was predicted using integrated rate laws of zero (2.2), first (2.3) and second (2.4) order reactions:

$C = C_0 - kt$ …….... (2.2)

$\ln C = \ln C_0 - kt$……..... (2.3)

$1/C = 1/C_0 + kt$ ……...……..... (2.4)

Where C, the concentration at time *t*; C_0, the concentration at time zero; *k*, the rate constant; *t*, the storage time (days).

2.6.9 **Determination of phenolic compounds, HMF and caffeine**

Reversed phase high performance liquid chromatography (RP–HPLC) was used to identify and quantify phenolic compounds, 5-hydroxymethylfurfural (HMF) and

caffeine. Fivefold diluted phenolic extract (2.6.1) was filtered through a 0.20 µm PTFE filter and then injected into amber HPLC vials. Sample extract (20 µL) was injected and separations were achieved on a Gemini C18 column (250 x 4.6 mm, 5 µm) operated at 35 °C. The method was performed as described by Pimpão et al.(2013) with some modifications. The mobile phase consisted of 0.1% (v/v) formic acid in deionised water (solvent A) and 80% (v/v) methanol (solvent B), employed at a flow rate of 0.5 mL/min. The gradient elution programme started at 10% B to reach 30% B at 15 min and remained 30% B until 45 min. In a final washing step 10% of solvent B was reached and maintained for 10 min. Compounds were identified by comparing retention time and UV spectra with their corresponding standards as well as carrying out the peak spiking of samples with their standards. Chromatograms of (+)-catechin, (-)-epicatechin, procyanidin B2 (-)-epigallocatechin-3-O-gallate, gallic acid, 5-hydroxymethylfurfural and caffeine were detected and recorded at the wavelength of 284 nm for quantification. All standards were dissolved in 80% (v/v) methanol and employed as the stock solutions. Calibration curves were plotted using peak area against concentration and quantification achieved using standard calibration curves. Results were expressed as milligram per 100 gram of fresh weight (mg/100 g FW). Retention time (min), linearity (R^2), wavelength (nm), and recoveries (%) were used to identify compounds and validate the method.

2.6.10 **Determination of pH**

The pH of baobab fruit samples was measured as described in AOAC (2005). Fruit pulp (4.0 g) was added to deionised water (150 mL), which was previously boiled and cooled to room temperature. The contents were homogenised for 60 s and transferred into a small

beaker for pH measurement using a pH meter. The pH of fruit juice was measured directly after equilibration at room temperature.

2.6.11 **Determination of moisture content**

To determine the moisture content in fresh baobab fruit samples, the hot oven method was used (AOAC, 2005). Accurately weighed fruit pulp (4.0 g) was enclosed in an oven set at 105 - 110 °C overnight, cooled in a desiccator for 30 minutes and reweighed. The procedure was repeated until a constant weight was obtained.

2.6.12 **Determination of water activity**

Water activity was determined using a Pawkit water activity meter. Baobab powder or juice was homogenised and transferred into a Pawkit sample cup until the bottom was completely covered. Water activity measurements were recorded after equilibration.

2.7 NMR sample preparation

An amount of 800 µL of baobab juice was transferred into a 1.5 ml Eppendorf tube and centrifuged at 10000 g for 15 min at 4 °C to remove any solid particles. Then, 550 µL of the supernatant solution was mixed with 150 µL of 1.5 M phosphate buffer solution (pH 7.4) containing 60 µL sodium 3-trimethylsilyl-(2,2,3,3-2H4)-1-propionate (TSP) (final concentration 0.8 mM). The mixture was vortexed and 650 µL was transferred into a 5 mm wide NMR tube.

2.8 ^{1}H NMR spectroscopy

The protocol was undertaken as described by Rosa et al.(2015) with some modifications. ^{1}H-NMR spectra were recorded at 300 K using a Varian Unity Inova 500 NMR spectrometer (Agilent Technologies, CA, USA) operating at 499.839. NMR spectra were acquired by using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with 512 scans, a spectral width of 6000 Hz, a relaxation delay of 2 s and a total CPMG time of 100 ms to attenuate broad signals from macromolecular components. A water pre-saturation delay was applied between pulses. After Fourier transformation with 0.3 Hz line broadening and a zero-filling to 64 K, ^{1}H spectra were phased and the baseline corrected.

2.9 Statistical data analysis

Results are presented as mean ± standard deviation (SD); $n \geq 3$. Analysis of variance (ANOVA) using Tukey's test ($p \leq 0.05$) was performed to evaluate the significance of differences between treatments and storage using IBM SPSS statistical software version 22. Correlations between antioxidant activity and variables (vitamin C, total polyphenol and phenolic compounds) were determined using Pearson's Correlation Coefficient Test to ascertain relationships.

Chapter 3 **Characterisation of bioactive compounds of fresh baobab**

Abstract

Bioactive compounds of baobab (*Adansonia digitata)* pulp from Malawi were investigated using reversed phase high performance liquid chromatography (RP-HPLC) while total phenol content (TPC) and total antioxidant activity (FRAP, ABTS and DPPH) were measured by spectrophotometry. Malawi baobab pulp showed high levels of ascorbic acid (351.92 ± 7.41 mg/100 g FW), vitamin C(465.8 ± 2.5 mg/100 g FW), citric acid (3300.84 ± 0.90 mg/100 g FW), malic acid (2364.98 ± 28.8 mg/100 g FW), procyanidin B2 (533.3 ± 22.6 mg/100 g FW), gallic acid (68.5 ± 12.4 mg/100 g FW) and (-)-epicatechin (43.0 ± 3.0 mg/100 g FW) and showed a maximum TPC of 1886.81 ± 1.61 mg GAE/100 g FW. These bioactive compounds likely contributed to a higher antioxidant activity observed in the pulp which was 2806.39 ± 92.8 mg TEAC/100 g FW for FRAP, 1516.09 ± 17.10 mg TEAC/100 g FW for ABTS and 50.93 ± 0.43% DPPH for DPPH. Results from the present study suggest that baobab fruit offers an opportunity for the development of quality novel functional foods which can help to improve nutrition and health status as well as commercialisation in Malawi.

Keywords: Baobab fruit; Thermal treatment; Bioactive compounds; Vitamin C; (-)-Epicatechin, Procyanidin B2; Total phenol content; Antioxidant activity; Malawi.

3.1 Introduction

Fresh fruits and derived products are a source of food and provide essential dietary micronutrients including polyphenols, vitamins, and minerals with several human health benefits (Prior, 2004; Igual et al., 2010; Gamboa-Santos et al., 2014). Research studies have reported that baobab fruit is a rich source of bioactive compounds including vitamin C, organic acids and procyanidin B2 (Shahat, 2006; Besco et al., 2007; Coe et al., 2013). However, quantitative information on quality attributes reported for baobab fruit products vary widely due to several factors including provenance, variety, genotype and analytical methods and techniques employed (Tembo, 2008).

3.2 Importance of the study

The quality of raw materials plays a significant role in overall product quality (Rössle et al., 2011; Pimpão et al., 2013) and determines postharvest handling, processing and preservation techniques to be undertaken. Before characterisation, optimised methods of identification, extraction and quantification are required to quantify quality attributes with high degree of accuracy.

There is sufficient knowledge and information on the nutritional profile and utilisation of commonly consumed fruits including oranges, apples and blueberries but such information is scarce for most indigenous fruits of Malawi (Tembo, 2008). Most of the available literature on the composition of baobab fruit is based on simple methods and techniques and may contribute to the wide variation in reported data. Further, dietary

intake of phenolic compounds including flavan-3-ols available in baobab fruit is largely unknown due to the lack of reliable knowledge and information.

3.3 Aim of the Chapter

The general aim of the present study was to quantify bioactive compounds and quality attributes in fresh baobab pulp and juice. Specific objectives were three-fold.

i. Optimisation of vitamin C and total phenol extraction and determination ,
ii. Identification of bioactive compounds (phenolic compounds, caffeine and 5-hydroxymethylfurfural in the fresh pulp) and
iii. Quantification of bioactive compounds, total phenol content and antioxidant activity in fresh baobab pulp

3.4 Vitamin C and main organic acids in fresh baobab pulp

Ascorbic acid (AA) is an important component of our nutrition and used as additive in many foods because of its antioxidant capacity (Burdurlu et al., 2006). Thus if well retained, other quality attributes as well as nutritional value of foods are also retained (Marfil et al., 2008). However ascorbic acid which is the main component of vitamin C (AA + DHA) is highly thermo labile compound and easily decomposes to DHA and other compounds under less desirable conditions (Fennema, 1996; Rawson et al., 2011). Degradation of ascorbic acid is through both aerobic and anaerobic pathways and influenced by many factors such as oxygen, heat, light, storage temperature and time as described in section 1.7. Structures of organic acids (ascorbic acid, dehydroascorbic,

citric, malic acid and tartaric acids) analysed in fresh baobab fruit pulp from Malawi using reversed phase high performance liquid chromatography (RP-HPLC) are presented in Figure 3.1A-E. Other quality attributes such as pH of juice and moisture of the pulp were also measured.

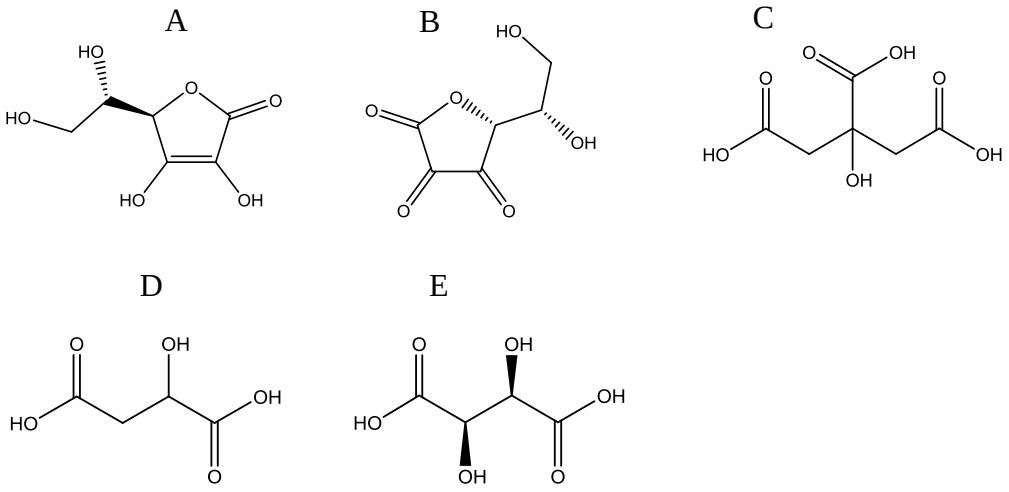


Figure 3.1 - Structures of main organic acids: ascorbic acid (A), dehydroascorbic acid (B), citric acid (C), malic acid (D) and tartaric acid (E) analysed in fresh baobab pulp from Malawi.

Organic acids were identified in fresh baobab pulp by comparison with chromatographic parameters (spectrum and retention time) of authentic standards. The chromatographic spectral peaks were sharp and retention times of compounds in baobab pulp extract were identical to spectrum of standards including ascorbic acid (AA), citric acid (CA), malic acid (MA) and tartaric acid (TA) (Figure 3.2).

Linearity, limit of detection and recoveries were performed to validate the RP-HPLC method. Results showed higher linearity ($R^2 \geq 0.99$) indicating that the response of RP-

HPLC method to known concentration of organic acids was good (Table 3.1). The limit of detection (LOD) was conducted to determine the selectivity and sensitivity of the method. The LOD was obtained from the analyte concentration that gave a signal equal to average background noise (y_{blank}) plus three times the standard deviation (s_{blank}) of the blank (LOD = $y_{blank} + 3s_{blank}$), while the limit of quantification (LOQ) was calculated by the expression LOQ = $y_{blank} + 10s_{blank}$ (Gosetti et al., 2007; Harris, 2007). The LOD for AA, CA, MA and TA was 10.6, 57.6, and 86.6, 24.3 mg/100 g respectively while the LOQ were 35.3, 92.0, 288.7, and 80.9 mg/100 g respectively. Any amount lower than LOQ was disregarded and ascribed to noise of the HPLC equipment. Spike recoveries were calculated to evaluate matrix effect. Higher recoveries were obtained (Table 3.1) indicating that the response of the method to baobab sample was as expected from calibration curve. Thus the selected RP-HPLC assay was sufficiently precise and appropriate for content evaluation (Fecka, 2009) of organic acids in baobab fruit.

Table 3.1 - Analytical parameters for identification of organic acids. Retention and recovery values are means of six determinations ± SD.

Standard compound	Retention time (min)	Wavelength (nm)	Linearity (R^2)	Recovery (%)
Ascorbic acid	3.97 ± 0.00	254	1.000	96.95 ± 1.44
Citric acid	5.01 ± 0.01	210	0.999	113.81 ± 4.13
Malic acid	3.58 ± 0.01	210	0.998	93.00 ± 0.31
Tartaric acid	3.15 ± 0.01	210	0.999	108.79 ± 6.47

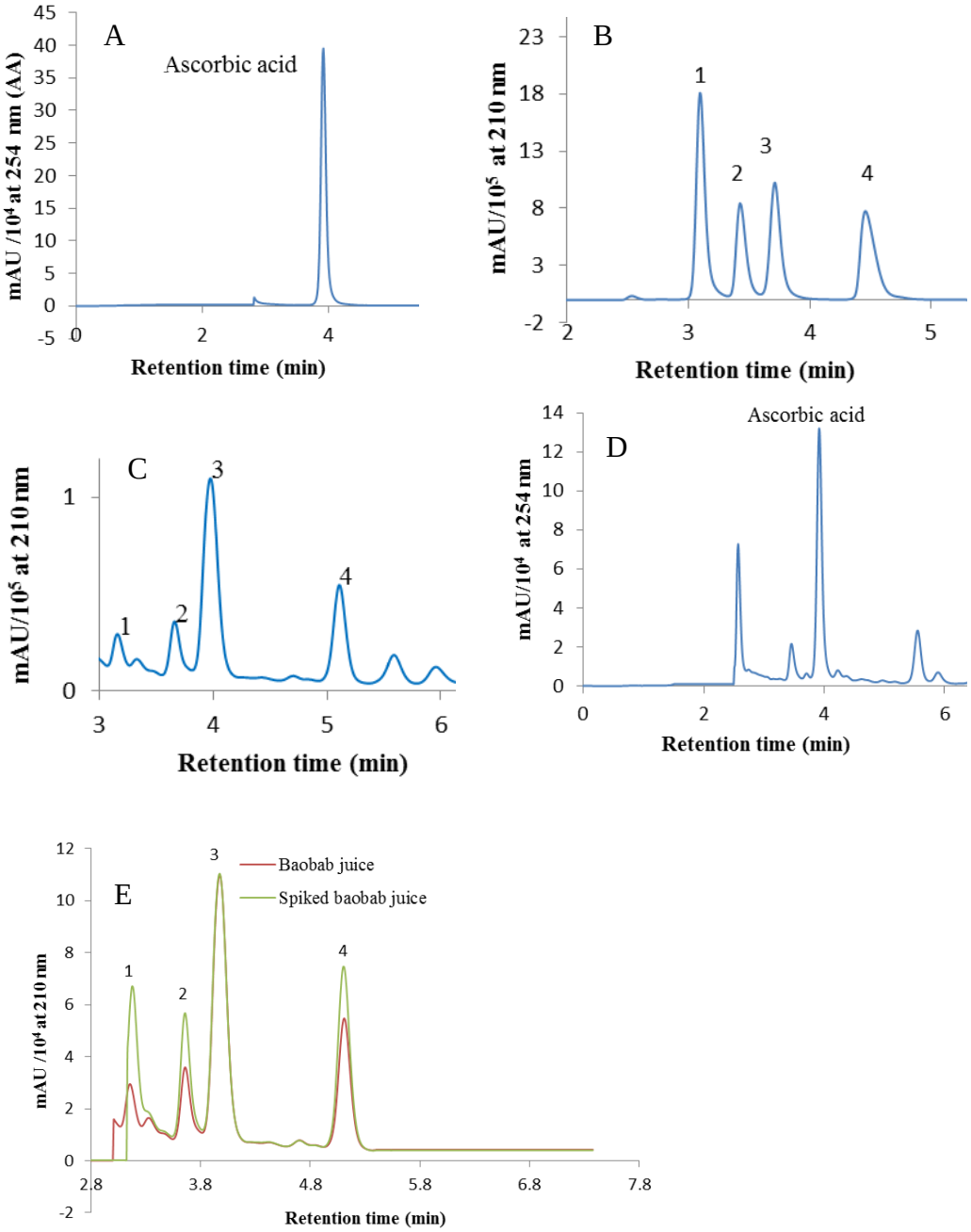


Figure 3.2 - RP-HPLC chromatograms for ascorbic acid standard at 254 nm (A), mixture of organic acids standards at 210 nm (B), baobab juice at 210 nm (C), baobab juice at 254 nm (D) and baobab juice spiked with organic acids at 210 nm (E). Peaks: 1, tartaric acid; 2, malic acid; 3, ascorbic acid; 4, citric acid.

3.4.1 **Standard calibration plots for ascorbic and organic acids**

Figure 3.3A shows the standard calibration curve (peak area versus concentration) for ascorbic acid that was used for quantification. The concentration of ascorbic acid covered a wide range (0 to 400 ppm) to ensure sample concentration fell within the range hence providing accurate quantitative information. The minimum standard concentration was predicted from LOD (10.6 ppm) for ascorbic acid in baobab pulp. The linearity ($R^2 \geq 0.999$) of calibration curve was good enabling accurate quantification of ascorbic acid in the samples. Calibration curves with organic acid concentration ranging from 20 to 800 ppm and good linearity ($R^2 \geq 0.999$) were used for quantification of citric, malic and tartaric acid in baobab pulp and juice (Figure 3.3B).

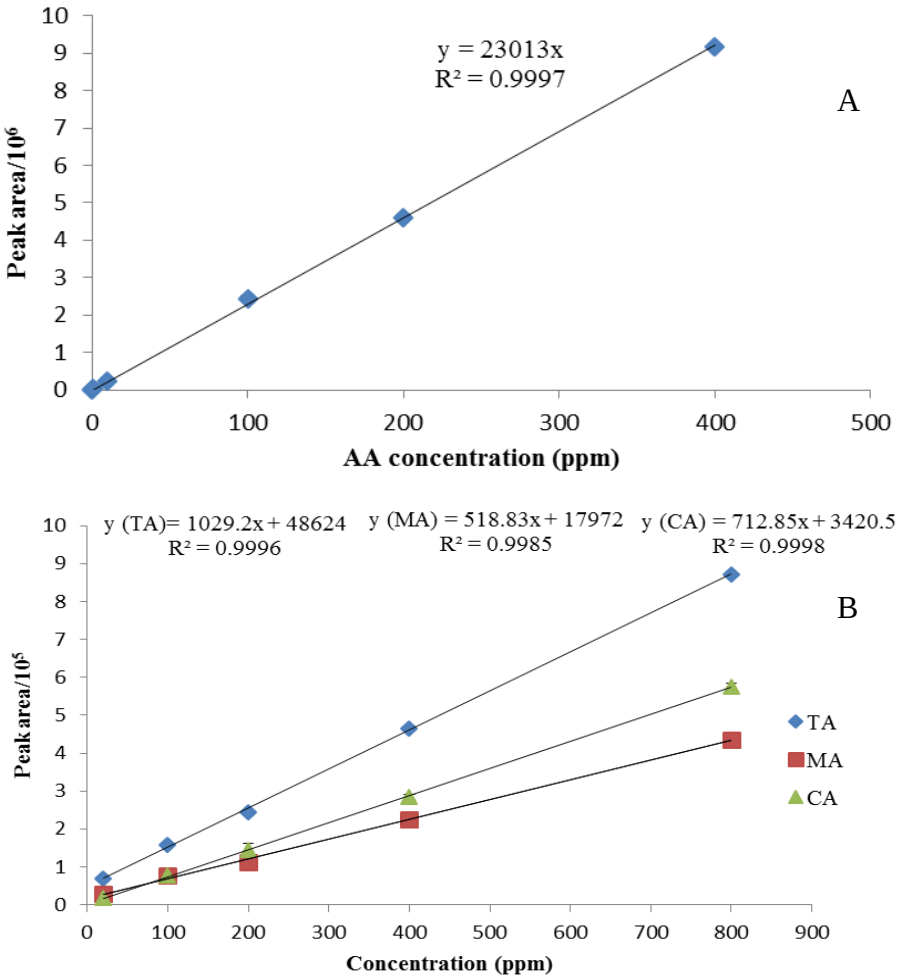


Figure 3.3 - RP-HPLC standard calibration curve for ascorbic acid (A) and citric acid (CA), malic acid (MA) and tartaric acid (TA) (B); n = 3.

3.4.2 **Optimisation of ascorbic acid extraction and determination of vitamin C**

Ascorbic acid is readily oxidized during sample preparation therefore it was necessary to optimise the extraction procedure to minimise losses and ensure accurate quantitative information of vitamin C in baobab products. The analytical method described in 2.6.8 was used for this experiment.

3.4.2.1 **Extraction time and stability of ascorbic acid**

Ascorbic acid may be easily lost or not fully extracted from plant foods during sample preparation and determination. The yield of ascorbic acid in the extract depends on several factors including extraction time, acidity of plant material, matrix and composition of extracting solvent (Tembo, 2008).

The study examined the effect of centrifugation time (10, 20 and 30 min) on the amount and stability of ascorbic acid in metaphosphoric acid, MPA (3 g/100 mL) extracting solvent including water as a reference. Results showed that there was no significant difference ($p > 0.05$) between the yield/amount of ascorbic acid obtained for the selected time interval. The initial amount of ascorbic acid obtained for centrifugation times of 10, 20 and 30 min were 474 ± 27, 469 ± 8 and 480 ± 6 mg/100 g respectively. Although the content of ascorbic acid was significantly higher ($p \leq 0.05$) at time zero and early storage time, levels decreased significantly with longer storage time. This may be attributed to degradation ascorbic acid influenced by water.

The stability of ascorbic acid in the extracts was evaluated at ambient temperature (21 °C) for a maximum period of 206 hours. Results for ascorbic acid in MPA (3 g/100 mL) centrifuged for 10 min remained higher during storage than samples extracted for 20 and 30 min. Therefore extraction time of 10 min was selected for use in all subsequent experiments. Similarly other authors (Chebrolu et al., 2012) used 10 min to extract ascorbic acid using MPA (3 g/100 mL) in grapefruit juice. On the other hand it was not surprising that the level of ascorbic acid decreased significantly ($p \leq 0.05$) after 44 h of

storage in water extract. Although the pH of baobab juice was low (3.1 ± 0.01), it is possible that metabolic and enzymatic activities were more responsible for ascorbic acid loss in the water extract. Metaphosphoric acid likely increased ionic strength thereby suppressing metabolic activity upon disruption of cells, inhibition of ascorbate oxidase, metal catalysts as well as precipitating interferences (proteins) (Sanmartin et al., 2000; Chebrolu et al., 2012) in the baobab extract. Ascorbic acid in water extract may easily get oxidised to several products including DHA as well as dicarbonyl compounds (erythrulose, 3-deoxythreosone, xylosone) as previously described. Degradation of DHA is more favoured at higher moisture content and water activity (Igual et al., 2010).

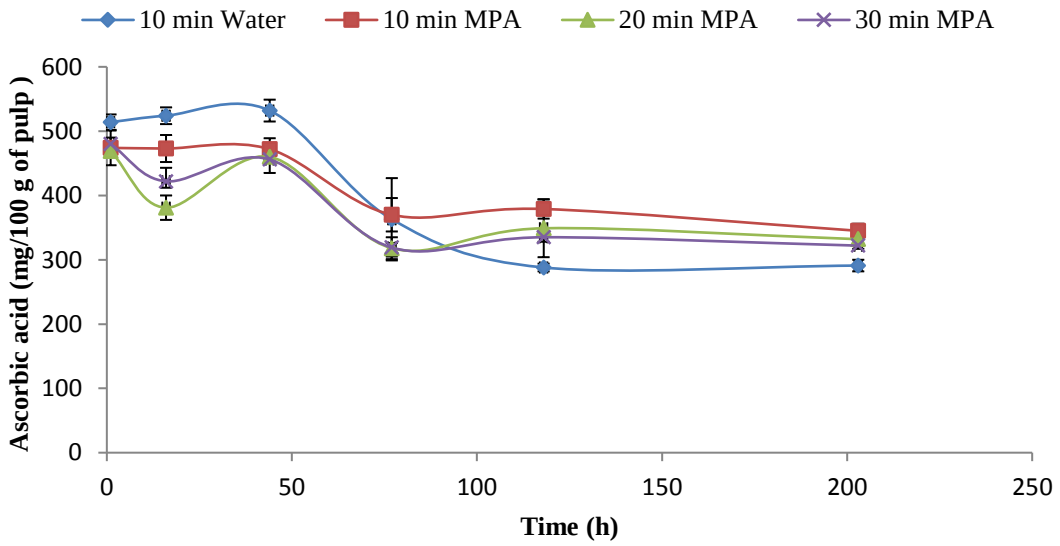


Figure 3.4 - Changes in ascorbic acid content in baobab extract during storage at 21 °C; n = 3.

3.4.2.2 **Effect of TCEP concentration on yield of total vitamin C**

Ascorbic acid (AA) is readily reduced to dehydroascorbic acid (DHA) during processing and even extraction from plant foods. The determination of DHA in real samples is

challenging due to instability and splitting of the DHA peak during RP-HPLC analysis. It is therefore practical to determine total vitamin C (AA and DHA) in plant foods through a pre-column derivatisation protocol using an effective reducing agent.

Chebrolu et al. (2012) recommended use of tris (2-carboxy ethyl) phosphine hydrochloride (TCEP) for reducing DHA to AA in fresh fruits such as kiwi, guava, strawberries and citrus fruits. However the optimum concentration may vary depending on composition, matrix or physicochemical properties of the fruit sample. An experiment was therefore conducted to assess the effectiveness of three concentrations of TCEP (2.5, 5.0 and 10 ppm) on the reduction of DHA to AA in baobab fruit samples. Results showed that maximum yield of total vitamin C was obtained using TCEP concentration of 5.0 ppm (Figure 3.5). Increasing concentration of TCEP to 10 ppm did not provide any significant increase in the yield of total vitamin C. It is possible that at a concentration of 10 ppm, TCEP was in excess and not used in the extract. For this reason a concentration of 5 ppm was selected and used for reduction of DHA during analysis of vitamin C (AA and DHA) in the rest of the samples.

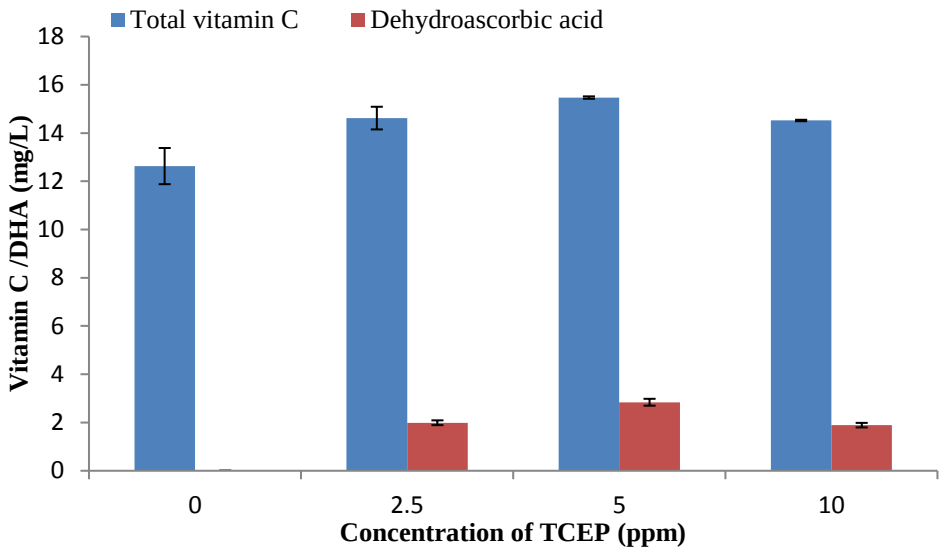


Figure 3.5 - Effect of 2-carboxy ethyl phosphine hydrochloride (TCEP) concentration on the yield of total vitamin C in fresh baobab pulp; n = 3.

3.4.3 **Physicochemical properties, vitamin C and organic acids**

Physicochemical parameters including pH, moisture content and water activity influence stability of chemical constituents, sensory and microbiological quality characteristics of fruits and derived products (Fennema, 1996). Results for vitamin C (AA and DHA) and organic acids in fresh baobab pulp are presented in Table 3.2.

Results showed that baobab pulp contains high levels of vitamin C and organic acids which are likely responsible for its acidic properties and a low mean pH value (3.11 ± 0.10). The acidic nature and lower mean moisture content (10.01 ± 0.30%) of fresh pulp contribute to extended vitamin C shelf life at ambient storage temperature (25 °C). Other researchers (Saka et al., 2007; Coe et al., 2013) reported pH values of 3.11 and 3.33 in

fresh baobab pulp. Therefore results obtained from this study are within the range of published data.

The mean total vitamin C content (AA & DHA) measured in the fresh baobab pulp (466.57 ± 8.1 mg/100 g) is more than eight fold higher than commonly consumed raw navel oranges (56.1 ± 1.0 mg/100 g) and raw lemon juice (42.9 ± 0.4 mg/100 g) reported by Scherer et al. (2012). Several authors have reported a wide range of vitamin C content (67 to 500 mg/100 g) in fresh baobab pulp (Tembo, 2008; Chadare et al., 2009; Kamatou et al., 2011; Ibrahima et al., 2013). Variation in composition and quantity of physicochemical properties of plant foods is attributed to several factors including variety, genotype, environmental conditions, ripening stage at harvest, postharvest handling, storage condition and analytical techniques used (Aron and Kennedy, 2008; Tembo, 2008). For instance, Chadare et al. (2009) reported an average vitamin C content of 283 mg/100 g pulp but also noted the tree-to-tree variability ranging from 150 – 500 mg/100 g. Titrimetric or spectrophotometric methods of measuring vitamin C are not accurate due to inaccuracies in determining end points and interferences in sample matrix (Scherer et al., 2012). The use of a selective and sensitive method (RP-HPLC-PDA) in the present study ensures accurate quantification of vitamin C (AA & DHA) and organic acids in baobab fruit products.

Addition of artificial preservatives including benzoic acids and derivatives to baobab juice may be significantly minimised due to high levels of citric (3300.84 ± 18.50 mg/100 g) and malic acid (2364.98 ± 28.76 mg/100 g) observed in fresh baobab pulp (Table 3.2).

Moreover organic acids are widely used as preservatives and acidulants to prevent discolouration and microbiological spoilage as well as improving organoleptic properties of most fruit products (Igual et al., 2010; Shahidi and Zhong, 2010; Scherer et al., 2012). The levels of organic acids in baobab pulp are significantly higher than levels in apples, oranges and grapefruit juice reported by several authors (Igual et al., 2010; Scherer et al., 2012).

Table 3.2 -RP-HPLC quantification of vitamin C and organic acids (mg/100 g FW) in baobab pulp. Results are means of triplicates (± SD); n = 3.

Organic acids	Content (mg/100 g FW pulp)
Vitamin C	465.8 ± 2.50
Ascorbic acid	351.92 ± 7.41
DHA	114.65 ± 1.10
Citric acid	3300.84 ± 0.90
Malic acid	2364.98 ± 28.8
Tartaric acid	173.93 ± 5.50

The present study further quantified specific bioactive compounds in fresh baobab pulp in order to fingerprint potential compounds that might be responsible or contribute significantly to total phenol content and antioxidant activity.

3.5 Identification of bioactive compounds

The determination of bioactive compounds in food systems is vital to fingerprint and track compounds responsible for functional properties both *in vitro* and *in vivo*. RP-HPLC analysis of phenolic compounds, caffeine and 5-hydroxymethylfurfural was conducted after optimising chromatographic conditions in order to identify predominant phytochemicals which may be associated with high total phenol content and antioxidant activity reported for baobab juice (Nhukarume et al., 2010). Although a few researchers have reported the presence of polyphenols including procyanidins (Shahat, 2006; Kamatou et al., 2011; Coe et al., 2013) in baobab fruit, limited quantitative information on several phenolic compounds is available. Therefore this study assessed the presence and levels of flavonol (quercetin), flavan-3-ols ((+)-catechin, (-)-epicatechin, procyanidin B2, (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin-3-O-gallate and (-)-epigallocatechin-3-O-gallate), phenolic acid (gallic acid) and hydroxycinnamates (p-coumaric acid, caffeic acid, ferulic acid and sinapic acid) in fresh baobab pulp.

Fresh baobab pulp showed presence of gallic acid and flavan-3-ols ((-)-epicatechin and procyanidin B2) as the most predominant bioactive compounds and very low levels of caffeine and HMF in 80% (v/v) methanolic extract of baobab pulp (Figure 3.6) using authentic standards.

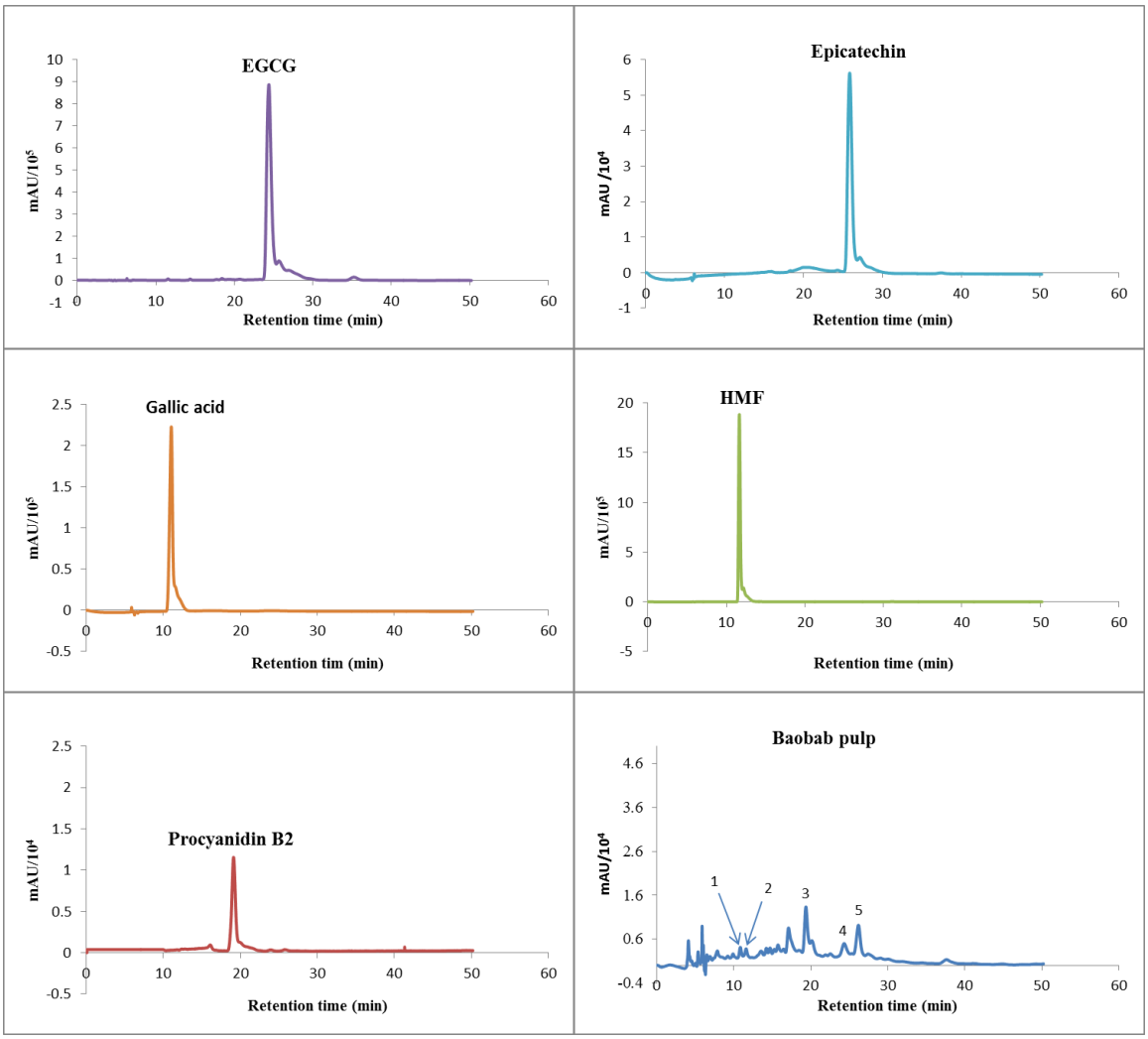


Figure 3.6 - RP-HPLC chromatograms of standard bioactive compounds and baobab pulp. Peaks of pulp extract: 1, gallic acid; 2, 5-hydroxymethylfurfural (HMF); 3, procyanidin B2; 4, epigallocatechin-3-O-gallate (EGCG); 5, (-)-epicatechin.

Chromatographic characteristics (shape of the peaks and retention times) of compounds in the sample were identical to spectral signature of standard compounds. The recoveries for GA, HMF, procyanidin B2, caffeine, EGCG and EC were approximately 106, 102, 100, 101, 106 and 96% respectively (Table 3.3). A minimum of three spiked samples at different concentration was included for each analysis to monitor any shift in peak retention time resulting from possible changes in pressure, temperature or column

condition (degradation). Structures of bioactive compounds identified in fresh baobab pulp are presented in Figure 3.7.

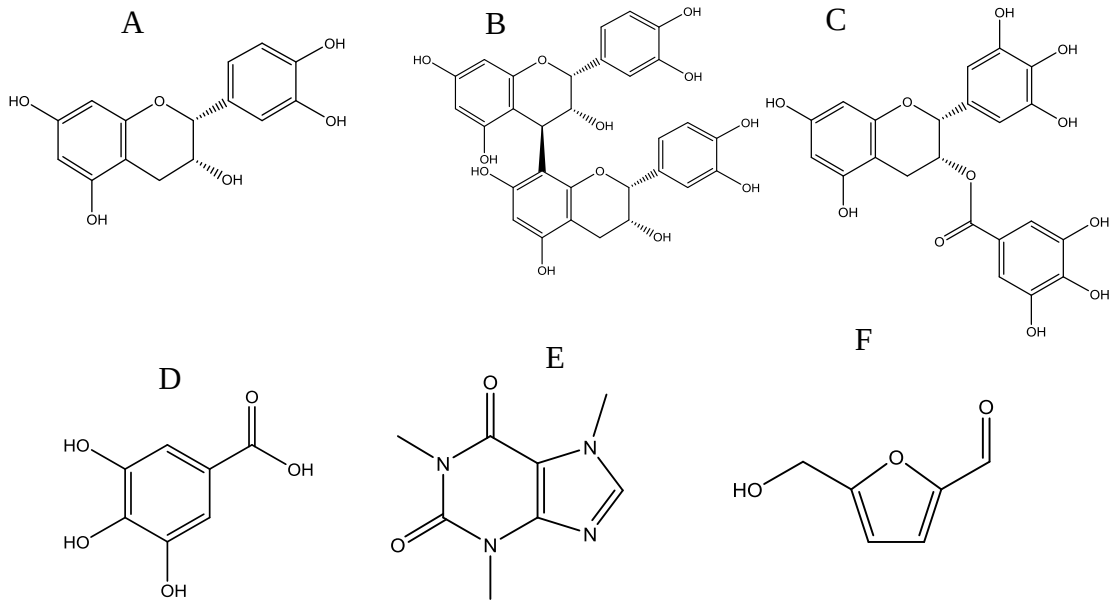


Figure 3.7 - Structures of bioactive compounds analysed in fresh baobab pulp: (-)-Epicatechin (A), procyanidin B2 (B), (-)-epigallocatechin-3-O-gallate (C), gallic acid (D), caffeine (E) and 5-hydroxymethylfuraldehye (F).

Table 3.3 - Analytical parameters for identification of bioactive compounds in fresh baobab pulp. Results are means of triplicates (± SD); n = 3.

Standard compound	Retention time (min)	Wavelength (nm)	Recovery (%)
(+)-Catechin	18.20 ± 0.01	284	ND
(-)-Epicatechin	25.80 ± 0.03	284	96.12 ± 0.66
Procyanidin B2	19.27 ± 0.29	284	100.29 ± 0.11
(-)Epicatechingallate	18.69 ± 0.02	280	ND
(-)-Epigallocatechin	18.40 ± 0.02	284	ND
EGCG	24.33 ± 0.03	284	106.21 ±16.15
Quercetin	28.85 ± 0.02	370	ND
Gallic acid	11.05 ± 0.01	284	106.28 ± 4.57
Caffeine	21.74 ± 0.02	284	100.84 ± 2.91
HMF	11.54 ± 0.01	284	102 ± 0.16

ND = Not detected

3.5.1 **Quantification of bioactive compounds**

Predominant bioactive compounds were quantified in fresh baobab pulp using standard calibration curves in order to compare the levels with commonly consumed fruits and evaluate their potential contribution to total phenol content and antioxidant activity of the fresh baobab pulp.

3.5.1.1 **Standard calibration curves for bioactive compounds**

Standard calibration curves used for quantification of bioactive compounds including galic acid (GA), 5-hydroxymethlyfurfural (HMF), procyanidin B2 (PRO), caffeine, (-)-epigallocatechin-3-O-gallate (EGCG) and (-)-epicatechin (EC) in baobab fruit samples are presented in Figures 3.8 and 3.9. Calibration curves covering a wide range of concentration with good linearity ($R^2 \geq 0.999$) which is a prerequisite for accurate quantification, was achieved.

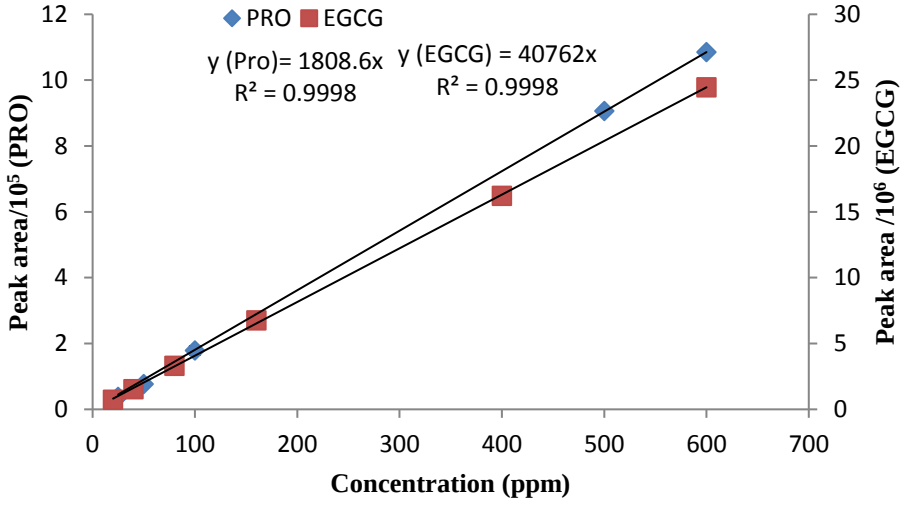


Figure 3.8 - Calibration curves for procyanidin B2 and EGCG at 284 nm used for quantification in baobab samples; n = 3.

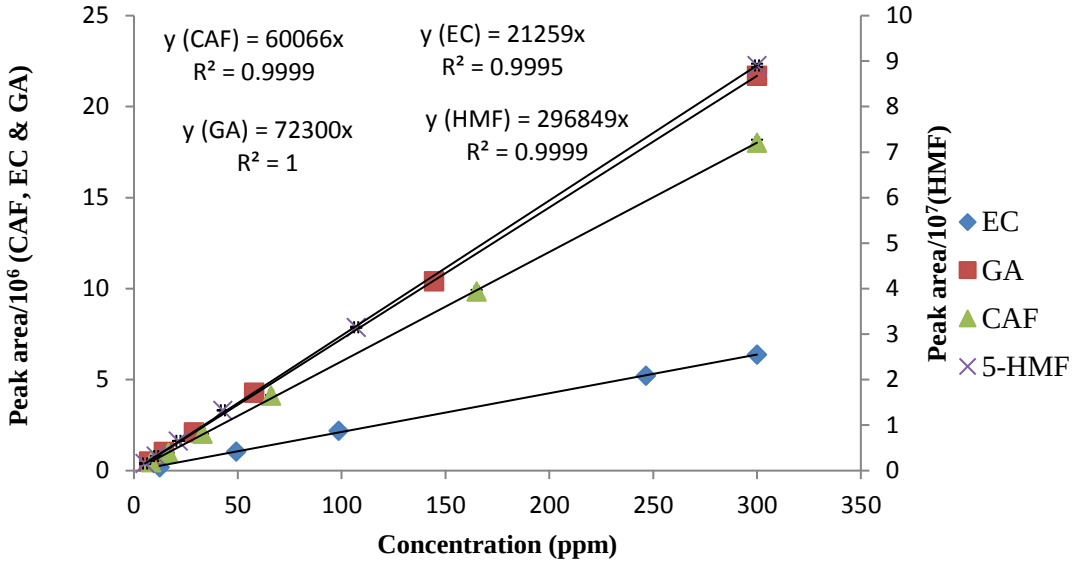


Figure 3.9 - Calibration curves for (-)-epicatechin (EC), caffeine (CAF), gallic acid (GA) and 5-hydroxymethlyfurfural (5-HMF) used for quantification in baobab samples; n = 3.

The content of procyanidin B2 (533.34 ± 2.63 mg/100 g), gallic acid (68.54 ± 12.41 mg/100 g) and (-)-epicatechin (42.95 ± 3.08 mg/100 g) determined in fresh baobab pulp were significantly higher ($p \leq 0.05$) than levels reported for many fruits. Fresh baobab seeds showed extremely high procyanidin B2 (1488.90 ± 7.43 mg/100 g FW) than levels observed in the pulp. Prior and Gu (2005) reported that choke berry, cranberry, blueberry and plum contain 663.7 ± 47.7, 418.8 ± 75.3, 255.8 ± 107.5 and 215.9 ± 50 mg/100 g total proanthocyanidin content respectively. Pimpão et al. (2013) reported that blueberry and blackberry contain 16.32 ± 3.10 and 6.08 ± 0.25 mg/100 g gallic acid respectively. Fresh baobab pulp from Malawi is thus an excellent source of procyanidin B2 and gallic acid and may be considered as a potential source of raw materials for novel functional foods. Moreover the level of (-)-epicatechin (42.95 ± 3.08 mg/100 g) was higher than levels reported by Pimpão et al. (2013) for blueberry, raspberry, blackberry

and Portuguese crowberry. It can therefore be envisaged that procyanidin B2, (-)-epicatechin and gallic acid contribute significantly to the total phenol content and antioxidant activity. Fresh baobab pulp expressed more than 39 fold less caffeine content (0.87 ± 0.04 mg/100 g) compared to decaffeinated roasted coffee (34 to 47 mg/100 g) reported by Fujioka and Shibamoto (2008).

Table 3.4-Selected bioactive compounds (mg/100 g FW) in fresh baobab pulp. Results are means of triplicates (±SD).

Bioactive compounds	Content	Bioactive compounds	Content
Quercetin	Not detected	(-)-Epicatechin	43.00 ± 3.10
(+)-Catechin	Not detected	Procyanidin B2	533.30 ± 22.6
(-)-Epicatechingallate	Not detected	EGCG	9.98 ± 0.08
(-)-Epigallocatechin	Not detected	Gallic acid	68.54 ± 12.4
		Caffeine	0.87 ± 0.04
		5-Hydroxymethylfurfural	1.10 ± 0.00

3.6 Effect of methanol concentration on total phenol content (TPC)

This experiment was conducted to examine the effect of methanol concentration on the yield of total phenolic content in baobab pulp and juice. Five methanol concentrations were evaluated. A calibration curve with gallic acid concentration ranging from 0 and 200

ppm and good linearity ($R^2 \geq 0.999$) was used for quantification of total phenol content in fresh baobab pulp (Figure 3.10).

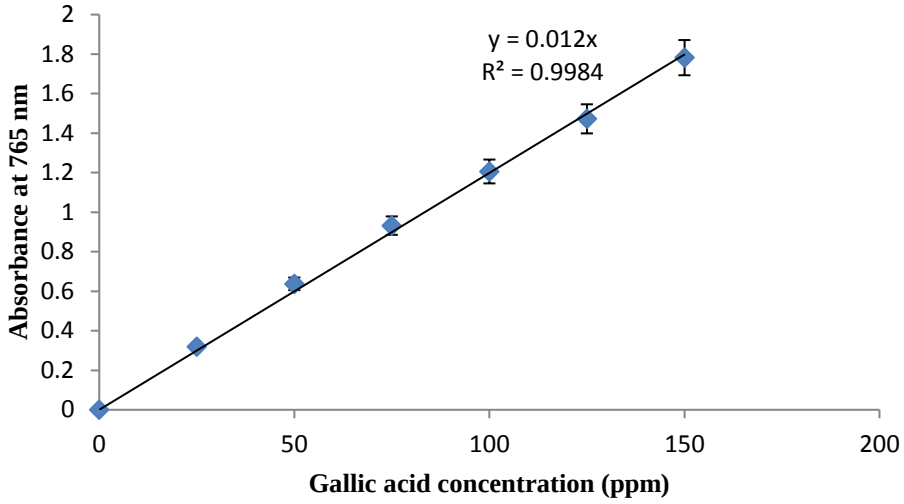


Figure 3.10 - Standard calibration curve for gallic acid used for quantification of total phenol content in baobab samples by the Folin assay; n = 3.

For this experiment, fresh pulp powder (10% moisture content) and baobab juice described in 2.5.3 (10% pulp content) were used. Results showed that methanol concentration significantly ($p \leq 0.05$) affected the yield of TPC extracted from pulp or juice (Figure 3.11). For the juice, TPC was increasing with methanol concentration. For instance, 80% (v/v) methanol/water gave significantly ($p \leq 0.05$) highest TPC (1862.14 ± 7.7 mg/100 g) of all extracting solvents including water (1539.60 mg/100 g). However, the trend was reversed for fresh pulp powder. TPC was decreasing with methanol concentration. For instance, 20% (v/v) methanol/water gave significantly highest ($p \leq 0.05$) TPC (1599.79 mg/100 g) than 80% (v/v) methanol/water (874.48 mg/100 g) in the pulp. From this experiment it can be suggested that baobab pulp contains both polar and

nonpolar compounds and optimisation of solvent concentration is necessary to maximise yield of TPC. The solvent 80% (v/v) methanol/water was selected and used for the extraction of phenolic compounds in baobab juice as described in chapter 2. Other researchers reported that 50% (v/v) of methanol/water or acetone/water gave significantly higher yield of TPC than concentrated or absolute counterparts (Wijekoon et al., 2011). Thus confirming variation of performance of solvent systems with species.

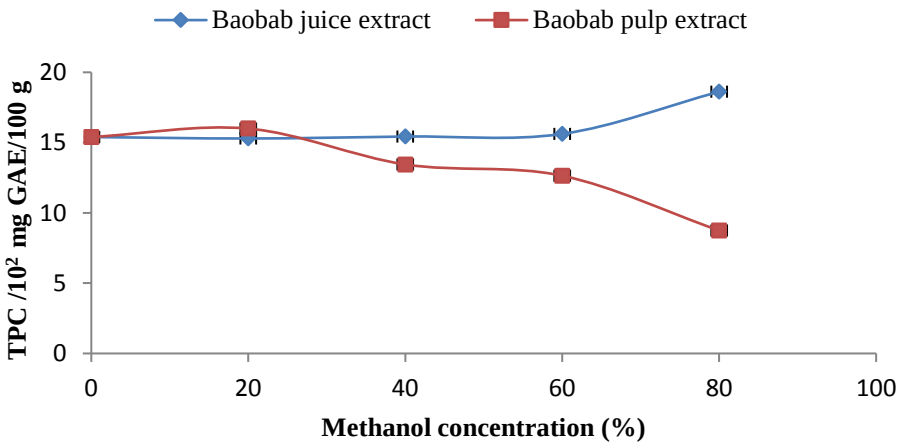


Figure 3.11 - Effect of methanol concentration (v/v) in water on TPC of baobab pulp and juice; n = 3.

3.6.1 **Total phenol content (TPC)**

Although the determination of TPC in samples using Folin-Ciocalteu reagent (FCR) assay has some limitations and interferences it is widely used for preliminary assessment of phenolic compounds (Pimpão et al., 2013). Results showed that fresh baobab pulp from Malawi contains higher total phenol content (1866.81 ± 1.61 mg /100 g FW) than some of the commonly consumed fruits considered as rich source of dietary polyphenols

including black chokeberry (1752 mg GAE/100 g), blackcurrant (821 mg GAE/100 g) and apple (205 mg GAE/100 g) according to Pérez-Jiménez et al. (2010). Ibrahima et al. (2013) reported lower value of TPC (1085 mg GAE /100 g) for baobab fruit pulp from Madagascar while Lamien-Meda et al. (2008) reported a much higher values of TPC (3518 – 4058 mg GAE/100 g) for baobab pulp from Burkina-Faso. Higher TPC is ascribed to bioactive compounds including procyanidin B2, (-)-epicatechin, galic acid and (-)-epigallocatechin-3-O-gallate observed in the fresh baobab pulp from Malawi (section 3.5). These compounds may have significant benefit to human health and nutrition by acting as antioxidants required by the immune system for the prevention of chronic diseases associated with oxidative stress (Crozier et al., 2009). Further analysis of the baobab extract including antioxidant activity was conducted.

3.6.2 **Antioxidant activity**

It is recommended to measure antioxidant activity using different methods because the activity of antioxidants in food and biological systems is dependent on a multitude of factors including colloidal properties of substrates, structural conformation of antioxidants, the condition and stage of oxidation and the localisation of antioxidants in different phases (Brand-Williams et al., 1995; Frankel and Meyer, 2000). For this reason, the total antioxidant activity of baobab fruit products was evaluated by three standard assays, the ferric reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2′-Azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging ability described in section 2.6 according to literature (Brand-Williams et al., 1995; Re et al., 1999; Benzie and Strain, 1996). Some dietary antioxidants are hydrophilic

while others are lipophilic therefore the use of FRAP, DPPH and ABTS assays helps to capture both thereby providing accurate total antioxidant activity information. In particular ABTS assay measures both hydrophilic as well as lipophilic antioxidants. Calibration curves (Figures 3.12-3.13), with Trolox concentration ranging from 10 to 250 mg L^{-1} and good linearity ($R^2 \geq 0.99$) were used for quantification of FRAP, DPPH and ABTS antioxidant activity.

Results show that the fresh of baobab pulp has very high antioxidant activity/capacity (FRAP, ABTS and DPPH). The FRAP and ABTS radical quenching ability in fresh baobab pulp were 4664.20 ± 11.81 and 1684.54 ± 17.00 mg TEAC/100 g respectively while DPPH radical scavenging activity was 50.93 ± 0.4% DPPH. FRAP values fell within the range (4171.81 – 5425.48 mg/100 g) reported by other authors (Coe et al., 2013) in fresh baobab pulp obtained from different locations. High level of antioxidant activity is attributed to higher vitamin C and total phenolic content in fresh baobab pulp. Strong positive relationship between total phenol content and antioxidant activity of fruit products is well documented for commonly consumed fruits (Almeida et al., 2011; Maizura et al., 2011; Park et al., 2011). Antioxidant activity in baobab fruit pulp cited in literature is vastly variable; from 2703 mg TEAC/100 g (Ibrahima et al., 2013) to 150174 - 175203 mg TEAC/100 g (Kamatou et al., 2011). The relatively low antioxidant activities of baobab pulp reported by Ibrahima et al. (2013) could be attributed to the lower content of ascorbic acid quoted in the same paper. Lamien-Meda et al. (2008) demonstrated differences in antioxidant activity when samples are extracted with different solvent systems. In the present work, 80% (v/v) methanol was selected for extraction of phenolic

compounds in the juice because it gave significantly higher yield of total phenol content as described in 3.6. It is also possible that the study which reported exceptionally high antioxidant capacities for baobab pulp employed a longer incubation period for these assays. Incubation time affects the extent of reaction between the oxidant, also known as probe ($[Fe\ (III)\ (TPTZ)_2]^{3+}$, $ABTS^{\cdot+}$ or $DPPH^{\cdot}$) and the antioxidants present in the sample. Further, the kinetic pattern of phenol oxidation is dependent on several factors (concentration, oxidation state) such that the reaction between phenolic antioxidants and probe may not reach completion within the time span of the protocol leading to underestimated TEAC values (Apak et al., 2016). Kaboré et al. (2011) described variations in nutrient and non-nutrient levels of baobab pulp with soil type, geographical location, age of fruit, processing and storage conditions, which may explain the differences in antioxidant activity reported in literature.

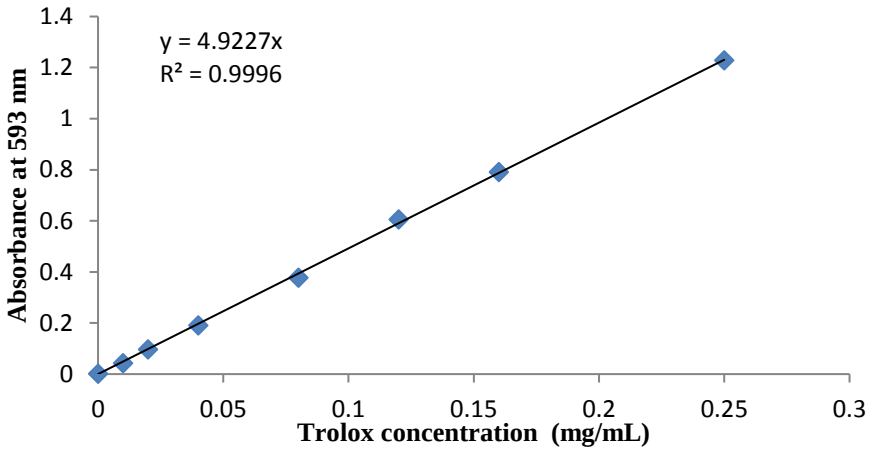


Figure 3.12 - Standard calibration curve for Trolox for quantification of FRAP in baobab products; n = 3.

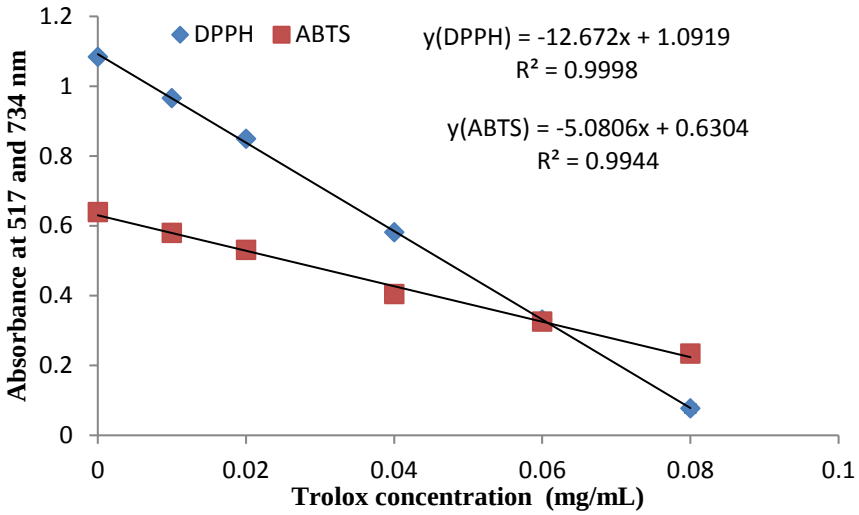


Figure 3.13 - Standard calibration curves for Trolox for quantification of antioxidant activity (ABTS and DPPH) in baobab products; n = 3.

3.7 Conclusions and recommendations

Results from this study showed that fresh baobab fruit pulp is rich source of vitamin C and contains higher levels of organic acids, (-)-epicatechin, procyanidin B2, gallic acid and (-)-epigallocatechin-3-O-gallate than some of the commonly consumed fruits. The presence of these bioactive compounds were likely responsible for higher total phenol content and antioxidant activity observed in the extract. Bioactive compounds identified in baobab pulp and seeds are associated with potential health benefits as a result of their antioxidant, antiviral, radical scavenging and metal chelating activities. Thus baobab fruit offers an opportunity for the development of quality novel functional foods and pharmaceuticals for improved nutrition and health status of the people of Malawi.

Chapter 4 **Influence of processing and storage on bioactive compounds of baobab juice**

Abstract

The effect of thermal processing and storage on selected bioactive compounds of baobab fruit pulp and juice from Malawi was evaluated using RP-HPLC while total phenol content (TPC) and antioxidant activity were measured by spectrophotometry. Heat treatment by microwaving and pasteurisation (72 °C, 15 s) did not significantly ($p \leq 0.05$) reduce vitamin C content in the pulp and juice respectively. However, vitamin C content of pasteurised juice was significantly ($p \leq 0.5$) lost during storage compared to untreated juice suggesting the need for advanced processing methods. The levels of (-)-epicatechin, procyanidin B2 and TPC were fluctuating during storage indicative of a series of chemical reactions which may affect juice quality. Although degradation of natural antioxidants was likely at high temperatures, the effect on overall antioxidant capacity was not as noticeable due to the presence of phenolic compounds and possibility of melanoidins (brown pigments). The increase in antioxidant activity towards the end of storage in processed juice coincided with the formation of melanoidins which were clearly visible at 30 °C. The antioxidant activity of baobab juice was more correlated with TPC compared to vitamin C. The findings from the present study supports the need for non-thermal pasteurisation to retain vitamin C as well as improve its stability during storage thus increasing nutritive value of baobab juice.

Keywords: Baobab juice; Thermal processing; Storage; Vitamin C; Bioactive compounds; Total phenol content; Antioxidant activity.

4.1 Introduction

A major motivation for fruit processing is in the preservation or shelf life extension by preventing undesirable changes in the nutritive value and sensory qualities (Ramaswamy and Marcotte, 2006). This is achieved by controlling the chemical, biochemical, physiological and microbiological activities using various techniques that include removal and addition of heat, removal of moisture, controlling water activity and addition of preservatives, sugar, salt and acids (Ramaswamy and Marcotte, 2006). Fruit processing is also aimed at product diversification to provide variety, taste and nutrition, and to facilitate marketing and production of nonconventional products (Igual et al., 2010). The majority of smallholder farmers and rural communities in Malawi benefit from baobab products through consumption and sale. However, utilisation is limited due to insufficient knowledge on processing for quality control. There are several studies on stability of vitamin C and other bioactive compounds in commonly consumed fruits (Klimczak et al., 2007; Derossi et al., 2010; Mølmann et al., 2015) but such information is missing for baobab fruit juice from Malawi. The current practice of processing baobab into juice relies heavily on uncontrolled thermal treatment and this may have great influence on the level, bioavailability and bioactivity of heat sensitive micronutrients. The high vitamin C content in fresh baobab pulp is significantly lost in the final juice due to excessive heating. High thermal pasteurisation (90 °C, 15 min) of baobab juice reduced levels of vitamin C by 56% (Ndabikunze et al., 2010). A solid understanding of the chemical composition,

kinetics and mechanism of food quality loss is a prerequisite for successful optimisation of processes and handling of the final products.

4.2 Aim of the chapter

The aim of the present study was to investigate the influence of thermal processing and storage on bioactive compounds and chemical properties of baobab pulp and juice. Specific objectives were three-fold.

i. To evaluate the ascorbic acid degradation kinetics in baobab juice,
ii. To evaluate the effect of thermal treatment on ascorbic acid content of baobab pulp and juice and
iii. To evaluate the influence of storage time and temperature on selected bioactive compounds of prepared baobab juice (PBJ).

4.3 Results and discussion

4.3.1 Effect of thermal treatment on ascorbic acid of baobab pulp

Retention studies of vitamins to assess the effects of food processing and storage on the nutritive value of foods are of great importance to food technologists. Vitamin C is thermolabile and therefore in fruit and vegetables it provides an indication of the loss of other vitamins and acts as a valid marker of deterioration of nutritional quality (Klimczak et al., 2007).

Baobab pulp was heat treated by microwaving and the effect of this heat treatment on ascorbic acid content of fresh pulp was evaluated. Results show that ascorbic acid was not significantly affected ($p > 0.05$) by microwaving. Fresh pulp and microwaved pulp showed mean ascorbic acid content of 309.3 ± 2.7 and 280.7 ± 0.9 mg/100 g FW respectively representing more than 91% retention. Similarly, Brewe et al. (2003) observed that microwave treatment at any power level for as little as 1 min reduced peroxidase activity and retained ascorbic acid content in broccoli, green beans and asparagus. Proper heat treatment of baobab pulp power may stabilise nutritional quality of this product which is widely available in UK supermarkets (Holland and Barret).

4.3.2 **Effect of thermal treatment on ascorbic acid of baobab juice**

For acidic fruit products, high temperature short time (HTST) pasteurisation is applied at temperatures around 72 °C with holding times of 15 s and above but low temperature long time (LTLT) pasteurisation (60 °C, 30 min; 60 °C, 60 min) is also widely used (Chen et al., 2013). Both methods may degrade taste, colour, flavour and nutritional quality of baobab juice. Therefore the effect of different pasteurisation treatments on quality attributes of baobab juice was investigated. Figure 4.1 shows ascorbic acid (AA) percent retention of pasteurised juice and untreated control (assigned 100%). As can be seen HTST pasteurised juice (72 °C, 15 s) showed significantly higher ($p \leq 0.05$) ascorbic acid than other treatments. For HTST (85 °C, 15 s), elevated temperatures might have contributed to an increased rate of degradation of AA while for LTLT regimes both temperature and time influenced degradation of ascorbic acid. Igual et al. (2010) observed no significant difference in the content of AA between freshly squeezed grapefruit juice

and conventionally HTST pasteurised juice (80 °C, 11 s). Ascorbic acid or total vitamin C degradation may vary in different fruit products due to differences in plant matrices, composition, concentration, pH and water activity (Fennema, 1996; Gamboa-Santos et al., 2014). For instance the presence of metal ions including Fe^{3+} and Cu^{2+} in the matrix may catalyse oxidation of ascorbate. The oxidation of AA to DHA is reversible and relative yield depends on reaction conditions. The pH dependence of oxidation of AA is governed mainly by the relative concentration of fully protonated ascorbate (AH_2), ascorbate monoanion (AH^-) and ascorbate dianion (A^{2-}) species hence pH or pK_a of the system. Ascorbic acid is stable at pH ≤ 2.5 while DHA is stable at pH 2.5 - 5.5 (Fennema, 1996; Sanmartin et al., 2000). Ripe and fresh baobab pulp has very low moisture content (10%) and low water activity which contribute to extended AA shelf life. Juice formation increases water activity depending on concentration may raise the pH shifting the AA/DHA equilibrium, favouring DHA formation which may easily undergo irreversible degradation with increased temperature (Fennema, 1996; Rawson et al., 2011) during thermal pasteurisation. The pH of baobab juice was 3.11 thus degradation of AA was more likely compared to DHA.

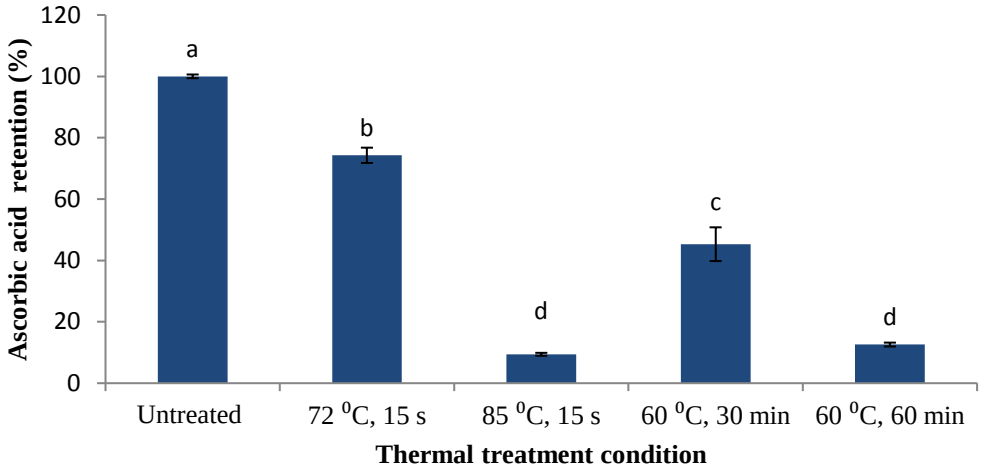


Figure 4.1 - Percent retention of ascorbic acid (AA) after pasteurisation. Results are means of triplicates (±SD).Values with different letters are significantly different (Tukey's test, $p \leq 0.05$).

4.3.3 **Vitamin C degradation kinetics in baobab juice**

Vitamin C degradation kinetics enables accurate prediction of vitamin C loss and shelf-life determination of juice. Such knowledge is important for the development of quality juice in terms of vitamin C content and associated functional properties including antioxidant activity. In the present study, the change of ascorbic acid concentration in baobab juice was evaluated as a function of time. Initial rates of degradation were presented as a function of concentration in order to obtain rate constants. Arrhenius plot was used to accomplish degradation kinetics of ascorbic acid in baobab juice.

4.3.3.1 **Initial rates of ascorbic acid degradation**

Initial rates of ascorbic acid degradation over 24 hours were conducted at 6, 15, 30 and 40 ºC storage temperatures. Figure 4.2 shows that the initial rates of AA degradation were directly proportional to ascorbic acid concentration ($R^2 \geq 0.9$) for each of the storage

temperatures. The rate constants (gradients, *k*) were 0.0094, 0.0198, 0.0332 and 0.0462 day^{-1} at 6, 15, 30 and 40 ºC respectively. The rate constant at 40 ºC was more than 4.9-fold of that at 6 ºC indicating accelerated rate of AA degradation. The temperature dependency of initial rate constants of ascorbic acid in baobab juice was determined using the Arrhenius plot (Figure 4.3). The activation energy, E_a (33.37 kJ/mol) of ascorbic acid was calculated from equation of the line ($\ln k = -\frac{E_a}{RT} + \ln[AA]$, $R^2 \geq 0.95$). Activation energy (E_a), hence rate constants for ascorbic acid in different plant foods may vary widely. For instance, other researchers reported E_a values for AA ranging from 98 to 112 kJ/mol in frozen green vegetables (Giannakourou and Taoukis, 2003); 82.1 kJ/mol in strawberries (Gambos-Santos et al., 2014); 53.42 to 106.23 kJ/mol in citrus juice concentrates (Karadeniz, 2004) and 51 to 135 kJ/mol for orange juice (Remini et al., 2015). Higher positive E_a means degradation of AA is more favourable with increasing temperature. If degradation reaction of a compound has zero E_a it means its rate is independent of temperature while a negative E_a means rate decreases when temperature is raised (Atkins, 2010). Therefore results from this study showed that increased temperature had less influence on ascorbic acid degradation compared to orange juice, strawberries and other commonly consumed juice. Phenolic compounds (procyanidin B2, gallic acid, (-)-epicatechin, (-)-epigallocatechin-3-O-gallate) available in high concentration in baobab pulp could be responsible for protective effect against ascorbic acid degradation through redox reaction. The mechanism may involve donation of hydroxyl protons or electrons to DHA slowing down loss of ascorbic acid in the process. Concentrated juice achieved by increasing pulp content rather than additives may be a

viable way of preventing ascorbic acid loss through auto-oxidation or thermal degradation during processing and storage for most fruit products.

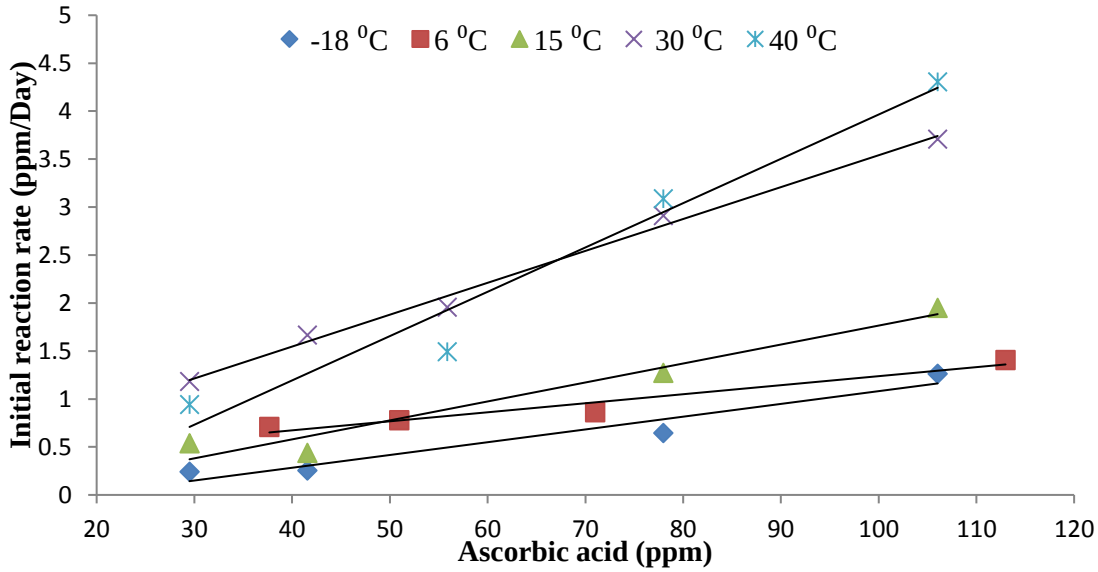


Figure 4.2 - Results for initial reaction rates of AA in baobab juice left at four storage temperatures; n = 3.

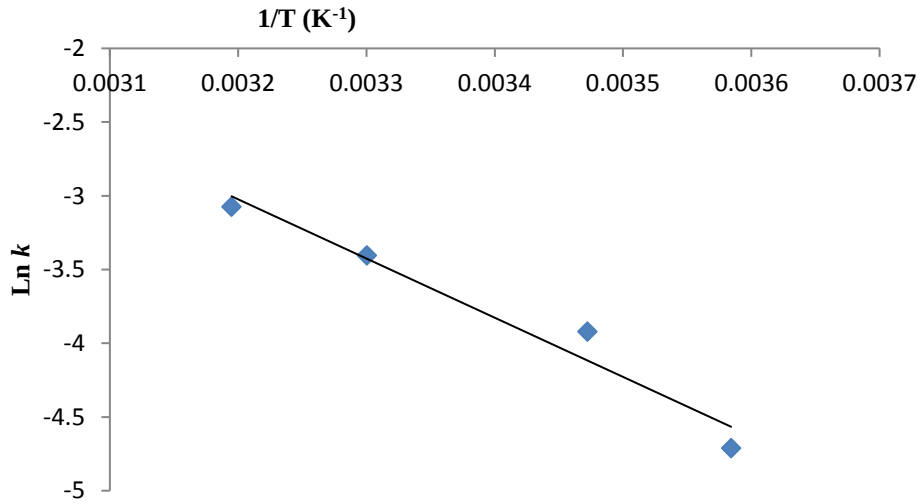


Figure 4.3 - Arrhenius plot (Ln *k* versus 1/T in Kelvin) of ascorbic acid degradation in baobab fruit juice; n = 3.

4.3.4 **Effect of storage on quality attributes of baobab juice**

Chemical composition and nutritional quality of plant foods are affected by several factors including processing and storage and are also influenced by plant species. It is therefore necessary to have sufficient information for individual plant species or variety for optimum utilisation. Results for changes in vitamin C, organic acids, total phenol content, antioxidant activity and selected bioactive compounds of untreated and processed baobab juice kept at isothermal temperature conditions (6, 15, 30 ºC) for a maximum period of 60 days are presented and described. These temperatures represent refrigeration (6 °C), medium (15 °C) and higher ambient temperature (30 ºC) usually attained in Malawi where raw materials were collected.

4.3.4.1 **Effect of storage temperature on ascorbic acid and vitamin C**

Table 4.1 shows results for ascorbic acid and vitamin C (AA & DHA) of untreated juice (UT), high temperature short time pasteurised juice (72 °C, 15 s) (HTST) and high temperature short time pasteurised juice (72 °C, 15 s) with added citric acid (0.5%) (CAHTST).

Table 4.1-Ascorbic acid and vitamin C concentration (ppm) in untreated (UT), pasteurised (HTST) and acidified (CAHTST) baobab juice. Results are means of triplicates (±SD).Mean values within a column with different letters are significantly different (Tukey's test, $p \leq 0.05$).

Storage condition / Treatment		*Organic acid concentration (ppm)*					
		UT		*HTST*		*CAHTST*	
Temperature *°C*	Time (Days)	*Ascorbic acid (ppm)*	*Vitamin C (ppm)*	*Ascorbic acid (ppm)*	*Vitamin C (ppm)*	*Ascorbic acid (ppm)*	*Vitamin C (ppm)*
6	0	87.09 ± 8.47^{a}	97.13 ± 0.89^{a}	75.80 ± 0.13^{a}	99.12 ± 0.95^{a}	68.32 ± 0.25^{a}	88.37 ± 0.95^{a}
	14	33.75 ± 1.00^{b}	87.41 ± 0.07^{b}	22.42 ± 1.08^{b}	61.44 ± 0.77^{b}	9.53 ± 1.73^{b}	43.58 ± 0.47^{b}
	28	15.28 ± 2.14^{d}	45.41 ± 3.65^{c}	6.57 ± 0.00^{c}	30.55 ± 1.57^{c}	9.31 ± 0.51^{b}	28.03 ± 1.20^{c}
	42	21.06 ± 1.01^{cd}	35.96 ± 1.08^{d}	3.45 ± 0.04^{efg}	16.08 ± 0.77^{de}	3.81 ± 0.26^{de}	14.93 ± 0.90^{d}
	60	17.71 ± 0.21^{d}	23.90 ± 0.19^{e}	2.15 ± 0.06^{gh}	14.04 ± 0.03^{ef}	2.32 ± 0.08^{e}	14.43 ± 0.44^{d}
15	*14*	27.19 ± 0.89^{bc}	45.06 ± 2.14^{c}	5.46 ± 0.47^{cd}	17.82 ± 0.53^{d}	5.38 ± 0.51^{cd}	15.54 ± 0.70^{d}
	28	5.27 ± 0.00^{e}	18.66 ± 2.09^{f}	3.53 ± 0.72^{efg}	17.28 ± 0.15^{d}	8.46 ± 0.34^{b}	25.55 ± 0.29^{c}
	42	7.47 ± 0.92^{e}	24.92 ± 0.26^{e}	3.29 ± 0.77^{fg}	8.12 ± 1.20^{g}	4.92 ± 0.44^{cd}	11.73 ± 1.31^{e}
	60	5.68 ± 0.00^{e}	12.48 ± 0.09^{g}	2.42 ± 0.11^{gh}	11.95 ± 0.22^{f}	4.85 ± 0.20^{cde}	7.16 ± 0.18^{g}
30	*14*	7.05 ± 1.48^{e}	9.76 ± 1.58^{gh}	4.71 ± 0.42^{de}	11.56 ± 1.47^{f}	4.58 ± 0.30^{cde}	9.51 ± 0.42^{efg}
	28	2.60 ± 0.30^{e}	4.53 ± 0.76^{i}	1.81 ± 0.20^{h}	3.71 ± 1.36^{i}	3.26 ± 0.93^{de}	9.78 ± 0.49^{ef}
	42	5.85 ± 0.53^{e}	7.36 ± 0.57^{hi}	4.76 ± 0.26^{de}	4.13 ± 0.40^{hi}	7.00 ± 2.18^{bc}	7.31 ± 1.72^{fg}
	60	3.89 ± 0.06^{e}	6.28 ± 1.12^{hi}	4.63 ± 0.22^{def}	6.73 ± 0.14^{gh}	8.25 ± 0.09^{b}	9.00 ± 0.24^{fg}

4.3.4.1.1 Changes in ascorbic acid and total vitamin C concentration

For untreated juice, the initial level of ascorbic acid (AA) and Vitamin C decreased significantly ($p \leq 0.05$) during storage. For instance, at 6 °C the level of AA decreased by 61.24 and 82.45% by day 14 and 28 respectively while vitamin C decreased by 10 and 53% during the same period. After day 28, the level of AA was very low and remained constant while vitamin C continued to decrease significantly ($p \leq 0.05$) until the end of storage time. At 15 °C, the level of AA decreased by 68.78 and 94.95% by day 14 and 28 respectively while vitamin C decreased by 53 and 81% during the same period. After day 28, the level of AA was very low and remained constant while vitamin C continued to decrease until the end of storage time. Finally, at 30 °C, initial level of AA dropped by 90.0 and 92.7% by day 14 and 28 respectively while vitamin C dropped by 90.0 and 95.3 % during the same period. After day 28, the level of both AA and vitamin C were too low.

From these results it was noted that for UT baobab juice the percentage loss of ascorbic acid was highest during early stages of storage but also the percentage loss increased with temperature. After day 28, the percent loss of ascorbic acid and vitamin C was gradual between successive storage times. The percent losses of ascorbic acid were higher compared to vitamin C at 6 and 15 °C whereas at 30 °C, both ascorbic acid and dehydroascorbic acid were almost completely lost. Lower losses in vitamin C may be ascribed to stability of DHA in baobab juice (pH 3.11 ± 0.10). Ascorbic acid requires more acidic conditions ($pH \leq 2.5$) for its stability.

Although there is an increasing demand for fresh, ready-to-eat and nutritious foods such as fresh-cut fruit and unpasteurised fruit juices, pasteurisation is necessary to inactivate naturally occurring microorganisms and decrease the activity of enzymes (Mosqueda-Melgar, 2012; Chen et al., 2013) which reduce quality attributes and shelf life of fresh fruits and fruit products. Ascorbate oxidase promotes the degradation of ascorbic acid to dehydroascorbic acid (Rawson et al., 2011) while pectin methylesterase (PME) catalyses pectin degradation and alters the colloid stabilising power of the pectin, which imparts the favourable appearance and mouth feel of juice (Igual et al., 2010). Since ascorbic acid has antioxidant properties and is one of the components in human nutrition its degradation means loss of other quality attributes as well as nutritional value of juice. High temperature short time (HTST) pasteurisation is recommended because apart from inactivation of enzymes and spoilage microorganisms it may retain thermolabile nutritional attributes including ascorbic acid and dehydroascorbic acid in some fruit juices. At all storage temperatures the initial concentration of both AA and vitamin C decreased significantly ($p \leq 0.05$). For instance, at 6 °C, the initial concentration of AA decreased by approximately 72 and 91% by day 14 and 28 respectively. After day 28, the level of AA was not significantly different ($p > 0.05$) during the remaining storage time. On the other hand, vitamin C decreased by about 38 and 69% by day 14 and 28 respectively and remained nearly constant after day 42 until the end of storage time. At 15 °C, the initial level of AA dropped significantly ($p \leq 0.05$) until day 28 then remained stable until the end of storage time. However, vitamin C concentration did not change significantly ($p > 0.05$) between day 14 and 28. It was interesting to note that the level of vitamin C in HTST juices kept at 6 and 15 °C were not significantly different ($p > 0.05$)

by the end of storage time. At 30 °C, ascorbic acid and vitamin C were the most affected compared to 6 and 15 °C and reached a minimum values by day 28. Higher storage temperature was more likely responsible for degradation of ascorbic and dehydroascorbic acid than other factors. After the first 14 days, the loss of vitamin C in HTST juice was generally more gradual compared to UT juice. This may be ascribed to the fact that HTST had lower level of vitamin C after 14 days compared to UT.

Citric acid is a natural antimicrobial and generally recommended as safe (GRAS) according to the U.S. Food and Drug Administration. Citric acid is one of the preservatives widely used in fruit products to stabilise pH and prevent deterioration caused by microbial activities (Mosqueda-Melgar, 2012). Baobab fruit juice was therefore acidified with 0.5% citric acid as described in 2.5.6. At 6 °C, AA decreased significantly ($p \leq 0.05$, 86%) by day 14, and then remained stable until day 28. The level of AA was not significantly different ($p > 0.05$) between day 42 and 60. Vitamin C decreased by 50.6% during the first 14 days, then dropped further by day 28, and finally remained stable until the end of storage. At 15 °C, the AA decreased by approximately 92% by day 14, and then remained stable until the end of storage. While vitamin C dropped by 82% during the first 14 days, increased on day 28, and finally continued to decrease until the end of storage. At 30 °C, the levels of both ascorbic acid and vitamin C were significantly lowest ($p \leq 0.05$) compared to levels at 6 and 15 °C. After day 14, ascorbic acid and vitamin C decreased by 98 and 88% respectively then became stable until the end of storage. According to several published data (Klimczak et al., 2007; Igual

et al., 2010), the level of ascorbic acid and vitamin C decrease during storage, depending on storage temperature, oxygen, light access, processing and preservation treatment.

In general results showed that the level of both ascorbic acid and vitamin C in all samples decreased with storage temperature. It is clear that pasteurisation and acidification did not improve overall vitamin C levels when compared to UT juice. It seems pasteurisation provoked degradation and loss of vitamin C during storage of baobab juice. For untreated juice degradative enzymes including ascorbate oxidase (AO), pectin methylesterase (PME) and polyphenol oxidase (PPO) were thought to be the most influential factors for the loss of AA and vitamin C. For pasteurised and acidified baobab juice, long exposure to oxygen and metallic plates contact during pasteurisation (heat exchanger) might have influenced initial degradation of ascorbic acid and vitamin C. Higher moisture content and water activity combined with elevated temperature were all responsible for accelerated degradation of ascorbic acid and vitamin C at 30 °C in all samples. DHA as well as dicarbonyl compounds formed during its degradation (DKGA, erythrulose, 3-deoxythreosone, xylosone) may participate in Strecker degradation with amino acids forming brown pigments (Fennema, 1996). Brown pigmentation was quite visible in juice samples stored at 30 °C. A possible mechanism for the formation of coloured compounds is predicted by the mechanism shown in Figure 4.4.

Results from this experiment indicated that consumption of untreated baobab juice (10%) left at 6 °C within 14 days may provide about 90% of initial vitamin C content. HTST pasteurisation and acidification with citric acid may still be required to improve sensory

and microbial characteristics of the juice. Results from this study are consistent with findings reported by other researchers (Burdurlu et al., 2006; Igual et al., 2010; Aka et al., 2013) regarding ascorbic acid and vitamin C degradation in citrus juice concentrates. It was reported that by the end of 8 weeks, the initial content of vitamin C decreased by 16, 78 and 83% at 28, 37 and 45 °C respectively for orange juice concentrate. During the same storage conditions, vitamin C decreased by 45, 76 and 80% for lemon juice concentrate; 30, 73 and 85% for grape fruit juice concentrate; and 34, 76 and 85% respectively for tangerine juice concentrate (Burdurlu et al., 2006). In all juice samples, the percent loss of vitamin C increases with storage temperature but is also influenced by fruit species.

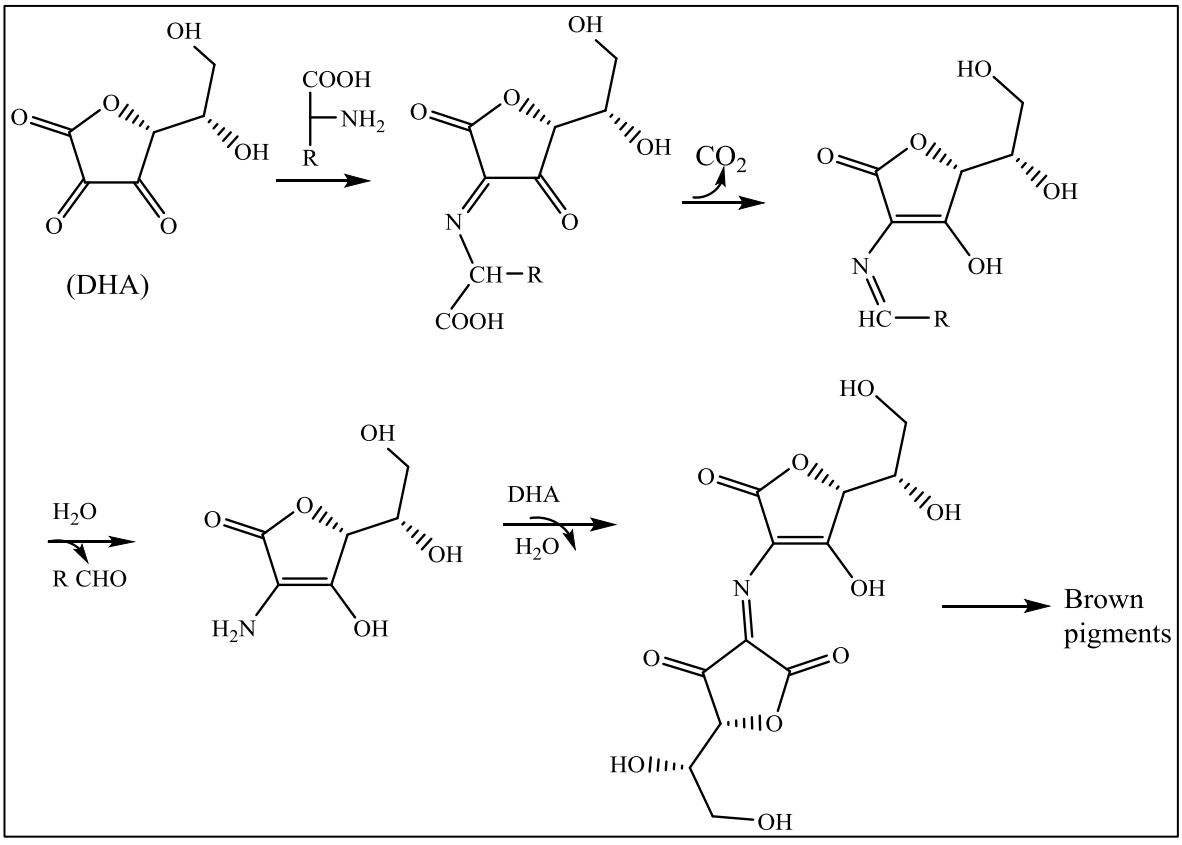


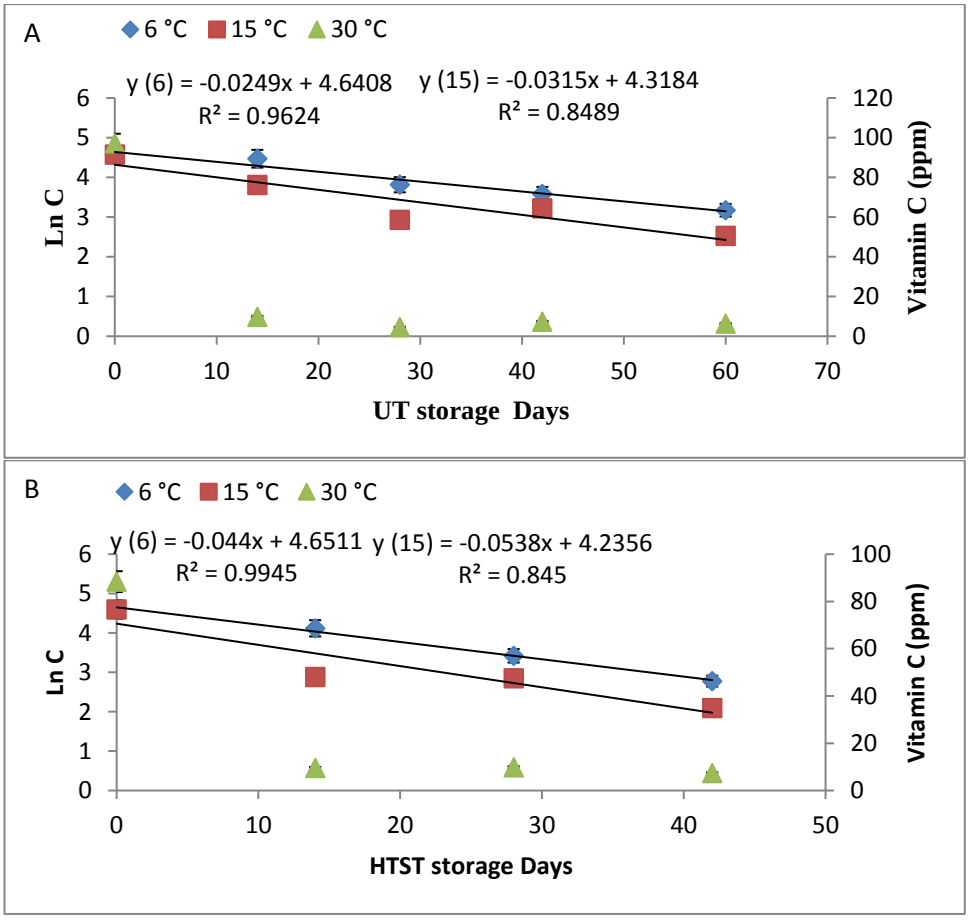
Figure 4.4 - Proposed mechanism for the degradation of dehydroascorbic acid and formation of brown pigments in juice (Belitz et al., 2009).

4.3.4.1.2 Overall vitamin C degradation kinetics in baobab juice

Several authors have successfully used integrated rate laws to describe changes in experimental data and estimate shelf life of quality attributes including vitamin C, polyphenols and antioxidant activities (Polydera et al., 2003; Blasco et al., 2004; Burdurlu et al., 2006; Odriozola-Serrano, 2009; Derossi et al., 2010; Gamboa-Santos et al., 2014). Such information is missing for baobab fruit and derived products from Malawi. Therefore, changes in vitamin C content during storage for untreated (UT) pasteurised (HTST) and acidified (CAHTST) juice samples were evaluated using integrated laws described in 2.6.8. The half-life ($t_{1/2}$) of vitamin C was derived from integrated rate laws and given by the expressions $C_o/2k$, ln (2)/k and $1/(k.C_o)$ for zero, first and second order reaction kinetics respectively.

The experimental data representation of Ln C versus storage time exhibited linear correlation at 6 and 15 °C ($R^2 \geq 0.9$) for all samples while at 30 °C loss of vitamin C highly likely followed zero order reaction kinetics (Figure 4.5A-C). Untreated juice still showed extended vitamin C half-life of all probably due to higher levels of other antioxidant compounds with protective role against its degradation. From rate constants (-k), the half-life of vitamin C at 6 and 15 °C was 27 and 22 days for UT, 15 and 12 days for HTST and 21 and 16 days respectively for CAHTST. Acidification of pasteurised juice inevitably extended the half-life of vitamin C in HTST juice by 40 and 33% at 6 and 15 °C respectively. Most researchers have also reported that vitamin C degradation follows first order reaction kinetics (Burdurlu et al., 2006; Odriozola-Serrano, 2009; Remini et al., 2015). The half-life of ascorbic acid or vitamin C reported for fruit juices varies

widely and depends on species, composition or concentration, processing and storage conditions. For instance, Burdurlu et al. (2006) reported a longer vitamin C half-life in citrus juice concentrates (orange, lemon, grapefruit and tangerines) while Remini et al. (2015) reported that only 20% of initial ascorbic acid in freshly squeezed blood orange juice was retained by day 28 of storage at 4 °C and complete degradation was reported for higher storage temperatures. Results from this study are therefore consistent with findings reported by other authors. From this study it seems stability of ascorbic acid and vitamin C in baobab juice is enhanced at low storage temperature (6 °C) for a period of 14 days.



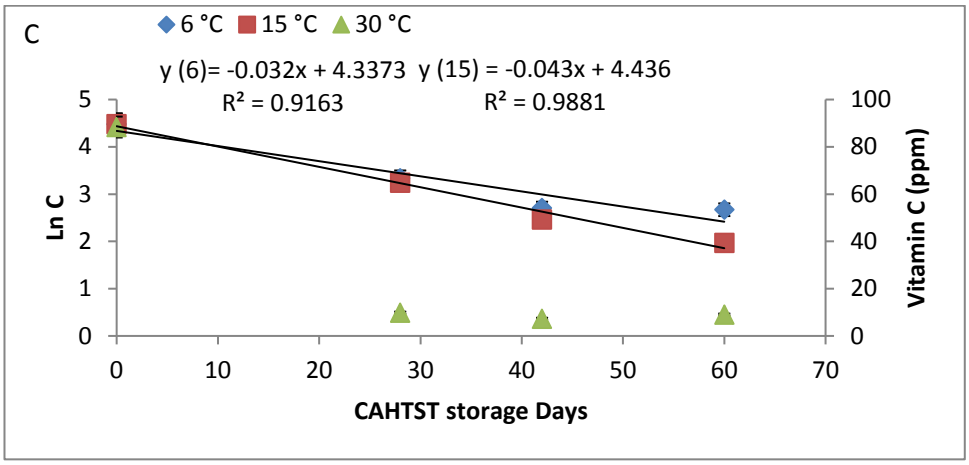


Figure 4.5 - Vitamin C degradation kinetics for 10% baobab juice which was untreated (A) pasteurised (B) and pasteurised with added citric acid (C); n = 3.

4.3.4.2 **Effect of storage on citric (CA) and malic acid (MA)**

Quantification of organic acids is important for proper comprehension of organoleptic and microbiological quality of fruits and derived products. Specifically organic acids influence flavour, colour and microbial stability in foods. Moreover organic acids are widely used as preservatives for a wide range of fruit products.

Appendix A shows experimental data for citric and malic acid in untreated (UT), high temperature short time pasteurised (HTST) and acidified high temperature short time pasteurised (CAHTST) juice samples stored at 6, 15 and 30 °C. In general, results showed that storage temperature and time had less influence on the levels of these two organic acids compared to the effect on ascorbic and dehydroascorbic acid.

For UT juice, the concentration of citric acid (CA) varied between 2934.17 ± 225.62 and 3318.73 ± 19.26 ppm. Apart from a significant drop ($p \leq 0.05$) on day 28, levels of CA were stable during storage. There was no significant difference ($p > 0.05$) between the

concentration of CA at the start and end of storage time. For HTST, the levels were also stable during storage. The concentration of CA varied between 3023.93 ± 167.55 and 3358.91 ± 31.79 ppm. Not surprising, levels of CA were very high in CAHTST juice and reflective of acidification with 0.5% citric acid. The concentration varied between 7575.39 ± 175.47 and 8970.26 ± 572.58 ppm. Apart from day 28, levels of CA were not significantly different ($p > 0.05$) during storage.

Evolution of malic acid (MA) at 6 °C was similar in all juice samples. For UT juice, the concentration varied between 1887.88 ± 188.65 and 2707.87 ± 24.73 ppm (Appendix A). There was a significant drop ($p \leq 0.05$) on day 14, which remained stable until day 28, and finally increased significantly ($p \leq 0.05$). For HTST, the concentration of MA varied between 1840.99 ± 45.77 and 2413.37 ± 43.12 ppm. A significant drop ($p \leq 0.05$) was observed on day 14, which then stabilised until day 42 and finally rose significantly on day 60. For CAHTST, MA concentration was within the range observed in UT and HTST samples. Apart from day 28 the concentration of MA was not significantly different ($p > 0.05$) during storage. Acidification might have enhanced stability of malic acid.

At 15 °C, evolution of both acids in all juice samples was similar to that observed at 6 °C. For UT, the concentration of citric acid was between 2986.30 ±150.50 and 3413.64 ± 22.57 ppm. The level of CA remained stable for the first 14 days, but dropped significantly ($p \leq 0.05$) on day 28. After 42 days, significantly high level of CA was observed, which stabilised until the end of storage. For HTST, the concentration of CA was between 2763.87 ± 190.73 and 3380.85 ± 15.34 ppm. Apart from a significant drop of day 42, the

concentration of CA was not significantly different ($p > 0.05$) between other storage days. For CAHTST, the concentration of CA was between 7353.06 ± 404.40 and 8973.36 ± 929.96 ppm. Apart from a significant drop on day 28, the concentration of CA was not significantly different ($p > 0.05$) between other storage days.

For UT juice, the concentration of malic acid (MA) was between 1886.71 ± 79.71 and 2597.85 ± 105.70 ppm. Levels remained stable during the first 28 days, which then increased on day 42, and stabilised again until the end of storage. For HTST juice, the concentration of MA was between 1512.02 ± 318.15 and 2413.37 ± 43.12 ppm. The level remained stable until day 60 when the significantly highest level ($p \leq 0.05$) was observed. For CAHTST, the concentration of MA was between 1846.14 ± 160.21 and 2374.17 ± 65.39 ppm. The level did not change significantly ($p \leq 0.05$) with storage time.

At 30 °C, all samples showed a similar pattern to that described for 6 and 15 °C storage. Fluctuation in the concentration of both acids was observed, although variations were rarely significant. The concentration of citric acid (CA) fluctuated between 2844.36 ± 220.00 and 3465.25 ± 19.52 ppm for UT; 2971.30 ± 212.90 and 3511.28 ± 27.85 ppm for HTST and 7497.63 ± 273.73 and 9117.09 ± 776.23 ppm for CAHTST. For all samples the level of CA was stable for the first 14 days, but dropped significantly ($p \leq 0.05$) on day 28. The level of CA increased on day 42, which then stabilised until the end of storage.

Finally the concentration of malic acid (MA) at 30 °C fluctuated between 1902.23 ± 132.49 and 2529.56 ± 120.13 ppm for UT; 1820.56 ± 387.45 and 2481.26 ± 20.95 for

HTST and 1836.56 ± 129.60 and 2374.17 ± 65.39 ppm for CAHTST. For UT juice, MA was stable for the first 14 days, but significantly ($p \leq 0.05$) decreased on day 28, and then increased on day 42, which then stabilised until the end of storage. For HTST and CAHTST the level of MA was stable during storage. The initial and final concentration of both acids in all samples (UT, HTST and CAHTST) was not significantly different ($p > 0.05$).

For better comprehension of the influence of storage on CA and MA, data was grouped by temperature and presented as a function of time for each treatment condition (Figures 4.6A-C and 4.7A-C). It is clear that both acids were not significantly influenced by storage temperature with few exceptions. For instance, the concentration of CA for UT on day 14 (3224.01 ± 6.09, 3197.47 ± 9.73 and 3465.25 ± 19.52 ppm) or day 60 (3318.73 ± 19.26, 3413.64 ± 22.57, and 3303.02 ppm) at 6, 15 and 30 °C respectively were not significantly different ($p > 0.05$).

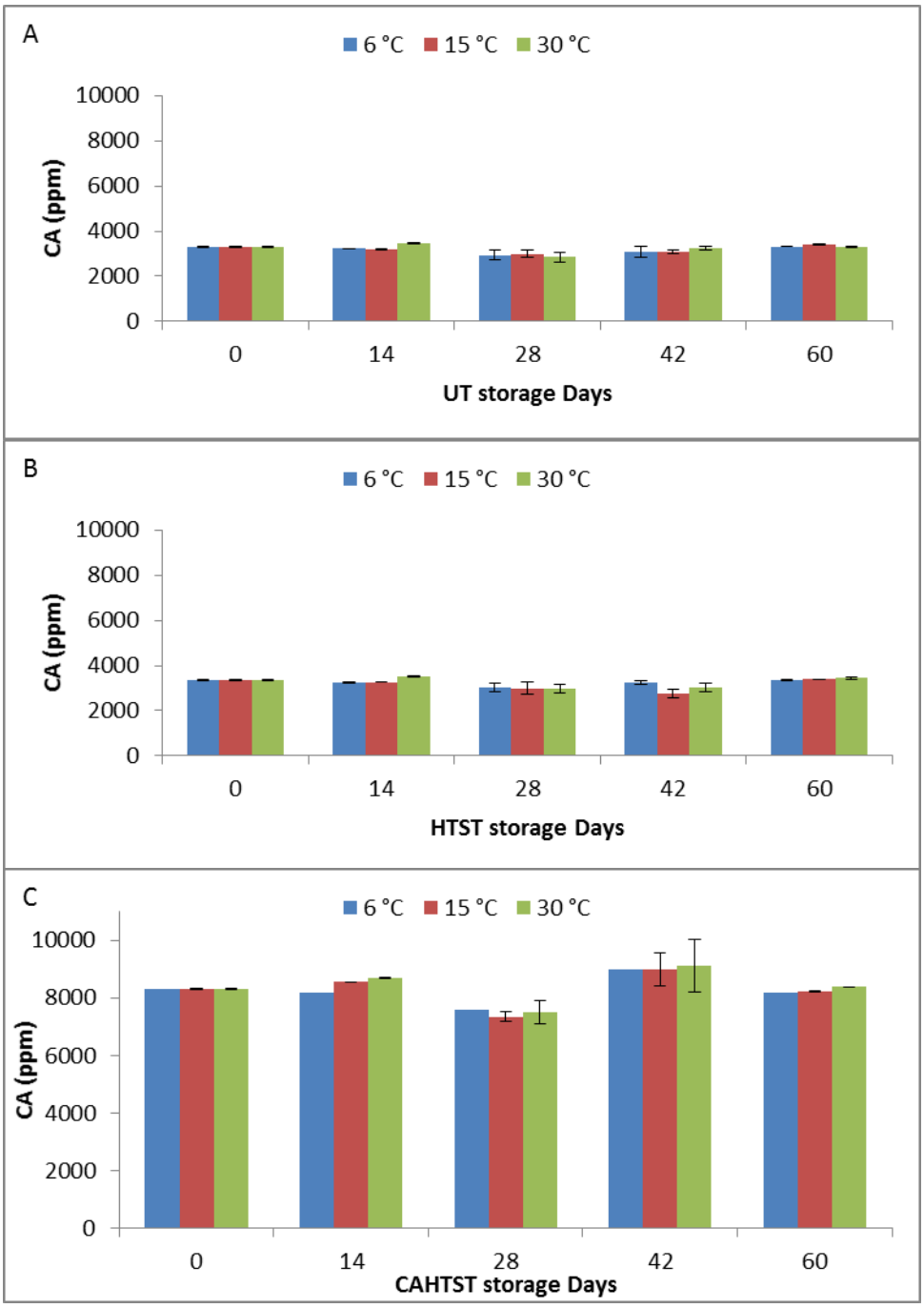


Figure 4.6 - Concentration of citric acid in UT (A), HTST (B) and CAHTST (C) baobab juice during storage at 6, 15 and 30 °C ; n = 3.

A similar trend was observed for malic acid (Figure 4.8A-C). For instance the level of MA for UT on days 14, 28 and 42 at 6, 15 and 30 °C was not significantly different ($p > 0.05$). A similar pattern was observed for HTST and CAHTST juice.

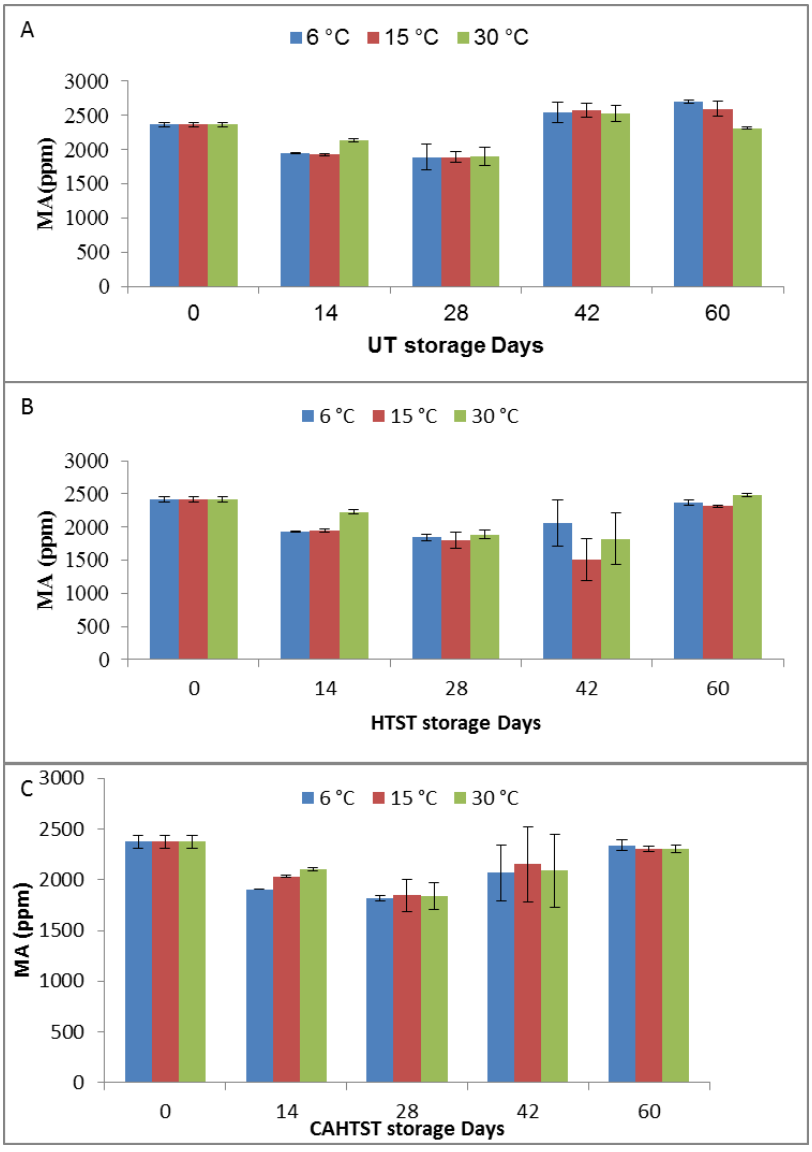


Figure 4.7 - Concentration of malic acid in UT (A), HTST (B) and CAHTST (C) baobab juice during storage at 6, 15 and 30 °C; n = 3.

Notable fluctuations of organic acids during storage could be attributed to degradation or reaction with other organic compounds including alcohol and phenolic compounds. Fermentation, esterification and oxidation are the most probable pathways for evolution of organic acids (Piras et al., 2014) especially for untreated juice. Some higher values of organic acids observed towards the end of storage at higher temperature could be attributed to degradation of carbohydrates and other phenolic compounds in the juice. In general citric and malic acid did not degrade during storage in all samples. The stability of organic acids in baobab juice during storage is by far better than ascorbic acid and vitamin C and it is not surprising that citric and malic acid are commonly used as preservatives. Results from present work are consistent with those reported by other investigators. For instance, Igual et al.(2010) reported that the content of both CA and MA of grapefruit were fluctuating but with no significant variation during storage. Under refrigeration conditions the level of citric acid in freshly squeezed, conventional (80 °C, 11 s) and microwave (900 W, 30 s) pasteurised grapefruit juice remained stable for the first 24 hours, but decreased in the next 3 days. Researchers reported that after 12 days, CA continued to decrease significantly in fresh squeezed grapefruit juice but remained constant for pasteurised counterparts (Igual et al., 2010). Fluctuations in the level of malic acid during storage of fresh and pasteurised grapefruit juice reported by Igual et al.(2010) were rarely significant. Results from this study are therefore in agreement with published research data.

In summary, this experiment provided quantitative information of the influence of thermal processing and preservation on organic acids in baobab juice. Overall, storage

temperature and time did not affect levels of malic and citric acid in the juice. Acidification somehow enhanced stability of malic acid and may be recommended to preserve some nutritional, organoleptic and microbiological properties of baobab juice.

4.3.4.3 **Effect of pasteurisation and storage on total phenol content (TPC)**

Stability of bioactive compounds and resultant total phenol content (TPC) may be influenced by processing and storage conditions. In this study, pasteurisation reduced the level of TPC significantly ($p \leq 0.05$) compared to untreated juice. The level of TPC in untreated and pasteurised juice was 1564.80 ± 1.60 and 1084.72 ± 3.67 mg GAE/100 g FW respectively representing about 31% loss (Appendices B1 and B2). Moreover a decrease of 16% in the level of TPC of fresh pulp (1866.81 ± 1.61 mg GAE/100 g FW) was already observed during juice formation. A significant decrease in the level of TPC with pasteurisation was also reported in grapefruit juice (Igual et al., 2010). The decrease in the level of TPC in untreated juice is attributed to low solubility of some phenolic compounds in water during juice formation while lower values of TPC in pasteurised juice is likely attributed to loss of heat sensitive phenolic compounds. It is therefore necessary to optimise processing and preservation conditions in order to ensure a higher retention of bioactive compounds in the final juice.

Storage studies recommend refrigeration of pasteurised fruit juice for shelf life extension of nutritional attributes and functional properties (Igual et al., 2011; Martínez-Flores et al., 2015). Results showed that storage (time and temperature) and treatment conditions

(UT, HTST & CAHTST) influenced the levels of TPC in baobab juice (Appendices B1-B3). In all juice samples the levels of TPC were fluctuating during storage.

For instance, at 6 °C, untreated juice showed significantly higher level ($p \leq 0.05$) of TPC than processed juice counterparts. For each treatment, higher levels of TPC were observed at the beginning and towards the end of storage. For UT, the minimum and maximum levels of TPC were observed on day 42 and 60 respectively. For both HTST and CAHTST the minimum and maximum levels were observed on day 28 and 42 respectively. At 15 °C, significantly higher level ($p \leq 0.05$) of TPC was observed in UT than HTST or CAHTST. TPC was more stable in CAHTST juice than in UT or HTST juice. Finally, at 30 °C, TPC were more unstable in UT than HTST or CAHTST juice. Clearly a wide range of variation was observed in UT while CAHTST juice showed a narrow range of variation. More than 44% of TPC was lost between day 28 and 42 for UT while only 20 and 3% of TPC was lost for HTST and CAHTST respectively during the same period.

A possible reason for the observed decrease in TPC during storage could be due to polyphenols reacting with sugars and sugar metabolites (Agbenorhevi and Marshall, 2012) present in baobab juice. For example gallic acid which was available in baobab juice can form complex sugar esters such as 2-O-digalloyl- tetra-O-galloyl-glucose (Rio et al., 2013). The decrease in TPC of juice kept at 30 °C could be largely attributed to the presence of heat sensitive phenolic compounds (-)-epicatechin which become more sensitive to chemical oxidation with increased temperature leading to the formation of compounds which are no longer detected in the Folin-Ciocalteu assay. Nevertheless, the

level of TPC in pasteurised juice remained significantly higher ($p \leq 0.05$) compared to untreated juice.

Chen et al. (2013) reported that thermal processing may cause complex physical and chemical reactions affecting the phenolic composition, including release of phenolic compounds from their bonded forms, degradation of polyphenols and the breakdown and transformation of phenolic compounds. Therefore in this study an increase in TPC may be attributed to transformation of phenolic compounds including proanthocyanidins (oligomeric and polymeric flavan-3-ols) to dimers or monomeric phenolic compounds or the formation of compounds over storage life which could also react with Folin-Ciocalteu reagent but which were not necessarily phenolic compounds (Piljac-Žegarac et al., 2009). For untreated juice it was also possible that microorganisms present in the juice produced metabolites capable of reacting with Folin-Ciocalteu reagent, facilitating an apparent increase in TPC.

Stability of TPC observed in HTST and CAHTST during storage could be attributed to inactivation of most degradative enzymes including PME and PPO. Acidification stabilised the pH of juice hence preventing microbial degradation of phenolic compounds. Results from this study are consistent with findings reported by other investigators. For instance Dawes & Keene (1999) found that high temperature short time (HTST) processing preserved TPC of kiwi juice due to the inactivation of polyphenol oxidase. In some instances like on day 60 (15 °C), compounds produced by moulds, which appeared more prevalent in untreated juice, may have slowed the apparent decrease in TPC in

comparison to HTST or CAHTST juice, which remained largely mouldy free during storage.

Similarly, other researchers have reported an increase (Klimczak et al., 2007), a decrease (Agbenorhevi and Marshall, 2012), fluctuation (Piljac-Žegarac et al., 2009) and stability (Igual et al., 2010) in values of TPC during storage in fresh and processed commonly consumed fruit products including kiwifruit, oranges and currant juice. It seems the trend of evolution of TPC during storage is dependent on fruit species, processing and preservation conditions but results are also affected by the method of analysis.

4.3.4.4 **Effect of storage on antioxidant activity**

The measurement of antioxidant activity of food extracts is important to evaluate the nutritive value (Apak et al., 2016a). Preservation methods are believed to be responsible for the depletion of naturally occurring antioxidants. Although processed fruit products are expected to have lower health protecting capacity than fresh ones, the functional properties of the former may be stable during storage (Nicoli et al., 1999).

The influence of pasteurisation and acidification on stability of antioxidant activity was evaluated in baobab fruit juice at three storage temperatures. Other researchers reported that because of multiple reaction characteristics and mechanisms involved in a mixed or complex system more methods should be used for the determination of antioxidant activity to reflect overall antioxidant activity of natural sources of antioxidants (Du et al., 2009). Because most natural antioxidants and phytochemicals are multifunctional (Frankel & Meyer, 2000) three optimised and commonly used antioxidant protocols

relevant to foods and biological systems were used to evaluate the antioxidant activity of baobab juice. For instance, DPPH assay only measures lipophilic antioxidants while ABTS considers both hydrophilic and lipophilic antioxidants (Apak et al., 2016a). Results for antioxidant activity (FRAP, DPPH and ABTS) for untreated (UT), pasteurised (HTST) and acidified (CAHTST) juice are presented in Appendices B1- B3.

4.3.4.4.1 Effect of storage on ferric reducing antioxidant power (FRAP)

Results showed that storage time and treatment condition influenced the levels of FRAP in baobab juice. In all juice samples the levels of FRAP were fluctuating with time. For instance, at 6 °C, higher values of FRAP were observed in UT juice while HTST juice showed the least values. In all treatments significant variations were observed but not consistent with time. For instance, higher FRAP values in UT and CAHTST juice samples were observed at the end of storage period. The initial FRAP value increased in UT and CAHTST juice samples by 23 and 14% respectively but dropped by 14% in HTST juice by the end of storage.

Similarly at 15 °C, highest FRAP value was observed in untreated juice samples. Notable significant variations were observed in all samples during storage. The initial FRAP value increased by 17.2% for UT juice but reduced by 4.7 and 2.8% for HTST and CAHTST respectively by the end of storage time. At 30 °C, FRAP was significantly affected in UT compared to other regimes. FRAP values were fairly stable in HTST and CAHTST juice samples. Initial FRAP values in UT, HTST and CAHTST decreased by 32, 3.6 and 12.7% respectively by the end of storage.

Significantly high levels of FRAP values observed in untreated (UT) and processed juice at time zero is attributed to higher vitamin C content observed in the same samples. Evolution of FRAP values during storage was consistent with changes in total polyphenol content. Vitamin C was lost during storage at all temperatures but the rate of degradation was highest at 30 °C. Therefore higher FRAP values observed during storage is indicative of the presence of stable phenolic compounds which are capable of exhibiting antioxidant activity. Phenolic compounds identified and analysed in baobab juice including procyanidin B2, (-)-epicatechin, (-)-epigallocatechin-3-O-gallate and gallic acid were likely contributing significantly to higher ferric reducing antioxidant power despite lower levels of vitamin C observed during storage. For untreated juice, phenolic compounds decreased during storage hence lower FRAP values were observed especially at elevated temperatures. For HTST and CAHTST juice samples phenolic compounds were protected from oxidation by the thermal inactivation of PPO or PME during pasteurisation of baobab juice hence higher and more stable FRAP values observed even at 30 °C. The increase in FRAP values may be attributed to formation of non-enzymatic reaction products (melanoidins) during high processing and storage temperatures. Other investigators reported that if antioxidant activity is increasing while phenolic compounds and vitamin C are decreasing, the increase in the antioxidant activity of stored juice may be ascribed to formation of non-enzymatic reaction products (Anese et al., 1999; Del Pino-Garcia et al., 2012).

Nonenzymic browning of juice on heating or on storage is usually due to a chemical reaction between reducing sugars and a free amino acid or a free amino group of an amino

acid that is part of a protein. The reducing sugar reacts reversibly with the amine to produce a glycosylamine which undergoes Amadori rearrangement to give in the case of D-glucose, a derivative of 1-amino-1-deoxy-D-fructose (Fennema, 1996). Non-enzymic browning of juice during thermal pasteurisation or storage is likely due to ascorbic acid browning (Fennema, 1996). The reaction continues especially at a lower pH to give an intermediate that dehydrates forming a furan derivative including 5-hydroxymethylfurfual (HMF). Baobab fruit pulp and resultant juice is acidic (pH < 3.01) and contains high levels of ascorbic acid and amino acids (Chadare et al., 2009). Protein accounts for 3.2% of the baobab fruit pulp, with tyrosine, glutamic acid and aspartic acid being the predominant amino acids (Osman, 2004), all of which can influence ascorbic acid browning at elevated temperatures and the melanoidins formed (Davies and Wedzicha, 1994) could account for higher antioxidant activity observed in stored juice. The increase in antioxidant activity towards the end of storage coincided with the formation of brown pigments which were clearly visible at 30 °C. Processing conditions that optimise the preservation of total phenols and AA will inevitably preserve the antioxidant capacity of baobab juice. Although some degradation of natural antioxidants was likely at high temperatures, the effect on overall antioxidant capacity was not as noticeable due to the presence of phenolic compounds and the antioxidant activity of the melanoidins.

From this study although some degradation of natural antioxidants was likely at high temperatures, overall losses in ferric reducing antioxidant activity was not as drastic in

HTST and CAHTST juice samples due to presence of flavan-3-ols and possible formation of melanoidins.

4.3.4.4.2 Effect of storage on DPPH radical scavenging activity

The DPPH assay was selected because it is an accurate, fast and easy electron transfer method, widely used for the determination of antioxidant activity in fruit and vegetable juices and recommended by many authors (Huang and Prior, 2005; Klimczak et al., 2007; Du et al., 2009; Piljac-Žegarac et al., 2009; Igual et al., 2010; Teh and Birch, 2014). Results showed that storage and treatment conditions significantly affected DPPH radical scavenging activity of baobab juice (Appendices B1-B3). The trend of antioxidant activity by DPPH assay was similar to that observed and described for FRAP assay. The DPPH radical scavenging activity values fluctuated during storage in all juice samples. Untreated juice showed higher DPPH values than other regimes but levels were significantly affected at 30 °C. At 6 °C, the initial DPPH values increased by 13.7 and 1.6% in UT and CAHTST respectively but dropped by 22.7% in HTST juice by the end of storage time. While at 15 °C, the initial DPPH values increased by 5.6% in UT but dropped by 5.6 and 3.6 % in HTST and CAHTST juice respectively. Not surprising, at 30 °C, UT juice was the most affected. The initial DPPH value dropped by 38% in UT juice versus 12.2 and 3% drop observed in HTST and CAHTST juice samples respectively.

The antioxidant activity (DPPH) of processed juice was slightly affected by storage temperature. Sudden drop in antioxidant activity of untreated juice at 30 °C could be

attributed to loss of both vitamin C and phenolic compounds. It seems DPPH antioxidant activity was more related to polyphenols than vitamin C. For instance, at 30 °C, levels of both DPPH and TPC were higher while ascorbic acid and vitamin C were significantly low. Pasteurisation and acidification retained phenolic compounds which were likely responsible for higher DPPH values during storage. The reduction in the values of DPPH observed on day 14 and day 42 at 15 and 30 °C could be attributed to reduction in naturally occurring antioxidants especially vitamin C and heat sensitive phenolic compounds and early melanoidins with pro-oxidant properties. The gain in DPPH values towards the end of storage coincided with the formation of brown pigments (melanoidins) which were more visible at 30 °C.

4.3.4.4.3 Effect of storage on ABTS radical scavenging activity

The advantage of the ABTS assay is that the extract reacts rapidly with ABTS radical permitting high throughput. Moreover ABTS assay measures antioxidant activity exhibited by both lipophilic and hydrophilic antioxidants including flavonoids in samples (Re et al., 1999; Apak et al., 2016a). Appendices B1-B3 show changes in antioxidant activity (ABTS) in untreated (UT), pasteurised (HTST) and acidified (CAHTST) baobab juice samples. In general, just like FRAP and DPPH, ABTS values were high, fairly stable with limited fluctuation behaviour observed during storage. Although untreated juice showed higher values of ABTS at 6 and 15 °C, it was significantly affected at 30 °C.

At 6 °C, the antioxidant activity (ABTS) was stable for all samples during storage apart from day 42 when significantly low ($p \leq 0.05$) values were observed. Although ABTS

values were fluctuating, the variations were rarely significant at 6 °C. By the end of storage time, initial values of ABTS in UT, HTST and CAHTST dropped by 1.1, 26.8 and 5.5% respectively.

At 15 °C, UT juice still showed significantly higher antioxidant activity during storage than HTST or CAHTST juice samples. Surprisingly ABTS values for UT were higher at 15 °C than at 6 °C. The initial and final ABTS values were not significantly different ($p > 0.05$). For HTST and CAHTST juice samples, the antioxidant activity (ABTS) was stable with the exception of values on day 42. By the end of storage time, initial ABTS values increased by 4.3% for UT, but decreased by 9.8 and 10.6% for HTST and CAHTST respectively.

Finally, at 30 °C, HTST juice showed significantly higher ($p \leq 0.05$) and more stable antioxidant activity (ABTS) than other regimes. Not surprising, levels of ABTS for UT dropped significantly ($p \leq 0.05$) to a minimum (856.78 ± 20.18 mg TEAC/100 g) by the end of storage. ABTS values were not significantly different ($p > 0.05$) in HTST and CAHTST between most storage days. The decrease in initial ABTS values for UT, HTST and CAHTST were 43.5, 5.9 and 22% respectively compared to initial values.

4.3.4.5 **Summary for evolution of antioxidant activity in baobab juice**

All antioxidant assays considered showed systematic fluctuation of antioxidant activity in baobab juice. It seems pasteurisation and acidification may help extend antioxidant activity of baobab juice. The significantly higher levels of antioxidant activity observed in untreated baobab juice at 6 °C in all assays (FRAP, DPPH and ABTS) may be ascribed

to higher levels of ascorbic and dehydroascorbic acid observed in the samples besides the presence of phenolic and other antioxidant compounds. For instance gallic acid and (-) - epigallocatechin-3-O-gallate (EGCG) were significantly higher ($p \leq 0.05$) in untreated juice than pasteurised juice. These compounds are present in high amounts in baobab fruit pulp (Kaboré et al., 2011; Kamatou et al., 2011) but are affected by processing and storage conditions.

The significant decrease in antioxidant activity at 30 °C for untreated juice is likely attributed to lack of protection against enzymatic degradation in addition to loss of heat sensitive antioxidant compounds. The reduction in antioxidant activity observed in treated juice samples (HTST & CAHTST) during storage could be attributed to reduction in vitamin C, heat sensitive phenolic compounds and early non-enzymatic reaction products with pro-oxidant properties.

Fluctuation in antioxidant activity of juice during storage may be attributed to several factors including the tendency of polyphenols to undergo polymerisation reactions whereby the resulting oligomers possess larger surface area available for charge delocalisation and Non-enzymic browning of juice due to ascorbic acid browning (Fennema, 1996). Baobab contains higher levels of (-)-epicatechin and procyanidin B2 (Shahat, 2006; Kamatou et al., 2011) and as shown in chapter 3 which may likely undergo polymerisation during storage under ideal conditions of temperature and pH leading to higher antioxidant activity. Higher antioxidant activity observed during storage could also be attributed to increased ability of partially oxidised phenolic compounds to donate an

electron or hydrogen atom from the aromatic hydroxyl group to a probe ([Fe (III) $(TPTZ)_2]^{3+}$, $ABTS^{\cdot+}$ or DPPH·) in the FRAP, ABTS and DPPH assays (Apak et al., 2016; Huang et al., 2005). When the degree of polymerisation exceeds a certain critical value the increased molecular complexity and steric hindrances reduce the availability of hydroxyl groups in reaction with the reagents (Piljac-Zegarac et al., 2009) thereby reducing antioxidant activity. The increase in antioxidant activity towards the end of storage coincided with the formation of brown melanoidins which were clearly visible at 30 °C. Protein accounts for 3.2% of the baobab fruit pulp, with tyrosine, glutamic acid and aspartic acid being the predominant amino acids, all of which can undergo non-enzymic browning reactions (Osman, 2004) at elevated temperatures with reducing sugars or polyphenols as described ealier.

Processing conditions that optimise the preservation of total phenols and ascorbic acid will inevitably preserve the antioxidant activity of baobab juice. Although some degradation of natural antioxidants was likely at high temperatures, the effect on overall antioxidant activity was not as drastic in pasteurised and acidified juice due to the presence of phenolic compounds and possible formation non-enzymatic browning reaction products during storage. Thus formation of brown pigments in baobab juice may be important to preserve the antioxidant activity despite the loss of vitamin C and heat sensitive phenolic compounds.

4.3.4.6 **Relationships amongst different antioxidant variables and assays**

The antioxidant activity of fruits and vegetables is mostly ascribed to ascorbic acid, flavonoids, hydroxycinnamates and carotenoids (Re and Pellegrini, 1999; Huang et al., 2005; Shahidi and Zhong, 2010; Martínez-Flores et al., 2015). The antioxidant activity of ascorbic acid is caused by the ease of its loss of electrons and subsequent stabilisation by the π-electron system (Sanmartin et al., 2000) while antioxidant activity of polyphenols is mainly because of their redox properties which enable them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kaur and Kapoor, 2001).These compounds may act independently or in combination in exerting antioxidant properties (Frankel and Meyer, 2000). For this reason, overall correlation analysis was conducted for all samples to explore the relationship between antioxidant activity and variables (vitamin C and total phenol content) (Table 4.2). A high positive correlation between assays (ABTS, DPPH and FRAP) was observed confirming presence of antioxidant compounds in baobab juice. Not surprising, the correlation between vitamin C and TPC was very poor.

Table 4.2 - Pearson's correlation coefficients for antioxidant variables and antioxidant activity.

Untreated Juice	TPC	FRAP	DPPH	ABTS
Vit C	0.512**	0.223	0.343*	0.447**
TPC		0.898**	0.928**	0.920**
FRAP			0.959**	0.907**
DPPH				0.933**
Pasteurised juice	**TPC**	**FRAP**	**DPPH**	**ABTS**
Vit C	0.011	0.259	0.351*	0.366*
TPC		0.876**	0.575**	0.530**
FRAP			0.786**	0.637**
DPPH				0.781**
Acidified HTST	**TPC**	**FRAP**	**DPPH**	**ABTS**
Vit C	0.071	0.139	0.177	0.525**
TPC		0.696**	0.711**	0.779**
FRAP			0.849**	0.619**
DPPH				0.541**

** Significant correlation at $p \leq 0.01$
* Significant correlation at $p \leq 0.05$

To further confirm the correlation between total phenol content and antioxidant activity (FRAP, DPPH and ABTS) data for CAHTST juice was presented as a function of TPC (Figure 4.8). Results showed a very close linear correlation between TPC and antioxidant activity. Higher linear regression for FRAP and ABTS ($R^2 \geq 0.90$) was indicative of a strong positive relationship with TPC. Results from this study confirm the presence of bioactive compounds in baobab fruit pulp with significantly high antioxidant properties which may contribute to antioxidant requirement of human defence system. Therefore consumption of quality baobab juice may contribute significantly to prevention of oxidative stress related health disorders. Apart from providing comprehensive information in terms of chemical composition and antioxidant activity this study may act

as a guide for further biochemical or clinical research to investigate bioactivity and bioavailability of specific bioactive compounds from baobab fruit both *in vitro* and *in vivo*.

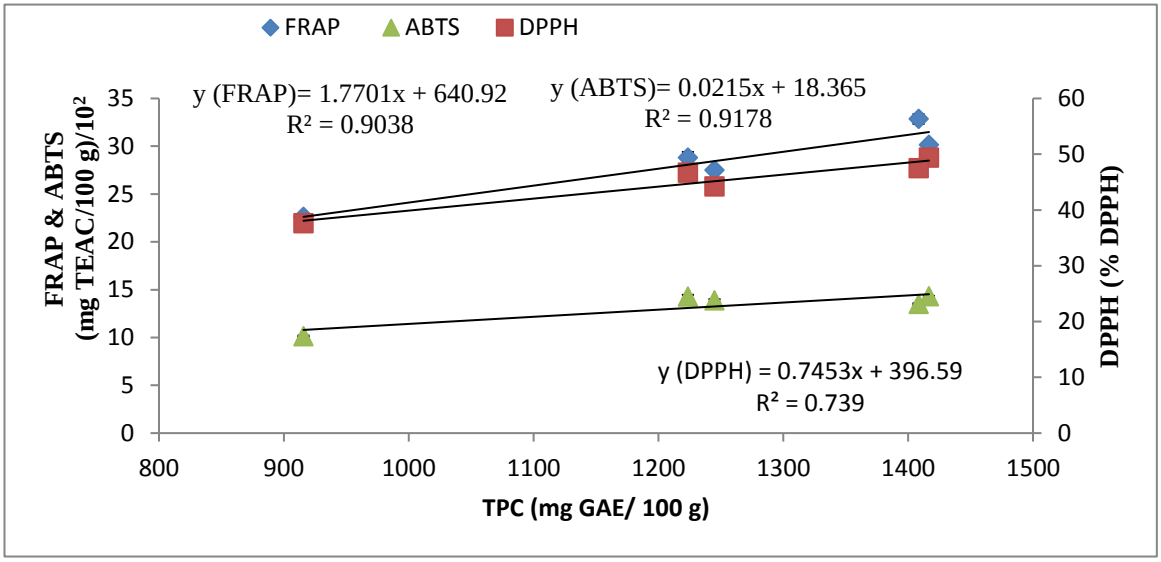


Figure 4.8 - Relationship between antioxidant activity and TPC of acidified juice; n = 3.

4.3.4.7 **(-)-Epicatechin and procyanidin B2**

Results from this study (chapter 3) and reports from other researchers show that baobab fruit contains high level of flavan-3-ols including (-)-epicatechin and procyanidin B2 (Shahat, 2006). The phenolic compounds however may suffer loss due to oxidation and polymerisation during storage. Recent experimental evidence indicates that storage conditions affect the content of phenolic compounds including flavan-3-ols since they can undergo hydrolysis, oxidation and complexation (Aron and Kennedy, 2008; Hernanz et al., 2009; Wojdyło et al., 2014). Moreover factors including origin, variety and degree of ripeness at harvest already influenced the content of phenolic compounds.

This study was therefore undertaken to evaluate the effect of storage on the content of (-)-epicatechin and procyanidin B2 in untreated (UT), pasteurised (HTST) and acidified (CAHTST) baobab juice from Malawi. Results for evolution of (-)-epicatechin and procyanidin B2 stored at three isothermal conditions (6, 15 and 30 °C) for a maximum a maximum period of 60 days including statistical analysis are presented in Appendix C. To describe the influence of storage, experimental data was presented as a function of time at constant temperature (Figures 4.9 to 4.11).

4.3.4.7.1 Effect of storage on untreated baobab juice (UT)

Results showed that storage time and temperature significantly ($p \leq 0.05$) affected levels of procyanidin B2 and (-)-epicatechin of UT juice samples (Figure 4.9). The initial level of both compounds was significantly lower compared to levels observed during storage. For instance, at 6 °C the level of procyanidin B2 and (-)-epicatechin was generally increasing. Procyanidin B2 increased steadily to highest level on day 28, dropped on day 42, then rose back to highest level by the end of storage, while (-)-epicatechin continuously increased until day 42, then dropped significantly ($p \leq 0.05$) by the end of storage. It was interesting to observe that on some occasions an increase in the level of procyanidin B2 was associated with a decrease in (-)-epicatechin. At 15 °C, the level of procyanidin B2 was fluctuating while (-)-epicatechin was continuously decreasing with storage time. Both compounds increased significantly ($p \leq 0.05$) on day 42 and finally dropped on day 60. At 30 °C, both procyanidin B2 and (-)-epicatechin were affected similarly during storage. Procyanidin B2 remained stable between day 0 and day 14, increased to significantly highest level on day 28; dropped to least level on day 42 and

increased again on day 60. (-)-Epicatechin increased between day 0 and day 28, and then dropped continuously until day 60.

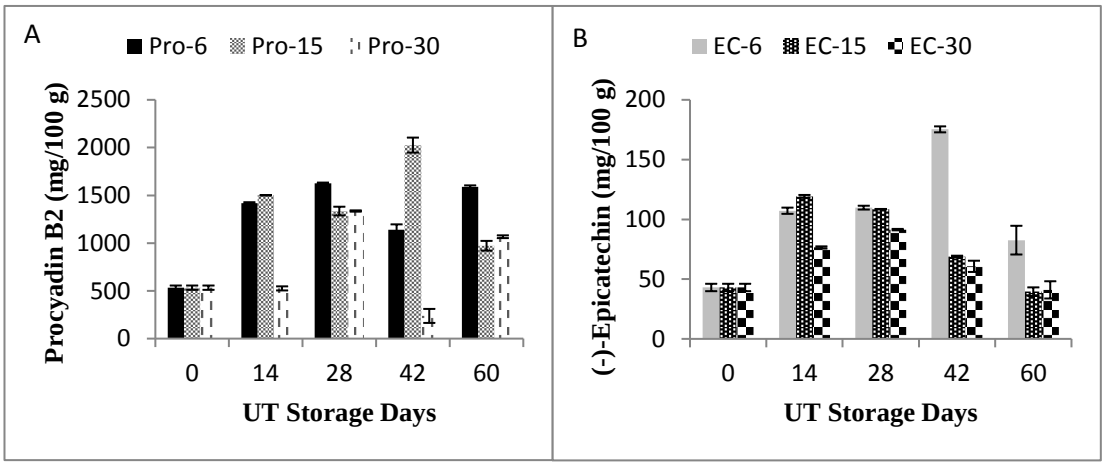


Figure 4.9 - Evolution of procyanidin B2 (A) and (-)-epicatechin (B) content of untreated baobab juice; n = 3.

4.3.4.7.2 Effect of storage on pasteurised juice (HTST)

Pasteurisation affected the level of procyanidin B2 more than (-)-epicatechin (Figure 4.10). Pasteurisation reduced the level of procyanidin B2 by 12.6% but increased the level of (-)-epicatechin by 10.9% compared to initial levels in untreated juice (Appendix C). Storage time and temperature affected levels of both procyanidin B2 and (-)-epicatechin.

At 6 °C, the level of procyanidin B2 and (-)-epicatechin increased significantly ($p \leq 0.05$) on day 42 by 269 and 107% respectively compared to initial levels. By the end of storage, the level of procyanidin B2 and (-)-epicatechin was 119.7 and 21.8% respectively higher compared to levels at time zero. At 15 °C, the level of procyanidin B2 increased on day 14, remained stable between day 14 and day 42 and finally dropped significantly ($p \leq$

0.05) on day 60 (Figure 4.10A). While the level of (-)-epicatechin ranged from 22.9 ± 0.7 to 100.0 ± 0.9 mg/100 g. (-)-Epicatechin increased between day 0 and day 14, remained stable until day 28, dropped significantly ($p \leq 0.05$) on day 42, and finally increased on day 60 (Figure 4.10B). By the end of storage, procyanidin B2 decreased by 70.6% while (-)-epicatechin increased by 103.3% compared to initial levels. At 30 °C the trend of procyanidin B2 between day 0 and day 42 was similar to that observed at 15 °C. The level increased significantly on day 14, remained stable until day 42 then finally increased significantly ($p \leq 0.05$) on day 60. While (-)-epicatechin increased between day 0 and day 14, then decreased continuously until day 42, and finally remained stable until the end of storage time. By the end of storage time, procyanidin B2 and (-)-epicatechin showed an increase of 250.1 and 18.0% respectively compared to levels at time zero.

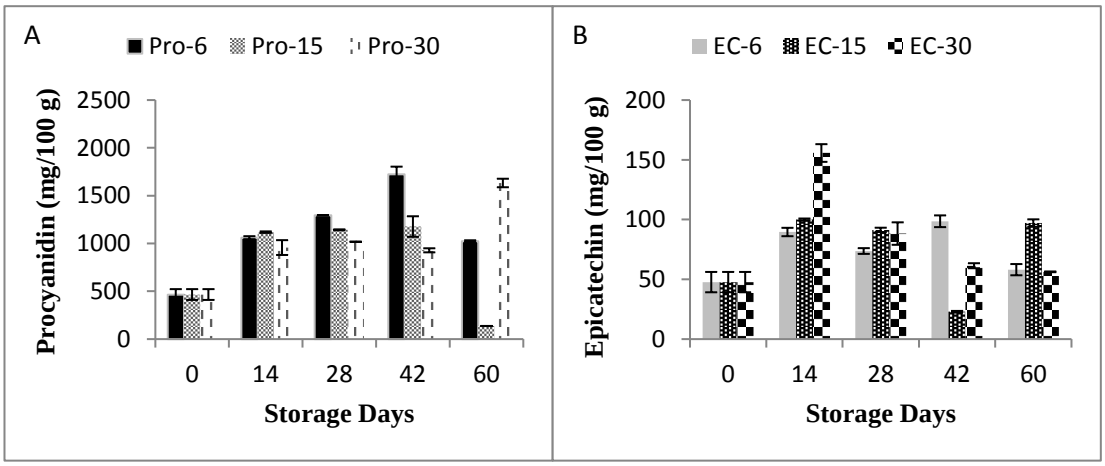


Figure 4.10 - Evolution of procyanidin B2 (A) and (-)-epicatechin (B) of pasteurised baobab juice; n = 3.

4.3.4.7.3 Effect of storage on acidified juice (CAHTST)

Acidification may be essential for preservation of bioactive compounds including vitamin C and phenolic compounds. The effect of storage temperature on procyanidin B2 and (-)-epicatechin content of CAHTST juice was evaluated. Results for evolution of procyanidin B2 and (-)-epicatechin at 6, 15 and 30 °C are described using Figure 4.11.

At 6 °C, results showed that levels of both procyanidin B2 and (-)-epicatechin were increasing between day 0 and day 42 and finally dropped on day 60. By the end of storage, the level of procyanidin B2 was 277.8% higher while (-)-epicatechin was 9.7% lower compared to levels at time zero. At 15 °C the level of procyanidin B2 was generally increasing while (-)-epicatechin was decreasing. By the end of storage, the level of procyanidin B2 was 347.6% higher while (-)-epicatechin was 12.8% lower compared to levels at time zero. At 30 °C, both procyanidin B2 and (-)-epicatechin increased significantly by day 14, then started decreasing till the end of storage time. The level of procyanidin B2 increased by more than 150% by day 14, then remained stable until day 42, and finally dropped significantly ($p \leq 0.05$) on day 60. The level of (-)-epicatechin increased by 351% on day 14, which then started decreasing significantly ($p \leq 0.05$) until day 60.

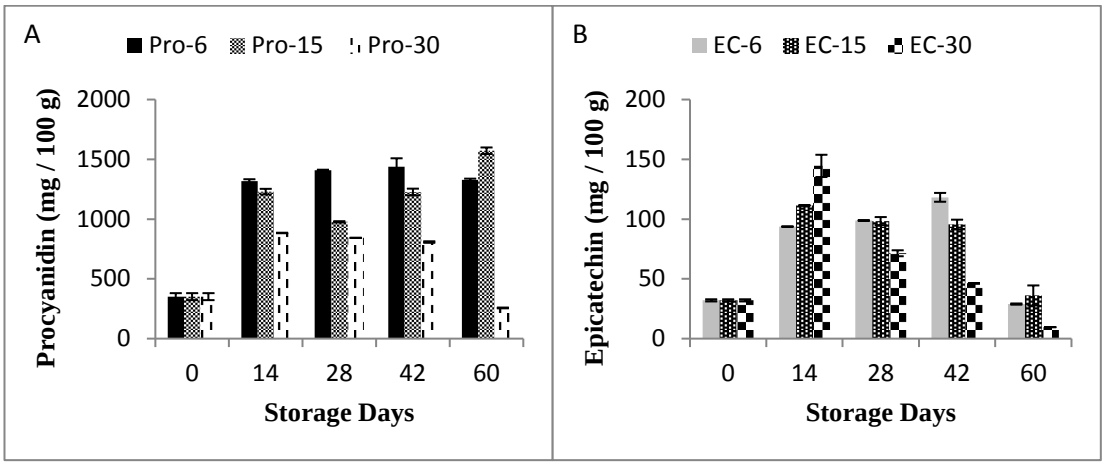


Figure 4.11 - Evolution of procyanidin B2 (A) and (-)-epicatechin (B) for acidified baobab juice; n = 3.

4.3.4.8 **Summary for evolution of procyanidin B2 and (-)-epicatechin**

Storage and treatment conditions significantly influenced levels of procyanidin B2 and (-)-epicatechin in baobab juice during storage. However procyanidin B2 was fairly stable compared to (-)-epicatechin in all samples during storage. Loss of procyanidin B2 and (-)-epicatechin in untreated juice could be attributed to enzymatic (PPO, PME) and microbial degradation. Phenolic compounds play a key role in enzymatic browning in fruits as they are preferred substrate of oxidative enzymes (Keenan et al., 2011). Residual enzymes in pasteurised juice could also account for loss of procyanidin B2 and (-)-epicatechin during storage. In all samples the initial level of procyanidin B2 and (-)-epicatechin increased after 14 days of storage, and then behaved differently afterwards based on treatment or storage conditions.

Evolution of procyanidin B2 and (-)-epicatechin has been previously described (1.8) and attributed to several reactions including depolymerisation of procyanidin polymers to oligomers (monomers, dimers, trimers, and tetramers), polymerisation, hydroxylation,

esterification, epimerisation, thermal degradation, enzymatic and nonenzymatic oxidation and microbial degradation (Kaur and Kapoor, 2001; Aron and Kennedy, 2008; Crozier et al., 2009; Keenan et al., 2011; Li et al., 2015). Figure 4.12 shows possible flavan-3-ols structures that may account for evolution of (-)-epicatechin and procyanidin B2 during storage of baobab juice.

The increase in both procyanidin B2 and (-)-epicatechin on day 14 and other storage days could be attributed to depolymerization of the higher oligomeric and polymeric proanthocyandins (condensed tannins), which constitute a much larger proportion of the flavan-3-ols (Keenan et al., 2011; Wojdyło et al., 2014). Other authors have suggested that unlike monomeric (-)-epicatechin, procyanidin B2 may bind to proteins in the juice thereby protecting it from degradation during storage (Wojdyło et al., 2014). Transformation of type A to type B procyanidin dimers (epimerisation) could have also contributed to higher levels of procyanidin B2. Further increase could be ascribed to improved release of phenolic compounds from their bonded forms (Chen et al., 2013) especially in pasteurised juice thereby enhancing extractability during analysis. Keenan et al. (2011) reported stability of procyanidin B2 in apple, strawberries and orange smoothies at lower temperatures.

The decrease in procyanidin B2 and (-)-epicatechin could be due polymerisation to trimers (Figure 4.12I), or tranformation to type A procyanidin (Figure 4.12K) through oxidative coupling between C2 and C7 of procyadin B2. (-)-Epicatechin (Figure 4.12A) may be hydroxylated to form (-)-epigallocatechin (Figure 4.12F); esterified with gallic

acid to form (-)-epicatechin-3-O-galate (Figure 4.12G) or (-)-epigallocatechin-3-O-gallate (Figure 4.12H). Fresh baobab juice showed substantial levels of gallic acid (chapter 3) therefore hydroxylation and esterification are the potential contributing factors for the loss of (-)-epicatechin observed during storage. (-)-Epicatechin may also isomerise to (-)-catechin (Figure 4.12D) and (+)-epicatechin (Figure 4.12E) during storage. Crozier et al.(2009) reported that these transformations may occur during food processing and storage.

Depolymerisation of procyanidin B2 to its monomers ((-)-epicatechin) is also possible and could account for the decrease in procyanidin B2 content during storage. The resultant monomers, (-)-epicatechin may subsequently undergo isomerisation leading to a lower percentage increase of (-)-epicatechin than corresponding percentage decrease in procyanidin B2 content observed in this study. Reactions including hydroxylation, esterification, and depolymerisation are favourable under acidic and low pH conditions typical of baobab fruit juice ($pH \leq 3.11$). Baobab fruit has significantly higher levels of organic acids which were stable during storage and may promote proposed reaction pathways for evolution of procyanidin B2 and (-)-epicatechin.

In conclusion, the rate of evolution of procyanidin B2 and (-) epicatechin are influenced by storage temperature. The percentage loss of procyanidin B2 was often times not proportional to percentage increase in (-)-epicatechin due to likelihood of instantaneous isomerisation to other flavan-3-ols monomers. The percentage increase of procyanidin B2 could be from depolymerisation of higher polymeric procyanidins or

proanthocyanidins (condensed tannins) and polymerisation of (-)-epicatechin hence much higher than corresponding percentage decrease of (-)-epicatechin observed in this study. It seems pasteurisation alone or with acidification enhanced stability of both compounds at 6 and 15 °C. Evolution of flavan-3-ols (procyanidin B2 and (-) epicatechin) during storage was consistent with total phenol and antioxidant activity observed in the same juice.

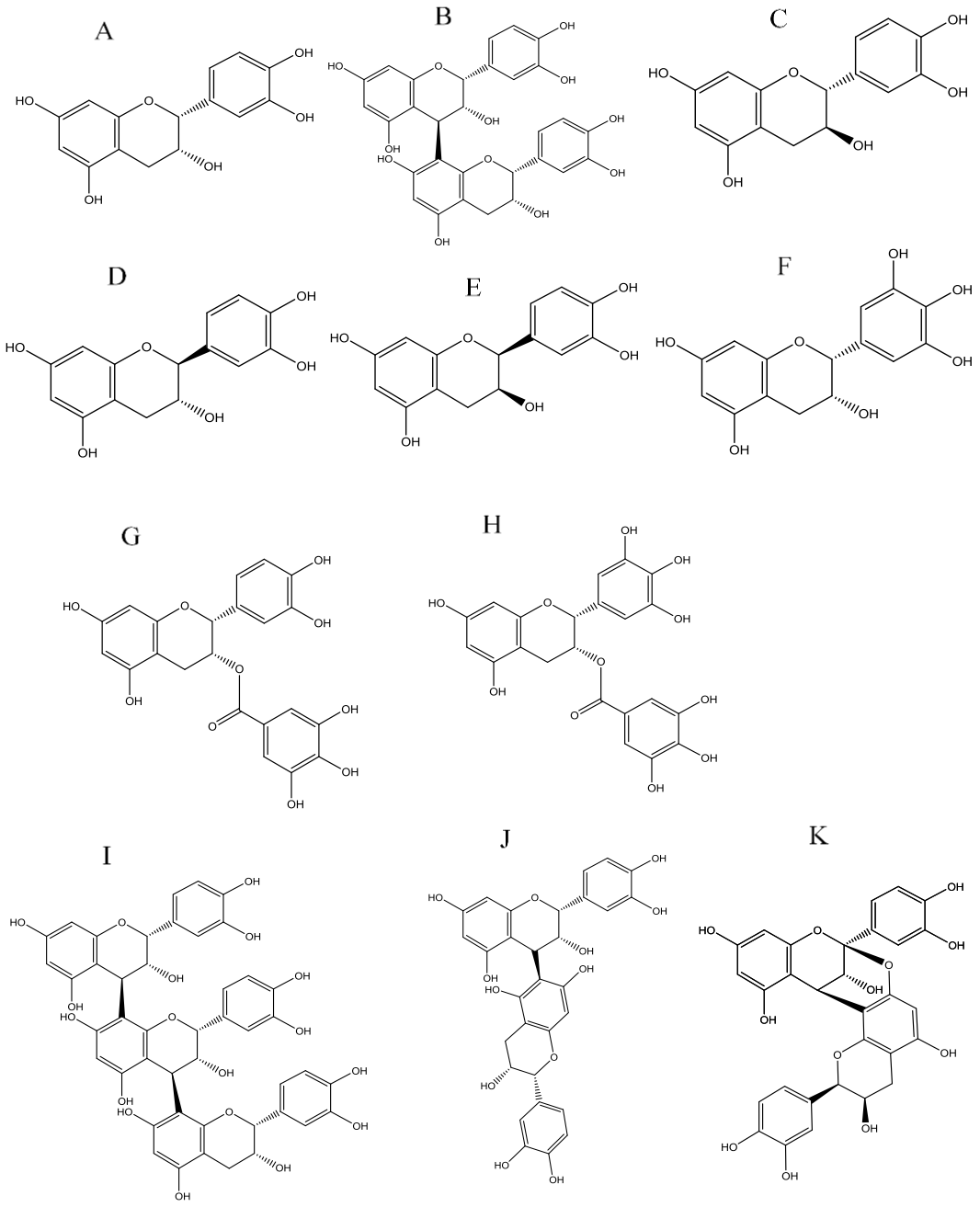


Figure 4.12 - Structures of (-)-epicatechin (A) and procyanidin B2 (B) and possible transformation products including (+)-catechin (C), (-)-catechin (D) and (+)-epicatechin (E), (-)-epigallocatechin(F), (-)-epicatechin-3-O-gallate (G),(-)-epigallocatechin-3-O-gallate (H), procyanidin C1 trimer (I), procyanidin B5 (J) and procyanidin A2 (K).

4.3.4.9 **Correlation Analysis**

Flavan-3-ols and their polymeric condensation products, the proanthocyanidins are reported to exhibit several health beneficial effects by acting as antioxidants. *In vivo* consumption of procyanidins has been implicated in improved antioxidant status (Crozier et al., 2009). In this study Pearson's correlation analysis was conducted to evaluate the relationship between antioxidant activity (FRAP, DPPH and ABTS) and bioactive compounds (procyanidin B2, (-)-epicatechin, total phenol content) of CAHTST baobab juice. Results showed that procyanidin B2 was significantly correlated to (-)-epicatechin, TPC and antioxidant activity (Table 4.3). The correlations between (-)-epicatechin and antioxidant activity were positive but lower compared to procyanidin B2. This suggests that procyanidin B2 contributed more than (-)-epicatechin to the overall antioxidant properties exhibited by baobab juice. Guendez et al. (2005) reported significantly higher correlation ($p \leq 0.05$) between antiradical potential and procyanidins (B1 and B2) from grape seeds ($r^2 = 0.7934$). Therefore results from this study are consistent with findings reported by other researchers regarding antioxidant properties of bioactive compounds.

Table 4.3 - Pearson's correlations between bioactive compounds and antioxidant activity.

CAHTST	EC	FRAP	DPPH	ABTS	TPC
Pro	0.271**	0.309**	0.252**	0.198*	0.212*
EC		0.053	0.009	0.055	0.060

** Significant correlation at $p \leq 0.01$

* Significant correlation at $p \leq 0.05$

4.3.5 **Conclusion**

Baobab fruit pulp is a rich source of vitamin C (ascorbic and dehydroascorbic acid), (-)-epicatechin, procyanidin B2, (-)-epigallocatechin-3-O-gallate, gallic acid and organic acids (citric, malic and tartaric) which have a wide range of application in the food industry as antioxidants and preservatives. Moreover these bioactive compounds have been reported to exhibit protective effects against onset of degenerative diseases and conditions by acting as antioxidants. Thermal treatment by microwaving did not affect levels of vitamin C in dry baobab pulp, thus this method may be used for by local fruit processors in Malawi for the development of a high quality intermediate dried product for sale. Moreover the techniques is cheaper and affordable by the resource poor communities.

Uncontrolled thermal treatment and storage temperature significantly affects the level of bioactive compounds, functional properties, and nutritive value of baobab juice. Low temperature long time (LTLT) (60 °C, 30 min and 60 °C, 60 min) and HTST (85 °C, 15 s) pasteurisation reduced levels of ascorbic acid in untreated juice. Although HTST (72 °C, 15 s) significantly ($p \leq 0.05$) reduced degradation of ascorbic during pasteurisation, the levels of ascorbic acid and vitamin C (AA + DHA) were lower compared to untreated juice samples suggesting further improvements required. Malawian fruit processors could combine microwaving of dry pulp powder and pasteurisation HTST (72 °C, 15 s) of juice to increase efficiency of enzyme inactivation. The final juice may retain vitamin C and improve its stability during processing and storage respectively.

The levels of organic acids were generally stable in all juice samples. In general all refrigerated samples kept at 6 °C showed higher level of ascorbic acid and vitamin C. All juice samples showed systematic fluctuation in the level of flavan-3-ols, total phenol content and antioxidant activity of baobab juice over a period of 60 days indicating possibility of a series of chemical reactions taking place. Total phenol content, procyanidin B2 and (-)-epicatechin were fairly more stable during storage compared to vitamin C and could be responsible for stable antioxidant activity of the juice. Moreover the antioxidant activity of baobab juice was more correlated with total phenol content than vitamin C, procyanidin B2 or (-)-epicatechin. The presence of amino acids and reducing sugars likely lead to non-enzymatic browning in baobab juice stored at elevated temperature (30 °C) and this may contribute to a higher antioxidant activity observed in the juice.

Low retention of vitamin C in the juice during thermal treatment (85°C, 15 s) and its loss in stored pasteurised (72°C, 15 s) juice increase the demand for advanced processing methods. Non-thermal pasteurisation methods including high hydrostatic pressure (HHP), ultrasound, and pulsed electric field (PEF) are therefore suggested in order to retain ascorbic acid during pasteurisation as well as increase its shelf-life and other quality attributes of baobab juice during storage. These methods have been successfully used in exotic fruits and their products (Rawson et al., 2011).

Chapter 5 **Characterisation of commercial baobab juice from Malawi**

Abstract

The chemical composition of commercial baobab juice (CBJ) from Malawi was investigated and compared to prepared baobab and commonly consumed fruit juices. Changes in the levels of ascorbic acid, vitamin C, organic acids (citric, malic and tartaric acid), procyanidin B2 and (-)-epicatechin were analysed by RP-HPLC while total phenol content (TPC) and antioxidant activity were measured by spectrophotometry during different storage conditions. The levels of ascorbic acid and vitamin C in CBJ were lower compared to prepared baobab and orange juices suggesting the need for improvement of pasteurisation method. The levels of (-)-epicatechin and organic acids were within the range observed in prepared baobab juice. Higher levels of procyanidin B2, TPC and antioxidant activity in CBJ compared to prepared juice were ascribed to high pulp content in the mucilage. The levels of most quality attributes including vitamin C, phenolic compounds hence antioxidant activity were higher during storage at 15 than 6 °C suggesting a higher solubility of these bioactive compounds. The present study provides important information to consumers and processors in order to determine quality of present juice and decide necessary improvements. The use of non-thermal pasteurisation is suggested to improve vitamin C retention and stability.

Keywords: Composition; Commercial baobab juice; Bioactive compounds; Storage condition; Antioxidant activity.

5.1 Introduction

Compositional analysis of fruit juice is necessary to determine the nutritive value and potential health benefit. There is great potential for the improvement of the nutritional and economic benefits of indigenous fruits through the development of quality products like juices that could be processed and commercialised as health drinks (Ndabikunze et al., 2010). In Malawi women dominate the utilisation and processing of baobab fruits. However the lack of nutritional data of fresh raw materials such as baobab fruit pulp and derived products limits full exploitation. Understanding changes in quality attributes during processing and storage of plant foods is important for quality product development in terms of safety, acceptability and nutritional values (Oms-Oliu et al., 2013). Moreover comprehensive nutritional information influences consumer demand and commercialisation of a food product. While there is sufficient knowledge and information on the influence of thermal processing and preservation of commonly consumed fruits including apples, grapes and oranges, such information is scarce for most indigenous fruits of Malawi.

Commercial baobab juice (CBJ) as prepared in Malawi is achieved by soaking roughly 10% baobab pulp (100 g/L) with seeds in water for 12 hours, filtering using a mesh bag to remove seeds and insoluble components, boiling (100 °C, 60 min), cooling to 45 °C, adding 10% sugar (100 g/L juice), 0.05% sodium benzoate (0.5 g/L) and finally filling pre-sterilised bottles. The resultant juice is viscous and usually fortified with commercial ascorbic acid to compensate for the loss during boiling. Commercial baobab juice is becoming popular and widely consumed by the Malawian population. While there is

sufficient nutritional information on most of tropical and exotic fruit juices found in the supermarkets of Malawi, such information is scarce for most indigenous fruit products of Malawi including commercial baobab juice.

5.2 Aim of the chapter

The main aim of the present study was to assess the chemical composition and investigate the influence of storage on quality attributes of commercial baobab juice available in Malawi. The level of vitamin C, total phenol content, (-)-epicatechin, procyanidin B2, antioxidant activity (FRAP, ABTS and DPPH) were measured and compared to prepared baobab juice (PBJ) and commonly consumed juices. Changes in quality attributes were monitored during storage at 6, 15 and 30 °C.

5.3 Results and discussion

5.3.1 Effect of storage on ascorbic acid and vitamin C

The initial level of ascorbic acid in commercial baobab juice (CBJ) was 5.09 ± 0.39 mg/100 mL. The level of ascorbic acid was lower than pasteurised prepared baobab juice (7.58 mg/100 mL) and commercial orange juice from concentrate (29.10 mg/100 mL) (USDA, 2015). The level of ascorbic acid in CBJ at 6 and 30 °C was less stable compared to juice samples stored at 15 °C as ascorbic acid extracted in samples left at 15 °C was significantly higher ($p \leq 0.05$) than other regimes (Figure 5.1). The loss of ascorbic acid from time zero was 79 and 73% at 6 and 30 °C respectively after 49 days of storage. It seems storage temperature showed less impact on ascorbic acid degradation for commercial baobab juice. Unlike PBJ the loss of ascorbic acid in CBJ was lower at 15 °C

compared to 6 °C. This could be attributed to higher solubility of ascorbic acid at 15 °C compared to 6 °C. Further, higher total solute concentration might be responsible for protection against ascorbic acid degradation which was significantly enhanced during storage at 15 °C, the reverse trend usually observed in juice without additives.

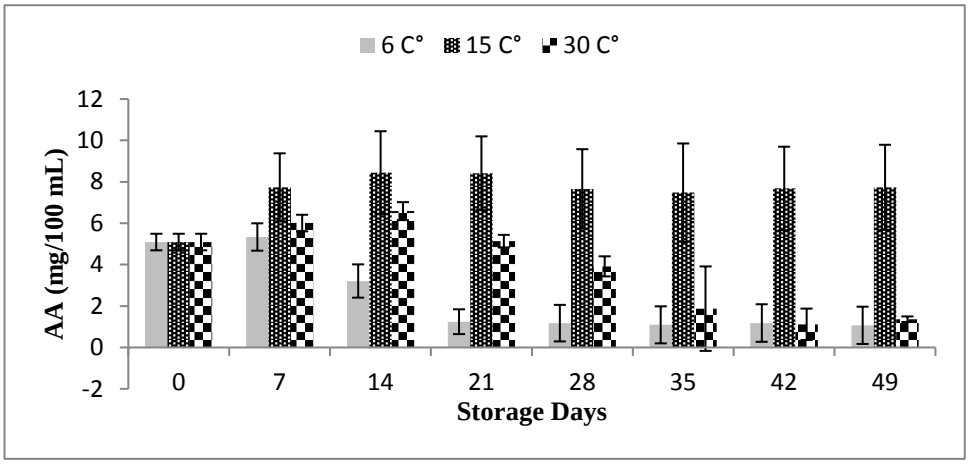


Figure 5.1 - Evolution of ascorbic acid (AA) content in commercial baobab juice at 6, 15 and 30 °C; n = 3.

The degradation of vitamin C (ascorbic acid and dehydroascorbic) is shown in Figure 5.2. The initial vitamin C content was 10.10 ± 0.24 mg/100 mL representing 5.01 mg/100 mL contribution from dehydroascorbic acid. The level of vitamin C was decreasing significantly ($p \leq 0.05$) during storage at 6 and 30 °C. Vitamin C content decreased after 7 days of storage at 15 °C then remained stable for rest of storage time. Samples stored at 6, 15 and 30 °C retained 21, 80 and 18% respectively of the initial vitamin C content. This showed that storage at 15 °C was more effective in terms of ascorbic acid and vitamin C preservation. This information may be important to local baobab fruit processors in Malawi.

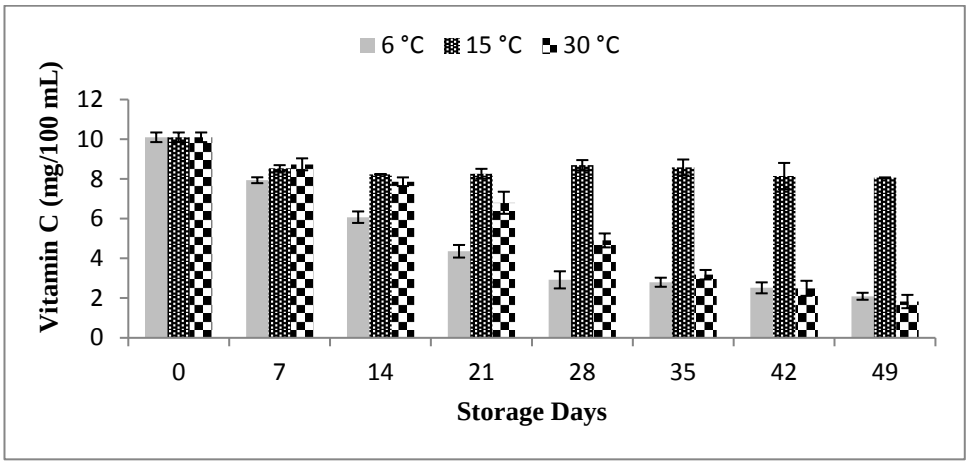


Figure 5.2 - Evolution of vitamin C content in commercial baobab juice at 6, 15 and 30 °C; n = 3.

5.3.2 **Effects of storage on organic acids**

Organic acids are present in fruits in different amounts. They are usually added to fruit juice as natural preservatives and also to give a sour taste. Evolution of organic acids in commercial baobab juice (CBJ) including citric (CA), malic (MA) and tartaric (TA) acid at three different temperatures is presented.

The initial CA content of all samples was 209.28 ± 30.15 mg/100 mL. The initial value of CA was lower but within the range observed in prepared baobab juice (330.08 mg/100 mL). Just as prepared baobab juice (PBJ), the level of CA in CBJ was generally stable during storage at all temperatures (Figure 5.3). For instance, samples stored at 15 and 30 °C retained 90 and 89% respectively of the initial CA content. The final CA content in CBJ stored at 6 °C was 12% higher compared to initial value.

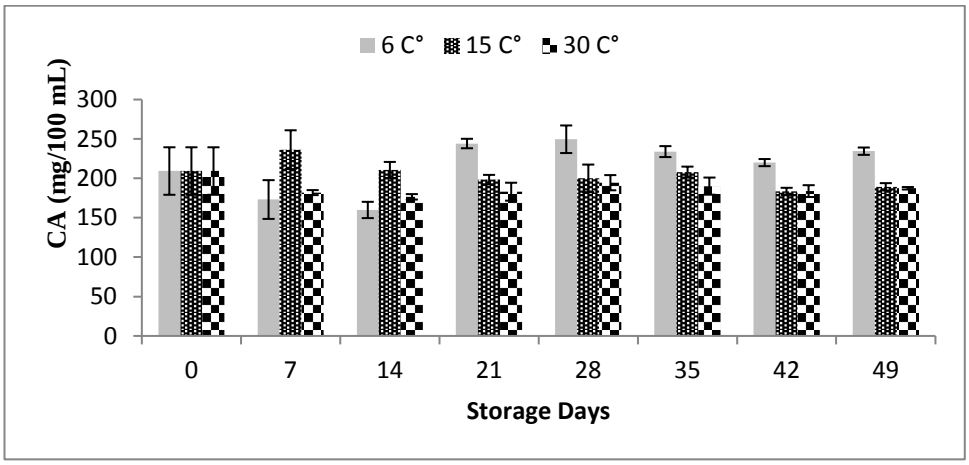


Figure 5.3 - Evolution of citric acid (CA) content in commercial baobab juice at 6, 15 and 30 °C; n = 3.

The initial level of malic acid (MA) was 276.80 ± 27.04 mg/100 mL and within the range observed in PBJ (236.50 ± 2.88 mg/100 mL). Results showed that at 6 °C, the level of MA decreased significantly ($p \leq 0.05$) by day 7, then remained stable until day 14 and finally increased continuously until the end of storage (Figure 5.4). Levels of MA at 15 and 30 °C were generally stable with notable homogenous groups. For instance, levels of MA on 14, 21, 28, 35 and 49 days were not significantly different ($p > 0.05$) at 15 °C. Similarly, after day 7, levels of MA were not significantly different ($p > 0.05$) during storage at 30 °C. Initial level of MA increased by 39.9% at 6 °C, but decreased by 31 and 36% at 15 and 30 °C respectively by the end of storage time.

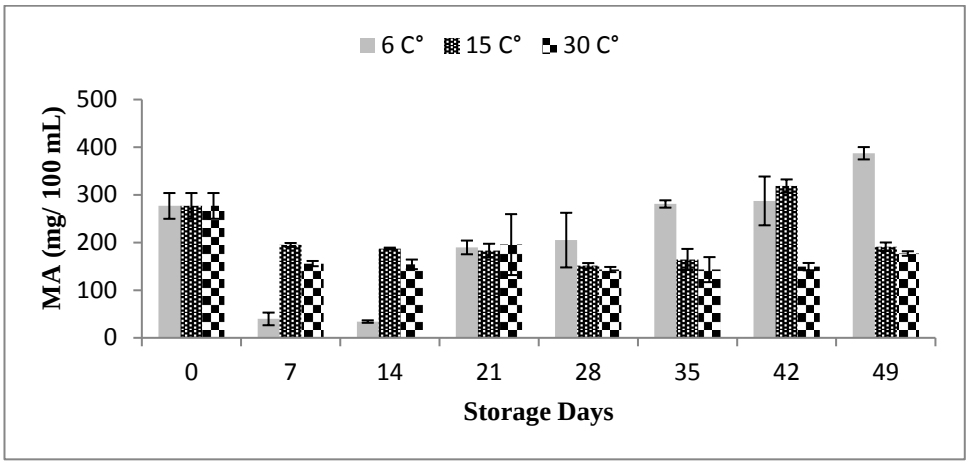


Figure 5.4 - Evolution of malic acid (MA) content in commercial baobab juice at 6, 15 and 30 °C; n = 3.

The initial content of tartaric acid (TA) in commercial baobab juice **(**CBJ) was 221.54 ± 20.26 mg/100 mL. The content of TA was fluctuating at 6 °C but was more stable at 15 and 30 °C during storage (Figure 5.5). For instance, the loss of TA in initial CBJ stored at 15 °C was 4.7% after 7 days, thereafter levels were not significantly different ($p > 0.05$). Levels of TA were not significantly different ($p > 0.05$) during storage at 30 °C.

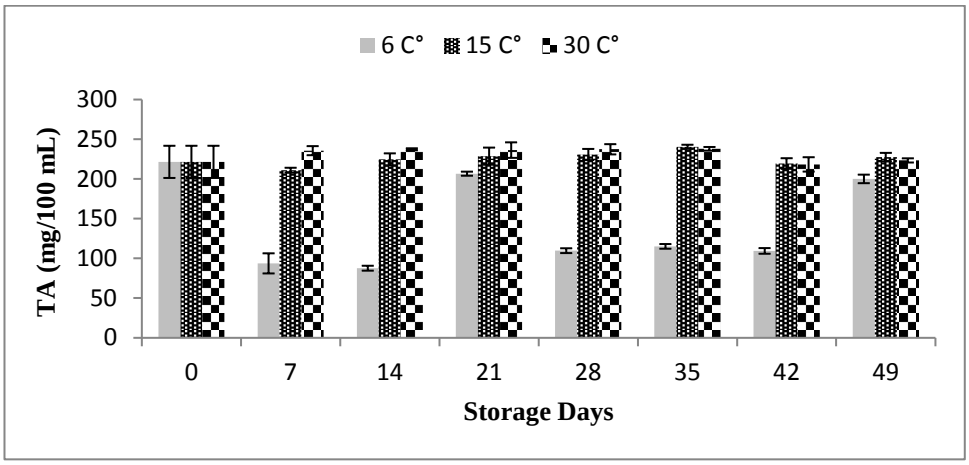


Figure 5.5 - Evolution of tartaric acid (TA) content in commercial baobab juice at 6, 15 and 30 °C; n = 3.

Results from present study shows that commercial baobab juice (CBJ) contains less ascorbic acid and vitamin C than prepared baobab juice (PBJ) but contains comparable levels of organic acids. Both juice samples contained significantly higher levels of organic acids than commonly consumed fruits.

5.3.3 **Effect of storage on total phenol content (TPC)**

In the present study, analysis of total polyphenol content was conducted for screening the presence of phenolic compounds and their evolution during storage in commercial baobab juice (CBJ) from Malawi.

The initial level of TPC in CBJ (1112.22 mg GAE/100 mL) was higher than levels observed in prepared baobab juice (260.80 ± 0.27 mg GAE/100 mL) and orange juice (68.42 mg GAE/100 mL) reported by other researchers (Klimczak et al., 2007). Higher

residual pulp content in CBJ was likely responsible for a higher total phenol content compared to PBJ and orange juice.

Results showed that storage time and temperature significantly affected the level of TPC (Figure 5.6) of CBJ. There was a significant decrease in TPC during storage at 6 and 30 °C. Initial level of TPC in CBJ decreased by 23 and 53% by the end of storage at 6 and 30 °C respectively. Surprisingly, initial level of TPC increased by 10% by the end of storage at 15 °C. The increase in TPC during storage had previously been observed and reported by other authors (Klimczak et al., 2007; Piljac-Žegarac et al., 2009). Other authors suggested that other compounds formed during storage may react with Folin-Ciocalteu reagent. The decrease in TPC could be due to the phenolic compounds reacting with sugars and sugar metabolites present in the juice (Agbenorhevi and Marshall, 2012). Moreover CBJ had higher sugar concentration deliberately added for preservation.

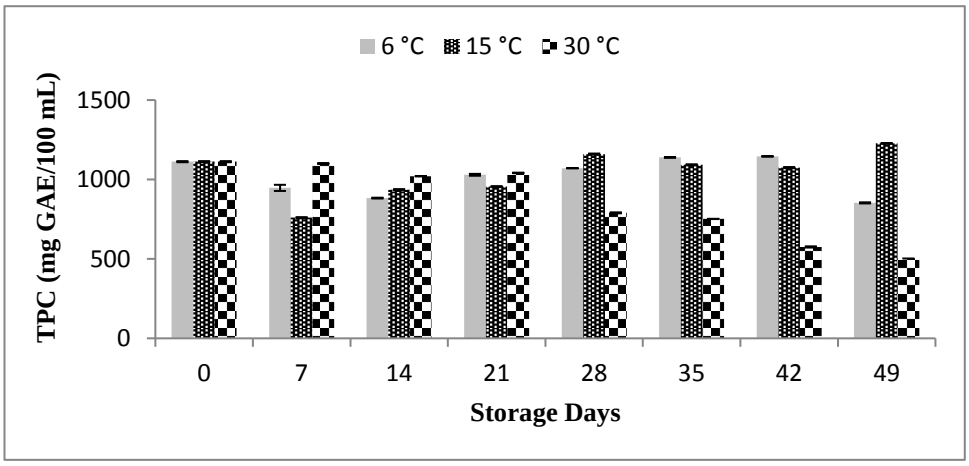


Figure 5.6 - Evolution of total phenol content in commercial baobab juice at 6, 15 and 30 °C; n = 3.

5.3.4 **Effect of storage on antioxidant activity**

The total antioxidant activity of CBJ was evaluated using FRAP, DPPH and ABTS assays. All antioxidant assays showed high antioxidant properties in the juice. The initial antioxidant activity (FRAP) was 17.83 mg TEAC/100 mL. The FRAP values in commercial baobab juice was lower than levels observed in PBJ and fruit smoothies reported by Agbenorhevi and Marshall (2012). Results showed that storage time and temperature significantly ($p \leq 0.05$) affected the ferric reducing antioxidant power (FRAP) of the juice (Figure 5.7). However, FRAP values were generally more stable at 15 °C. The trend in FRAP values was similar to that observed for TPC. Initial levels of FRAP decreased by 7.45 and 48% by the end of storage time at 6 and 30 °C respectively. However, an increase of 17.9% in FRAP values was observed by the end of storage at 15 °C. The increase in FRAP values is likely attributed to higher levels of total phenol content, ascorbic acid, as well as vitamin C observed in the same juice sample.

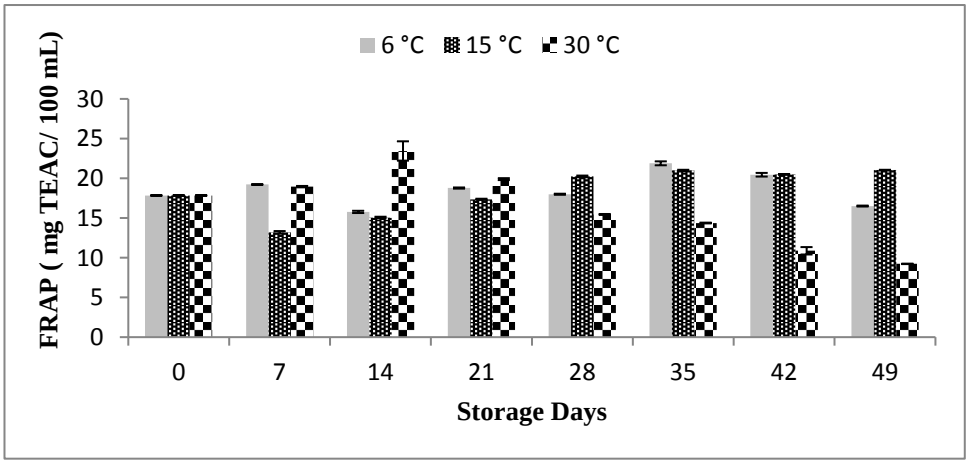


Figure 5.7 - Evolution of antioxidant activity (FRAP) in commercial baobab juice at 6, 15 and 30 °C; n = 3.

Results for evolution of antioxidant activity (DPPH) in CBJ during storage are presented in Figure 5.8. The initial DPPH value (80.94 ± 0.72%) was significantly higher ($p \leq 0.05$) than levels reported for PBJ and commonly consumed fruits. Too high DPPH values observed could be attributed to higher TPC and sugar metabolites. Antioxidant activity (DPPH) was significantly affected during storage under all experimental conditions. By the end of storage time, initial DPPH values in the juice stored at 6, 15, and 30 °C was reduced by 2.3, 4.1 and 54.9% respectively. The reduction of DPPH free radical scavenging activity was consistent with changes in ascorbic acid, vitamin C and total phenol content. The variation of antioxidant activity with total phenol content was also reported by other authors (Agbenorhevi and Marshall, 2012; Martínez-Flores et al., 2015).

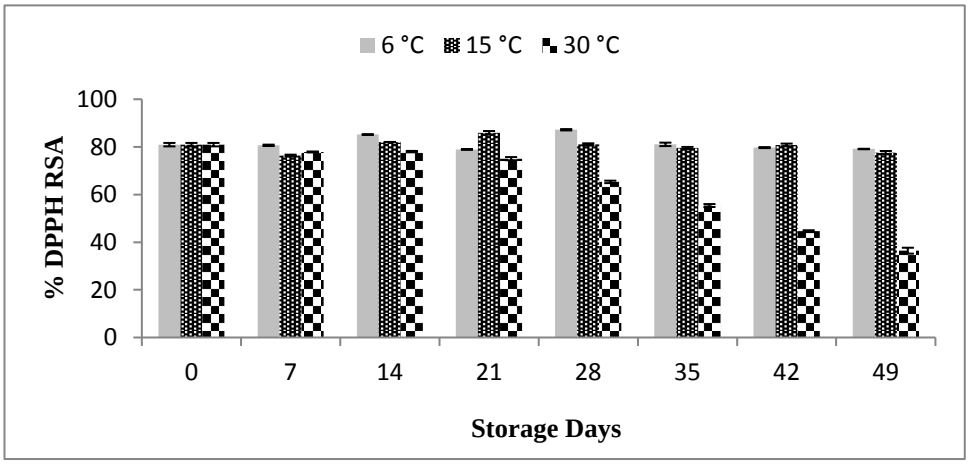


Figure 5.8 - Evolution of antioxidant activity (DPPH) in commercial baobab juice at 6, 15 and 30 °C; n = 3.

Finally, results for evolution of antioxidant activity (ABTS) are presented in Figure 5.9. The initial ABTS value was 112.62 ± 0.45 mg TEAC/100 mL. The observed value was

lower than those observed in prepared baobab juice but higher than levels reported for other fruits including quince juice (Wojdyło et al., 2014) and jujube (Kou et al., 2015). The ABTS free radical scavenging ability of juice varied significantly during storage under all experimental conditions. There was a general decrease in ABTS values with storage time and temperature. For instance, the level of ABTS ranged from 111.00 ± 0.30 to 117.53 ± 0.39 at 6 °C; 103.70 ± 0.40 to 115.27 ± 0.59 at 15 °C and 69.51 ± 0.30 to 112.62 ± 0.45 mg TEAC/100 mL at 30 °C. Essentially, the initial ABTS values in the juice left at 6, 15 and 30 °C reduced by 1.4, 7.5 and 38.3% respectively. Likewise, degradation of antioxidant activity (ABTS) was likely attributed to loss of vitamin C and phenolic compounds. Although commercial baobab juice showed considerably higher antioxidant activity than most fruits, levels were generally lower than prepared baobab juice. Prolonged heating and addition of sugar might have affected quality characteristics of the juice.

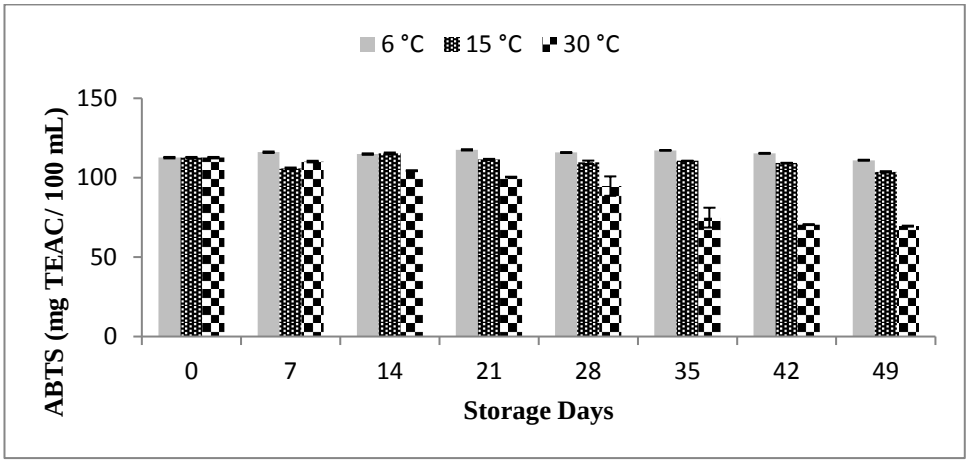


Figure 5.9 - Evolution of antioxidant activity (ABTS) in commercial baobab juice; n = 3.

5.3.5 **Effect of storage on flavan-3-ols of commercial baobab juice**

Flavan-3-ol is one of the main subclasses of dietary flavonoids (Crozier et al., 2009). Flavan-3-ols contain diverse structures including (-)-epicatechin and procyanidin B2. *In vivo* consumption of procyanidin B2 has been implicated in improved antioxidant status and decreased DNA damage in humans (Crozier et al., 2009). Procyanidin B2 is also involved in sensory characteristics of juice. Therefore their presence in foods greatly affects food quality (Aron and Kennedy, 2008). For these reasons and the interest to compare with prepared baobab juice (PBJ), a study was undertaken to quantify and investigate the influence of storage on (-)-epicatechin and procyandin B2 in commercial baobab juice. Results for procyanidin B2 evolution at 6, 15 and 30 °C are presented in Figure 5.10.

The initial level of procyanidin B2 (98.47 ± 11.33 mg/100 mL) was higher than levels observed in prepared baobab juice (53.33 ± 2.26 mg/100 mL for UT; 46.63 ± 5.65 mg/100 mL for HTST and 35.12 ± 2.92 mg/100 mL for CAHTST juice) and values reported for other beverages including red wine (2.7 ± 0.5 mg/100 mL) (Crozier et al., 2009). The level of procyanidin B2 was affected similarly to observations reported for prepared baobab juice. Lower levels were observed for samples at 6 °C with values ranging from 67.75 ± 11.88 to 98.47 ± 11.33 mg/100 mL. Significantly highest levels ($p \leq 0.05$) of procyanidin B2 were observed in the juice kept at 15 °C with value ranging from 98.47 ± 11.33 to 355.35 ± 53.29 mg/100 mL. Samples stored at 30 °C showed a wide variation (16.60 ± 1 to 224.89 ± 11.54 mg/100 mL) indicating high rate of degradation of procyanidin B2 to other compounds. In all cases, the level of procyanidin B2 was

fluctuating during storage. By the end of storage time, the initial level of procyanidin B2 at 6 and 30 °C decreased by 31 and 83% respectively, while at 15 °C levels increased more than two-fold. Therefore the level of procyanidin B2 was directly related to total phenol content and antioxidant activity observed in CBJ described previously.

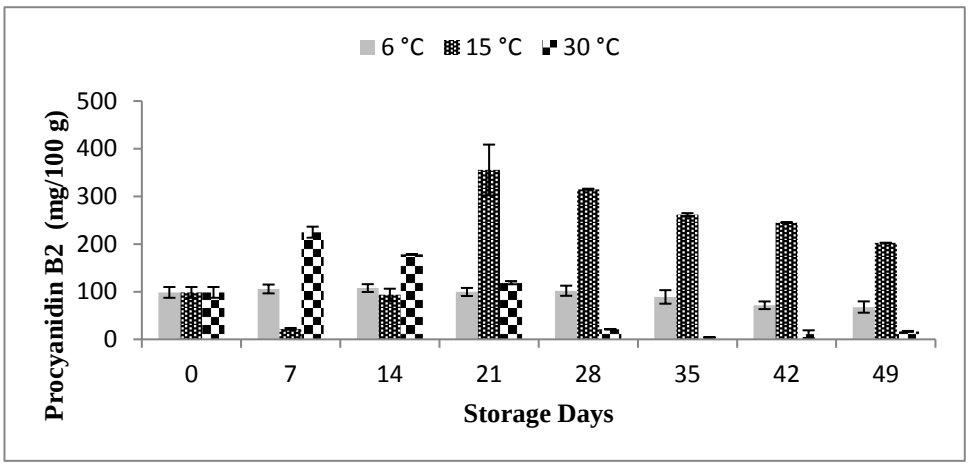


Figure 5.10 - Evolution of procyanidin B2 in commercial baobab juice at 6, 15 and 30 °C; n = 3.

Results for the content of (-)-epicatechin and changes during storage are presented in Figure 5.11. The initial content of (-)-epicatechin in CBJ (6.0 ± 6.33 mg/100 mL) was within the range observed in prepared baobab juice (4.30 ± 0.31 mg/100 mL for UT; 4.77 ± 0.85 mg/100 mL for HTST and 3.2 ± 0.09 mg/100 mL for CAHTST juice). However, green tea still stands out to contain the highest levels of (-)-epicatechin (73.8 ± 1.7 mg/100 mL) while the level in black tea (1.1 ± 0.02 mg/100 mL) reported in the same article (Crozier et al., 2009) was lower than levels observed in commercial or prepared baobab juice.

(-)-Epicatechin content was significantly affected by storage temperature. Samples stored at 15 °C showed highest levels of all with a general increase during storage (Figure 4.28). (-)-Epicatechin degraded at a higher rate for samples stored at 30 °C compared to samples at 6 °C. By the end of storage, the initial level of (-)-epicatechin in the juice stored at 6 and 30 °C decreased by 54 and nearly 100% respectively. The level of (-)-epicatechin showed an increase of 128% at 15 °C by the end of storage time. Evolution of (-)-epicatechin was consistent with of total phenol content and antioxidant activity.

The increase or decrease in procyanidin B2 and (-)-epicatechin has been previously attributed to depolymerisation, isomerisation, hydroxylation, esterification reactions and these reactions may be enhanced by higher solute concentration including sugar metabolites (Aron and Kennedy, 2008; Crozier et al., 2009; Keenan et al., 2011).

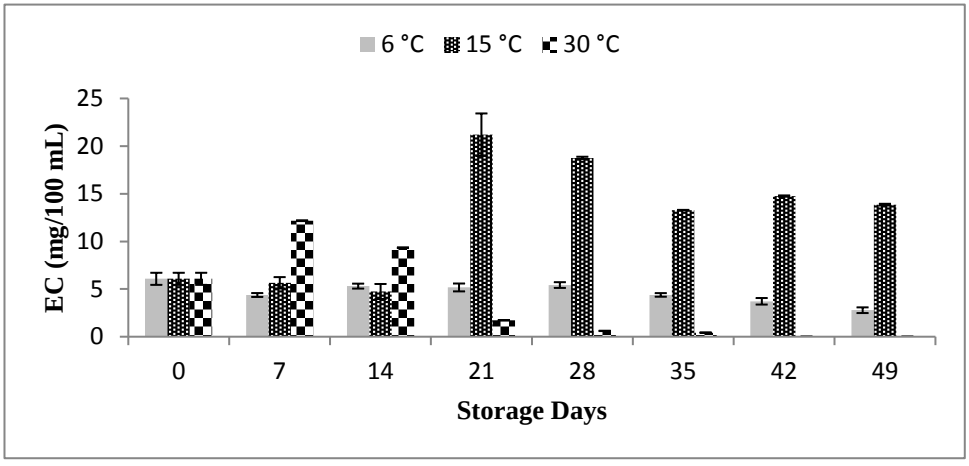


Figure 5.11 - Evolution of (-)-epicatechin in commercial baobab juice at 6, 15 and 30 °C; n = 3.

5.3.6 **Relationships amongst different variables in commercial juice**

Correlation analysis is important to establish relationships between variables and methods employed. It also helps to validate findings as most naturally occurring chemical compounds are well known to possess specific chemical or biological properties. Pearson's correlation coefficients between nutritional attributes measured in commercial baobab juice are presented in Table 5.1.

Table 5.1 - Pearson correlation coefficients (r) between quality attributes of commercial baobab juice purchased from Malawi.

	Vit. C	TPC	PRO	EC	FRAP	DPPH	ABTS	CA	MA	TA
AA	0.84**	0.32**	0.62**	0.66**	0.28**	0.37**	0.25*	-0.25*	-0.14	0.41**
Vit.C		0.45**	0.62**	0.65**	0.38**	0.50**	0.41**	-0.29*	-0.22	0.34**
TPC			0.61**	0.57**	0.90**	0.79**	0.76**	0.17	0.23	-0.15
PRO				0.96**	0.83**	0.53**	0.43**	-0.17	-0.02	0.23
EC					0.51**	0.52**	0.42**	-0.09	0.01	0.24
FRAP						0.71**	0.66**	0.04	0.17	-0.12
DPPH							0.95**	0.25*	0.14	-0.33
ABTS								0.32*	0.18	-0.41**
CA									0.55**	-0.13
MA										0.12

** Significant correlation at $p \leq 0.01$

* Significant correlation at $p \leq 0.05$

Results showed that antioxidant activity and bioactive compounds were positively and significantly correlated ($p \leq 0.05$). Moreover antioxidant activity assays were highly correlated ($P \leq 0.01$). The correlation coefficients between FRAP and DPPH, FRAP and ABTS, and DPPH and ABTS were 0.71, 0.66 and 0.95 respectively. Ascorbic acid was significantly correlated with vitamin C, total phenol content, procyanidin B2, (-)-epicatechin, FRAP, DPPH, ABTS and tartaric acid (Table 5.1). Vitamin C showed a similar trend to ascorbic acid. Total phenol content showed significant correlation with procyanidin B2 ($r = 0.61$, $p \leq 0.01$), epicatechin ($r = 0.57$, $p \leq 0.01$), FRAP ($r = 0.90$, $p \leq 0.01$), DPPH ($r = 0.79$, $p \leq 0.01$) and ABTS ($r = 0.76$, $p \leq 0.01$). The correlation coefficients of procyanidin B2 with FRAP, DPPH and ABTS were 0.83, 0.53 and 0.43 respectively while the correlation coefficients of (-)-epicatechin with FRAP, DPPH and ABTS were 0.51, 0.52 and 0.42 respectively. Procyanidin B2 showed stronger positive correlation with antioxidant activity than (-)-epicatechin. Not surprising organic acids were generally not strongly correlated with other variables and antioxidant activity.

Procyanidin B2 and (-)-epicatechin showed strong positive correlation ($r = 0.96$, $p \leq 0.01$). Levels of (-)-epicatechin and procyanidin B2 increased proportionally ($R^2 > 0.92$) during storage at 15 °C (Figure 5.12). This may suggest that depolymerisation of higher proanthocyanidins to procyanidin B2 and (-)-epicatechin was more favourable at 15 °C.

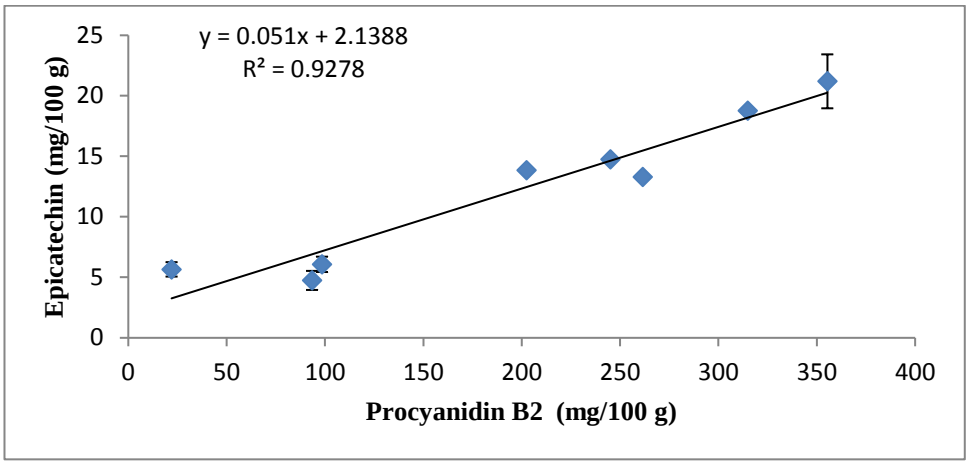


Figure 5.12 - Relationship between evolution of procyanidin B2 and (-)-epicatechin; n = 3.

Pearson's correlation analysis of CBJ indicates that when the level of ascorbic acid is high, vitamin C, TPC, procyanidin B2, (-)-epicatechin, FRAP, DPPH and ABTS are likewise. Ascorbic acid, vitamin C, total phenol and flavan-3-ols (procyanidin B2 and (-)-epicatechin) showed a strong positive influence on antioxidant activity of commercial juice. Further, results showed that TPC had more influence on antioxidant activity of the juice similar to observations made in prepared juice. The correlation of TPC with vitamin C or ascorbic acid is lower than with flavan-3-ols due to thermal instability of vitamin C. Phenolic compounds are fairly stable during storage. Other researchers also reported significant positive correlation amongst ascorbic acid, total phenol content, flavan-3-ols and antioxidant activity (FRAP, DPPH and ABTS) in different food products including fruits and vegetables (Thaipong et al., 2006; Tuberoso et al., 2010; Rössle et al., 2011; Kou et al., 2015). Therefore results from this study are consistent with published data. It seems keeping commercial juice at 15 °C was better and enhanced the levels of bioactive

compounds (total phenols, procyanidin B2 and (-)-epicatechin) which were reflected in the increased antioxidant activity.

5.3.7 **Conclusion**

The use of more sensitive and selective analytical techniques (RP-HPLC) revealed that commercial baobab juice (CBJ) contains lower ascorbic acid and vitamin C compared to prepared baobab juice (PBJ). The level of organic acids (citric, malic and tartaric acids) in CBJ were within the range observed in PBJ. However the level of total phenol content, flavan-3-ols (procyanidin B2 and (-)-epicatechin) and DPPH were significantly higher in CBJ compared to PBJ and values reported by several authors (Gu et al., 2004) for commonly consumed fruit juices (orange, grape and apple) and red wine.

The level of ascorbic acid and vitamin C was more stable during storage at 15 °C compared to 6 and 30 °C. This could be attributed to increased solubility of ascorbic acid at 15 °C compared to 6 °C. The other possibility could be increased bioavailability of phenolic compounds which may protect vitamin C by donating hydrogen thereby converting dehydroascorbic acid back to ascorbic acid in redox reaction (Agbenorhevi and Marshall, 2012). This was consistent with significantly higher levels of total phenol content, procyanidin B2 and (-)-epicatechin observed in CBJ during storage at 15 °C compared to 6 and 30 °C. Higher levels of bioactive compounds and total phenol content likely contributed significantly to higher antioxidant activity (DPPH) observed in the juice kept at 15 °C. This could be attributed to increased release of phenolic compounds from juice matrix at a higher temperature. Similarly, Choi et al. (2006) observed that a

higher temperature influenced release of bound polyphenolic compounds from the matrix of Shiitakes (*Lentinus edodes*) and lead to higher total phenol content and antioxidant activity.

Results from this study will thus provide knowledge and information regarding composition of commercial baobab juice and influence of storage on important quality attributes. Thus present data may act as a guide to possible improvements. For instance non-pasteurisation and use of citric acid could be sufficient to preserve vitamin C and other quality attributes rather than boiling at 100 °C for 60 min, addition of excess sugar and use of sodium benzoate as is currently done. Moreover the use of sugar and sodium benzoate are associated with potential adverse effects on health (Shahidi and Zhong, 2010). Thus the current demand of food preservation is the use of natural additives.

Chapter 6 ^{1}H NMR metabolic profiling of baobab juice from Malawi

Abstract

In this study the effect of thermal treatment and storage on the metabolic profile and composition of untreated (UT), pasteurised (HTST) and acidified baobab juice (CAHTST) from Malawi was characterised and evaluated by ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy. The ^{1}H NMR spectra of aqueous extract of juice confirmed presence of a wide range of metabolites in the amino and organic acids, sugars and phenolic regions. Principal Component Analysis (PCA) of ^{1}H NMR data produced distinct clusters separated according to treatment and storage conditions. PCA loading plots revealed presence of very high concentration of ethanol in untreated juice at 30 °C, reasonably indicative of the occurrence of an intense fermentation activity. Further orthogonal variation of the partial least squares (OPLS) regression analysis showed evidence of a temporal dynamic change of juice metabolome at each storage temperature. The corresponding loadings line plots revealed metabolites that most significantly accumulated in response to time and with the reverse response. For CAHTST juice, the most important accumulating metabolites were fructose, glucose and galacturonic acid, while sucrose decreased over time. The more rapid production of ethanol in the absence of any thermal treatment is a clear indication that pasteurisation of juice alone or in combination with the addition of citric acid can minimise fermentation in baobab juice.

Keywords: Baobab fruit juice; Metabolic profile; Thermal processing; Storage condition, ^{1}H NMR; Principal Component Analysis.

6.1 Metabolomics

Metabolomics is an emerging field of "omics" research that focuses on high-throughput characterisation of low molecular weight molecules (metabolites) in biological matrices. Being able to measure the chemical changes that occur in food as it is being processed or prepared and linking those changes to specific characteristics such as sensory characteristics or food safety, thus metabolomics has a wide range application in food science and nutrition (Wishart, 2008; Cuthbertson et al., 2012; Chin and Slupsky, 2013; Brennan, 2014; Cubero-Leon et al., 2014; Richards and Holmes, 2015; Trimigno et al., 2015). It can be used for compound identification (authentication and pathogen detection), characterisation of bioactive compounds (Li et al., 2012; Mannina et al., 2012), predicting sensory attributes and evaluating the impact of food processing, storage and safety (Le Gall et al., 2004; De Oliveira et al., 2014). Whereas in nutrition, metabolomics may be used to investigate the effects of nutrients or foods at the metabolic level; and more recently the interaction of nutrients with the gut microbiota (Richards et al., 2015; Trimigno et al., 2015). Metabolomics can also be used to assess the effects of diet on health, mapping dietary patterns as well as a tool for observational and interventional studies into the effects of diet and nutrition on health (Nieman et al., 2012; Richards et al., 2015).

Different technologies and equipment are used in metabolomics including nuclear magnetic resonance (NMR) spectroscopy, gas chromatography - mass spectrometry (GC-MS), liquid chromatography – mass spectrometry (LC-MS) and capillary electrophoresis – mass spectrometry (CE-MS) (Wishart, 2008). Each of the techniques and equipment outlined has advantages and disadvantages. New software programs are available to rapidly process spectral or chromatographic patterns of acquired data. In food metabolomics, in particular, NMR spectroscopy is recognised as one of the main analytical methodologies giving a complete view of the foodstuffs metabolites and, together with suitable statistical analysis, providing relevant results in terms of foodstuffs quality, processing, raw material, safety and so on (Mannina et al., 2012). The strength of the NMR spectroscopy approach is the capacity to look at all the components of a mixture at once allowing a metabolite qualitative and quantitative analysis and thus generating huge data from which it is necessary to extract the required information (Mannina et al., 2012). Further, NMR spectroscopy is robust, non-destructive, fast, it requires neither derivatisation nor separation of compounds, has a large body of software and databases for metabolite ID and is compatible with liquid and solid food samples.

The metabolomics approach can be organised into two main analytical classes: targeted and untargeted analysis. In quantitative metabolomics or targeted profiling approach, the aim is to identify and/or quantify in the sample as many compounds as possible. This is usually done by comparing the sample's NMR or MS spectrum to a spectral reference library obtained from pure compounds (Weljie et al., 2006). After identification and quantification of metabolites, data is subjected to multivariate statistical analysis to

identify the most important biomarkers or informative metabolic pathways (Weljie et al., 2006).

Untargeted analysis, on the other hand, determines patterns or fingerprints of metabolites to identify the relevant spectral features that distinguish sample classes and does not necessitate identifying specific compounds (Trygg et al., 2007; Chin and Slupsky, 2013). Statistical comparisons and feature identification techniques involve unsupervised clustering such as Principal Component Analysis (PCA) or supervised classification such as Partial Least Square Discriminant Analysis (PLS-DA). Principal component analysis is a multivariate technique that analyses a data table in which observations are described by several inter-correlated dependent variables (Abdi and Williams, 2010). It is a dimensional reduction technique and permits one to easily plot, visualise and cluster metabolomics data sets based on linear combinations (called principal components) of shared features (Izquierdo-García et al., 2011). PCA is commonly used to identify how one sample is different from another, which variables contribute significantly to the difference and whether those variables contribute in the same way (correlated) or independently (uncorrelated) from each other. In contrast to PCA, PLS-DA is a supervised classification technique that can be used to enhance the separation between groups of observations by rotating PCA components such that a maximum separation among classes is obtained (Trygg et al., 2007). The basic principles behind PLS-DA are similar to that of PCA, but in PLS-DA a second piece of information is used, namely, the labelled set of class identities. Although PCA and PLS-DA, on their own, do not permit the direct identification or quantification of compounds, they still allow an unbiased (or

untargeted), chemically comprehensive comparison to be made among different samples (Wishart, 2008).

Metabolomics can be further categorised into discriminative, informative, or predictive analysis. Discriminative analysis finds differences between sample groups, while informative analysis identifies and quantifies specific (targeted) or non-specific (untargeted) metabolites. Predictive analysis relies on statistical models that are based on the metabolic profile to predict which class an observation (sample), that may otherwise be difficult to classify, may belong to (Chin and Slupsky, 2013).

In the present study, targeted ^{1}H NMR-based metabolomics was used to identify and compare the metabolome of untreated juice (UT), high temperature short time pasteurised juice (HTST) and high temperature short time pasteurised with added citric acid juice (CAHTST) samples and to analyse the temporal changes upon different storage conditions. The results complement the chromatographic (RP-HPLC) and spectrophotometric dataset described in previous chapters (3 and 4) for comprehensive characterisation of baobab fruit juice from Malawi.

6.2 Chemometric analysis of ^{1}H NMR data

The NMR spectra were processed using MestReNova (Version 10.0, Mestrelab Research SL, Santiago de Compostela, Spain) and corrected for misalignments in chemical shift primarily due to pH-dependent signals. Each NMR spectrum was used to construct a data matrix by subdividing it into regions (bins) having an equal size of 0.0025 ppm over a chemical shift range of 0.5-9.5 ppm. The regions of the spectra containing the TSP (from

-0.5 to 0.5 ppm) and water (δ 4.6-5.2) resonances were excluded. Bins were normalized to the sum of total spectral area to compensate for the overall concentration differences. The final data set was automatically reduced to ASCII files, converted into an Excel file and then imported to SIMCA version 14.0 (Umetrics, Umeå, Sweden) for statistical analysis. Data were Pareto scaled and analyzed using Principal Component Analysis (PCA) and Orthogonal projection to latent structures (OPLS).

PCA is an unsupervised pattern recognition method, commonly performed to examine the intrinsic variation in the data set. Essentially, the aim of PCA is to express the main information contained in the initial variables in a lower number of uncorrelated variables, called principal components (PCs), which are combinations of the initial measurements but highlight the variance within the data set and remove redundancies (Izquierdo-García et al., 2011). Successive PCs account for decreasing amounts of variance, and most of the information is contained in the first few PCs. Graphically, the output from the PCA analysis consists of score plots, giving an indication of any grouping in the data sets in terms of metabolomic similarity, and loading plots, providing an indication as to which variables are important with respect to the patterns obtained in the score plots. PCA model performance was evaluated using the correlation coefficient R^2 and the default method of 7-fold internal cross-validation of SIMCA to extract the coefficient Q^2. Both R^2 and Q^2 vary between 0 and 1. R^2 is defined as the proportion of variance in the data explained by the model and indicates goodness to fit. Q^2 is defined as the proportion of variance in the data predictable by the model and indicates the goodness of prediction. A good prediction

model is achieved when $Q^2 > 0.5$, and if $Q^2 > 0.9$, it is regarded as an excellent predictive ability.

OPLS model tries to find a linear relationship between an X predictor matrix (e.g. spectrometric data of biological samples) and a Y response matrix (e.g. clinical results, treatment. . .). It is a modification of partial least squares (PLS), which separates the systematic variation in X into two parts, one that is linearly related to Y (predictive information) and one that is unrelated to Y (orthogonal information). The quality of the model is described by the parameter R^2Y and Q^2Y, which represent the fraction of the variation of Y-variable and the predicted fraction of the variation of the Y-variable, respectively. The statistical significance of R^2Y and Q^2Y values is estimated through response permutation testing (Jackson, 1991;Trygg and Wold, 2002; Boccard and Rutledge, 2013; Marincola et al., 2015). In this test, the Y-matrix is randomly re-ordered (200 times in this study), while the X-matrix is kept constant. This means that the Y-data remain numerically the same, but their positions are shifted by random shuffling. Each time a new OPLS model is fitted using X and the permuted Y matrix, providing a reference distribution of R^2Y and Q^2Y for random data. An intercept of the Q^2Y regression line below zero and an R^2Y intercept value significantly lower than the corresponding original one are indicative of a valid model. The contribution of variables to the model can be evaluated by plotting the OPLS coefficient (covariance) using a method which maintains the original format of NMR spectra, thereby improving interpretation of the model (Cloarec et al., 2005). In this method, the OPLS coefficients are back-scaled by multiplying all values by the standard deviation and then plotted

against their respective chemical shift. The direction of the signals in the plots relative to zero indicates positive or negative covariance. The colour code is calculated from the correlation matrix and represents the weight of the variable in the model, from blue to red for a low and high weight, respectively.

6.3 Results and discussion

6.3.1 ^{1}H NMR spectra

High resolution NMR spectroscopy has been shown to be a valuable method for the analysis of low molecular weight compounds in fruit juices, allowing simultaneously the detection of a wide range of species such as several sugars, organic acids, amino acids and other minor components such as phenolic compounds. Figure 6.1 shows a representative ^{1}H NMR spectrum of baobab juice and in Figure 6.2 the main assignments in the different spectral regions are detailed. As reported in Table 6.1, 25 compounds were identified on the basis of data published in the literature (Shahat, 2006; Chadare et al., 2008; Caluwé et al., 2010; Mannina et al., 2012; Ibrahima et al., 2013; De Oliveira et al., 2014; Rosa et al., 2015), and by using the database of Chenomx NMR Suite 7.1 (free version).

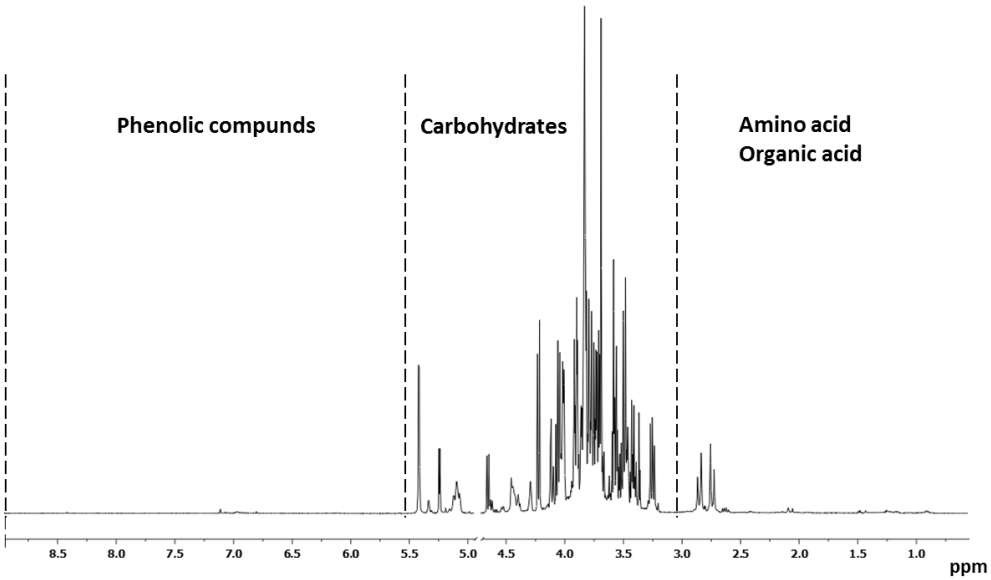


Figure 6.1 - Representative ^{1}H NMR spectrum of a baobab juice (10% D_2O) over a chemical shift ranging from 0.5-9.5 ppm.

The high-field region of the NMR spectrum (0.8-3.0 ppm) shows signals arising from aliphatic groups of free amino acids and organic acids. In particular, signals representing leucine (Leu), isoleucine (Ile), valine (Val) and alanine (Ala) were identified. The predominant organic acid identified was citric acid (Cit). In addition, malic (Mal), and lactic (Lac) acids were also present. In the mid-field region of the spectrum (3.0-5.5 ppm) the main contributions arise from the strongly overlapped signals of carbohydrates: sucrose, fructose and glucose. Some of the signals in the low-field region (5.5-9.0 ppm) were assigned to the aromatic compounds trigonelline and epicatechin and to formic acid. Given the structural similarity between phenolic compounds and the singlet nature of some NMR signals, spectral assignment in this region was not straightforward. Similarly, ^{1}H NMR spectrum of orange juice showed presence of sucrose, glucose, fructose, citric

acid in high concentration than amino acids and phenolic metabolites (De Oliveira et al., 2014).

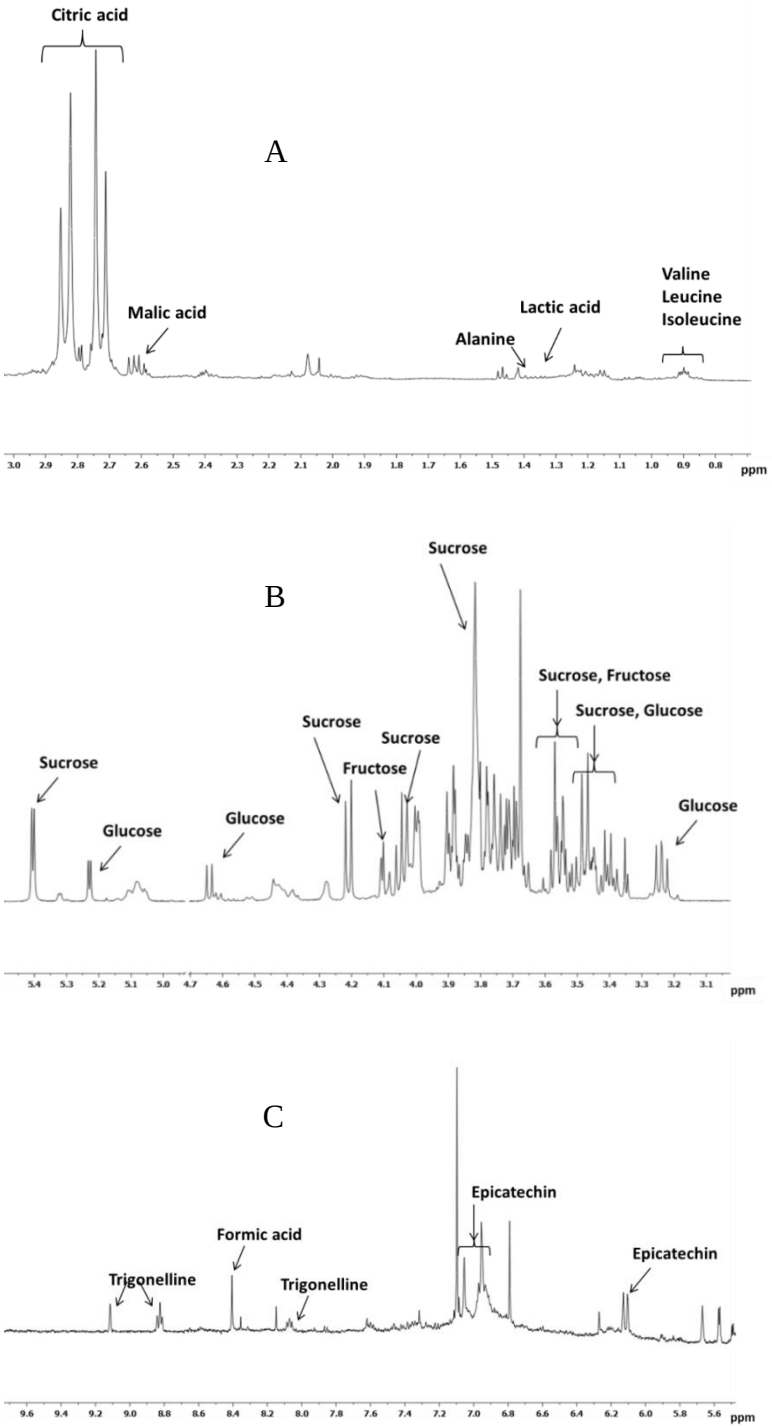


Figure 6.2 - Spectral area assignments of a representative ^{1}H NMR spectrum of a baobab juice (10% D_2O).

Table 6.1 ^{1}H chemical shifts of metabolites identified in baobab juice

Compound	*Group*	^{1}H *(ppm)*[a]	^{1}H *Multiplicity*[b]
Acetic acid (Ace)	CH_3	1.91	*s*
Alanine (Ala)	β CH_3	1.46	*d*
Arginine (Arg)	β CH_2/γCH_2	1.92/1.70	*m/m*
Ascorbic acid	CH	4.01	*m*
Caffeine (Caf)	N-CH_3	3.94	*s*
Citric acid (Cit)	CH_2	2.85/2.74	*d/d*
Epicatechin (EC)	CH	7.10/6.96/6.11/6.08	*d/m/d/d*
Ethanol	CH_2/ CH_3	3.67/1.17	*q/t*
Ferulic acid	γ CH_3	3.9	*s*
Formic acid	HCOO-	8.46	*s*
Fructose	CH_2/CH_2/CH	3.53/3.57/4.09	*m/m/m*
Fumaric acid	CH	6.47	*s*
Gallic acid	β CH	7.04	*s*
Glucose (Glc)	β CH /αCH	5.24/4.63	*d/d*
Histidine (His)	Ar-H	7.84/7.08	*s/s*
Isoleucine (Ile)	δ-CH_3	0.95/1.02	*t/d*
Lactic acid (Lac)	CH_3	1.32	*d*
Leucine (Leu)	δ CH_3, δ CH_3	0.96	*d*
Malic acid (Mal)	CH_2	2.37	*dd*
Methanol	CH_3	3.35	*s*
NPhenylacetylphenylalanine	CH_2	3.55	*dd*
Succinic acid (Suc)	α, β CH_2	2.40	*s*
Sucrose	CH	4.19	*d*
Threonine (Thr)	γ CH_3	1.32	*d*
Valine (Val)	γ'CH_3/γ CH_3	1.04/0.98	*d/ d*

[a] ^{1}H chemical shifts are reported with respect to TSP signal (0.00 ppm). [b]Multiplicity definitions: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; m, multiplet.

6.3.2 **Multivariate Data Analysis (MVA).**

Visual analysis of all NMR spectra (all treatments and storage times) showed some similarities and differences in metabolite composition amongst untreated (UT), high temperature short time pasteurised (HTST) and high temperature short time pasteurised with added citric acid (CAHTST) juice samples (Fig. 6.3). However, due to the complexity of spectra and the high number of samples, the use of MVA tools was warranted. An exploratory analysis of the data set was carried out by applying a PCA. As described previously, PCA is an unsupervised technique and requires no information about class membership; it looks just for inherent variation in the data set (Jackson, 1991; Nord et al., 2004; Pereira et al., 2005; Eriksson et al., 2006; Weljie et al., 2006; Izquierdo-García et al., 2011; McKenzie et al.,2011; Frank and Engel, 2013; Stevens, 2013; Marincola et al., 2015).

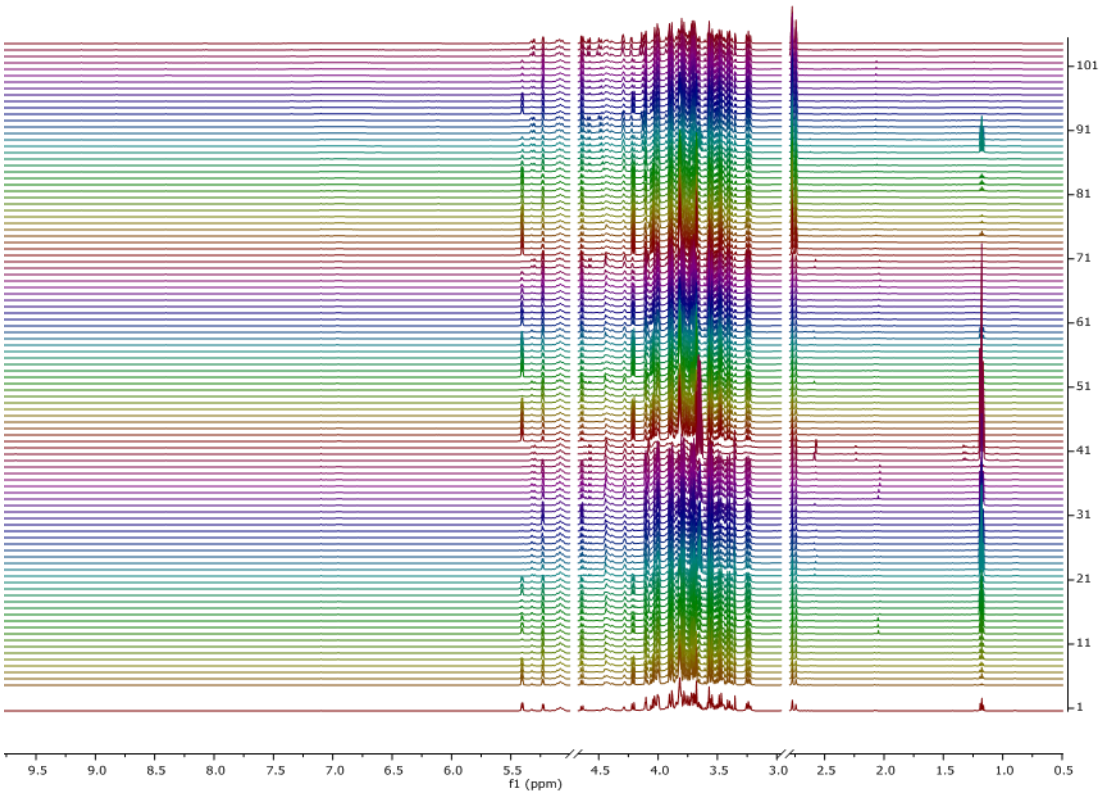


Figure 6.3 - Stacked ^{1}H NMR spectra of untreated (UT), pasteurised (HTST) and pasteurised with added citric acid (CAHTST) baobab juice used to generate data tables for statistical analysis, n = 104.

Figure 6.4 shows the scores plot for the PCA model built with the complete ^{1}H-NMR data set of baobab juice samples (UT, HTST and CAHTST). PC1 and PC2 components explained 34.1% and 24.2% of the total variation, respectively. The plot shows the presence of three outliers, corresponding to UT samples kept at 30 °C for 60 days. The visual inspection of the corresponding NMR spectra revealed a very high concentration of ethanol, reasonably indicative of the occurrence of an intense fermentation activity. These outliers were excluded for further statistical treatment and PCA repeated. The corresponding scores plot is shown in Figure 6.5.

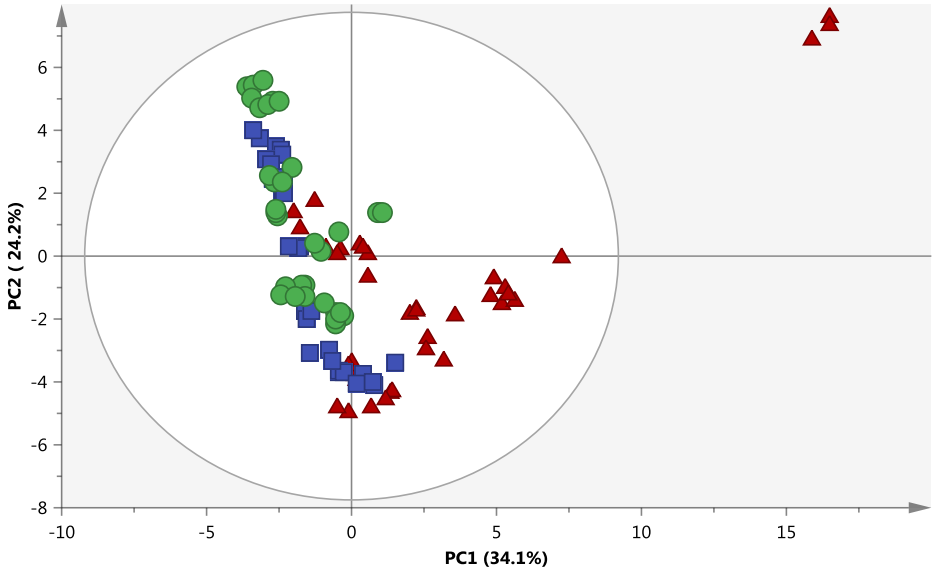


Figure 6.4 - Scores plot of the PCA model built using the overall data set of ^{1}H NMR spectra of baobab juice (n = 104): Symbols denote the treatment condition: UT (triangle); HTST (square); CAHTST (circle).

Despite the strong overlapping of scores, the PCA score plot in Figure 6.5 shows clear differentiations of baobab juices, indicating metabolic differences among the samples. In particular, as can be seen, the scores of CAHTST samples (circle symbol) are mainly distributed in the lower side of the plot (along negative PC2 values), while those relative to UT (triangle) and HTST (square) samples are located on the upper side of the plot (along positive PC2 values). Moreover, in terms of storage time, a progressive shift of scores can be observed from the right to the left side of the plot as storage days increase. It seems therefore that the first PC could be considered as representative of the storage temperature, while the second PC axis of the processing treatments.

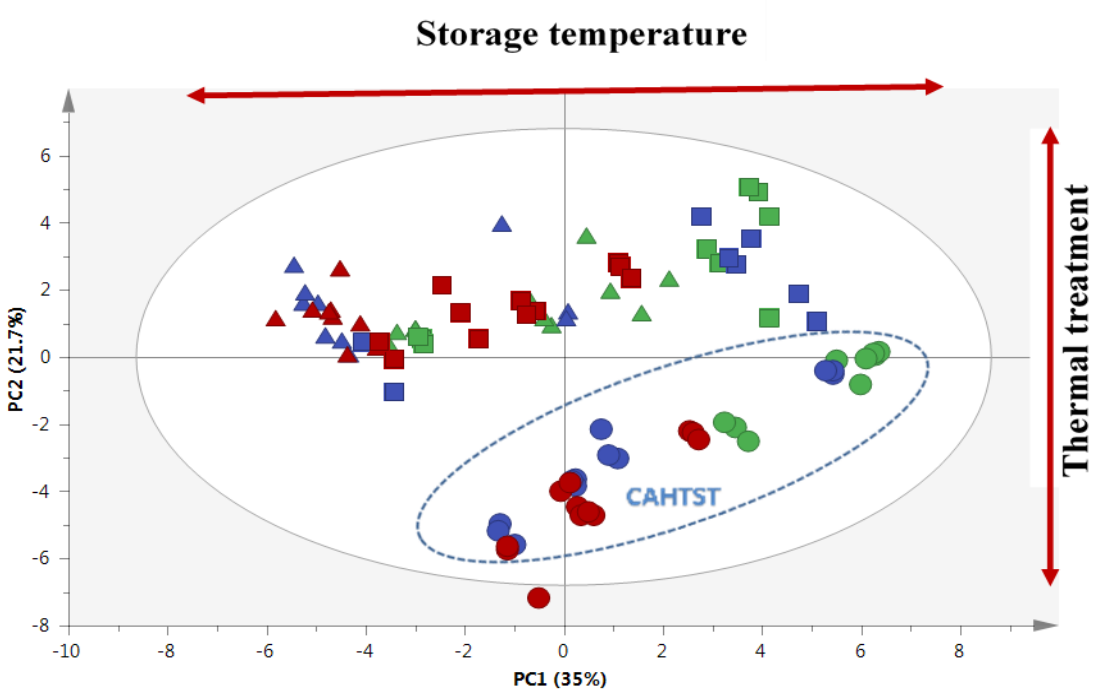


Figure 6.5 - Scores plot of the PCA model built from the data set of ^{1}H NMR spectra of baobab juice (n = 101). The plot is coloured-coded according to storage temperature (°C): 6 (green); 15 (blue); 30 (red). Symbols denote the treatment condition: UT (triangle); HTST (square); CAHTST (circle).

In order to obtain deeper insights on the biochemical consequences arising at each storage temperature as a function of time, MVA was performed separately for each thermal treatment. In particular, the time related information content of the NMR data acquired on juice was exploited by the orthogonal variation of the partial least squares (OPLS) regression analysis. This analysis finds the best correlations between the NMR spectral features and time. Figure 6.6 shows the OPLS scores plots of the model built for UT (Fig. 6.6A), HTST (Fig. 6.6B) and CAHTST (Fig. 6.6C). As it can be noted, all graphs evidence a temporal dynamic change of juice metabolome at each storage temperature, scores shifting from the left to the right along the PC1 axis as the storage time increase. Analysis of the corresponding loading line plots (Fig. 6.7) revealed metabolites that most

significantly accumulated in response to time and with the reverse response. The direction of the ^{1}H NMR peaks in these plots represents the relative differences in metabolites among samples: peaks with positive (negative) phase indicate the most abundant metabolites in samples on the right (left) side of the scores plot. Furthermore, plots are coloured with the modelled correlation [p(corr)] that contains information on the statistical significance of this change: warmed colours signals, such as red, indicates metabolites that contribute most significantly to the model than do the metabolites associated with cool coloured signals, such as blue. The absolute values of p (corr.) greater than 0.5 was defined as cutoff for statistical significance.

The loading plots derived from the OPLS models showed that the major compounds that contributed to sample separation were ethanol and carbohydrates. In particular, in the case of UT samples levels of ethanol increased over time, while carbohydrates decreased with time (Fig. 6.7A). In the case of HTST (Fig. 6.7B) and CAHTST (Fig. 6.7C) samples, the most important accumulating metabolites were fructose, glucose and galacturonic acid, while sucrose decreased over time. The decrease in sucrose could be attributed to hydrolysis of this sugar to glucose and fructose during storage. The presence of ethanol in baobab juice samples is indicative of the fermentation process of carbohydrates caused by microorganisms. Its content was higher in UT than in HTST and CAHTST samples. The more rapid production of ethanol in the absence of any thermal treatment is a clear indication that pasteurisation of juice alone or in combination with the addition of citric acid can minimise fermentation in baobab juice. Furthermore, the highest levels of

ethanol content was observed in UT samples stored at 30 °C for 60 days, this finding supporting that warmer temperatures enhance the fermentative process in baobab juices.

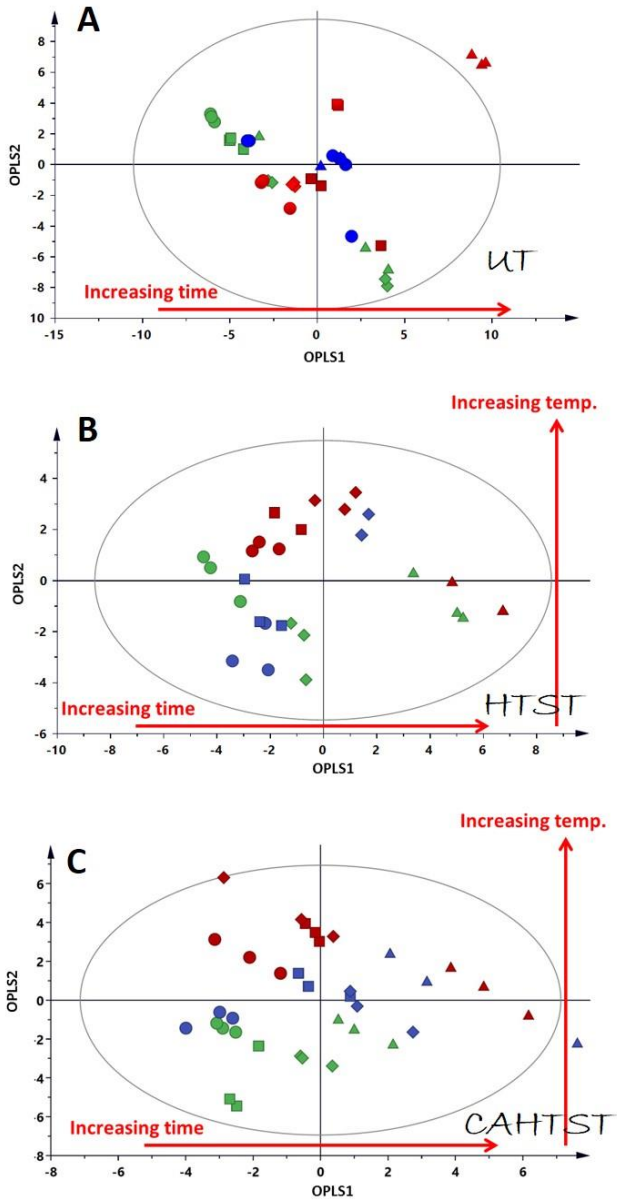


Figure 6.6 - OPLS applied to the ^{1}H NMR spectral data of UT (A), HTST (B) and CAHTST (C) baobab juice sampled at different storage times and temperatures. The plot is coloured-coded according to storage temperature (°C): 6 (green); 15 (blue); 30 (red). Arrows are arbitrarily drawn and represent time and temperature-related trajectories.

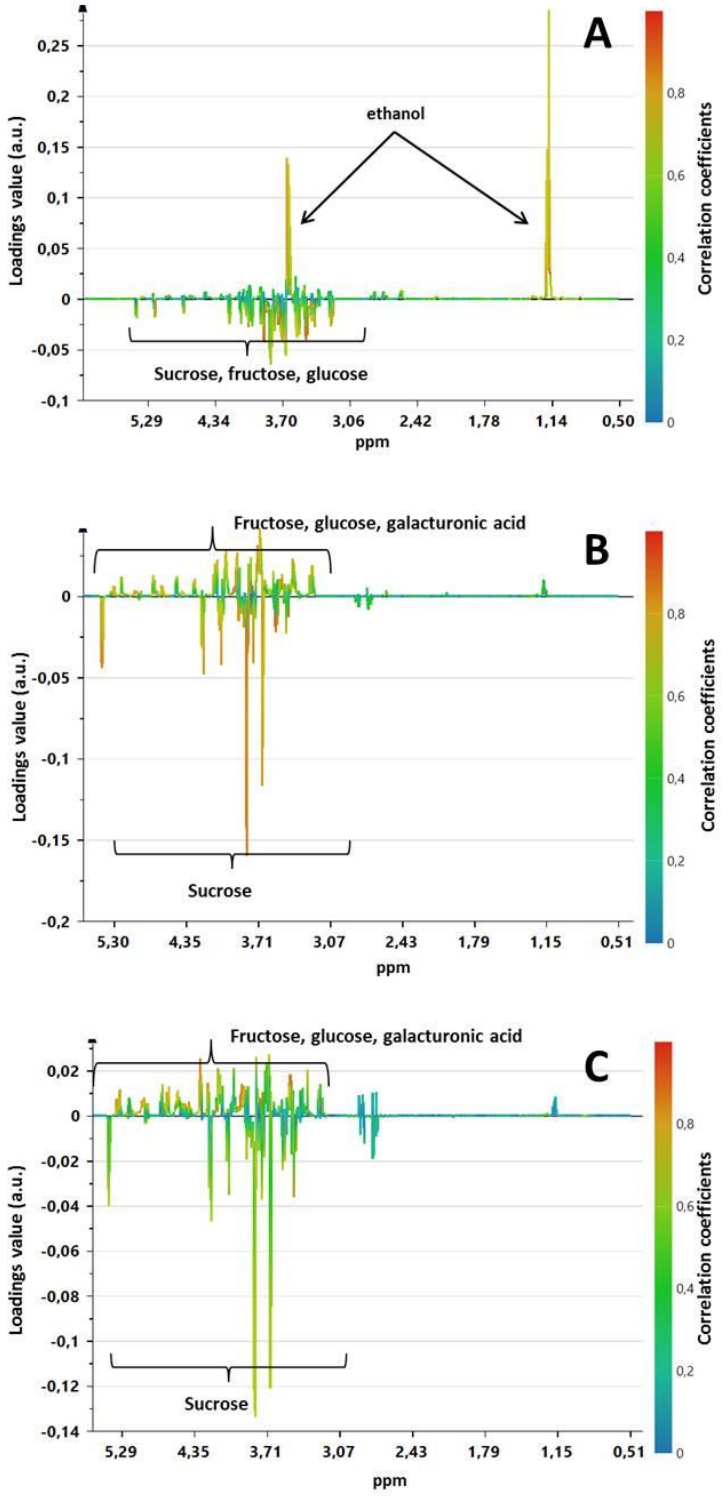


Figure 6.7 - OPLS loadings plot applied to the ^{1}H NMR spectral data of UT (A), HTST (B) and CAHTST (C) baobab juice samples.

Similarly, storage conditions of fresh orange juice provoked evolution of metabolic compounds including production of formic acid and ethanol (De Oliveira et al., 2014). The increase of ethanol was higher at 24 °C (3.6 times) than at 14 °C (2.4 times) compared to initial levels (De Oliveira et al., 2014). Therefore results from this study are consistent with published data.

6.3.3 **Conclusions and recommendations (done)**

^{1}H NMR coupled with chemometrix tools enabled complete analysis of baobab juice in terms of metabolic composition and changes with processing and during storage. The ^{1}H NMR spectra confirmed presence of a wide range of metabolites such as essential amino acids, organic acids, carbohydrates and phenolic compounds. For instance high level of organic acids (citric, malic and tartaric acid) and flavan-3-ols ((-)-epicatechin) analysed in baobab pulp and juice by RP-HPLC (chapters 3 and 4) were confirmed by ^{1}H NMR metabolomics. Pasteurisation and acidification reduced fermentation activity which was more evident in untreated juice stored at 30 °C for 60 days. Presence of high concentration of sugar metabolites (glucose, and fructose) and amino acids confirmed possibility of formation of non-enzymatic reaction products responsible for increased antioxidant activity observed in processed juice stored at higher temperature (chapter 4). Transformation of metabolites during storage was evident from change of relative spectra information and concentration. Presence of high relative concentration of ethanol in untreated juice samples left at 30 °C was evident for higher rate of fermentation. Not surprising juice samples at 6 °C showed higher relative sucrose concentration than monosaccharides (glucose and fructose). This indicates lower hydrolysis of disaccharides

including sucrose at lower storage temperature. Ethanol and sugars were largely responsible for PCA scores and clustering of a model which means they were changing significantly with storage time and temperature. This knowledge and information is necessary for the development of quality juice from baobab fruit pulp for improved health as well as commercialisation in Malawi.

Chapter 7 **Influence of seed liquor on quality attributes of baobab juice**

Abstract

In this study the influence of seed liquor on stability of bioactive compounds, total phenol content and antioxidant activity of baobab juice was evaluated using standard analytical methods described in Chapter 2. The addition of seed liquor at the current level neither enhanced nor reduced vitamin C in the juice. Marginal protective role against vitamin C degradation was observed with increasing percent seed liquor at 15 °C. Procyanidin B2, (-)-epicatechin, total phenol content (TPC) were significantly increased by addition of seed extracts. Juice samples with seed liquor stored at 15 °C showed higher total phenol content, flavan-3-ols and antioxidant activity suggesting a high bioavailability of these compounds. Thus seed liquor (2 or 3%) may be used to enhance phenolic compounds and antioxidant activity of baobab juice. The use of higher percent seed liquor may show protective role against vitamin C degradation due to antioxidant properties of phenolic compounds from roasted seeds.

Keywords: Baobab fruit juice; Seed liquor; Vitamin C; Bioactive compounds; Total phenol content; Antioxidant activity.

7.1 Introduction

Autoxidation in food and biological systems is responsible for adverse effects and implications in human health as well as in food stability and preservation (Shahidi and Zhong, 2010). Various methods are used to extend shelf life and ensure widespread availability. Common preservation methods include physical (drying, freezing, chilling, vacuum, modified atmosphere, sterilisation, pasteurisation, ultra-high temperature, ohmic and blanching) and chemical/additives (salting, sugar, antioxidants, fermentation and acidification). Food quality loss is mainly influenced by autolytic enzymes and spoilage microorganisms including bacteria, moulds and fungi (Aneja et al., 2014). Environmental conditions such as temperature, pressure, oxygen supply, pH and catalysts affect the rate of degradation (Fennema, 1996). Therefore each preservation method is aimed at preventing factors responsible for food spoilage or quality loss. Antioxidants either natural or synthetic are widely used as food preservatives. However synthetic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are associated with carcinogenesis (Shahidi and Zhong, 2010). Therefore the use of natural antioxidants such as ascorbic acid, tocopherols, and gallic acid esters as well as plant extracts is being promoted (Serra et al., 2008; Shahidi and Zhong, 2010; Rupasinghe and Yu, 2012; Aneja et al., 2014).

Baobab seeds are a rich source of bioactive compounds including flavan-3-ols (Shahat, 2006) and show very high total phenol content and antioxidant activity. Seed oil is rich in vitamin A, polyunsaturated fats and contains high concentrations of linoleic and oleic

acids (Kamatou et al., 2011). Seed oil extract has been shown to extend ascorbic acid shelf life of baobab pulp (Donkor et al., 2014).

7.1.1 Aim of the chapter

Based on prior experiments and literature that baobab seeds are a rich source of bioactive compounds it was envisaged that direct addition of roasted seed liquor (SL) to baobab juice may have protective effect against vitamin C degradation and improve overall quality of the juice including total phenol content and antioxidant activity.

A research study was therefore undertaken to evaluate the influence of seed liquor on bioactive compounds, total phenol content and antioxidant activity of baobab juice during storage. The specific objectives were twofold:

(i) Influence of seed liquor on stability of vitamin C and

(ii) Influence of seed liquor and storage on total phenol content, antioxidant activity, procyanidin B2 and (-)-epicatechin content of baobab juice.

7.2 Results and discussion

7.2.1 Influence of seed liquor on vitamin C degradation kinetics

Table 7.1 shows results for changes in vitamin C concentration in HTST pasteurised juice (72 °C 15 s) with added seed liquor (SL) with concentration ranging from 0 to 3%. To investigate the influence of seed liquor on degradation kinetics of vitamin C, juice samples were stored at 6, 15 and 30 ºC for a maximum period of 60 days. Results were

compared to HTST pasteurised (72 °C, 15 s) and acidified juice (0.5% citric acid) (CAHTST). Extremely low values observed in unexpected instances (day 28) may be considered as outliers due to instability of ascorbic acid. Exposure to light, air and metal contamination may enhance degradation of vitamin C as previously described.

Table 7.1- Vitamin C concentration (ppm)* in baobab fruit juice with citric acid (CAHTST) and different percent concentration of seed liquor (SL).

Temp. (°C)	*Time Days*	*CAHTST*	*HTST*	*HTST + SL (1%)*	*HTST + SL (2%)*	*HTST + SL (3%)*
	0	26.51 ± 0.29^{b}	29.74 ± 0.28^{a}	25.92 ± 0.27^{b}	25.40 ± 0.03^{b}	25.0 ± 0.07^{b}
	14	11.45 ± 0.24^{de}	14.12 ± 0.11^{c}	12.29 ± 0.10^{cd}	12.28 ± 0.03^{cd}	12.79 ± 0.06^{cd}
	28	10.20 ± 1.67^{e}	7.71 ± 0.33^{f}	12.82 ± 2.19^{cd}	6.33 ± 0.50^{fg}	7.95 ± 0.73^{f}
6	*42*	5.18 ± 0.12^{gh}	4.71 ± 0.05^{ghi}	3.9 ± 0.21^{hi}	4.41 ± 0.29^{hi}	4.38 ± 0.17^{hi}
	60	3.76 ± 0.06^{hi}	3.88 ± 0.02^{hi}	3.23 ± 0.34^{i}	3.94 ± 0.04^{hi}	3.92 ± 0.10^{hi}
	14	4.31 ± 0.13^{ghikjl}	4.16 ± 0.69^{hijklm}	5.29 ± 0.03^{efghi}	6.08 ± 0.08^{ef}	6.05 ± 001^{ef}
	28	7.66 ± 0.09^{d}	5.71 ± 1.28^{efg}	1.18 ± 0.46^{n}	1.30 ± 0.06^{n}	3.00 ± 1.23^{lm}
15	*42*	3.08 ± 0.16^{lm}	3.00 ± 0.54^{lm}	4.45 ± 0.08^{ghijk}	4.75 ± 0.50^{fghij}	6.23 ± 0.52^{de}
	60	2.87 ± 0.09^{m}	2.32 ± 0.02^{mn}	3.19 ± 0.18^{klm}	3.69 ± 0.05^{jklm}	5.47 ± 0.04^{efgh}
	14	3.09 ± 0.44^{fghi}	1.85 ± 0.16^{hij}	3.96 ± 0.39^{fghi}	4.23 ± 0.48^{fg}	4.07 ± 0.32^{fgh}
	28	2.19 ± 0.41^{ghij}	0.30 ± 0.13^{j}	6.57 ± 2.96^{de}	7.91 ± 0.16^{cd}	8.98 ± 0.47^{c}
30	*42*	3.63 ± 0.25^{fghi}	3.43 ± 0.70^{fghi}	4.89 ± 0.08^{ef}	4.40 ± 0.0^{efg}	4.45 ± 0.0^{efg}
	60	1.89 ± 0.05^{hij}	2.71 ± 1.66^{fghi}	1.74 ± 0.04^{ij}	2.59 ± 0.04^{fghij}	2.44 ± 0.11^{ghij}

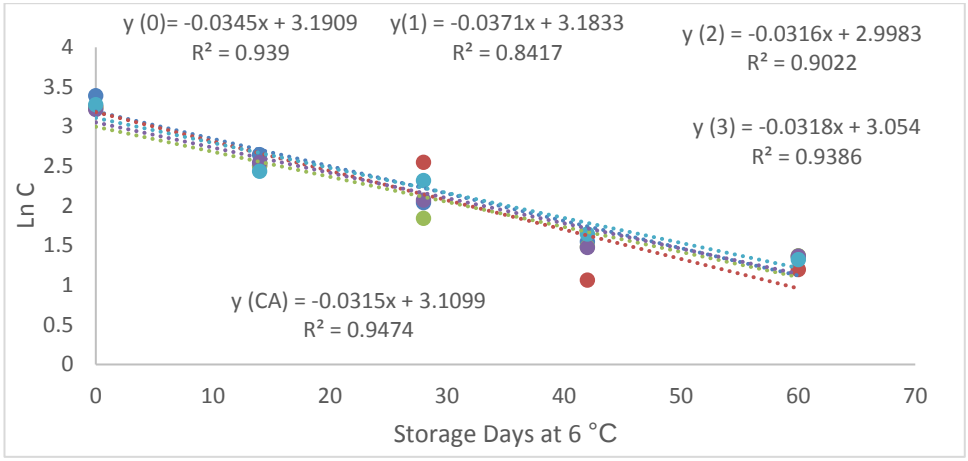
* Results are means of triplicate (±SD) and values with same letters at a constant temperature are not significantly different ($p > 0.05$).

Results showed that storage temperature and time had a significant effect on the level of vitamin C in the juice. The loss of vitamin C was significantly increasing ($p \leq 0.05$) with storage time and temperature. Juice samples containing seed liquor slowed down the degradation process in some instances especially at 15 °C. As can be seen, the final level

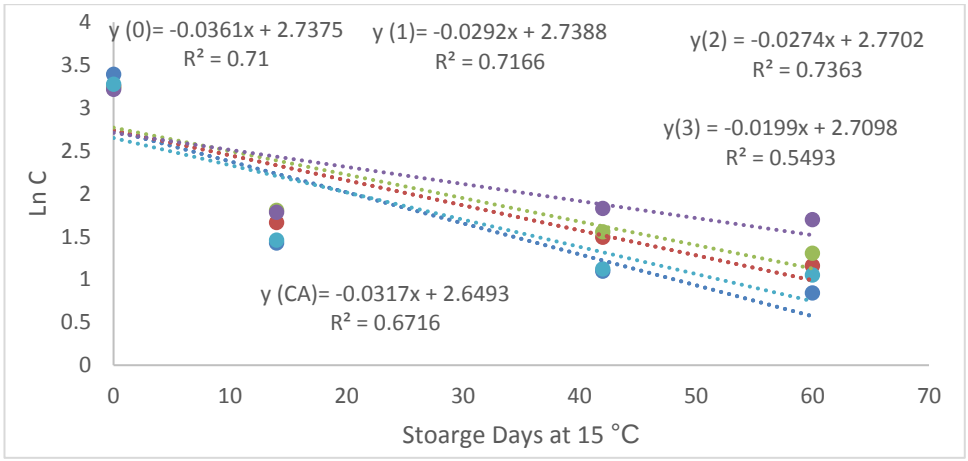
of vitamin C in the juice with seed liquor was significantly higher ($p \leq 0.05$) compared to CAHTST juice. It seems vitamin C preservation was generally increasing with percent seed liquor at 15 °C. For instance, juice with 3% seed liquor contained more than 47.5% vitamin C (5.47 ± 0.04 ppm) than CAHTST juice (2.87 ± 0.09 ppm) by the end of storage time at 15 °C. Phenolic compounds available in baobab seeds including (-)-epicatechin and procyanidin B2 observed in this study and as reported by other authors (Shahat, 2006) are likely ascribed to protection of vitamin C against autoxidation. These compounds may exert antioxidant properties by reducing dehydroascorbic acid to ascorbic acid thereby enhancing vitamin C stability. Other authors have reported that addition of baobab seed oil to baobab pulp enhanced vitamin C stability (Donkor et al., 2014). The present study provides an alternative method that is cheaper and probably a simpler way of achieving the same. Moreover, roasted and ground baobab seed liquor is already consumed like coffee drink in most parts of Malawi. Availability of phenolic compounds in plant derived foods is normally increased with temperature. At 15 °C phenolic compounds from seed liquor may be more readily available for redox reactions including antioxidant behavior against ascorbic acid degradation. This could explain why higher vitamin C stability was observed in the juice containing 3% SL stored at 15 °C compared to 6 °C.

The experimental data representation of Ln C versus storage time exhibited linear correlation at 6 and 15 °C (Figure 7.1). Generally, the first order reaction kinetics model showed lower values of rate constants for juice containing seed liquor suggesting reduced rate of vitamin C degradation. Rate constants values were within the range observed for CAHTST juice. At 6 °C, the half-life (Ln 2/k) of vitamin C with seed liquor ranged from

18.68 to 21.94 days. The half-life of vitamin C in the juice containing 2 and 3% SL and CAHTST juice was approximately the same (22 days). At 15 °C, the half-life of vitamin C in the juice was increasing with percent seed liquor. For instance, the half-life of vitamin C in the juice containing 0, 1, 2 and 3% SL was 19.20, 23.74, 25.30 and 34.83 days respectively compared to 21.87 days for CAHTST juice. Higher vitamin C half-life of samples stored at 15 °C compared to 6 °C could be attributed to higher levels of total phenol content and flavan-3-ols observed in the same juice.



A



B

Figure 7.1-Degradation kinetics of vitamin C in baobab juice with different percent seed liquor. Series: 0% SL (deep blue); 1% SL (red); 2% SL (green); 3% SL (purple); 0.5% citric acid (light blue) at 6 °C (A) and 15 °C (B); n = 3.

7.2.2 **Effect of seed liquor on total phenol content**

Figure 7.2(A-C) shows the level of total phenol content in baobab juice with seed liquor and added citric acid (CAHTST). Results showed that TPC varied with percent seed liquor and storage time. The level of TPC with seed liquor generally remained high during storage. For instance, the mean values of TPC in the juice containing 2% SL between day 0 and 60 were not significantly different ($p > 0.05$).

At 15 °C, juice with seed liquor showed higher TPC compared to juice with added citric acid. Juice samples containing 1 and 3% SL also showed higher levels of TPC compared to equivalent samples at 6 °C. Although fluctuations in TPC were notable in all samples, seed liquor generally enhanced stability of phenolic compounds and compared well with acidified juice.

At 30 °C, TPC in all samples were lower compared to samples stored at 6 or 15 °C. Juice samples with 3% SL showed highest TPC of all. In most cases, the level of TPC was decreasing significantly ($p \leq 0.05$) with storage time. It seems phenolic compounds in acidified juice were more stable at 30 °C. Higher citric acid concentration stabilised pH of the juice and possibly reduced chemical reactions responsible for the loss of phenolic compounds at higher temperatures. For instance, means of TPC on day 0, 28, and 60 were not significantly different ($p > 0.05$). From this experiment, it seems keeping juice with seed liquor at 15 °C is recommended in terms of stability of phenolic compounds.

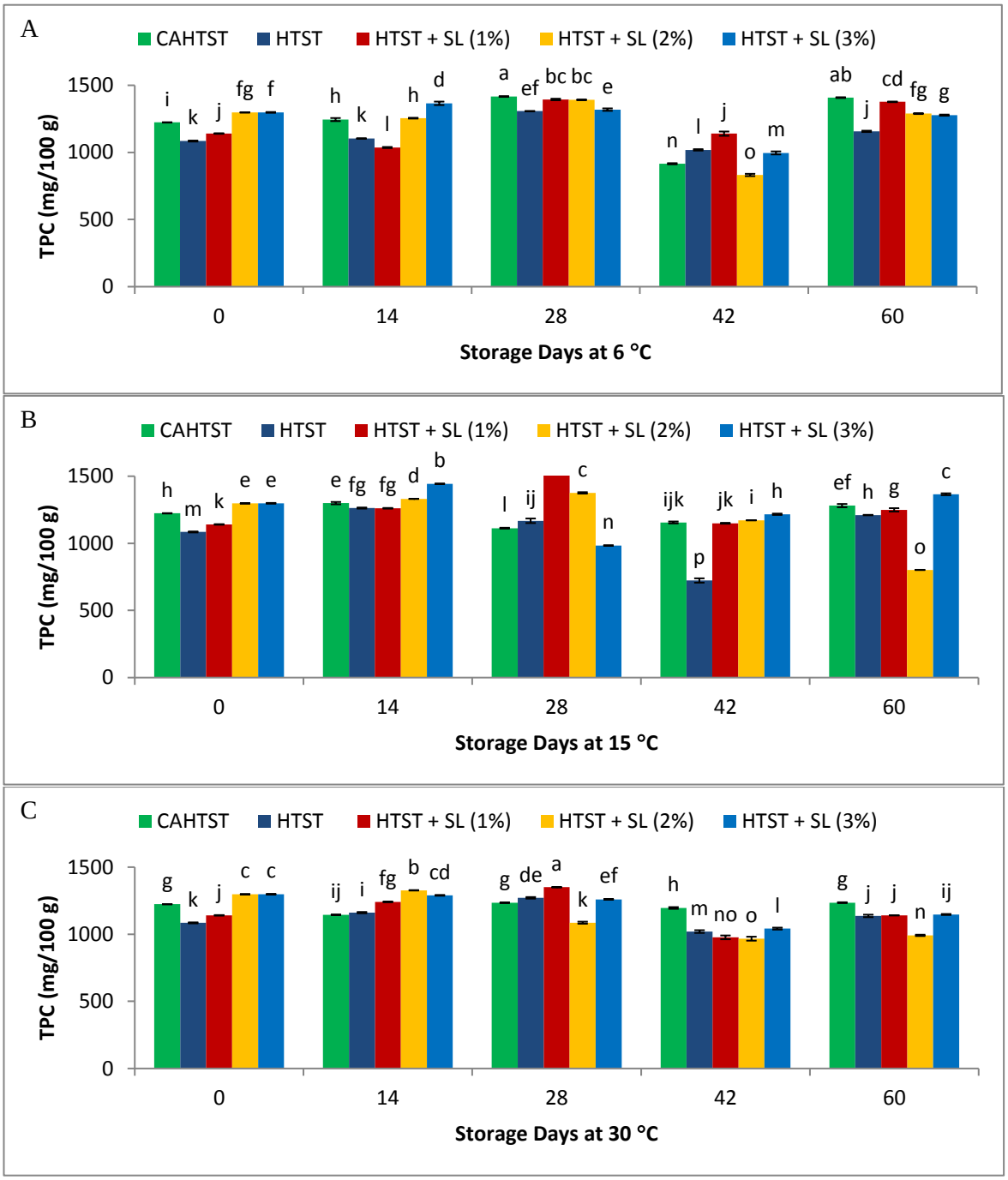


Figure 7.2 - Changes in total phenol content in baobab juice with different levels of seed liquor stored at 6 (A), 15 (B) and 30 °C (C). Levels with same letters are not significantly different ($p > 0.05$); n = 3.

7.2.3 **Effect of seed liquor on antioxidant activity**

Bioactive compounds available in the seeds and those evolved during roasting of seeds enhanced overall antioxidant activity of the juice. Results for the effect of seed liquor on antioxidant (FRAP, DPPH ABTS) of baobab juice containing different percent seed liquor are described.

7.2.3.1 **Ferric reducing antioxidant power (FRAP)**

Table 7.2 shows antioxidant activity (FRAP) in baobab juice with different percent seed liquor and 0.5% citric acid (CAHTST). In general, results showed a significant increase ($p \leq 0.05$) in FRAP values with % SL in all samples. FRAP values were more stable in juice samples stored at 6 and 15 °C compared to 30 °C. Fluctuations in FRAP values were also notable during storage. FRAP values in the juice containing 3% SL and 0.5% citric acid (CAHTST) were not significantly different ($p > 0.05$) on day 28 and 42 of storage. The increase in FRAP values with % SL is expected and attributed to increased concentration of phenolic compounds. Although FRAP values in all samples remained high during storage, fluctuations during storage has been previously attributed to other chemical reactions of phenolic compounds including hydroxylation, polymerization and possibility of Maillard reactions. The resultant products may exhibit higher or lower antioxidant activity.

At 15 °C, juice with seed liquor showed significantly higher ($p \leq 0.05$) antioxidant activity compared to acidified juice. Just like TPC, ferric reducing antioxidant activity was more enhanced in samples stored at 15 °C compared to 6 °C.

At 30 °C, all juice samples showed significantly lower ($p \leq 0.05$) antioxidant activity (FRAP) compared to 6 or 15 °C. Nevertheless, juice samples with seed liquor showed higher FRAP values than those with 0.5% CA in most cases. The mean FRAP values between 14, 28 and 60 days were significantly higher ($p \leq 0.05$) for juice containing 3% SL than acidified juice. Despite formation of non-enzymatic reaction products, it seems the loss of heat sensitive compounds had greater influence on overall FRAP values of the juice at 30 °C.

Table 7.2-Effect of seed liquor on antioxidant activity (FRAP) of baobab juice during storage. Results are means of triplicates (±SD). Values with different letters at a constant temperature are significantly different ($p \leq 0.05$).

	HTST	Time *(Days)*				
T (°C)	Treatment	0	14	28	42	60
	CA (0.5%)	2882.00±58.28^{ef}	2751.09±3.91^{gh}	3015.17± 3.91^{bc}	2262.42 ± 5.17^{m}	3284.90±50.82^{a}
	SL (0%)	2738.67±13.54^{hi}	2395.59±7.05^{l}	2878.61±16.00^{ef}	2382.05± 11.89^{l}	2365.12± 8.52^{lm}
	SL (1%)	2695.79±7.05^{hij}	2577.29±13.68^{k}	3108.84±3.91^{b}	2865.07 ± 130.97^{ef}	2852.66±22.54^{fg}
	SL (2%)	2906.83±16.70^{def}	2614.53±10.34^{jk}	3014.04±8.52^{bc}	2020.91±27.71^{n}	2898.93±13.68^{ef}
6	SL (3%)	2743.19±30.72^{h}	2634.85±3.91^{ijk}	2959.87±10.34^{cde}	2262.42± 23.05^{m}	3007.27±18.65^{bcd}
	CA (0.5%)	2882.00±58.28^{def}	2681.12±12.21^{ij}	2317.72±51.27^{l}	3160.75±96.73^{b}	2800.74±5.17^{fg}
	SL (0%)	2738.67±13.54^{ghi}	2626.95±21.14^{j}	2445.25±9.77^{k}	1852.75±37.29^{m}	2608.89±15.27^{j}
	SL (1%)	2695.79±7.05^{hij}	2629.20±22.54^{j}	3064.83±32.18^{bc}	2740.93±22.54^{ghi}	2752.22±32.65^{ghi}
	SL (2%)	2906.83±16.70^{j}	2757.86±10.88^{ghi}	2792.84±37.24^{fgh}	2812.03±23.05^{efg}	3649.42±13.54^{a}
15	SL (3%)	2743.19±30.72^{ghi}	3107.71±11.73^{b}	2975.67±13.54^{cd}	2955.36±3.39^{d}	2891.03±17.92^{def}
	CA (0.5%)	2882.00±58.28^{ab}	2474.59±14.76^{k}	2572.78±8.96^{ij}	2800.74±9.77^{cde}	2516.35±12.82^{jk}
	SL (0%)	2738.67±13.54^{efg}	2658.55±3.91^{h}	2740.93±22.54^{defg}	2268.06±35.35^{l}	2640.49±39.05^{hi}
	SL (1%)	2695.79±7.05^{fgh}	2801.87±10.88^{cde}	2679.99±13.68^{h}	2183.42±5.86^{m}	2234.21±8.96^{lm}
	SL (2%)	2906.83±16.70^{a}	2809.77±39.05^{cd}	2568.26±17.04^{j}	2238.72±11.89^{lm}	2274.84±14.76^{l}
30	SL (3%)	2743.19±30.72^{defg}	2752.22±22.20^{cdef}	2817.67±5.17^{bc}	2456.53±7.82^{k}	2553.59±15.64^{j}

7.2.3.2 **DPPH radical scavenging activity**

Results showed that compounds from seed liquor (SL) improved the DPPH radical scavenging antioxidant activity of the juice (Table 7.3). At 6 °C all samples containing seed liquor showed significantly higher ($p \leq 0.05$) antioxidant activity (DPPH) compared to juice with 0.5% CA (CAHTST). The % DPPH in most juice samples with seed liquor was stable during storage at 6 °C. Significantly high levels ($p \leq 0.05$) of antioxidant activity (DPPH) were observed in juice with SL stored at 15 °C. DPPH values ranged from 37.05 ± 0.77 to 49.32 ± 0.16 for 0% SL, 45.34 ± 0.19 to 57.33 ± 0.43 for 1% SL, 48.51 ± 0.11 to 61.37 ± 0.22 for 2% SL and 50.06 ± 0.09 to 57.33 ± 0.09% DPPH for 3% SL while DPPH values in juice with 0.5% CA ranged from 38.29 ± 0.23 to 46.71 ± 0.09% DPPH. Clearly, the DPPH radical antioxidant activity of the juice increased with seed liquor and showed more stability during storage compared to juice with 0.5% CA. Higher DPPH antioxidant activity was consistent with higher values of vitamin C, TPC and FRAP observed in the same juice. At 30 °C, all juice samples showed lower DPPH antioxidant activity values compared to 6 or 15 °C storage. The DPPH radical scavenging activity was more stable between day 0 and 14 for juice containing 2 and 3% SL compared to acidified juice during the same period.

Table 7.3-Effect of seed liquor on antioxidant activity (DPPH) of baobab juice during storage. Results are means of triplicates (±SD). Values with different letters at a constant temperature are significantly different ($p \leq 0.05$).

	HTST	Time *(Days)*				
T (°C)	Treatment	0	14	28	42	60
	CA (0.5%)	46.71± 0.09^{kl}	44.22 ± 0.11^{m}	49.41±0.09^{fgh}	37.64± 0.11^{o}	47.48± 0.27^{jk}
	SL (0%)	49.32± 0.16^{fgh}	42.67±0.60^{n}	49.07± 0.22^{ghi}	41.89± 0.14^{n}	38.11±0.72^{o}
	SL (1%)	50.96± 0.46^{de}	48.79±0.23^{hi}	54.53±0.09^{b}	45.87±0.28^{l}	48.17± 0.05^{ij}
	SL (2%)	49.84± 0.14^{fg}	46.43±0.19^{l}	55.65± 0.16^{a}	38.11±0.05^{o}	51.15± 0.71^{d}
6	SL (3%)	50.06± 0.09^{ef}	49.50± 0.16^{fgh}	50.93± 0.19^{de}	46.06± 0.25^{l}	52.76± 0.18^{c}
	CA (0.5%)	46.71± 0.09^{ij}	41.15± 0.61^{l}	38.29± 0.23^{m}	46.96± 0.23^{hi}	45.03± 0.19^{k}
	SL (0%)	49.32± 0.16^{efg}	41.74± 0.47^{l}	45.96± 1.55^{ijk}	37.05± 0.77^{m}	46.58± 0.33^{ijk}
	SL (1%)	50.96± 0.46^{d}	45.34± 0.19^{jk}	57.33± 0.43^{b}	50.00± 0.14^{defg}	51.30± 0.11^{d}
	SL (2%)	49.84± 0.14d^{efg}	48.51± 0.11^{gh}	49.04± 0.25^{fg}	50.87± 0.14^{de}	61.37± 0.22^{a}
15	SL (3%)	50.06± 0.09d^{efg}	54.66± 0.19^{c}	50.50± 1.34^{def}	54.84± 0.28^{c}	57.33± 0.09^{b}
	CA (0.5%)	46.71± 0.09^{cde}	40.87± 0.05^{l}	42.86± 0.69^{jk}	47.33± 0.14^{c}	45.25± 0.69^{fgh}
	SL (0%)	49.32± 0.16^{b}	43.29± 0.19^{ij}	46.93± 0.30^{cd}	44.19± 0.25^{hi}	46.46± 0.51^{cdef}
	SL (1%)	50.96± 0.46^{a}	49.53± 0.14^{b}	45.62± 0.19^{defg}	42.73± 0.23^{jk}	42.52± 0.76^{jk}
	SL (2%)	49.84± 0.14^{ab}	50.06± 0.00^{ab}	40.53± 1.26^{l}	41.55± 0.38^{kl}	44.41± 0.19^{ghi}
30	SL (3%)	50.06± 0.09^{ab}	49.13± 0.09^{b}	45.50± 0.32^{efgh}	44.38± 0.09^{ghi}	45.90± 0.22^{def}

7.2.3.3 **ABTS**

In general, results showed that the ABTS cation reducing ability of juice samples was increasing with percent seed liquor (Table 7.4). Antioxidant activity (ABTS) in the juice with seed liquor was generally stable during storage. For example, ABTS values in the juice containing 1 or 2% SL on day 0, 14 and 60 were not significantly different ($p > 0.05$).

Significantly higher levels ($p \leq 0.05$) of ABTS were observed in samples stored at 15 °C. All juice samples containing seed liquor showed higher stability of antioxidant activity (ABTS) during storage compared to juice with 0.5% citric acid preservative. Although antioxidant activity (ABTS) was significantly affected in all samples at 30 °C, juice samples containing 3% SL still afforded the significantly highest ($p \leq 0.05$) antioxidant activity of all.

Table 7.4-Effect of seed liquor on antioxidant activity (ABTS) of baobab juice during storage. Results are means of triplicates (±SD). Values with different letters at a constant temperature are significantly different ($p \leq 0.05$).

	HTST	Time *(Days)*				
T (°C)	Treatment	0	14	28	42	60
	CA (0.5%)	1428.83±17.10^{g}	1386.82±.00^{h}	1432.06±3.23^{fg}	1012.99±7.46^{m}	1350.19±7.46^{i}
	SL (0%)	1495.62±1.87^{cde}	1287.70±13.06^{j}	1520.40±18.66^{c}	1102.41±9.70^{l}	1094.87±17.80^{l}
	SL (1%)	1510.71±7.46^{c}	1497.78±4.94^{cd}	1614.13±11.35^{a}	1275.85±1.87^{j}	1512.86±3.23^{c}
	SL (2%)	1460.07±12.24^{efg}	1466.54±15.27^{def}	1577.50±3.23^{b}	941.89±3.73^{n}	1511.78±21.03^{c}
6	SL (3%)	1619.51±22.39^{a}	1439.60±6.73^{fg}	1495.62±3.73^{cde}	1196.13±14.09^{k}	1502.09±8.13^{cd}
	CA (0.5%)	1428.83±17.10^{f}	1413.75±10.39^{fg}	1149.81±1.87^{n}	1200.44±3.73^{m}	1276.93±14.09^{l}
	SL (0%)	1495.62±1.87^{cd}	1383.58±5.60^{h}	1266.16±7.46^{l}	1001.14±12.24^{o}	1349.11±13.46^{i}
	SL (1%)	1510.71±7.46^{c}	1565.65±6.73^{b}	1501.00±4.94^{c}	1290.93±1.87^{kl}	1340.49±3.73^{ij}
	SL (2%)	1460.07±12.24^{e}	1507.47±4.94^{c}	1396.51±8.55^{gh}	1314.64±3.73^{jk}	1467.61±3.23^{de}
15	SL (3%)	1619.51±22.39^{a}	1623.82±9.87^{a}	1515.01±6.73^{c}	1385.74±1.87^{gh}	1461.15±11.20^{e}
	CA (0.5%)	1428.83±17.10^{de}	1212.29±6.46^{j}	1205.83±17.10^{j}	1210.14±1.87^{j}	1114.26±6.73^{k}
	SL (0%)	1495.62±1.87^{bc}	1384.66±7.46^{f}	1272.62±31.89^{h}	1042.08±3.73^{m}	1229.53±14.57^{ij}
	SL (1%)	1510.71±7.46^{b}	1426.68±4.94^{def}	1473.00±7.46^{bc}	1092.71±32.32^{kl}	1116.41±1.87^{k}
	SL (2%)	1460.07±12.24^{cd}	1471.92±3.73^{bc}	1260.77±12.93^{hi}	1062.55±1.87^{lm}	1133.65±6.73^{k}
30	SL (3%)	1619.51±22.39^{a}	1407.28±21.52^{ef}	1316.79±1.87^{g}	1211.21±4.94^{j}	1256.46±9.87^{hi}

7.2.4 **Effect of seed liquor on procyanidin B2 and (-)-epicatechin**

7.2.4.1 **Procyanidin B2**

Baobab seeds contain high levels of phenolic compounds including procyanidin B2 and (-)-epicatechin (Shahat, 2006) while fresh pulp is also an excellent source along with vitamin C and other organic acids. Thus baobab juice with seed liquor was expected to have higher levels of both flavan-3-ols and organic acids. Significantly high levels of procyanidin B2 was observed in juice containing seed liquor (Table 7.5). In some cases the level of procyanidin B2 was fluctuating during storage. At 6 °C, the level of procyanidin B2 was generally increasing in all samples during storage.

At 15 °C, juice containing seed liquor showed significantly higher ($p \leq 0.05$) levels of procyanidin B2 than juice with 0.5% CA. With few exceptions, the level of procyanidin B2 increased steadily during storage. Procyanidin B2 content fluctuated between 466.27 ± 56.51 and 1177.38 ± 107; 479.91 ± 29.05 and 2570.15 ± 6.64; 302.90 ± 46.82 and 3094.67 ± 13.47; and 1332.44± 30.41 and 1744.05 ± 0.07 mg/100 g for juice containing 0, 1, 2 and 3% SL respectively versus 351.21± 29.24 and 1571.82 ± 27.38 mg/100 g for juice with 0.5% CA. A possible suggestion especially for the decrease in procyanidin B2 with percent seed liquor or during storage may be attributed to its protective role of vitamin C (redox reactions) and other chemical reactions described previously such as polymerization, hydroxylation and epimerisation during storage.

At 30 °C, all samples showed lower values of procyanidin B2 compared to those stored at 6 or 15 °C. The highest ($p \leq 0.05$) level of procyanidin B2 (2435.69 ± 69.01 mg/100 g)

was observed in juice with 3% SL on day 14. Unexpected higher values of procyanidin B2 in the juice without seed liquor or during storage may be attributed to epimerisation of procyanidin dimer (from type A to B), polymerisation of (-)-epicatechin or depolymerisation of polymeric proanthocyanidins (condensed tannins).

Table 7.5- Effect of seed liquor on procyanidin B2 content (mg/100 g) of baobab juice during storage. Results are means of triplicates (± SD).

	HTST	Time *(Days)*				
T (°C)	Treatment	0	14	28	42	60
	CA (0.5%)	351.21 ± 29.24	1317.94 ±.15.49	1407.87 ± 5.17	1438.56 ±69.99	1327.15 ± 11.20
	SL (0%)	466.27 ±56.51	1060.24 ±16.07	1295.21 ± 1.81	1721.91 ± 81.73	1024.30 ± 9.28
	SL (1%)	479.91 ± 29.05	1412.08 ± 72.34	1193.4 6 ± 3.90	1186.00 ± 22.30	663.92 ± 49.47
	SL (2%)	302.90 ± 46.82	1271.24 ± 99.22	1205.38 ± 11.46	1533.17 ± 42.70	1656.86 ±76.83
6	SL (3%)	1332.44 ± 30.41	1515.32 ± 8.90	1177.54 ± 13.10	1624.91 ± 4.42	1910.87 ± 28.05
	CA (0.5%)	351.21 ± 29.24	1229.70 ± 23.38	976.86 ± 5.36	1226.57 ± 28.04	1571.82 ± 27.38
	SL (0%)	466.27 ± 56.51	1118.72 ±7.68	1143.12 ± 4.50	1177.38±107.37	137.15 ± 0.62
	SL (1%)	479.91 ± 29.05	1330.01 ± 0.92	1193.46 ± 3.90	1996.52 ± 5.82	2570.15 ± 6.64
	SL (2%)	302.90 ± 46.82	1261.19 ± 10.37	1470.78 ± 35.06	2270.40 ± 55.29	3094.67 ± 13.47
15	SL (3%)	1332.44 ± 30.41	1193.65 ± 17.62	1280.55 ± 23.75	1193.71 ± 11.43	1744.05 ± 0.07
	CA (0.5%)	351.21 ± 29.24	883.75 ± 7.88	843.14 ± 3.95	807.95 ± 60.78	256.69 ± 1.79
	SL (0%)	466.27 ± 56.51	957.52 ± 77.51	1018.05 ± 0.93	928.64 ± 21.20	1632.74 ± 44.54
	SL (1%)	479.91 ± 29.05	1556.40 ± 3.06	1446.02 ± 19.55	442.66 ± 38.82	83.43 ± 7.48
	SL (2%)	302.90 ± 46.82	1975.36 ± 2.05	1175.27 ± 94.54	695.36 ± 17.77	184.75 ± 21.80
30	SL (3%)	1332.44 ± 30.41	2435.69 ± 69.01	1854.83 ± 116.69	1547.54 ± 13.84	80.24 ± 11.41

7.2.4.2 **(-)-Epicatechin**

Table 7.6 shows levels of (-)-epicatechin during storage in baobab juice samples with different levels of seed liquor. At 6 °C, higher levels of (-)-epicatechin were observed in samples containing seed liquor. Just like procyanidin B2, the level of (-)-epicatechin increased significantly ($p \leq 0.05$) between day 0 and 42. Absolute values ranged from 47.67 ± 8.52 to 98.61 ± 4.95; 31.37 ± 6.35 to 161.03 ±17.17; 23.52 ± 2.81 to 144.0 ± 1.30 and 85.16 ± 2.48 to 98.98 ± 1.02 mg/100 g for juice containing 0, 1, 2 and 3% SL respectively versus 32.03 ± 0.85 to 118.19 ± 3.66 mg/100 g for acidified juice.

In contrast to procyanidin B2, the level of (-)-epicatechin in juice samples with seed liquor was lower at 15 °C than at 6 °C. It is possible that some (-)-epicatechin were polymerised to procyanidin B2. Nevertheles (-)-epicatechin content was generally higher in the juice with seed liquor than acidified juice. The level of (-)-epicatechin ranged from 22.93 ± 0.73 to 100.01 ± 0.91; 31.37 ± 6.35 to 111.27 ±1.94; 23.52 ± 2.81 to 110.92 ± 1.06 and 45.43 ± 2.51 to 109.44 ± 2.42 mg/100 g for juice containing 0, 1, 2 and 3% SL respectively versus 32.03 ± 0.85 to 111.38 ± 0.33 mg/100 g for juice with 0.5% CA. Despite notable fluctuation behavior of (-)-epicatechin during storage, levels remained fairly high.

At 30 °C, the level of (-)-epicatechin was significanly reduced by the end of storage time. In all samples, the level of (-)-epicatechin increased significantly ($p \leq 0.05$) between day 0 day and 14 then dropped again by day 28. Although (-)-epicatechin was stable in the

juice with seed liquor between day 28 and 42, it degraded significantly ($p \leq 0.05$) by the end of storage.

Table 7.6 - Effect of seed liquor on (-)-epicatechin content of baobab juice during storage. Results are means of triplicates (±SD).

	HTST	Time *(Days)*				
T (°C)	Treatment	0	14	28	42	60
	CA (0.5%)	32.03 ± .85	93.68 ± 0.23	98.77 ± 0.15	118.19 ± 3.66	28.94 ± 0.47
	SL (0%)	47.67 ± 8.52	89.63 ± 3.48	73.67 ± 2.37	98.61 ± 4.95	58.11 ± 4.72
	SL (1%)	31.37 ± 6.35	85.57 ± 0.16	62.80 ± 8.72	161.03 ±17.17	72.67 ± 0.23
	SL (2%)	23.52 ± 2.81	74.24 ± 3.05	99.00 ± 7.42	144.0 ± 1.30	29.31 ± 1.76
6	SL (3%)	85.16 ± 2.48	100.26 ± 0.47	90.62 ± 1.47	98.98 ± 1.02	63.77 ± 3.69
	CA (0.5%)	32.03 ± 0.85	111.38 ± 0.33	98.26 ± 3.38	95.51 ± 3.98	36.14 ± 8.38
	SL (0%)	47.67 ± 8.52	100.01 ± 0.91	91.43 ± 1.76	22.93 ± 0.73	97.14 ± 3.07
	SL (1%)	31.37 ± 6.35	106.34 ± 0.58	82.41 ± 2.51	111.27 ± 1.94	63.08 ± 0.63
	SL (2%)	23.52 ± 2.81	56.96 ± 4.27	97.45 ± 5.54	110.92 ± 1.06	37.81 ± 1.08
15	SL (3%)	85.16 ± 2.48	45.43 ± 2.51	101.72 ± 3.62	57.55 ± 1.38	109.44 ± 2.42
	CA (0.5%)	32.03 ± 0.85	144.53 ± 9.26	71.37 ± 2.50	46.18 ± 0.22	9.65 ± 0.00
	SL (0%)	47.67 ± 8.52	155.71 ± 7.39	88.27 ± 9.36	61.42 ± 2.07	56.35 ± 0.34
	SL (1%)	31.37 ± 6.35	95.84 ± 0.42	74.82 ± 0.30	98.06 ± 27.37	1.89 ± 0.09
	SL (2%)	23.52 ± 2.81	142.12 ± 6.06	82.05 ± 2.85	82.86 ± 0.28	2.07 ± 0
30	SL (3%)	85.16 ± 2.48	119.17 ± 0.33	85.81 ± 6.24	68.26 ± 5.24	0000

Results from this experiment showed that addition of seed liquor increased the level of both procyanidin B2 and (-)-epicatechin. The levels of both compounds increased during storage at 6 and 15 °C. Although (-)-epicatechin was more affected with storage temperature, overall these compounds were fairly stable compared to vitamin C and are likely responsible for the higher total phenol content and antioxidant activity (FRAP, DPPH and ABTS) observed in the juice. The antioxidant properties of phenolic compounds from seed liquor likely minimised the loss of vitamin C by preventing autoxidation of ascorbic acid or reducing dehydroascorbic acid to ascorbic acid through a redox reaction mechanism. Several authors (Coe et al., 2013; Donkor et al., 2014) have recommended the use of baobab products including seed oil or pulp powder for the preparation of novel functional foods. Therefore, in this study, addition of roasted seed liquor to baobab juice is recommended to improve quality properties including antioxidant activity. Consumption of quality baobab juice will undoubtedly promote the health status of the people of Malawi.

7.3 Conclusions and recommendation

Higher levels of bioactive compounds from seeds (Shahat, 2006) are likely responsible for increased total phenol content and antioxidant activity of baobab juice with added seed liquor during storage. From this experiment, it is suggested that the use of higher seed liquor concentration (more than 3%) could provide a clearer picture regarding vitamin C preservation. The level of bioactive compounds and antioxidant activity of baobab juice were more enhanced at 6 and 15 °C. The use seed liquor is easier, cheaper

and affordable by the resource poor communities in Malawi especially women who rely heavily on production of baobab juice for family consumption and sale. The current practice of using synthetic additives and their salt derivatives to preserve quality attributes of baobab juice may be minimised and health risks associated with them avoided.

Chapter 8 **General discussions and recommendations, suggestions for future work and conclusion**

8.1 Summary of key findings

Recently phytochemicals present in fruits and vegetables have attracted a growing attention because of their potential role in preventing diseases associated with oxidative stress (Kaur and Kapoor, 2001). It is therefore imperative to identify and quantify phytochemicals which are capable of providing physiological health benefits (bioactive compounds). For example polyphenols are reducing agents and together with other dietary reducing agents such as vitamin C, vitamin E and carotenoids they protect the body tissues against oxidative stress (Scalbert and Williamson, 2000). However, the compositional profile and functional properties of phytochemicals are affected by several factors such as species, provenance, processing and storage conditions (Kaur and Kapoor, 2001; Agbenorhevi and Marshall, 2012; De Oliveira et al., 2014; Trimigno et al., 2015). Fruit juices are not always consumed immediately after production, therefore understanding changes in the levels of bioactive compounds and metabolic profile is necessary to provide comprehensive information for quality control. This study was therefore undertaken to investigate the presence of bioactive compounds in fresh baobab fruit pulp from Malawi and evaluate the influence of processing and preservation techniques using reversed phase high performance liquid chromatography (RP-HPLC) and spectrophotometry. NMR-based metabolomics was used to compare the metabolic profile of untreated (UT), high temperature short time (HTST) pasteurized and high

temperature short time pasteurized with added citric acid (CAHTST) baobab juice samples and to analyse the temporal changes upon different storage conditions.

8.2 Characterisation of baobab products

Analysis of fresh baobab pulp showed high levels of ascorbic acid, vitamin C and organic acids (citric, malic and tartaric acid) while procyanidin B2, (-) –epicatechin and gallic acid were the most predominant phenolic compounds. ^{1}H NMR spectroscopy revealed twenty-five compounds (carbohydrates, organic acids, amino acids and phenolic compounds) in untreated juice. ^{1}H NMR metabolomics provided a more detailed and comprehensive molecular picture that complemented information obtained from RP-HPLC and spectrophotometry analysis of baobab juice. For instance, organic acids (citric, malic and tartaric acids) and phenolic compounds (flavan-3-ols) were confirmed in ^{1}H NMR spectrum of baobab juce and provided extra compositional information of juice samples at time zero and during storage.

The mean vitamin C content (AA + DHA) measured in the fresh baobab pulp (467 ± 8.1 mg/100 g FW) was more than eight fold higher than commonly consumed raw navel oranges (56.1 ± 1.0 mg/100 g) and raw lemon juice (42.9 ± 0.4 mg/100 g) reported by Scherer et al. (2012). The presence of high levels of vitamin C means an encouragement for the consumption of quality baobab products can enhance reduction of vitamin C deficiency symptoms such as scurvy which is more prevalent in the rural communities of Malawi. Local fruit processors may blend baobab fruit pulp with other priority indigenous or tropical fruits such as mobala plums (*Parinari curatellifolia),* monkey oranges

(Strychnos cocculoides) and mangoes (*Mangifera indica)* in order to improve vitamin C content. This will likely improve nutritional quality and extend shelf life of resultant juice by preventing quality loss caused by auto-oxidation such as browning common in fruit juices with lower levels of ascorbic acid and other organic acids. The pharmaceutical industry may extract and manufacture vitamin C tablets from locally available natural resources (baobab fruit pulp). Thus vitamin C tablets can be available at relatively affordable price especially to those in need of vitamin C supplements. Organic acids are important for organoleptic and microbiological quality of fruits and derived products. Specifically, organic acids influence taste, flavour, colour and microbial stability in foods. The significantly high levels of organic acids including citric (3300.84 ± 0.90 mg/100 g FW), malic (2364.98 ± 28.8 mg/100 g FW) and tartaric acid (173.93 ± 5.50 mg/100 g FW) in baobab fruit pulp were likely responsible for a low pH (3.11 ± 0.01), lemon taste and extended microbiological shelf life of baobab fruit juice reported by Ndabikunze and co-workers (Ndabikunze et al., 2010). The current practice of addition of artificial preservatives such as sodium benzoate can be greatly minimised due to presence of higher levels of these natural preservatives in baobab pulp.

Analysis of methanol/water (80:20, v/v) extract of baobab fresh pulp from Malawi revealed that procyanidin B2 (533 ± 22.6 mg/100 g FW), gallic acid (68.5 ± 12.4 mg/100 g FW) and (-)-epicatechin (43.0 ± 3.08 mg/100 g FW) are the most predominant phenolic compounds. Observed levels were significantly higher ($p \leq 0.05$) than values reported for many commonly consumed fruits including choke berry, cranberry, blueberry and plum considered as good sources of proanthocyanidins (Prior and Gu, 2005; Pimpão et al.,

2013). Higher total phenol content and antioxidant activity of fresh baobab pulp and resultant juice observed in this study as well as reported by other researchers (Kamatou et al., 2011; Coe et al., 2013) can therefore be ascribed to higher vitamin C, procyanidin B2, (-)-epicatechin and gallic acid besides other antioxidant compounds. Fresh baobab pulp from Malawi is thus an excellent source of bioactive compounds and may be considered as a potential source of raw material for the development of novel functional foods within Malawi and elsewhere.

From this study, scientific evidence for the presence of bioactive compounds will likely promote initiatives for the development of novel functional foods through optimisation of baobab products such as juice or fortification of others with baobab pulp powder. The Malawi food/beverage industry may use baobab pulp to enhance antioxidant properties of several products such as juices and confectionary thereby improving their nutritive value. The Malawi government may invest or engage foreign investor industries to extract, isolate and purify procyanidin B2, gallic acid and (-)-epicatechin which have wide application in the food/beverage and pharmaceutical industries as well as research institutions. This will inevitably promote commercialisation and international trade for Malawi.

Analysis of commercial baobab juice purchased from Malawi showed lower levels of ascorbic acid and vitamin C compared to experimental prepared baobab juice. Prolonged heating during preparation was likely responsible for the loss of vitamin C. Commercial juice showed significantly higher ($p \leq 0.05$) levels of procyanidin B2, total phenol content

and DPPH radical scavenging antioxidant activity compared to experimental juice. The level of (-)-epicatechin was within the level of experimental juice. Higher level of procyanidin B2, total phenol content antioxidant activity was attributed to higher residual pulp content in the final juice since filtration of commercial juice is achieved by a sack cloth with larger pore sizes. The present study has provided reliable quantitative information of commercial baobab juice which is useful for quality control during processing of baobab fruit pulp. Optimisation of baobab fruit processing offers an opportunity for the development of quality juice in terms of vitamin C and phenolic compounds which are both required by the immune system of human body.

For commercial baobab juice it is recommended that juice extraction from pulp powder be done instantly rather than after socking pulp overnight. Vitamin C is highly water soluble and may readily be extracted from pulp. Overnight socking may enhance oxidation of ascorbic acid and reduce its level in the resultant juice. The rural communities may be encouraged to form larger groups/cooperatives and seek support from governments or non-governmental organisations for non-thermal pasteurisation methods and techniques. Non-thermal methods such as high hydrostatic pressure (HHP), high pressure homogenisation (HPH) and ultrasound (US) retain ascorbic acid and provide fresh-like and safe final fruit products (Ramaswamy and Marcotte, 2006). Quality indigenous fruit products can help vulnerable groups of people in a community including malnourished children, pregnant women and people living with HIV/AIDS (Ndabikunze et al., 2010). If the Malawian baobab fruit processors are well trained and given extra

support including advanced processing methods, they can produce quality products for consumption and sale.

8.3 Influence of processing and storage on quality attributes

8.3.1 Effect of thermal treatment on vitamin C

The quality of raw material affects nutritional content and product shelf life. Non-thermal and thermal treatment methods are used to prevent quality loss caused by oxidation and microorganisms before final product development. Baobab pulp was microwaved and the effect of this heat treatment on ascorbic acid content was evaluated. Results showed that the content of ascorbic acid in the fresh baobab pulp was not significantly affected by microwaving.

Experimental baobab juice was prepared in the laboratory by dissolving untreated baobab pulp (100 g) in cooled boiled drinking water (1 L) to mimick the initial production step commonly used by local fruit processors in Malawi with some modification. Instead of socking pulp containing seeds in water for overnight, juice extraction from pure pulp was undertaken immediately to reduce loss of vitamin C (ascorbic acid and dehydroascorbic acid) influenced by water. Vitamin C is contained in the pulp, thus prior separation of pulp from seeds and increasing its content during extraction in water would likely increase its content and permit accurate quantification per 100 g of fresh weight. It is therefore recommended that Malawian fruit processors separate pulp from baobab fruit using domestic mortar, pestle and sieves rather than the current practice. Baobab pulp which is naturally dry could be further dried or sterilised and kept in well-capped and sealed plastic

containers before juice preparation and further treatment or sold as an intermediate product.

HTST (72 °C, 15 s) reduced degradation of ascorbic acid during pasteurization compared to other methods investigated. For HTST (85 °C, 15 s) pasteurisation, elevated temperatures might have contributed to an increased rate of AA degradation while for LTLT regimes (60 °C, 30 min; 60 °C, 60 min) both temperature and time influenced degradation of ascorbic acid. Other authors also reported a higher percent loss of ascorbic acid with higher temperature pasteurisation in baobab juice (Ndabikunze et al., 2010).

From these findings the Malawian fruit processors could microwave baobab pulp powder to inactive spoilage enzymes and microorganisms for increasing the shelf life and maintaining high level of ascorbic acid. After juice preparation, non-thermal pasteurisation could be used to retain ascorbic acid in the final juice. There is sufficient evidence that non-thermal methods are more superior to thermal methods of pasteurisation in terms of both ascorbic acid retention as well as microbiological safety and shelf life extension of fruit juices (Aneja et al., 2014). Moreover, non-thermal techniques such as power ultrasound has been identified as potential technology to meet US Food and Drug Administration (USFDA) requirement of 5 log reduction of *Escherichia coli* in fruit juices (Aneja et al., 2014). In addition non-thermal pasteurisation methods are associated with lower 5-hydroxymethylfurfural (HMF) concentration and browning index than heat-treated juices throughout storage (Aguilo-Aguayo and Oms-Oliu, 2009). Thus an investment towards the use of these methods would enhance full

exploitation of baobab fruits of Malawi which are currently underutilised due to lack of proper processing methods and techniques. Consumption of quality baobab fruit juice may help to reduce malnutrition and HIV/AIDS opportunistic diseases amongst the affected communities of Malawi.

8.3.2 **Effect of storage on quality attributes**

Fruit juices are not always consumed immediately after preparation. Several changes leading to quality loss in terms of flavour, colour, nutritional quantities and safety occur in untreated and processed juice during storage. In this study changes in the content of vitamin C, organic acids, phenolic compounds and antioxidant activity in HTST (72 °C, 15 s) pasteurised and acidified baobab juice left at storage conditions (6, 15 and 30 ºC) for a maximum period of 60 days were evaluated and compared against untreated juice. The findings were compared with metabolic profile of same juice samples for comprehensive analysis.

8.3.2.1 **Effect of storage on vitamin C**

Two commonly occurring degradation in fruit juices are non-enzymatic browning and ascorbic acid (AA) loss (Johnson et al., 1995), however enzymatic loss is also possible in untreated juice. Results from present study showed that the level of ascorbic acid and vitamin C (AA + DHA) was decreasing with storage time and temperature in all samples. AA was more affected than vitamin C (AA + DHA) suggesting the loss of AA to dehydroascorbic acid (DHA) was greater than the loss of DHA to 2,3-diketogulonic acid (DKGA). The loss of vitamin C in pasteurised juice during storage is mainly attributed to

non-enzymatic pathways since enzymatic degradation is eliminated during processing (Burdurlu and Karadeniz, 2003).

In the present study pasteurisation and acidification had less influence on the stability of ascorbic acid and vitamin C (AA + DHA) in baobab juice during storage as the level of these quality attributes remained significantly higher ($p \leq 0.05$) in untreated juice. It is possible that some of the degradative enzymes (ascorbate oxidase and ascorbate peroxidase) were not completely inactivated and contributed to the loss of ascorbic acid and vitamin C (AA + DHA) in pasteurised and acidified juice. From these findings it is suggested that further optimisation should include microwaving or sterilising pulp, production of baobab juice concentrate using the common evaporators used in citrus industry for producing orange juice concentrate. During this process, degradation of ascorbic acid is minimised and its concentration increased by the use of a low-pressure low- boiling temperature technique to remove of excess water from the juice. Concentrated baobab juice may be stable and no further treatment required. Alternatively, the concentrated baobab juice may be subjected to a higher conventional HTST pasteurisation such as 80 °C for 11 s employed by other researchers (Igual et al., 2010) in grapefruit juice. Higher pasteurisation temperature may ensure complete inactivation of degradative enzymes. A higher vitamin C and organic acid concentration in stored juice may enhance preservative role. Other quality attributes such as taste and colour would be maintained and microbiological shelf life increased. Moreover the Malawian industry may afford these approaches. Local fruit processors may increase vitamin C concentration in the juice by increasing pulp content in the water during juice extraction. Thermal

pasteurisation applied on a more concentrated juice would likely retain vitamin C and organic acids which would essentially increase nutritive value and extending shelf-life.

8.3.2.2 **Effect of storage on organic acids**

Overall, storage time and temperature did not significantly ($p \leq 0.05$) affect levels of citric and malic acids in all juice samples. Rare fluctuation in organic acid concentration observed especially in untreated juice was likely ascribed to fermentation, esterification and oxidation of organic acids (Piras et al., 2014). A fermentation activity in untreated juice samples was confirmed by ^{1}H NMR-based metabolomics. A higher concentration of ethanol was observed in untreated juice. Higher values of organic acids observed towards the end of storage at higher storage temperatures (15 and 30 °C) could be attributed to degradation of carbohydrates and other phenolic compounds in the juice.

From this study, stability of organic acids (citric and malic acid) in baobab juice means increasing their concentration through production of concentrated juice provides a cheaper option for achieving a stable product. The current practise of using synthetic preservatives including benzoic acid and sodium benzoate would be minimised. The appearance of higher ethanol concentration and fluctuation of organic acids in untreated juice suggest that pasteurisation is still recommended to prevent fermentation activities despite that it showed higher ascorbic acid and vitamin C (AA + DHA) content as described in the previous section.

8.3.2.3 **Effect of storage on procyanidin B2 and (-)-epicatechin**

Storage conditions influenced levels of procyanidin B2 and (-)-epicatechin in baobab juice. Results showed that procyanidin B2 was more stable than (-)-epicatechin during storage. Higher losses were observed in samples stored at 30 °C compared to 6 and 15 °C. Significant loss of these compounds in untreated juice at higher storage temperature was attributed to enzymatic (PPO, PME) degradation where phenolic compounds play a key role in enzymatic browning as they are preferred substrate of oxidative enzymes (Keenan et al., 2011). In treated juice samples, evolution of procyanidin B2 and (-)-epicatechin was ascribed to hydroxylation, esterification, epimerisation, thermal degradation and non-enzymatic oxidation during storage as described in previous sections.

From present study, it seems the content of procyanidin B2 generally increase during storage at 6 and 15 °C while levels decrease significantly at 30 °C. It is therefore recommended to keep baobab juice at lower temperature to preserve these bioactive compounds which may exert several health benefits due to their antioxidant properties. Conditions that preserve vitamin C will inevitably preserve flavan-3-ols.

8.3.2.4 **Effect of storage on total phenol content and antioxidant activity**

Results showed that storage time and temperature influenced the level of total phenol content (TPC) in the juice. In all samples, the level of TPC was fluctuating during storage. Polyphenols reacting with sugars and sugar metabolites (Agbenorhevi and Marshall, 2012) and formation of complexes with proteins (Ozdal et al., 2013) were all possible

reasons for evolution of TPC in baobab juice. The interaction may be attributed to the fact that phenolic group is an excellent hydrogen donor that forms hydrogen bonds with the carboxyl group of the protein (Mulaudzi et al., 2012). Juice samples left at 6 and 15 °C showed higher levels of total phenol content and antioxidant activity. Accelerated decrease in TPC of untreated juice stored at 30 °C after 42 days was attributed to loss of heat sensitive phenolic compounds and enzymatic activity. Higher levels of flavan-3-ols contributed to higher levels of TPC.

All antioxidant activity assays considered showed few systematic fluctuations of antioxidant activity in baobab juice. Untreated juice showed higher antioxidant activity at lower storage temperatures. Pasteurisation stabilised antioxidant activity of baobab juice at higher storage temperature. The significantly high level of antioxidant activity was consistent with vitamin C, total phenol content or procyanidin B2 level in the same juice. Correlation analysis between bioactive compounds and antioxidant activity confirmed these observations. Production of a more concentrated juice achieved as described for preservation of vitamin C would likely afford a higher total phenol content and antioxidant activity.

8.3.3 ^{1}H NMR metabolic profile of baobab juice during storage

NMR spectroscopy approach helped to look at all the components of baobab juice at once and confirmed presence of metabolites and chemical reactions ascribed to evolution of compounds during storage. Temporal dynamic change of juice metabolome at each storage temperature was observed. Analysis of the corresponding loading line plots

revealed metabolites that most significantly accumulated in response to time and with the reverse response. The loading plots derived from the OPLS models showed that the major compounds that contributed to sample separation were ethanol and carbohydrates. In particular, in the case of untreated (UT) juice samples levels of ethanol increased over time, while carbohydrates decreased with time. In the case of pasteurised (HTST) and acidified (CAHTST) juice samples, the most important accumulating metabolites were fructose, glucose and galacturonic acid, while sucrose decreased over time. The presence of ethanol in juice samples was indicative of the fermentation process of carbohydrates caused by microorganisms. Its content was higher in UT than in HTST and CAHTST samples. Furthermore, the highest levels of ethanol content was observed in UT samples stored at 30°C for 60 days, supporting that warmer temperatures enhanced the fermentative process.

The more rapid production of ethanol in the absence of any thermal treatment was a clear indication that pasteurisation of baobab juice alone or in combination with the addition of citric acid minimised fermentation. The presence of amino acid and reducing sugar metabolites were likely responsible for brown pigmentation at elevated temperature. While a higher concentration of ethanol in untreated justifies presence of enzymes. Thus ^{1}H NMR metabolomics complement information obtained using RP-HPLC and spectrophotometry analysis of baobab juice. Malawi fruit processors would be encouraged to pasteurise baobab juice using non-thermal or HTST pasteurisation to prevent spoilage caused by fermentation activities. Very high levels of ethanol in stored juice would reduce its quality despite having higher vitamin C content. Results from the

present knowledge will likely provoke the demand for non-thermal methods of pasteurisation to add value to a huge natural resources which is currently underutilised by the Malawian industry and population.

8.3.4 **Future work**

The presence of procyanidin B2 and (-)-epicatechin in fresh baobab pulp provides a possibility that other proanthocyanidins may be available in other forms (oligomeric, polymeric or isomeric). Future work should include screening for potential oligomers of proanthocyanidins (procyanidin B1, B5 and A2) as well as monomeric isomers ((-)-catechin, (+)-epicatechin) that were not checked due to time limit and unavailability of standards. Further, most dietary phenolic compounds of potential health benefit mainly exist in conjugated forms with sugar via O-glycosidic bonds or with other polyols as esters (Pimpão et al., 2013). Therefore future work should consider characterisation of these in baobab pulp and juice after hydrolysis. This would inevitably facilitate analysis of individual phenolic compounds including phenolic acids and tannins. For instance, gallic acid occurs widely as sugar esters in gallotannins like 2-O-digalloyl- tetra-O-galloyl-glucose (Crozier et al., 2009). Thus, in absence of hydrolysis protocol, the level of gallic acid and other phenolic compounds hence total phenol content and antioxidant activity may be underestimated. Although fresh baobab pulp did not show presence of hydroxycinnamates (p-coumaric acid, caffeic acid, ferulic acid and sinapic acid), it could be possible they are present as conjugates of tartaric acid and quinic acids (chlorogenic acids). Direct analysis of chlorogenic acids (3-O-, 4-O- and 5-O-caffeoylquinic acids) in baobab extract may also be considered. Fresh baobab pulp showed reasonable amount of

tartaric acid (173.93 ± 5.48 mg/100 g), hence formation of corresponding chlorogenic acids may be possible. Further it would be important to characterise fresh baobab pulp powder using NMR-based metabolomics prior to preparation and during processing and storage of the juice for comprehensive compositional information. In this way quality changes that occur at each stage can be monitored and optimised.

8.4 Influence of seed liquor on quality attributes of baobab juice

Preservatives are aimed at extending shelf life of fruit juices. However consumer demand for fresh and safe foods without chemically synthesised preservatives leads to an increase in the use of natural preservative (Aneja et al., 2014). Addition of seed liquor (SL) to the baobab juice showed increased total phenol content and antioxidant activity compared to baobab juice without SL despite the fact that no clear pattern for protective role against vitamin C was observed. At least no destructive role of seed liquor on vitamin C was observed. Higher levels of procyanidin B2, (-)-epicatechin and total phenol content were generally observed in juice with seed liquor which were likely responsible for higher antioxidant activity (FRAP, DPPH & ABTS).

The Malawian fruit processors could blend baobab juice with seed liquor in order to enhance antioxidant activity of the juice. This would contribute and encourage full exploitation of baobab seeds adding to other uses including addition to soups and stew as a thickener, production of fermented products and extraction of essential oil (Chadare et al., 2008; Kaboré et al., 2011).

8.4.1 **Future work**

Further research is recommended to investigate the influence of different percent seed liquor on organoleptic properties of the juice. This is an important component during product development to balance functional, safety and sensory characteristics. Future research study is also needed to evaluate the influence of seed liquor on microbial shelf life of the juice. This may involve enumeration of lactic acid bacteria, or yeast and moulds which are common in acidic fruit products. This may include isolation and quantification using conventional microbiology.

8.5 Conclusion

There were five objectives of this study. The first was optimisation of analytical methods and techniques. The second was characterisation of baobab products including commercial baobab juice. The third was to investigate the influence of processing and storage on the levels of bioactive compounds, total phenol content and antioxidant activity of baobab juice. The fourth was to evaluate changes in metabolic profile of baobab juice during processing and preservation. While the fifth was to evaluate the influence of seed liquor on vitamin C, bioactive compounds, total phenol content and antioxidant activity of baobab juice.

RP-HPLC showed that baobab fruit pulp and resultant juice contain high levels of vitamin C, (-)-epicatechin, procyanidin B2, (-)-epigallocatechin-3-O-gallate, gallic acid and organic acids. ^{1}H NMR spectroscopy confirmed presence of organic acids (citric, malic and tartaric acids) and revealed more metabolites including amino acids (leucine,

isoleucine, valine and alanine), carbohydrates (sucrose, fructose and glucose), and main phenolic compounds (flavan-3-ols).

Heat treatment by microwaving of baobab pulp powder did not significantly reduce vitamin C and could be used during preservation. The technique is faster and cheaper thus affordable by the resource poor fruit processors of Malawi. The production of a stable and high quality baobab powder in terms of vitamin C composition would likely promote utilisation and commercialisation in Malawi. High temperature short time pasteurisation (72 °C, 15 s) retained ascorbic acid and vitamin C in the final juice compared to low temperature long time pasteurisation regimes (60 °C, 30 min and 60 °C, 60 min) at time zero, however levels of ascorbic acid and vitamin C (AA + DHA) were lower during storage. The antioxidant activity of baobab juice was highly correlated with total phenol content suggestion presence of more bioactive compounds including procyanidin B2 and (-)-epicatechin quantified in the juice. Total phenol content, procyanidin B2 and (-)-epicatechin were fairly stable during storage compared to ascorbic acid and vitamin C and could be responsible for stable antioxidant activity of the juice. Non-enzymatic browning enhanced antioxidant activity of treated juice kept at higher temperature despite having lower levels of ascorbic acid and vitamin C (AA + DHA). DHA as well as dicarbonyl compounds formed during its degradation (DKG, erythrulose, 3-deoxythreosone, xylosone) can undergo ascorbic acid browning (non-enzymic browning) via a Strecker-like degradation reaction with amino acids forming brown pigments (Fennema, 1996) including HMF. Brown pigmentation was quite visible in baobab juice stored at 30 °C. The likelihood of ascorbic acid browning was the decrease of vitamin C

(AA + DHA) during storage and presence of amino acids observed in the ^{1}H NMR spectra of the juice.

The NMR spectra showed some similarities and differences in metabolites composition amongst untreated, pasteurised (HTST) and acidified (CAHTST) juice samples suggesting that processing and storage had an influence on metabolic profile of baobab juice. Untreated juice showed higher level of ethanol than pasteurised and acidified juice during storage at elevated temperature (30 °C) indicative of an intense fermentation activity. Thus despite higher vitamin C content observed in untreated juice, pasteurisation using non-thermal methods and further treatment such as increasing organic acid content are still necessary to achieve an ethanol free juice. The level of procyanidin B2, (-)-epicatechin, total phenol content and antioxidant activity was enhanced in baobab juice containing seed liquor. Influence of seed liquor on vitamin C needs further investigation to obtain a clear picture.

From this study the following suggestions and recommendation are made in order to optimise processing of baobab fruit:

(i) Fruit processors in Malawi could microwave baobab pulp powder, package it appropriately and sale as an intermediate product which is nutritious in terms of vitamin C.

(ii) Use of more concentrated baobab juice and non-thermal pasteurisation are suggested to improve vitamin C retention as well as stability during storage.

(iii) Addition of roasted seed liquor to baobab juice as an alternative means of enhancing bioactive compounds and antioxidant activity of the juice. Further investigation into influence of seed liquor on vitamin C need to be explored. This may be achieved by increasing percent seed liquor while assessing the impact on organoleptic properties of resultant juice (taste and colour).

(iv) All samples kept at 6 °C showed higher levels of ascorbic acid, vitamin C (AA + DHA) and phenolic compounds and lower ethanol concentration thus refrigeration could minimise loss of overall quality of baobab juice.

Regular consumption of quality baobab juice may contribute significantly to the recommended daily intake of vitamin C and together with other bioactive compounds may help to improve the health status of vulnerable communities of Malawi including malnourished children, pregnant women and people living with HIV/AIDS. Quality baobab products will likely promote utilisation and commercialisation in Malawi.

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Appendix A

Citric and malic acid concentration (ppm) in untreated (UT), pasteurised (HTST) and acidified (CAHTST) baobab juice. Results are means of triplicates (±SD).Values with different letters in columns are significantly different (Tukey's test, $p \leq 0.05$).

Storage condition / Treatment		*Organic acid concentration (ppm)* *UT*		*HTST*		*CAHTST*	
T *°C*	Time (Days)	*Citric acid (ppm)*	*Malic acid (ppm)*	*Citric acid (ppm)*	*Malic acid (ppm)*	*Citric acid (ppm)*	*Malic acid (ppm)*
6	0	3300.84 ± 18.51^{abc}	2364.98 ± 28.76^{bc}	3338.52 ± 21.18^{abc}	2413.37 ± 43.12^{ab}	8302.91 ± 16.45^{abcd}	2374.17 ± 65.39^{a}
	14	3224.01 ± 6.09^{abcd}	1946.09 ± 12.93^{d}	3245.28 ± 3.09^{abc}	1924. 63 ± 10.70b^{cde}	8201.98 ± 1.53^{abcd}	1905.99 ± 0.79^{abc}
	28	2934.17± 225.62^{de}	1887.88 ± 188.65^{d}	3023.93 ± 167.55^{bcd}	1840.99 ± 45.77^{de}	7575.39 ± 175.47^{bcd}	1818.62 ± 24.69^{c}
	42	3089.29 ± 233.75^{bcde}	2548.10 ± 146.87^{ab}	3229.07 ± 73.00^{abc}	2058.69 ± 349.38^{abcd}	8970.26 ± 572.58^{a}	2070.34 ± 274.58^{abc}
	60	3318.73 ± 19.26^{abc}	2707.87 ± 24.73^{a}	3358.91 ± 31.79^{abc}	2367.98 ± 34.87^{abc}	8175.14 ± 24.46^{abcd}	2339.69 ± 55.34^{ab}
15	*14*	3197.47 ± 9.73^{abcde}	1929.00 ± 15.64^{d}	3262.12 ± 6.43^{abc}	1942.25 ± 20.49b^{cde}	8550.51 ± 17.81^{abc}	2032.46 ± 10.78^{abc}
	28	2986.30 ± 150.50^{cde}	1886.71 ± 79.71^{d}	2987.69 ± 283.84^{bcd}	1798.58 ± 127.88^{de}	7353.06 ± 404.40^{d}	1846.14 ± 160.21^{bc}
	42	3094.80 ± 79.32^{bcde}	2578.17 ± 98.71^{ab}	2763.87 ± 190.73^{d}	1512.02 ± 318.15^{e}	8973.36 ± 929.96^{a}	2152.97 ± 369.54^{abc}
	60	3413.64 ± 22.57^{ab}	2597.85 ± 105.70^{ab}	3380.85 ± 15.34^{bc}	2313.22 ± 22.64^{abcd}	8240.57 ± 6.86^{abcd}	2305.50 ± 22.67^{abc}
30	*14*	3465.25 ± 19.52^{a}	2132.75 ± 23.37^{cd}	3511.28 ± 27.85^{a}	2230.02 ± 39.83^{abcd}	8683.54 ± 13.66^{ab}	2101.51 ± 13.77^{abc}
	28	2844.36 ± 220.00^{e}	1902.23 ± 132.49^{d}	2971.30 ± 212.90^{cd}	1888.68 ± 68.44^{cde}	7497.63 ± 273.73^{cd}	1836.56 ± 129.60^{bc}
	42	3259.15 ± 76.31^{abcd}	2529.56 ± 120.13^{ab}	3030.84 ± 176.32^{bcd}	1820.56 ± 387.45^{de}	9117.09 ± 776.23^{a}	2088.93 ± 359.58^{abc}
	60	3303.02 ± 18.79^{abc}	2316.39 ± 22.76^{bc}	3440.71 ± 36.94^{a}	2481.26 ± 20.95^{a}	8377.48 ± 27.26^{abcd}	2306.34 ± 38.62^{abc}

Appendix B

B.1 Total phenol content (TPC) and antioxidant activity in untreated juice. Results are means of triplicates (±SD). Values with different letters in columns are significantly different (Tukey's test, $p \leq 0.05$).

Storage		*Content and radical scavenging activity*			
Temperature (°C)	*Time (Days)*	*TPC (mg GAE/ 100 g)*	*FRAP (mg TEAC/100 g)*	*DPPH (%)*	*ABTS (mg TEAC/ 100 g)*
6	0	1564.81 ± 1.60^{b}	2806.39 ± 92.78^{e}	50.93 ± 0.43^{de}	1516.09 ± 17.10^{ab}
	14	1399.07 ± 1.60^{d}	2914.73 ± 8.96^{d}	52.52 ± 0.05^{c}	1564.57 ± 6.46^{ab}
	28	1393.52 ± 1.60^{d}	3091.91 ± 16.00^{c}	52.39 ± 0.09^{c}	1534.41 ± 8.13^{ab}
	42	1000.46 ± 14.98^{i}	2496.03 ± 18.65^{f}	42.95 ± 0.38^{g}	1207.98 ± 81.85^{d}
	60	1593.52 ± 4.24^{a}	3450.79 ± 23.78^{a}	57.89 ± 0.19^{a}	1498.85 ± 8.13^{b}
15	*14*	1430.56 ± 0.00^{c}	2842.50 ± 3.91^{de}	50.43 ± 0.09^{e}	1582.88 ± 16.58^{a}
	28	1361.11 ± 5.01^{e}	2916.99 ± 3.91^{d}	51.15 ± 0.05^{de}	1404.05 ± 8.13^{c}
	42	1201.85 ± 5.26^{g}	2786.07 ± 18.85^{e}	51.49 ± 0.11^{d}	1359.88 ± 32.53^{c}
	60	1352.31 ± 6.85^{ef}	3289.41 ± 7.82^{b}	53.79 ± 0.34^{b}	1580.73 ± 6.46^{a}
30	*14*	1175.46 ± 3.21^{h}	2568.26 ± 5.17^{f}	48.66 ± 0.28^{f}	1332.95 ± 3.73^{c}
	28	1339.81 ± 0.80^{f}	3139.31 ± 7.05^{c}	50.53 ± 0.16^{e}	1534.41 ± 10.39^{ab}
	42	740.28 ± 14.50^{k}	1666.54 ± 15.64^{h}	30.40 ± 0.58^{i}	931.11 ± 9.70^{e}
	60	810.19 ± 9.25^{j}	1883.23 ± 40.68^{g}	31.34 ± 0.49^{h}	856.78 ± 20.18^{e}

B.2 Total phenol content and antioxidant activity in HTST pasteurised juice. Results are means of triplicates (±SD).Values with different letters in columns are significantly different (Tukey's test, $p \leq 0.05$).

Storage Temperature (°C)	*Time (Days)*	*Content and radical scavenging activity*			
		TPC (mg GAE/100 g)	*FRAP (mg TEAC/100 g)*	*DPPH* (%)	*ABTS (mg TEAC/100 g)*
6	0	1084.72 ± 3.67^{f}	2738.67 ± 13.54^{b}	49.32 ± 0.16^{a}	1495.62 ± 1.87^{a}
	14	1103.70 ± 0.80^{f}	2395.59 ± 7.05^{de}	42.67 ± 0.60^{de}	1287.70 ± 13.06^{bc}
	28	1307.41 ± 1.60^{a}	2878.61 ± 16.00^{a}	49.07 ± 0.22^{a}	1520.40 ± 18.66^{a}
	42	1018.98 ± 5.26^{g}	2382.05 ± 11.89^{de}	41.89 ± 0.14^{e}	$1102.41 \pm 9.70d^{e}$
	60	1156.94 ± 4.81^{de}	2365.12 ± 8.52^{e}	38.11 ± 0.72^{f}	1094.87 ± 17.80^{de}
15	*14*	1262.96 ± 4.01^{b}	2626.95 ± 21.14^{c}	41.74 ± 0.47^{e}	1383.58 ± 5.60^{ab}
	28	1168.06 ± 16.84^{d}	2445.25 ± 9.77^{d}	45.96 ± 1.55^{bc}	1266.16 ± 7.46^{bcd}
	42	723.15 ± 16.10^{h}	1852.75 ± 37.29^{g}	37.05 ± 0.76^{f}	1001.14 ± 12.24^{e}
	60	1210.65 ± 1.60^{c}	2608.89 ± 15.27^{c}	46.58 ± 0.33^{b}	1349.11 ± 13.46^{ab}
30	*14*	1161.11 ± 4.81^{d}	2658.55 ± 3.91^{c}	43.29 ± 0.19^{de}	1111.02 ± 121.33^{cde}
	28	1271.30 ± 5.78^{b}	2740.93 ± 22.54^{b}	46.93 ± 0.30^{b}	1315.71 ± 61.66^{b}
	42	1019.91 ± 10.24^{g}	2268.06 ± 35.35^{f}	44.19 ± 0.25^{cd}	1422.37 ± 61.91^{ab}
	60	1136.57 ± 9.25^{e}	2640.49 ± 39.05^{c}	46.46 ± 0.51^{b}	1407.28 ± 154.87^{ab}

B.3 Total phenol content and antioxidant activity in acidified baobab juice (CAHTST). Results are means of triplicates (±SD).Values with different letters in columns are significantly different (Tukey's test, $p \leq 0.05$).

Storage		*Content and radical scavenging activity*			
Temperature (°C)	*Time (Days)*	*TPC (mg GAE/100 g)*	*FRAP (mg TEAC/100 g)*	*DPPH (%)*	*ABTS (mg TEAC/100 g)*
6	0	1223.61 ± 1.39^{c}	2882.00 ± 58.28^{d}	46.71 ± 0.09^{b}	1428.83 ± 17.10^{a}
	14	1244.91 ± 11.31^{c}	2751.01 ± 3.91^{ef}	44.22 ± 0.11^{c}	1386.82 ± 0.00^{b}
	28	1416.67 ± 2.78^{a}	3015.17 ± 3.91^{c}	49.41 ± 0.09^{a}	1432.06 ± 3.23^{a}
	42	915.74 ± 4.24^{h}	2262.42 ± 5.17^{i}	37.64 ± 0.11^{f}	1012.99 ± 7.46^{h}
	60	1408.33 ± 2.78^{a}	3284.90 ± 50.82^{a}	47.48 ± 0.27^{b}	1350.19 ± 7.46^{c}
15	*14*	1299.54 ± 8.82^{b}	2681.12 ± 12.21^{fg}	41.15 ± 0.61^{e}	1413.75 ± 10.39^{ab}
	28	1112.96 ± 3.21^{g}	2317.72 ± 51.27^{i}	38.29 ± 0.23^{f}	1149.81 ± 1.87^{f}
	42	1155.56 ± 7.22^{f}	3160.75 ± 96.73^{b}	46.96 ± 0.23^{b}	1200.44 ± 3.73^{e}
	60	1281.02 ± 12.13^{b}	2800.74 ± 5.17^{de}	45.03 ± 0.19^{c}	1276.93 ± 14.09^{d}
30	*14*	1144.91 ± 2.12^{f}	2474.59 ± 14.76^{h}	40.87 ± 0.05^{e}	1212.29 ± 6.46^{e}
	28	1234.72 ± 1.39^{cd}	2572.78 ± 8.96^{fg}	42.86 ± 0.69^{d}	1205.83 ± 17.10^{e}
	42	1195.83 ± 6.36^{e}	2800.74 ± 9.77^{de}	47.33 ± 0.14^{b}	1210.14 ± 1.87^{e}
	60	1235.19 ± 3.21^{cd}	2516.35 ± 12.82^{h}	45.25 ± 0.69^{c}	1114.26 ± 6.73^{g}

Appendix C

Procyanidin B2, and (-)-Epicatechin content (mg/100 g FW) in untreated, pasteurised and acidified baobab juice. Results are means of triplicates (±SD).Values with different letters in columns are significantly different (Tukey's test, $p \leq 0.05$).

Storage condition / Treatment		*UT*		*HTST*		*CAHTST*	
Temperature °C	*Time (Days)*	*PRO*	*EC*	*PRO*	*EC*	*PRO*	*EC*
	0	533.3 ± 22.6^{h}	43.0 ± 3.1^{g}	466.3 ± 56.5^{f}	47.7 ± 8.5^{d}	351.2 ± 29.2^{h}	32.0 ± 0.9^{f}
	14	1418.2 ± 9.9^{d}	107.2 ± 2.6^{b}	1060.2 ± 16.1^{cde}	89.6 ± 3.5^{b}	1317.9 ± 15.5^{cd}	93.7 ± 0.2^{c}
6	*28*	1626.9 ± 6.8^{b}	109.8 ± 1.6^{b}	1295.2 ± 1.8^{b}	73.7 ± 2.4^{c}	1407.9 ± 5.2^{bc}	98.8 ± 0.1^{c}
	42	1140.0 ± 56.2^{f}	175.3 ± 2.5^{a}	1721.9 ± 81.7^{a}	98.6 ± 4.9^{b}	1438.6 ± 70.0^{b}	118.2 ± 3.7^{b}
	60	1588.3 ± 16.1^{bc}	82.6 ± 12.0^{cd}	1024.3 ± 9.3^{de}	58.1 ± 4.7^{d}	1327.1 ± 11.2^{c}	28.9 ± 0.5^{f}
	14	1501.0 ± 0.4^{cd}	119.2 ± 1.2^{b}	1118.7 ± 7.7^{cd}	100.0 ± 0.9^{b}	1229.7 ± 23.4^{de}	111.4 ± 0.3^{b}
	28	1335.4 ± 45.4^{e}	108.5 ± 0.2^{b}	1143.1 ± 4.5^{cd}	91.4 ± 1.8^{b}	976.9 ± 5.4^{f}	98.3 ± 3.4^{c}
15	*42*	2026.0 ± 78.5^{a}	68.8 ± 0.8^{ef}	1177.4 ± 107.4^{bc}	22.9 ± 0.7^{e}	1226.6 ± 28.0^{e}	95.5 ± 4.0^{c}
	60	972.3 ± 50.8^{g}	39.5 ± 3.6^{g}	137.2 ± 0.6^{g}	97.1 ± 3.1^{b}	1571.8 ± 27.4^{a}	36.1 ± 8.4^{ef}
	14	525.9 ± 21.4^{h}	76.5 ± 0.9^{e}	957.5 ± 77.5^{e}	155.7 ± 7.4^{a}	883.8 ± 7.9^{g}	144.5 ± 9.3^{a}
	28	1336.0 ± 6.6^{e}	91.4 ± 0.6^{c}	1018.1 ± 0.9^{de}	88.3 ± 9.4^{b}	843.1 ± 4.0^{g}	71.4 ± 2.5^{d}
30	*42*	237.5 ± 73.3^{i}	60.7 ± 4.7^{f}	928.6 ± 21.2^{e}	61.4 ± 2.1^{cd}	808.0 ± 60.8^{g}	46.2 ± 0.2^{e}
	60	1066.7 ± 14.4^{fg}	41.0 ± 7.2^{g}	1632.7 ± 44.5^{a}	56.3 ± 0.3^{d}	256.7 ± 1.8^{i}	9.6 ± 0.0^{g}